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Identification of differentially expressed genes of *Haloxylon ammodendron* in response to salinity stress

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Abstract

Haloxylon ammodendron (C.A. Mey.), an endangered desert tree with excellent drought and salinity tolerance, provides a unique genotype to characterize and understand the tolerance mechanisms. In this study, four RNA-Seq libraries were constructed and sequenced from *H. ammodendron* under salinity stress. Total 12 027 differentially expressed genes (DEGs) were identified, in which 4 023, 3 517, 4 487 genes were differentially expressed under light salinity stress (200 mM NaCl), moderate salinity stress (400 mM NaCl), and severe salinity stress (800 mM NaCl), respectively. The up-regulated DEGs included several transcription factors (*e.g.*, *MYB* and *bHLH*), hormone-related genes (*e.g.*, *cytochrome P450*), protein kinases (*e.g.*, *Atpk2-Atpk19 like*), and genes involved in carbon metabolism (*e.g.*, *UDP glycosyltransferase*), osmotic regulation (*e.g.*, proline transporter), and ubiquitin proteasome system (*e.g.*, ubiquitin-conjugating enzymes). Heat shock proteins were identified as positive regulators of salinity tolerance in *H. ammodendron*. The expression patterns of 13 DEGs verified by real-time quantitative PCR were identically consistent with the variations in transcript abundance identified by RNA-Seq. Our results provide new insights into molecular mechanism of *H. ammodendron* in response to salinity stress.

Additional key words: carbon metabolism, drought and salinity tolerance, heat shock proteins, hormone-related genes, NaCl, RNA-Seq, transcription factors.

Introduction

Salinity stress is one of the main environmental factors limiting plant growth and crop productivity (Flowers 2004). The mechanisms of salt tolerance in plants can be divided into three main classes: osmotic adjustment, ion exclusion, and tissue tolerance (Roy *et al*. 2014). Transcript regulation in response to salinity stress and different gene expression under salinity stress have been characterized in many plants such as *Arabidopsis* (Jakoby *et al*. 2002), wheat (Niu *et al*. 2012), rice (Kawasaki *et al*. 2001, Fang *et al*. 2008), and maize (Andjelkovic and Thompson 2006). Recent research showed that the tissue-selective signaling and hormone crosstalk are related to the salinity stress (Wu *et al*. 2015, You *et al*. 2016). Several gene families and metabolic pathways, such as the *NAC* (*NAM* - no apical meristem, *ATAF* - *Arabidopsis* transcription activation factor, and *CUC* - cup-shaped cotyledon) transcription factor family (Fang *et al*. 2008) and cation/proton antiporters (*CPA*) family (Jia *et al*. 2018), have been characterized for their salt tolerance. In *Arabidopsis*, three *NAC* genes were induced by drought, salinity, and/or low temperature (Tran *et al*. 2004). *Arabidopsis NAC* gene *AtNAC2* can be induced by high salinity and the over-expression of *AtNAC2* altered lateral root development and enhanced salt tolerance (He *et al*. 2005). The drought

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Abbreviations: ATAF - *Arabidopsis* transcription activation factor; AP2/EREBP - APETALA2/ethylene-responsive element binding proteins; bHLH - basic helix-loop-helix; bZIP - basic leucine zipper; COG - clusters of orthologous groups of proteins; CPA - cation/proton antiporters; CUC - cup-shaped cotyledon; CYP - cytochrome protein; DEG - differentially expressed gene; GO - gene ontology; HSP - heat shock protein; MYB - myeloblastosis family of transcription factors; KEGG - Kyoto encyclopaedia of genes and genomes; LSS - light salinity stress; MSS - moderate salinity stress; NAC - NAM (no apical meristem) ATAF and CUC; qPCR – quantitative PCR; RNA-Seq - RNA-based next generation sequencing; SSS - severe salinity stress; TF - transcription factor; UPS - ubiquitin proteasome system; WRKY - containing a conserved WRKYGQK domain.
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resistance and salt tolerance in the transgenic rice are significantly improved by the over-expression of stress responsive gene *SNAC1* (Hu *et al*. 2006). The *CPA* family comprises Na^+/H^+ exchanger (*NHX*), K^+-efflux antiporter (*KEA*), and cation/H^+ exchanger (*CHX*) (Jia *et al*. 2018). There are about 40 *CPA* genes in *Arabidopsis* and some of their homologues have been identified in other plant species (Jia *et al*. 2018). Members of the *CPA* gene family, such as *NHX1*and *NHX2*, were reported to play a role in salt tolerance at cellular, organ, or whole-plant levels (Barragán e*t al*. 2012). Several novel soybean *CHX* genes were identified to have a protective function under salinity stress (Jia *et al*. 2017).

