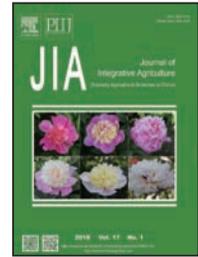




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RESEARCH ARTICLE

Transcriptome analysis of salt-responsive genes and SSR marker exploration in *Carex rigescens* using RNA-seq

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Abstract

Carex rigescens (Franch.) V. Krecz is a wild turfgrass perennial species in the *Carex* genus that is widely distributed in salinised areas of northern China. To investigate genome-wide salt-response gene networks in *C. rigescens*, transcriptome analysis using high-throughput RNA sequencing on *C. rigescens* exposed to a 0.4% salt treatment (Cr_Salt) was compared to a non-salt control (Cr_Ctrl). In total, 57 742 546 and 47 063 488 clean reads were obtained from the Cr_Ctrl and Cr_Salt treatments, respectively. Additionally, 21 954 unigenes were found and annotated using multiple databases. Among these unigenes, 34 were found to respond to salt stress at a statistically significant level with 6 genes up-regulated and 28 down-regulated. Specifically, genes encoding an EF-hand domain, ZFP and AP2 were responsive to salt stress, highlighting their roles in future research regarding salt tolerance in *C. rigescens* and other plants. According to our quantitative RT-PCR results, the expression pattern of all detected differentially expressed genes were consistent with the RNA-seq results. Furthermore, we identified 11 643 simple sequence repeats (SSRs) from the unigenes. A total of 144 amplified successfully in the *C. rigescens* cultivar Lüping 1, and 69 of them reflected polymorphisms between the two genotypes tested. This is the first genome-wide transcriptome study of *C. rigescens* in both salt-responsive gene investigation and SSR marker exploration. Our results provide further insights into genome annotation, novel gene discovery, molecular breeding and comparative genomics in *C. rigescens* and related grass species.

Keywords: salt stress, *Carex rigescens*, transcriptome, differentially expressed genes, SSR markers

1. Introduction

Abiotic stresses such as high salinity have been shown to limit growth, distribution and yield in many plant species (Shabala and Cuin 2008). It has been shown that more than eight million square kilometers of land are affected by salt throughout the world (FAO 2008). These impacted soils account for more than 6% of the total land. In China, saline soils cover over 360 thousand square kilometers,

Received 10 April, 2017 Accepted 15 August, 2017
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doi: 10.1016/S2095-3119(17)61749-0

amounting to 4.88% of the total available land (NSSO 1998). Soil salinization has severe effects on ecological construction and environmental control especially in arid and semi-arid regions in northern China. High salt concentrations in soils create an ion imbalance leading to plant stresses such as oxidative damage, slowed growth rate, cell dehydration, chlorophyll degradation or even cell death (Munns and Tester 2008). Understanding plant salt stress response mechanisms and the genetic basis for plant growth and development under salt stress is imperative. Plants defend themselves against salt stress by regulating a series of physiological responses *via* altering salt stress-specific gene expression. During the past decades, studies have focused on exploring plant salt tolerance mechanisms including plant sensory mechanisms, transcription factor (TF) regulation, Na⁺ transport network processes and low Na⁺ maintenance in the cytoplasm based on sodium hydrogen exchange (NHX) and salt overly sensitive (SOS) pathways (Deinlein et al. 2014). Nevertheless, salt stress response mechanisms in plants remain poorly understood due to the complexity of the response process and the genetic variability among plant species.

Next-generation high-throughput sequencing techniques have become an increasingly useful tool for exploring whole plant genomes (Deshmukh et al. 2014), providing a means for analyzing plant molecular regulatory mechanisms in specific environments such as various abiotic stress conditions. Many independent studies have been conducted exploring salt stress response mechanisms in a variety of plant species, such as *Medicago sativa* (Postnikova et al. 2013; Long et al. 2015), *Medicago truncatula* (Long et al. 2015), *Halogeton glomeratus* (Wang et al. 2015), and *Caragana korshinskii* (Li S et al. 2016). These studies have advanced our knowledge on how the genome, transcriptome and genes function and interact in plants under salt stress. However, few turfgrass species have been studied in regards to salt stress response mechanisms on the molecular level using RNA sequencing techniques. A database was created to confer the global transcript resource of Japanese lawngrass (*Zoysia japonica* Steud.) (Xie et al. 2015) and the highly polyploidy, facultative apomictic Kentucky bluegrass (*Poa pratensis* L.) (Bushman et al. 2016) using transcriptome technology. Furthermore, using these RNA-seq databases, candidate genes involved in salt tolerance have been identified in the halophytic turfgrass *Sporobolus virginicus* (Yamamoto et al. 2015) and in both shoot and root tissues of Kentucky bluegrass (Bushman et al. 2016). The protective roles of exogenous melatonin in response to salt stress have been illustrated in the widely planted turfgrass bermudagrass (*Cynodon dactylon*) (Shi et al. 2015). These results have strengthened the knowledge on salt stress response and tolerance mechanisms as well as the genetic basis of these mechanisms in turfgrass species

but the molecular mechanisms regarding the other important turfgrass species respond to salt stress still remain largely unexplored.

Carex rigescens (Franch.) V. Krecz. is a perennial plant that belongs to the *Carex* genus and is widely distributed in China's northern regions such as Liaoning, Hebei, Henan, Shandong and Inner Mongolia. This grassy plant possesses many adaptations for survival in various soil types such as tolerances to cold, drought, and heat stresses (He et al. 1993). As a wild grass, *C. rigescens* has the potential to be utilized as a genetic resource and has been genetically utilized for integration into important turfgrass species in Beijing, China (Wang 1997). Presently, research endeavors on *C. rigescens* focus on breaking seed dormancy (Liang et al. 2011), improving seed germination rates (Sun et al. 2011), salinity threshold calculations and pharmaceutical usage (Gao et al. 2009; Zhang et al. 2017). Few studies have been devoted to *C. rigescens* genomics and gene expression profiles in response to salt stress. In this study, two RNA sequence libraries, one control and one salt-treated, were constructed using *de novo* Illumina sequencing. The transcriptome analyses were used to characterize *C. rigescens* and discriminate its gene expression profiles in response to salt stress. To our knowledge, this is the first study on *C. rigescens* response to salt stress using transcriptome technology. Our findings provide insight into novel gene discovery, molecular breeding and comparative genomics of *C. rigescens*.

2. Materials and methods

2.1. Plant materials and sample preparation

C. rigescens Lüping 1 was used in this study. The seeds of Lüping 1 were sown into pots and incubated in a regime of 16 h light/8 h dark in 25°C light/20°C dark for 30 days. For the salt treatment, seedlings were irrigated with 50 mL of 0.4% NaCl dissolved in sterilized water (Cr_Salt). Seedlings irrigated with an equal volume of sterilized water were used as a control (Cr_Ctrl). This salt concentration was chosen as it has been shown to be crucial to plant growth (Mohammadi-Nejad et al. 2010; Yang et al. 2014). Whole fresh seedlings from the control and treatment groups (four seedlings each) were pooled for sample collection at 24 h after irrigation. Samples were stored at -80°C after flash-freezing with liquid nitrogen.