Haloxylon ammodendron is a kind of xerophytic desert tree and the source of *Cistanche deserticola,* a precious herbal medicine. *Haloxylon ammodendron* has an excellent drought and salinity tolerance and plays a very important role in maintaining the ecological balance of entire desert ecosystem in which it grows (Long *et al*. 2014). Previous studies on *H. ammodendron* were mainly focused on its physiology or the expression of a small amount of its genes in response to salinity stress (Xie *et al*. 2008, Gao *et al*. 2010, Yang *et al*. 2011). However, the molecular mechanism of salinity tolerance in *H. ammodendron* is still unclear. In this study, the RNA-Seq data of *H. ammodendron*s in response to salinity stress was generated, transcriptional differences of potential salinity tolerance genes were characterized, and their putative functions were deduced in the patterns in which their transcript abundance were described under different salinity treatments. Our assembled, annotated transcriptome datasets and gene expression profiles of *H. ammodendron* will facilitate genetic and genomic studies on understanding the molecular mechanism of salinity tolerance in *H. ammodendron* as well as provide transcriptome datasets to develop new plant cultivars with novel salinity tolerance through genetic engineering.

Materials and methods

Plants and salinity treatments: *Haloxylon ammodendron* (C.A. Mey.) seeds were germinated in plastic pots with a sufficiently moistened *Cornell Peat-Lite* mix. The seedlings were grown in a growth cabinet with day/night temperatures of 24/20 °C, a 16-h photoperiod, a 45 % relative air humidity, and a photon flux density of 350 μmol m^{-2} s^{-1}. A completely randomized design was used with 20 uniform plants. After three months of growth, three salinity treatments were applied: light salinity stress (LSS, 200 mM NaCl), moderate salinity stress (MSS, 400 mM NaCl), and severe salinity stress (SSS, 800 mM NaCl). Five plants were assigned to each treatment and the control (CK, without salinity stress). The soil salinity was kept at the predetermined levels by weighting the individual pot and rehydrating it every day. The plants stressed for three months in each treatment were sampled and sequenced.

Isolation of RNA, cDNA library construction and sequencing: To investigate the differentially expressed genes (DEGs) of *H. ammodendron* in response to salinity stress, total RNA was extracted from whole seedlings and the cDNA was reversely transcribed from mRNA of each treatment according to our previous publication (He *et al*. 2013). Three replicates for each treatment were applied for cDNA sequencing. The *Illumina* paired-end sequencing technology (*Illumina Hi Seq 2000* platform) was used to sequence and analyze raw RNA-Seq data and the base calling were produced by the *Illuminain* software analyzer at the *HUADA Gene Company* (Sheng Zhen, China).