2.2. RNA extraction and sequencing

Total RNA was isolated from *C. rigescens* seedlings using Trizol Reagent (Biomed Biotech, Beijing, China) according to the manufacturer's instruction. The RNA purity and

integrity were assessed using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) and RNA Nano 6000 Assay Kit in an Agilent Bioanalyzer 2100 System (Agilent Technologies, CA, USA), respectively. A Qubit® RNA Assay Kit and a Qubit® 2.0 Fluorimeter (Life Technologies, CA, USA) were used to determine RNA concentration. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit from Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on an Illumina HiSeq 2000 platform and paired-end reads were generated.

2.3. Unigene assembly, functional annotation and differential unigene expression analysis

Raw data (raw reads) in fastq format were first processed through in-house perl scripts (Hu *et al.* 2015). To obtain clean data (clean reads), the adapter sequences, poly-N nucleotides and low quality reads were removed from the raw data. The Q20, GC-content and two sequence duplication level of the clean data were calculated. All downstream analyses used were based on the clean data. Transcriptome assembly was accomplished based on the pooled files for each treatment using Trinity (Grabherr *et al.* 2011) with `min_kmer_cov` set to 2 with all other parameters being set to default. For data analysis, FPKM (fragments per kilobase million) was used as it considers both the influence of sequencing depth and the gene length of reads. Gene function was annotated based on the following databases: Nr (NCBI non-redundant protein sequences); Nt (NCBI non-redundant nucleotide sequences); Pfam (protein family); KOG/COG (clusters of orthologous groups of proteins); Swiss-Prot (a manually annotated and reviewed protein sequence database); KO (KEGG orthology database) and GO (gene ontology). For differential gene expression analysis, the read counts of each library were adjusted using the edgeR Program (Robinson *et al.* 2010) package through one scaling normalized factor. Differential expression analysis of the two samples was performed using the DEGseq R package (Wang *et al.* 2010). The *P*-value was adjusted using the *q*-value (Storey and Tibshirani 2003). A *q*-value < 0.005 and $|\log_2(\text{fold change})| > 1$ were set as the threshold for significant differential expression.

2.4. Quantitative real-time PCR (qRT-PCR) validation

RNA samples of *C. rigescens* Lüping 1 seedlings were

reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa Biomedicals, Japan) after being treated with RNase-free DNase I (TaKaRa). Quantitative RT-PCR reactions were performed using the Applied Biosystems 7300 Sequence Detection System (Carlsbad, CA, USA) with SYBR® Premix *Ex Taq*™ II (TaKaRa). The *CrActin* gene (GenBank no. KX343900) was used as an internal control and triplicate quantitative assays were performed. Primer sequences for the selected genes are listed in Table 1. Primers were designed using NCBI's primer designing tool (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The PCR cycling conditions comprised an initial polymerase activation step of 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 31 s. To confirm the specificity of the product a dissociation curve was designed after each PCR run. The formula $2^{-\Delta\Delta C_t}$ was used to calculate the relative transcript levels (Livak and Schmittgen 2001). All data are shown as the means ± SD (*n*=3).

2.5. DNA extraction and SSR marker exploration

DNA was extracted from two different genotypes of *C. rigescens* Lüping 1 (samples 1 and 2) using a Plant Genomic DNA Extraction Kit (TaKaRa). Simple sequence repeats (SSRs) were predicted by MISA (1.0) (<http://pgrc.ipkgatersleben.de/misa/misa.html>) according to the unigenes obtained from the transcriptome data. The primers for the SSRs were designed using Primer3 (version 2.3.5). The PCR amplification reactions contained 1 μL *Taq* DNA polymerase, 0.1 mmol L⁻¹ dNTP, 2 mmol L⁻¹ Mg²⁺, 1 μL 10× buffer (without Mg²⁺), 1 μmol L⁻¹ primers, 100 ng DNA, and ddH₂O was added to the 10 μL reaction. Thermocycling was performed at 94°C for 3 min; 10 cycles with 94°C for 30 s, 60.5°C for 30 s (each loop reduced 0.5°C), 72°C for 3 min; 23 cycles with 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and an additional polymerization step at 72°C for 5 min. After separated in 10% PAGE electrophoresis, the PCR products were visualized by silver staining and imaged using a UMAX 2100XL Scanner (UMAX Technologies, USA).

3. Results

3.1. Transcriptome sequencing and assembly

The RNA extracted from whole plants of *C. rigescens* with or without salt treatment was sequenced using the paired-end technique to compare gene expression patterns. In total, 108 307 484 raw reads were obtained (Table 2), including 59 544 878 and 48 762 606 from the *Cr_Ctrl* and *Cr_Salt* samples, respectively. After removing the adapter sequences, ambiguous nucleotides and low-quality

sequences, a new total of 104 806 034 clean reads were identified, consisting of 57 742 546 in the Cr_Ctrl samples and 47 063 488 in the Cr_Salt samples. When combining the reads from the two sample groups, 81 860 transcripts with a mean length of 1 046 bp and an N50 of 1 679 bp were generated using the Trinity software. A total of 43 847 unigenes were annotated with a mean length of 858 bp and an N50 of 1 561 bp. The length distribution of unigenes and transcripts were analyzed and were presented as a function of the different interval lengths (Fig. 1).

3.2. Blast analysis

All assembled unigenes were blasted against public databases, including NCBI, KO, Swiss-Prot, Pfam, GO and KOG for similarity searches using the Blast algorithm (E-value<1E-5). A total of 21 954 unigenes were annotated in at least one searched database, accounting for 50.06% of the total unigenes obtained. Among them, 20 330 (46.36%) and 5 497 (12.53%) assembled unigenes showed identity with sequences in the Nr and Nt databases, respectively. The percentage of assembled unigenes homologous to sequences in KO, Swiss-Prot, Pfam, GO and KOG databases were 14.84, 35.46, 35.88, 39.38 and 19.68%, respectively (Table 3).

3.3. Functional annotation and pathway assignment

When the GO database was used to classify unigene functions, all 17 268 GO database results matched the Cr_Ctrl or Cr_Salt group unigenes. These were then classified into three functional categories: biological processes, cellular components and molecular functions (Fig. 2). Within the biological processes category, 22 subcategories were created to further classify the matched unigenes. The two dominant subcategories were cellular processes and metabolic processes. In the cellular components category, these sequences were further divided into 14 classifications. The most represented subcategories were cell and cell part. Among the molecular functions terms, these unigenes were classed into 11 classifications. The largest subcategory was binding and the second largest was catalytic activity (Fig. 2).

To get a better understanding of the biological relevance of the systemic function categories from the annotated *C. rigescens* genes, the KEGG pathway mapping database was searched. According to the KEGG pathway analysis, 6 075 unigenes were assigned to five pathways: cellular processes (688, 11.3% of unigenes), environmental information processing (517, 8.5% of unigenes), genetic information processing (1 558, 25.6% of unigenes), metabolism (2 925, 48.1% of unigenes) and organism

Table 1 Primers used in quantitative RT-PCR for validation of differentially expression genes

Gene ID	Primer (5'→3')	Tm (°C)	Product size (bp)
KX343900	F: CCGACCGTATGAGCAAGGA R: TGAGTGAGGCGAGGATGGA	85.5	120
comp35285_c0	F: GGTGGTCTCGGCATCTCA R: TGGTTTGATCCGGCCATTGT	81.0	105
comp42718_c0	F: CACTGACACCAGCACCAGTT R: TCCTTCACTTCGCCACAAGC	86.0	115
comp44196_c0	F: TACCAAGCCAGACCCGAGAT R: CTCGACCTTATCCGCTCTCAC	86.0	184
comp46251_c0	F: TGGCGGTCTGGATTTGGTTT R: GGCATGGCTGCTCAAATTCC	87.5	199
comp36473_c0	F: GGGGATGCAGCTGGAAAAATG R: CCAGCCCAATCACACACCTTA	81.5	165
comp39685_c0	F: TCGGATAGCGAGTTGTGCGAG R: TGAACGTGTGTGCATCCTTGA	81.0	184
comp45194_c0	F: ATACTCCTACAAGCGCAAAGCA R: GGTGGAACGGGCATCACTAA	78.0	189
comp40218_c1	F: TACCGACAACAGGCGAACC R: GCTTGGGCATTCTCACTTCAGG	84.0	176

Table 2 Summary of assembled transcripts and unigenes for all *Carex rigescens* samples

Metric	Total	Total length (bp)	Mean length (bp)	N50
Row reads	108 307 484	–	–	–
Clean reads	104 806 034	–	–	–
Transcripts	81 860	85 636 807	1 046	1 679
Unigenes	43 847	37 620 355	858	1 679

–, absent; N50, the size of the contig above which the assembly contains at least 50% of the total length of all the contigs.

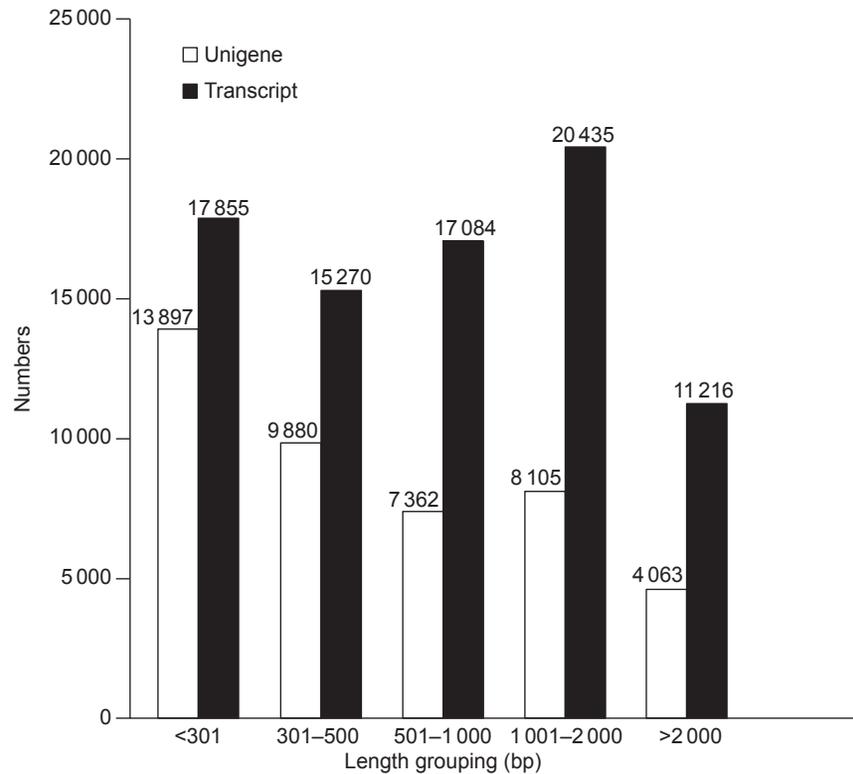


Fig. 1 *Carex rigescens* unigene and transcript lengths identified using transcriptome databases.

Table 3 Blast analysis of assembled unigenes using various databases

Database ¹⁾	Number of unigenes	Percent (%)
Nr	20330	46.36
Nt	5479	12.53
KO	6075	14.84
Swiss-Prot	15549	35.46
Pfam	15735	35.88
GO	17268	39.38
KOG	8633	19.68

¹⁾ Nr, NCBI non-redundant protein sequences; Nt, NCBI non-redundant nucleotide sequences; KO, KEGG orthology database; Swiss-Prot, a manually annotated and reviewed protein sequence database; Pfam, protein family database; GO, gene ontology; KOG, eukaryotic orthologous groups.

system (967, 15.9% of unigenes) (Fig. 3). The largest group accounting for 48.1% of analyzed unigenes belonged to the metabolism category and included genes involved in carbohydrate metabolism (559), amino acid metabolism (459), energy metabolism (472), lipid metabolism (317), biosynthesis of other secondary metabolites (233) and metabolism of cofactors and vitamins (192). The genetic information processing category representing 25.6% of the unigenes was the second largest group containing genes involved in translation (670), folding, sorting and degradation (495), transcription (254) and replication and repair (175).

The third largest group was the organism system comprising 15.9% of the unigenes and included genes involved in environmental adaptation (234) and immune system (188). The cellular processes and environmental information processing pathways were also represented in the unigenes from *C. rigescens* but with low percentages of 11.3 and 8.5%, respectively.

3.4. Gene expression analysis

Using the RNA-seq results, total unigenes were obtained from the Cr_Ctrl or Cr_Salt samples (Table 2). After standardizing the read counts, DEGseq was used for the data analysis within the threshold value of [q -value<0.005 and $|\log_2(\text{fold change})|>1$]. Thirty-four unigenes were identified that were differentially expressed after salt stress (Table 4), of which 6 were up-regulated and 28 were down-regulated. The $\log_2(\text{fold changes})$ of the differentially expressed genes (DEGs) ranged from -4.15 to 1.74 (Table 4).