Transcriptome *de novo* assembly and sequence annotation: The quality of raw sequence reads for the control and three salinity treatments from the image

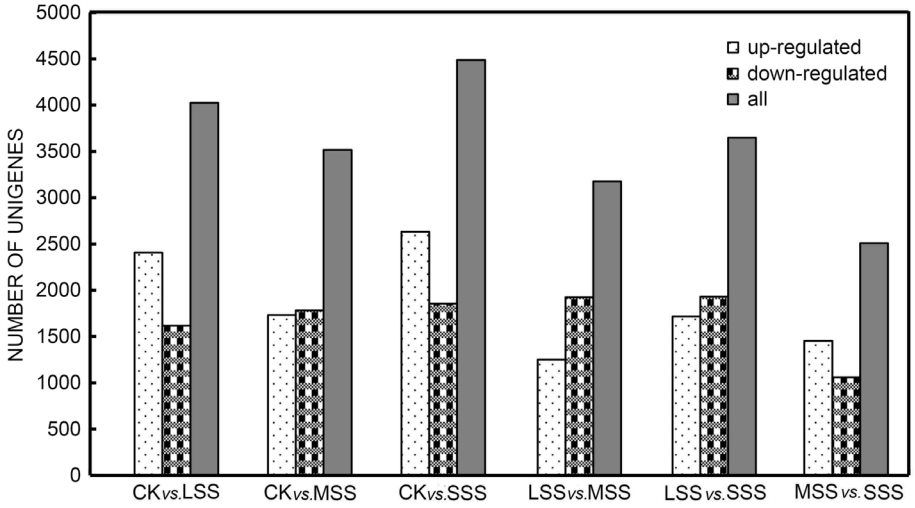

Fig. 1. Differentially expressed genes (DEGs) from six pairwise comparisons of four different RNA-Seq libraries of *Haloxylon ammodendron* in response to salinity stress. Up-regulated genes, down-regulated genes, and total DEGs were identified and characterized. LSS - light salinity stress (200 mM NaCl); MSS - moderate salinity stress (400 mM NaCl); SSS - severe salinity stress (800 mM NaCl).

data output of the *Illumina* sequencing facility were checked by *HUADA Gene Company*. The ribosomal RNA contamination, low quality bases, and adapters were trimmed and removed. The *de novo* assembly of *H. ammodendron* transcriptomes was accomplished by following the online instructions of *Trinity* (http://trinityrnaseq.sourceforge.net/). Reads per kilobase per million reads was used to analyze the gene expression. To validate the assembly reliability, all unigenes were annotated through *BLASTX* alignment against the *Nr* or *Uni Prot* database and NCBI non-redundant using the *BLAST 2.2.28+* program with an e-value threshold of $1e^{-5}$.

Characterization of differentially expressed genes: Read counts for each gene were calculated to identify DEGs and then were modeled as the Poisson distribution (Audic and Claverie 1997). The DEGs were identified requiring a false discovery rate of 0.001 and an absolute value of log 2 (fold-change). Cluster analysis of gene expression patterns was performed by using the *Cluster* software (De Hoon *et al*. 2002) and Java Tree View (Saldanha 2004). To better understand the functions of these specifically regulated genes and gain more insights into these genes, *BLAST* analysis and gene ontology (*GO*) analysis were performed. In the analysis of gene expression profiles, functional assignments were mapped onto *GO* terms (Harris *et al*. 2004). The *GO* enrichment analysis was constructed using *WEGO* (Ye *et al*. 2006). Significantly enriched Kyoto Encyclopedia of Genes and Genomes (*KEGG*) pathways were identified according to the *P* values and enrichment factors (Ye *et al*. 2006) using a *BLAST* search against the *KEGG* database and then mapped onto *KEGG* pathways.

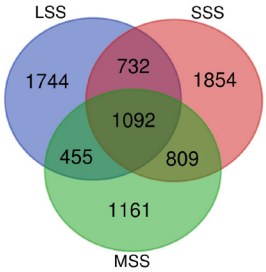

Fig. 2. The summary of *Haloxylon ammodendron* differentially expressed genes under three salinity treatments. LSS - light salinity stress (200 mM NaCl); MSS - moderate salinity stress (400 mM NaCl); SSS - severe salinity stress (800 mM NaCl).