3.5. Cluster analysis of differentially expressed genes

To determine the clustering model of the DEG expression profiles under salt stress, a hierarchical cluster analysis

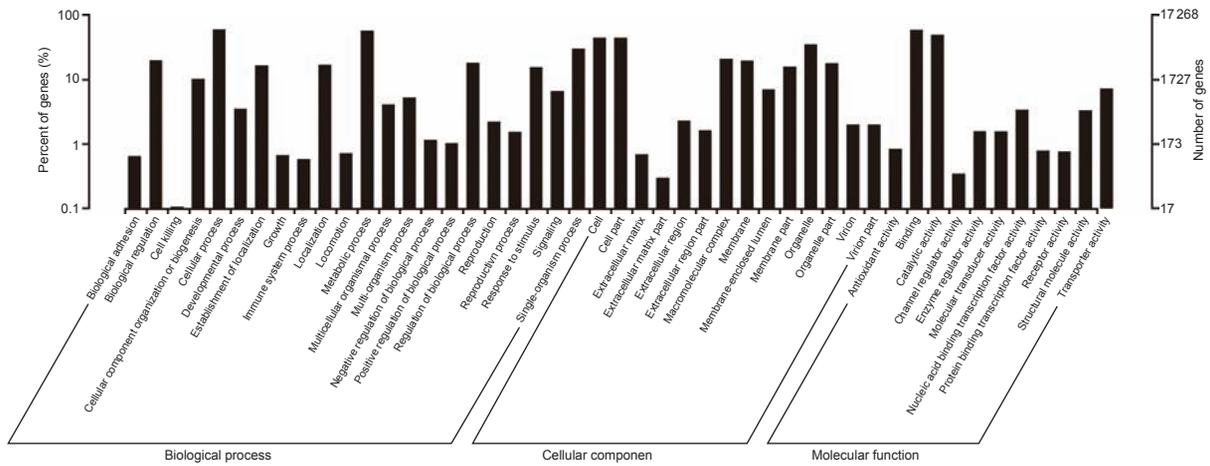


Fig. 2 Gene ontology (GO) categorization of assembled unigenes in *Carex rigescens*. These unigenes were separated into metabolic categories based on biological processes, cellular components and molecular functions.

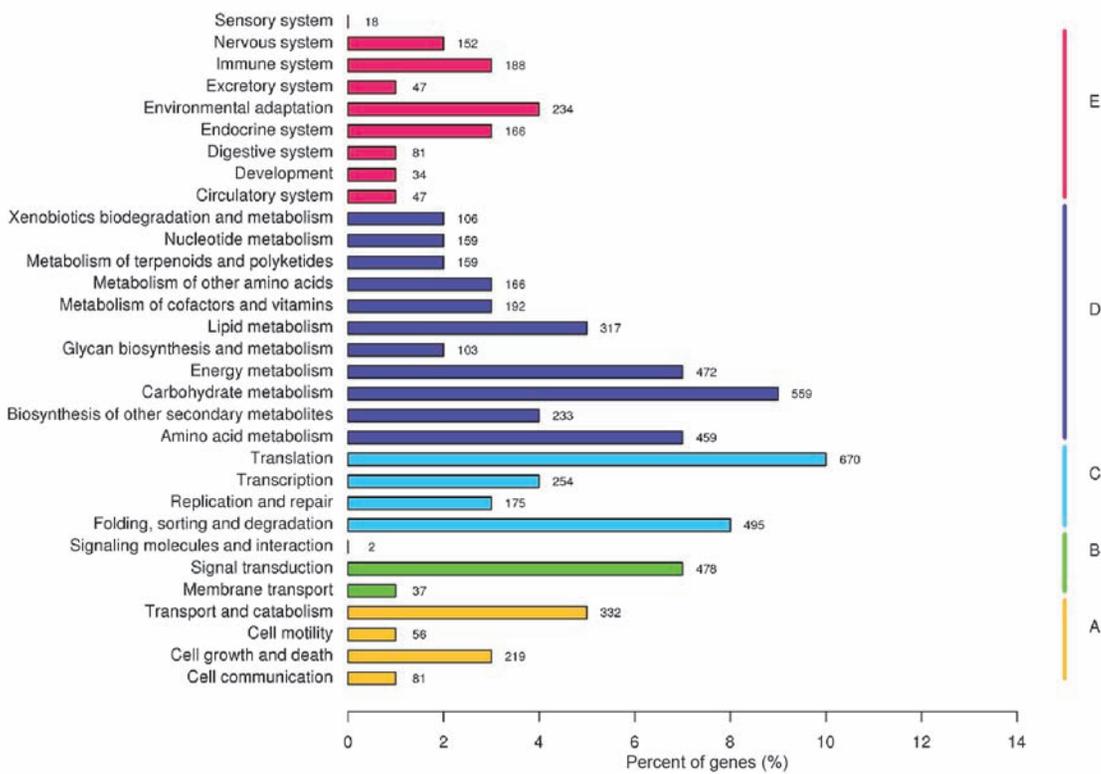


Fig. 3 Kyoto encyclopedia of genes and genomes (KEGG) classification of assembled unigenes in *Carex rigescens*. The unigenes identified using the KEGG database were further parsed into metabolic categories and presented as the percent of each subcategory of the total genes identified. The total amount of genes can be seen to the right of each subcategory. A, cellular processes pathway. B, environmental information processing pathway. C, genetic information processing pathway. D, metabolism pathway. E, organism system pathway.

was performed based on sample's $\log_{10}(\text{FPKM}+1)$. The average reads of the two replicates were used for each experiment. Our results suggested that 34 DEGs were differentially expressed after salt stress as clear expression

variations were observed between the Cr_Ctrl and Cr_Salt samples (Fig. 4).

The red bands represent the high gene expression quantity and the blue represent low gene expression quantity

Table 4 Differentially expressed genes under salt stress¹⁾

Gene ID	log ₂ (fold change)	Pfam ID	Pfam description
comp39578_c0	1.7397	PF07690//PF00083	Major facilitator superfamily//Sugar (and other) transporter
comp39197_c0	1.6499	PF03222	Tryptophan/tyrosine permease family
comp36020_c0	1.5682	PF01546	Peptidase family M20/M25/M40
comp42878_c0	1.1821	–	–
comp41592_c0	1.1077	PF03435	Saccharopine dehydrogenase
comp39830_c0	1.0638	PF00394//PF07731//PF07732	Multicopper oxidase//Multicopper oxidase//Multicopper oxidase
comp43735_c0	–1.0090	PF00847	AP2 domain
comp44364_c0	–1.0326	PF13606//PF00642//PF00023	Ankyrin repeat//Zinc finger C-x8-C-x5-C-x3-H type (and similar)//Ankyrin repeat
comp30048_c0	–1.1141	PF02135//PF00651	TAZ zinc finger//BTB/POZ domain
comp42018_c0	–1.1458	PF06293//PF07714//PF01737//PF03822//PF00196//PF00069	Lipopolysaccharide kinase (Kdo/WaaP) family//Protein tyrosine kinase//YCF9//NAF domain//Bacterial regulatory proteins, luxR family//Protein kinase domain
comp45091_c0	–1.2453	PF13537	Glutamine amidotransferase domain
comp31909_c0	–1.2502	PF00228//PF06463	Bowman-Birk serine protease inhibitor family//Molybdenum cofactor synthesis C
comp40218_c1	–1.3091	PF00445	Ribonuclease T2 family
comp37266_c0	–1.3673	PF00293	NUDIX domain
comp39974_c0	–1.4614	–	–
comp45194_c0	–1.6050	–	–
comp38506_c0	–1.6225	PF04827	Plant transposon protein
comp43984_c0	–1.6988	–	–
comp16207_c0	–1.7431	PF13499//PF13405//PF00036//PF10591//PF13202	EF-hand domain pair//EF-hand domain//EF hand//Secreted protein acidic and rich in cysteine Ca binding region//EF hand
comp39606_c0	–1.7489	PF13639//PF12861//PF00097//PF14634//PF12678//PF12906	Ring finger domain//Anaphase-promoting complex subunit 11 RING-H2 finger//Zinc finger, C ₃ HC ₄ type (RING finger)//Zinc-RING finger domain//RING-H2 zinc finger//RING-variant domain
comp39685_c0	–1.8530	PF06507//PF05891//PF00435//PF00891//PF08100	Auxin response factor//AdoMet dependent proline dimethyltransferase//Spectrin repeat//O-methyltransferase//Dimerisation domain
comp36473_c0	–1.8939	–	–
comp46251_c0	–1.9010	PF03328//PF01274	HpcH/Hpal aldolase/citrate lyase family//Malate synthase
comp44196_c0	–2.1148	PF00847	AP2 domain
comp42718_c0	–2.1689	PF00096//PF01363//PF06397	Zinc finger, C ₂ H ₂ type//FYVE zinc finger//Desulfoferrodoxin, N-terminal domain
comp46702_c0	–2.1864	PF01477//PF00305	PLAT/LH2 domain//Lipoxygenase
comp35445_c0	–2.2433	PF00847	AP2 domain
comp42460_c0	–2.2735	PF07819//PF01764//PF02683	PGAP1-like protein//Lipase (class 3)//Cytochrome C biogenesis protein transmembrane region
comp41495_c0	–2.3744	PF13405//PF13499//PF10591//PF00036//PF13202	EF-hand domain//EF-hand domain pair//Secreted protein acidic and rich in cysteine Ca binding region//EF hand//EF hand
comp42934_c0	–2.6706	PF08558//PF13202//PF13405//PF13499//PF00036	Telomere repeat binding factor (TRF)//EF hand//EF-hand domain//EF-hand domain pair//EF hand
comp16291_c0	–3.0117	–	–
comp46858_c1	–3.0239	–	–
comp35285_c0	–3.6040	PF04827	Plant transposon protein
comp41898_c0	–4.1518	PF03051//PF00112	Peptidase C1-like family//Papain family cysteine protease

¹⁾ Pfam, protein family database; –, absent.

as deduced from expression fold changes.

3.6. Differential expression validation using quantitative RT-PCR

To validate the transcriptome data, the expression profiles

of eight candidate DEGs from *C. rigescens* were analyzed using qRT-PCR. The results showed that all eight DEGs had significant changes in transcript expression after exposure to salt stress. These transcripts were quantified using qRT-PCR and were consistent with the RNA-seq data (Table 5 and Fig. 5). The qRT-PCR validation analysis

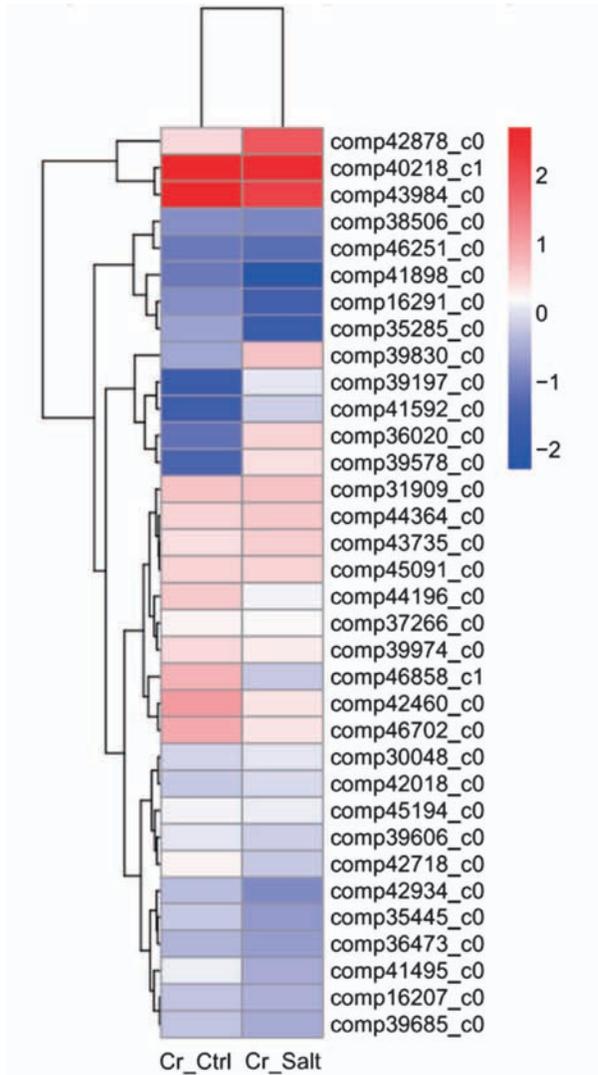


Fig. 4 Hierarchical cluster analysis of the differentially expressed genes under salt stress.

provided additional support that our transcriptome results were reliable.

3.7. Potential SSR marker exploration

A total number of 43 847 sequences were examined for putative SSR markers using MISA (1.0) (Nagaraja Reddy *et al.* 2011). From these sequences, 11 643 SSR motifs were predicted. The SSR motifs that were identified possessed repeated structures ranging from a mono-motif unit to a hexa-motif unit. The motif units were parsed into repeat types based on the amount of nucleotides in each repeat. These repeats were placed into categories comprised of 5–8, 9–12, 13–16, 17–20, and 21–26 nucleotides and separated further by motif units (Fig. 6). The SSR-specific primers amplified clear target bands in 144 of the 193 pairs

of primers tested (Appendix A). The primers that yielded clear amplicons made up 74.61% of the total primers tested and of this percentage, 69 primer pairs (47.92%) reflected polymorphisms between the two different genotypes of *C. rigescens* Lüping 1 (Fig. 7, Table 6, and Appendix A). These results provided potential SSR markers related to salt stress and our working primers offer a foundation for further exploration of these polymorphisms.

4. Discussion

The natural habitat of *C. rigescens* confines this species to drought stressed regions in northern China, resulting in its high tolerance to water deficit. Water availability, the most important abiotic factor affecting plants, is highly related to salt stress, particularly in irrigated land. To determine the gene expression profiles of *C. rigescens* under salt stress on a genome-wide scale, full plant tissues from salt-treated or untreated samples were used to test transcript abundance *via* high-throughput RNA-seq.

Results showed that several genes changed in their expression profiles after the salt treatment. These genes likely have an important function in salt stress response in *C. rigescens*. For example, the EF-hand domain genes (comp16207_c0, comp41495_c0 and comp42934_c0) have been shown to be involved in high-affinity Ca^{2+} -binding helix-loop-helix structures and are conserved Ca^{2+} sensors (Jing *et al.* 2016; Zhang *et al.* 2016). These genes were also found to respond to salt stress in our system. These Ca^{2+} sensors play crucial roles in response to environmental and developmental stimulations, including calcineurin B-like proteins (CBLs), calmodulin-like proteins (CMLs), calmodulins (CaM), and calcium dependent protein kinases (CDPKs) (Kudla *et al.* 2010). Previous studies have illustrated that these EF-hand domains contain Ca^{2+} sensors that have regulatory roles in response to salt stress in many plant species such as *Arabidopsis* (Li P *et al.* 2016), maize (Wang and Shao 2012; Zhang *et al.* 2016), rice (Jing *et al.* 2016) and *Glycine soja* (Chen *et al.* 2015). In our study, the EF-hand domain gene expression changed after exposure to salt stress. This altered expression could reflect the EF-hand domain's involvement in signal sensing and transduction to an important second Ca^{2+} messenger in *C. rigescens*. However, the putative EF-hand domain regulatory function in response to salt stress calls for further study in *C. rigescens*.