Real-time quantitative PCR validation: Gene expression patterns were validated by real-time quantitative PCR (qPCR) to measure the digital transcript abundance and 13 DEGs were randomly selected and analyzed using the real-time qPCR. Primers for 13 DEGs and a reference gene were designed by the *Primer 5* software based on the target genes (Table 1 Suppl.). Following the manufacturer's instructions, total RNA was treated with genomic DNA eraser to remove gDNA contamination, and then about 0.4 μg of total RNA was used for cDNA synthesis by a *PrimeScript™ RT* reagent kit with a gDNA eraser sample kit (*TaKaRa*, Tokyo, Japan). The 0.01 cm^3 of quadruplet PCR reaction solutions contained 0.005 cm^3 of real-time *SYBR Premix Ex Taq* (*TaKaRa*), 5 μM forward primers, 5 μM reverse primers, and 20 ng of cDNA templates. All reactions were run as duplicates in 96-well plates. The quadruplet PCR reactions were performed on a *Lightcycle 480* (*Roche*, Basel, Switzerland) using the following cycle regime: 55 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The *18S*

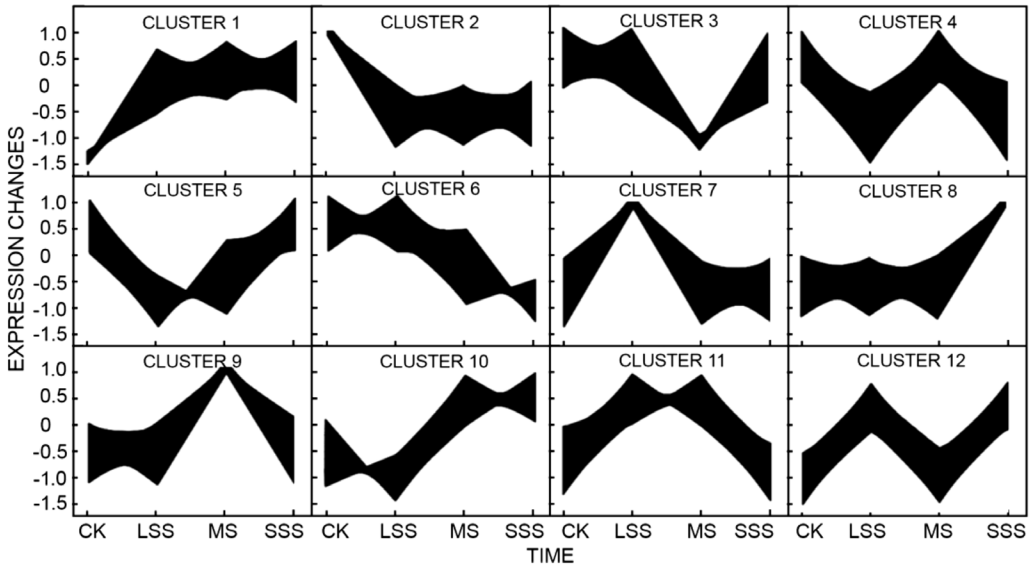

Fig. 3. Clusters and gene ontology enrichment of differently regulated genes of *Haloxylon ammodendron* in response to salinity stress. Genes with certain degree of regulation were grouped via hierarchical clustering. Expression values were normalized and scaled between -1.5 and 1.5 (*y*-axis). LSS - light salinity stress (200 mM NaCl); MSS - moderate salinity stress (400 mM NaCl); SSS - severe salinity stress (800 mM NaCl).