Another group of genes whose change in expression in *C. rigescens* were found after salt stress was the zinc finger protein (ZFP) related genes (comp44364_c0, comp30048_c0, comp42718_c0, and comp39606_c0). The zinc finger proteins are one of the most common TFs and play critical roles in response to abiotic stress *via* transcriptional

Table 5 Verification of differentially expressed genes by qRT-PCR¹⁾

Gene ID	RPKM			qPCR		
	Cr_Salt	Cr_Ctrl	Fold change	Cr_Salt	Cr_Ctrl	Fold change
comp35285_c0	5.46	66.35	0.08	2.58±0.30	7.02±0.92	0.37
comp42718_c0	23.34	104.94	0.22	1.10±0.10	3.23±0.07	0.34
comp44196_c0	30.57	132.43	0.23	0.37±0.06	1.04±0.13	0.36
comp46251_c0	12.46	46.55	0.27	0.64±0.06	1.70±0.15	0.38
comp36473_c0	11.96	44.46	0.27	1.40±0.02	3.37±0.44	0.42
comp39685_c0	35.54	128.39	0.28	0.12±0.01	0.29±0.03	0.41
comp45194_c0	24.63	74.92	0.33	0.51±0.03	1.52±0.13	0.34
comp40218_c1	527.68	1307.52	0.40	0.69±0.12	1.91±0.18	0.36

¹⁾RPKM, reads per kilobase per million, obtaining from the transcriptome data; Cr_Salt, transcript expression level in salt treated *Carex rigescens*; Cr_Ctrl, transcript expression level in control *C. rigescens*. Values are means±SD.

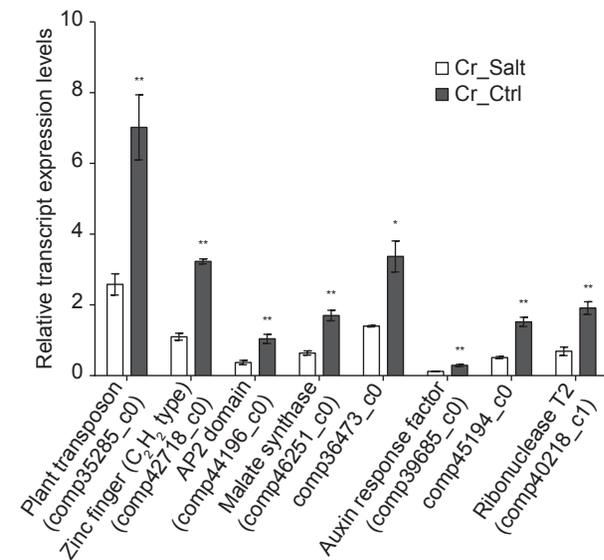


Fig. 5 Relative transcript expression level of the eight salt-responsive candidate genes. Cr_Salt, transcript expression level in salt treated *Carex rigescens*; Cr_Ctrl, transcript expression level in control *C. rigescens*. Data are shown as means±SD ($n=3$). Asterisks indicate statistically significant difference at * $P<0.05$ and ** $P<0.01$ respectively using ANOVA test.

regulation of the downstream genes responsible for plant stress tolerance (Kielbowicz-Matuk 2012). The C₂H₂-type ZFPs are some of the best characterized TFs and their involvement in plant development and stress resistance processes have been largely confirmed (Tang *et al.* 2013; Ma *et al.* 2016). The C₂H₂-type ZFP gene (comp42718_c0) in our study was down-regulated, indicating its role in salt stress response in *C. rigescens*. Identification and expression of the C₂H₂-type ZFP gene has been conducted in many plant species, such as alfalfa (Tang *et al.* 2013), *Carica papaya* (Jiang and Pan 2012), and chrysanthemum (Gao *et al.* 2012), highlighting its role in regulating abiotic stress. Additionally, the BTB/POZ domain ZFP gene

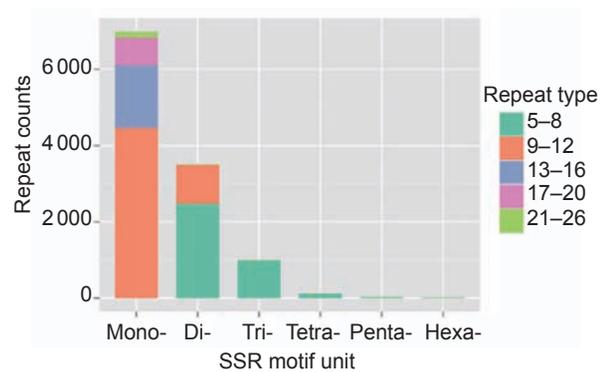


Fig. 6 Distribution of simple sequence repeats (SSRs) identified from *Carex rigescens*.

(comp30048_c0) was up-regulated in our study. This is consistent with previous studies regarding salt responses where this ZFP acts as a regulatory mechanism for transcriptional control of hormone processes such as ABA and ethylene (Weber and Hellmann 2009; Kim *et al.* 2016). Another gene in *C. rigescens* that responded to salt exposure was the C₃HC₄-type RING ZFP gene (comp39606_c0). This gene has been shown to have an essential role in regulating actin as a general stress modulator and its expression was able to be altered to elevate salt stress tolerance (Jung *et al.* 2013). In addition, the C-x8-C-x5-C-x3-H type ZFP gene (comp44364_c0) was also down-regulated in *C. rigescens*. Overexpression of the CCCH type gene *AtZFP1* in *Arabidopsis* has been shown to enhance salt tolerance by maintaining ionic balance and limiting oxidative and osmotic stresses (Han *et al.* 2014). We posit that the change in expression of the C-x8-C-x5-C-x3-H type ZFP could have a similar ionic balance mechanism to relieve salt stress in *C. rigescens*.