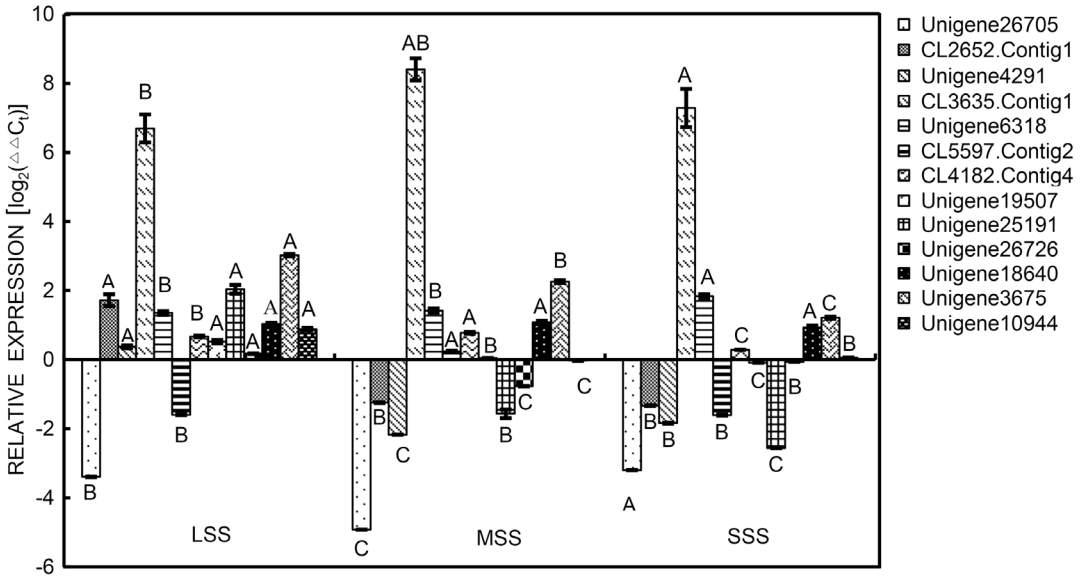

Fig. 4. Real-time PCR analysis on differently expressed genes of *Haloxylon ammodendron* in response to salinity stress. The *18S* was used to normalize the expression of all target genes. Means ± SDs, n = 3; means with different letters are significantly different at a 1 % level. LSS - light salinity stress (200 mM NaCl); MSS - moderate salinity stress (400 mM NaCl); SSS - severe salinity stress (800 mM NaCl).

gene was amplified in parallel as an internal reference gene. Relative expressions of the amplified products were calculated using the $2^{-\Delta\Delta Ct}$ method (Kanehisa *et al.* 2006). Three technical replicates were used for each sample and the data are shown as means ± standard deviations (SDs).

Results and discussion

Previous research verified that plants can enhance their salinity tolerance through modulation of gene transcription (Li *et al*. 2016). In this study, DEGs in *H. ammodendron* were analyzed under different salinity treatments and identified by pairwise comparisons of four RNA-Seq libraries, *i.e.*, CK *vs.* LSS, CK *vs.* MSS, CK *vs.* SSS, LSS *vs*. MSS, LSS *vs*. SSS, and MSS *vs*. SSS (Fig. 1). Our results indicated that the largest number of DEGs (4 487) were identified from the comparison of CK *vs*. In SSS, 2 631 genes were up-regulated and 1 856 genes were down-regulated. The second largest number of DEGs (4 023) was identified from the comparison of CK *vs*. LSS, in which 2 406 genes were up-regulated and 1 617 down-regulated. The third largest number of DEGs (3 647) was detected from the comparison of LSS *vs*. SSS, followed by CK *vs*. MSS (3 517), LSS *vs*. MSS (3 147), and MSS *vs*. SSS (2 510). We found that 455 DEGs were overlapped in LSS and MSS, 732 DEGs were overlapped in LSS and SSS, and 809 DEGs were overlapped in MSS and SSS (Fig. 2). Total 1 092 DEGs were identified to be differently regulated under all 3 salinity treatments indicating that these stress-responsive genes may be potentially involved in biological process and/or metabolic pathways for salinity tolerance in *H. ammodendron*.

To investigate the co-expressed genes in response to salinity stress, we applied the statistical clustering analysis to all genes that were differentially expressed under three salinity treatments compared to the control. Total 12 clusters of regulated genes were detected, comprising 61 599 genes that showed some degree in their different expressions (Fig. 3). The largest group (Cluster 8) included 8 544 genes (13.87 %) that had no significant changes in response to LSS (200 mM NaCl) and MSS (400 mM NaCl), but a significant increase in response to SSS (800 mM NaCl). The second largest group (Cluster 7) contained 7 495 genes (12.16 %) that increased in response to LSS but continually decreased in response to MSS and SSS. The third largest group (Cluster 9) contained 6 582 genes (10.68 %) and their expressions did not change notably in response to LSS and SSS except for a significant increase in response to MSS. The fourth largest group (Cluster 2) contained 6 346 genes (10.03 %), with a significant decrease in response to LSS and then they were stable in response to MSS and SSS. The fifth largest group (Cluster 10) contained 5929 genes (9.62 %) with a slight decrease in response to LSS, but a significant increase in response to MSS stress and then were stable in response to SSS. The smallest group (Cluster 6) contained 3 586 genes (5.82 %), and their expressions did not notably change in LSS, but continually decreased in response to MSS and SSS.

To validate the RNA-Seq-based gene expressions, real-time qPCR was performed on 13 randomly selected genes with different expressions in different salinity treatments. The qPCR results of these tested DEGs showed a similar trend that was observed in RNA-Seq data although the magnitudes of these gene expressions were different (Fig. 4). By comparing to the control, we found that the gene

CL3635.Contig1 was the most significantly up-regulated in all three salinity treatments, following by Unigene3675, Unigene6318, and Unigene18640, whereas Unigene26705 was significantly down-regulated in all salinity treatments used.

Transcription factors (TFs) are important upstream regulatory proteins and play significant roles in plant responses to abiotic and biotic stresses. The role of various TFs, such as MYB (the myeloblastosis family of transcription), NAC, bHLH (basic helix-loop-helix), AP2/EREB (APETALA2/ethylene-responsive element binding proteins), WRKY (containing a conserved WRKYGQK domain), and bZIP (basic leucine zipper), in regulating salinity and drought responses has been previously reviewed (Jiang *et al*. 2017). The over-expression of these transcription factors can enhance the drought or salinity tolerance of transgenic plants (Seki *et al*. 2002, Jeong *et al*. 2010, Takasaki *et al*. 2010, Hao *et al*. 2011). In this study, we found that many TFs, such as 3 *AP2-EREB* TFs (Unigene13909, Unigene9944, CL4042.Contig2), *bHLHs* (CL213.Contig1), *bHLH74* (CL350.Contig2), *bZIP* (Unigene24130), *C3H* (CL2305.Contig2), 3 *MYBs* (CL6074.Contig2, Unigene14210, Unigene10242), and *NAC* (Unigene11417), were significantly up-regulated under all salinity treatments (Table 2 Suppl.). Ethylene-responsive transcription factors *ERTF003* (Unigene17144) and *ERTF*034-like (CL4042.Contig2) were up-regulated in different salinity treatments, however, *ERTF012* (Unigene3675) was down-regulated under salinity stress. Our results indicate that some ethylene-responsive TFs may function as positive or negative regulators of salinity tolerance in *H. ammodendron*.