We also found that genes with the AP2 domain (comp43735_c0, comp44196_c0, and comp35445_c0) showed a change in expression after salt stress in *C. rigescens*. It has been demonstrated that the TFs of the

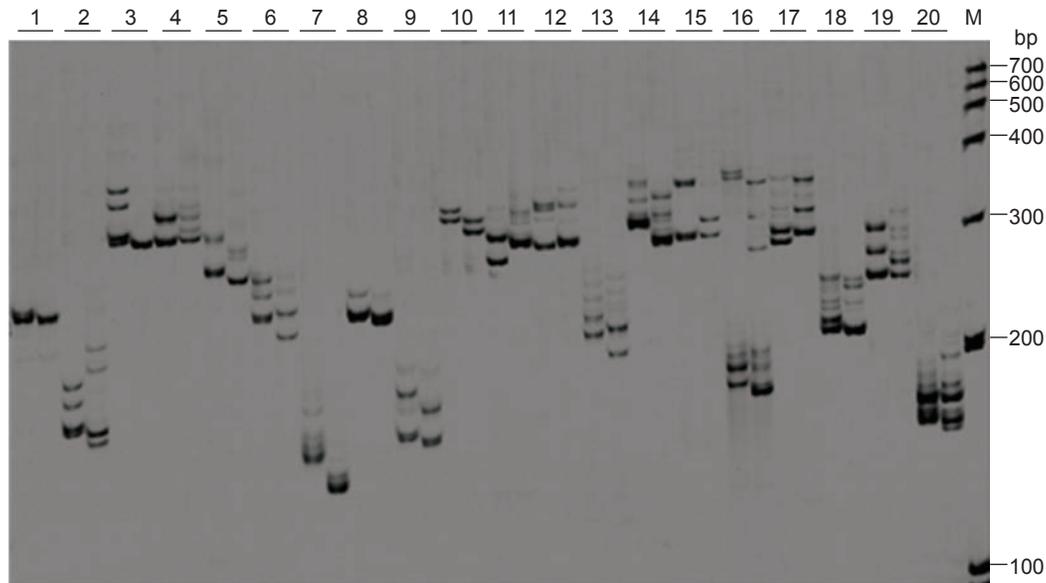


Fig. 7 Polymorphism analysis of 20 simple sequence repeat (SSR) loci using PCR. A representative polyacrylamide gel electrophoresis presented the PCR results of 20 putative SSR loci. For each SSR locus, two individual DNA samples were tested from genotypes of *Carex rigescens* Lüping 1 and 2, in the left and right lane, respectively. The 20 loci (1–20) from left to right were: Cr2, Cr21, Cr31, Cr33, Cr43, Cr44, Cr105, Cr118, Cr119, Cr124, Cr150, Cr157, Cr169, Cr185, Cr189, Cr20, Cr151, Cr156, Cr184 and Cr187; M was DNA maker I. Cr1 was used as the negative control showing no polymorphism between the two samples.

Table 6 Diversity analysis of simple sequence repeat (SSR) in 20 loci

Marker no.	Loci ¹⁾	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')	Product size (bp)	Tm (°C)	Polymorphism ²⁾
1	Cr2	GGAGGGCATCGATACAAGCA	TCCAATGGCATCGTCATCGT	208	60	NA
2	Cr20	TCCAACCTCATAGACTCCTCTCT	GAGGCGGGGAAGAAGAAGAC	187	60	YA
3	Cr21	AGGGAGTTGAGGCTGGTACT	GCTCCTCCTCCCTCCCTAA	148	60	YA
4	Cr31	CCCTGTGCGCTCCCTCTTT	TGGGGGTGAAATCACTACGC	260	61	YA
5	Cr33	GGTGTCTCTGGAGGCCTTC	TGGTGCCATTGTGTGTAGCA	258	60	YA
6	Cr43	TGGTACCCACAAACCCATCAG	CTTATACGCAGCCACGTCGA	232	60	YA
7	Cr44	ATGGCGAAGATGTAGGTGGC	TCCCCTTCTCCCCTCTCTG	213	60	YA
8	Cr105	ATCAGGCGTTAGTTGGTGCA	CACCGTGTGGATGGGACTAG	133	60	YA
9	Cr118	CAGGAGCCGCACCATTAAGA	AGCTCTGCTCAGCCCAATTT	206	60	NA
10	Cr119	AAGGTGGCAACTAGGGATGC	TGCCTCAATTACTCACTCCACC	147	60	YA
11	Cr124	ATGACGAGGACAGTGATGGC	CCGTATCGGCTACACCTACC	260	60	YA
12	Cr150	AAAACCTAACCTTCGCAGCG	AGGTAGCATGTGAAACGCCA	260	60	YA
13	Cr151	AAAACAACGGCCATGAAGC	TCACGAATCCCAGCCACTTC	276	60	YA
14	Cr156	AAGCCCAGGCACTCAATCTC	GTTGGCGCAGATTGAGCAAT	210	60	YA
15	Cr157	AGCTAACTCGGAGGGAACAA	AAAACCCACAGCAAGACCCA	259	59	YA
16	Cr169	CCCTGTCCATGGATGACACC	GTCCGACCATACACCATCTCC	203	60	YA
17	Cr184	GCAGCACTCAACACAAGTGG	GGGCCTACGATTCAGAGGTG	244	60	YA
18	Cr185	CGGATTGGTCCAGTAGGTGCG	CCAAGTGGTTTCTGTTTTCCCT	277	59	YA
19	Cr187	GCAGACTTTGTTTGCAACA	TGCTCCCAATGCACTGTTTT	163	58	YA
20	Cr189	TGAAGCGTGACGTACCAGTC	GGCACTTGCTGGTTCTGTTG	271	60	YA

¹⁾ All 69 loci mentioned in the text are listed in Appendix A.

²⁾ NA represents no SSR polymorphism, while YA represents the presence of a SSR polymorphism between the two samples of tested loci.

AP2/ERF family are able to regulate diverse processes in plant development and stress responses (Xu *et al.* 2011). In rice, the AP2/ERF domain-containing genes such as *OsAP21* (Jin *et al.* 2013) and *OsRAVs* (Duan *et al.* 2016)

have been shown to have a crucial role in salt stress response as verified in over-expression experiments using *Arabidopsis* and the CRISPR/Cas9 system, respectively. In addition, the *JcERF2* gene, a novel AP2/ERF TF in *Jatropha*

curcas, has been shown to confer salt tolerance (Wang *et al.* 2015). Accordingly, homologous genes were identified in *C. rigescens* and also had a change in expression after salt treatment. These homologs could be important AP2/ERF TFs that respond to salt stress or have involvement in salt stress adaptation.

An additional TF that showed down-regulated expression in *C. rigescens* was the auxin response factors (ARF) (comp39685_c0) that has been known to bind auxin response elements to specifically regulate the expression of auxin response genes. Changes in ARF after salt stress have been studied in *Vigna unguiculata*, *Arabidopsis* and maize, and using a *miR160* target transcript, ARF was shown to better aid in root development and auxin signaling under salt stress (Liu *et al.* 2008; Paul *et al.* 2011).

Many other genes had also shown a change in expression post-salt stress in *C. rigescens*. One of these genes was the MFS (comp39578_c0), which encodes a Na⁺/H⁺ antiporter that is considered a molecule transporter sensor in higher plants and acts as a distinct mechanism for salt-stress adaptation (Reddy *et al.* 2012; Sekhwal *et al.* 2013). Another pertinent example was the gene encoding saccharopine dehydrogenase (comp41592_c0) that was up-regulated after salt treatment in *C. rigescens*. Though saccharopine dehydrogenase has long been known to be up-regulated under osmotic stress in *Brassica napus* (Moulin *et al.* 2000), its behavior under salt stress has only been reported in the seed developmental stage of maize where significant changes in the saccharopine pathway occurred (Kiyota *et al.* 2015).