Previous studies indicated that a number of hormone-related genes, such as cytochrome P450 in ABA biosynthesis pathway, are significantly up-regulated in response to salinity stress (Mao *et al*. 2013, Zhang *et al*. 2014, Wang *et al*. 2017). There are 244 cytochrome 450 (*CYP450*) genes in *Arabidopsis* genome and the transfer of *Arabidopsis AtCYP78A7* into rice can increase the drought tolerance of transgenic rice (Nam *et al*. 2013). Other studies indicated that the disruption of *CYP707A3* in *Arabidopsis* can increase the drought tolerance, but the over-expression of this gene can reduce drought tolerance (Bak *et al*. 2011) suggesting that *CYPs* in different plants can be positive or negative regulators of drought tolerance. In this study, we found that salinity stress induced the transcript increases of *CYP* genes, such as *CYP450* (Unigene20778), *CYP450-86B1-like* (Unigene15178), and *CYP450-82C4-like* (Unigene20780), indicating that the *CYPs* may be positive regulators of salinity tolerance in *H. ammodendron* (Table 2 Suppl.).

Responses of carbon or starch metabolism to drought stress have been found in many plants such as alfalfa (Naya *et al*. 2007) and maize (Kakumanu *et al*. 2012). In *H. ammodendron*, one sugar transport protein (Unigene7883) and four sucrose UDP glucosyltransferase proteins, *i.e.*, UDP-glycosyl transferase 85A2-like (CL4049.Contig2), UDP-glycosyltransferase 85A3-like (Unigene23953), UDP-glycosyltransferase 85A2-like (CL1406.Contig1), and UDP-glycosyl transferase (Unigene35723), were significantly up-regulated under all salinity treatments (Table 2 Suppl.). These expression data suggest that sugar or carbon metabolism as well as various sugar-related signaling pathways are influenced by salinity stress in *H. ammodendron*. Salinity tolerance of most plants is consistent with the transport of substances involved in osmotic adjustment (such as proline and other amino acids) (Cockburn *et al*. 1996, Chang *et al*. 2014). In yeast, amino acid permeases modulate the efficient transport of proline, alanine, and valine, but the expression of most amino acid permease gene family members is repressed under high salinity (Rentsch *et al*. 1996). Our results show that the transcription of amino acid permease 2-like (CL2149.Contig1) was induced in NaCl-treated *H. ammodendron*. We found the continuous increase in the expressions of proline iminopeptidase (CL4680.Contig2), solute carrier family 25 (CL5895.Contig2), solute carrier family 13 (CL5828.Contig1), solute carrier family 40 (CL2892.Contig1), solute carrier family 15 (CL602. Contig1), and solute carrier family 2 (CL2997 contig3) in response to salinity stress (Table 2 Suppl.). Therefore, *H. ammodendron* may have the distinct ability to synthesize and transport various osmotic protectants, such as proline, osmotin, and amino acids, to maintain cell pressure potential under osmotic stress, which has not been reported in the *Arabidopsis* or other eukaryotes.

Heat shock proteins (HSPs) can regulate the activity of Na^+ or K^+-ATP enzymes. Many *HSP* genes are generally not expressed, but can be expressed if they are stimulated by external stimuli. Therefore, *HSPs* are important substances for defensing the external stimulus. In this study, a heat shock cognate 70 kDa protein isoform1 (CL2574. Contig13), a heat shock 70 kDa protein (CL801.Contig2), a small heat shock 17.4 kDa protein (Unigene11667), a small heat shock protein (Unigene8639), a small heat shock protein.18.1 (Unigene22310) and a heat shock protein 83-like (CL4411.Contig1) were significantly up-regulated under different salinity treatments (Table 2 Suppl.) indicating that *HSP*s may be a positive regulators of salinity tolerance in *H. ammodendron*.

Phosphorylation of protein kinases can activate many target proteins in response to osmotic stress in higher plants (Umezawa *et al*. 2004). In this study, a serine-threonine kinase (Unigene22252), a CBL-interacting serine/threonine-protein kinase 6-like protein (CL4751. Contig1), and L-type lectin-domain-containing receptor kinases were differentially up-regulated under different salinity treatments, which is consistent with the up-regulated expression pattern of a *Glycine soja* G-type lectin S-receptor-like serine/threonine-protein kinase (GsSRK) that is significantly increased under $NaHCO_3$ treatment (Ge *et al*. 2010) and under NaCl and polyethylene glycol (PEG) treatments (Sun *et al*. 2013). Our results indicate that the serine/threonine-protein kinase Atpk2-Atpk19 like (CL1544.Contig2) was significantly up-regulated in response to salinity stress similarly to the previous study that the *AtLPK1* expression in *Arabidopsis* is strongly induced by stress hormones and salinity treatments, and the *AtLPK1* over-expression in *Arabidopsis* can enhance salinity tolerance (Huang *et al*. 2013). However, the serine/

threonine-protein kinase sx32 (Unigene18472) was down-regulated under different salinity treatments in this study. Therefore, serine-threonine kinases can have a positive or negative effect to the abiotic stress. In *H. ammodendron*, a large number of protein kinases may play an essential role in sensing external salinity signals and regulating gene expression at the cellular level, however, the genes encoding protein kinases in response to stresses (*e.g.*, salinity or drought) are different from other plants.

Ubiquitin proteasome system (UPS), an important regulator of a series of life processes within a cell, is closely related to the occurrence and development of diseases (Powell *et al*. 2012). The energy produced by cellular metabolism can stimulate cells to replicate themselves and complete the self-metabolism and repair functions. In this study, ubiquitin extension protein (CL7928.Contig2) ubiquitin-conjugating enzyme E2 (CL4959.Contig2), ubiquitin-conjugating enzyme (CL5816.Contig1), E3 ubiquitin-protein ligase *AIP*2-like (Unigene10144,) and E3 ubiquitin-protein ligase *UPL*1-like (CL448.Contig1) were significantly up-regulated under different salinity treatments (Table 2 Suppl.). Our results indicate the ubiquitin-conjugating enzyme can promote the salinity stress at various degrees. As few studies related to ubiquitin-conjugating enzymes in response to the abiotic stresses have been reported, it would be valuable to further characterize and understand the structures and functions of ubiquitin genes newly identified in this study.

Resistance proteins contain an appropriate avirulence protein, and it guards the plant against pathogens and a direct or indirect interaction with the avirulence protein, consequently, triggering a defense system which restricts the pathogen growth. In this study, most disease resistance protein genes, such as disease resistance *RPP*13-like protein 1-like (Unigene2395), disease resistance protein *RGA*3-like (Unigene5608), disease resistance protein *RGA*4-like (unigene25823), disease resistance protein *RGA*1(Unigene2076), disease resistance protein *RPM*1 (CL8031.Contig1), and disease resistance protein *RPS2* (Unigene527), were significantly down-regulated under different salinity treatments indicating that the disease resistance of *H. ammodendron* was weakened by the salinity stress.

Conclusions

In this study, the transcriptome data for *H. ammodendron* obtained by RNA-Seq was used to identify and annotate a large number of DEGs regulated under salinity stress, which provides an excellent abundant dataset for further genetic and genomic research. Genes responsible for salinity stress and their expression profiles under different salinity treatments were further analyzed. The up-regulated DEGs that are related to salinity tolerance include some previously reported genes (*e.g.*, *MYB*, *bHLH*, *C3H*, and *AP2EREBP*) and some newly identified genes (*e.g.*, those encoding cytochrome P450, ubiquitin-conjugating enzymes, and resistance proteins). Furthermore, a certain amount of target candidate genes would serve as prospective functional studies on the salinity stress process. We found the significant changes of carbon metabolic pathways, hormone-related genes, TFs, protein kinases, and UPS in *H. ammodendron* under salinity stress. These changes mainly worked as the upstream of salinity responses and then led to hormone signalling changes, which further triggered TF expression changes and salinity tolerance in *H. ammodendron*. Our results provide new insights into the molecular mechanism of *H. ammodendron* in response to salinity stress and can be used as a valuable reference or resource to further characterize candidate genes potential for the development of new plant genotypes with novel salinity tolerance through genetic engineering.

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