5. Conclusion

In this study, we have annotated 21 954 unigenes of the total generated 43 847, and identified 34 differentially expressed genes in response to salt stress in the *C. rigescens* variety Lüping 1 using RNA-seq. We also verified 144 SSR markers and 69 of them reflected a polymorphism between the two different genotypes of *C. rigescens* variety Lüping 1. This is the first study examining gene expression profiles and SSR markers in *C. rigescens* in response to salt stress. Our results may provide further insight into genome annotation, novel gene discovery, molecular breeding and comparative genomics in *C. rigescens* and related species.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31472139).

Appendix associated with this paper can be available on <http://www.ChinaAgriSci.com/V2/En/appendix.htm>

References

- Bushman B S, Amundsen K L, Warnke S E, Robins J G, Johnson P G. 2016. Transcriptome profiling of Kentucky bluegrass (*Poa pratensis* L.) accessions in response to salt stress. *BMC Genomics*, **17**, 48.
- Chen C, Sun X, Duanmu H, Zhu D, Yu Y, Cao L, Liu A, Jia B, Xiao J, Zhu Y. 2015. *GsCML27*, a gene encoding a calcium-binding Ef-Hand protein from *Glycine soja*, plays differential roles in plant responses to bicarbonate, salt and osmotic stresses. *PLOS ONE*, **10**, e0141888.
- Deinlein U, Stephan A B, Horie T, Luo W, Xu G, Schroeder J I. 2014. Plant salt-tolerance mechanisms. *Trends Plant Science*, **19**, 371–379.
- Deshmukh R, Sonah H, Patil G, Chen W, Prince S, Mutava R, Vuong T, Valliyodan B, Nguyen H T. 2014. Integrating omic approaches for abiotic stress tolerance in soybean. *Frontiers in Plant Science*, **5**, 244.
- Duan Y B, Li J, Qin R Y, Xu R F, Li H, Yang Y C, Ma H, Li L, Wei P C, Yang J B. 2016. Identification of a regulatory element responsible for salt induction of rice *OsRAV2* through *ex situ* and *in situ* promoter analysis. *Plant Molecular Biology*, **90**, 49–62.
- FAO. 2008. FAO land and plant nutrition management service. [2016-03-24]. <http://www.fao.org/ag/agl/agll/spush>
- Gao C, Rong R, Zhang Z Q, Guo P J, Meng H. 2009. Experimental research of extracting extracorporeal anti-herpes virus. *China Pharmaceuticals*, **18**, 5–6. (in Chinese)
- Gao H, Song A, Zhu X, Chen F, Jiang J, Chen Y, Sun Y, Shan H, Gu C, Li P, Chen S. 2012. The heterologous expression in *Arabidopsis* of a chrysanthemum Cys2/His2 zinc finger protein gene confers salinity and drought tolerance. *Planta*, **235**, 979–993.
- Grabherr M G, Haas B J, Yassour M, Levin J Z, Thompson D A, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q D, Chen Z H, Muceli E, Hacohen N, Gnirke A, Rhind N, di Palma F, Birren B W, Nusbaum C, Lindblad-Toh K, Friedman N, *et al.* 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, **29**, 644–652.
- Han G L, Wang M J, Yuan F, Sui N, Song J, Wang B S. 2014. The CCCH zinc finger protein gene *AtZFP1* improves salt resistance in *Arabidopsis thaliana*. *Plant Molecular Biology*, **86**, 237–253.
- He S, Xing Q, Yin Z. 1993. *Flora of Beijing*. Beijing Publishing House, China. pp. 1293–1314. (in Chinese)
- Hu L, Li H, Chen L, Lou Y, Amombo E, Fu J. 2015. RNA-seq for gene identification and transcript profiling in relation to root growth of bermudagrass (*Cynodon dactylon*) under salinity stress. *BMC Genomics*, **16**, 575.
- Jiang L, Pan L J. 2012. Identification and expression of C2H2 transcription factor genes in *Carica papaya* under abiotic and biotic stresses. *Molecular Biology Reports*, **39**, 7105–7115.
- Jin X, Xue Y, Wang R, Xu R, Bian L, Zhu B, Han H, Peng R, Yao Q. 2013. Transcription factor *OsAP21* gene increases

- salt/drought tolerance in transgenic *Arabidopsis thaliana*. *Molecular Biology Reports*, **40**, 1743–1752.
- Jing P, Zou J, Kong L, Hu S, Wang B, Yang J, Xie G. 2016. OsCCD1, a novel small calcium-binding protein with one EF-hand motif, positively regulates osmotic and salt tolerance in rice. *Plant Science*, **247**, 104–114.
- Jung Y J, Lee I H, Nou I S, Lee K D, Rashotte A M, Kang K K. 2013. BrRZFP1 a *Brassica rapa* C3HC4-type RING zinc finger protein involved in cold, salt and dehydration stress. *Plant Biology*, **15**, 274–283.
- Kielbowicz-Matuk A. 2012. Involvement of plant C₂H₂-type zinc finger transcription factors in stress responses. *Plant Science*, **185–186**, 78–85.
- Kim H, Kim S H, Seo D H, Chung S, Kim S W, Lee J S, Kim W T, Lee J H. 2016. ABA-HYPERSENSITIVE BTB/POZ PROTEIN 1 functions as a negative regulator in ABA-mediated inhibition of germination in *Arabidopsis*. *Plant Molecular Biology*, **90**, 303–315.
- Kiyota E, Pena I A, Arruda P. 2015. The saccharopine pathway in seed development and stress response of maize. *Plant Cell and Environment*, **38**, 2450–2461.
- Kudla J, Batistic O, Hashimoto K. 2010. Calcium signals: The lead currency of plant information processing. *The Plant Cell*, **22**, 541–563.
- Li P, Zhang G, Gonzales N, Guo Y, Hu H, Park S, Zhao J. 2016. Ca²⁺-regulated and diurnal rhythm-regulated Na⁺/Ca²⁺ exchanger AtNCL affects flowering time and auxin signalling in *Arabidopsis*. *Plant, Cell & Environment*, **39**, 377–392.
- Li S, Fan C, Li Y, Zhang J, Sun J, Chen Y, Tian C, Su X, Lu M, Liang C, Hu Z. 2016. Effects of drought and salt-stresses on gene expression in *Caragana korshinskii* seedlings revealed by RNA-seq. *BMC Genomics*, **17**, 200.
- Liang F, Bai S Y, Dong A X, Wu N. 2011. Biological character comparison with the seeds of 18 plant species in *Carex* genus. *Pratacultural science*, **28**, 1825–1830. (in Chinese)
- Liu H H, Tian X, Li Y J, Wu C A, Zheng C C. 2008. Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. *RNA — A Publication of the RNA Society*, **14**, 836–843.
- Livak K J, Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔC_T} method. *Methods*, **25**, 402–408.
- Long R C, Li M N, Kang J M, Zhang T J, Sun Y, Yang Q C. 2015. Small RNA deep sequencing identifies novel and salt-stress-regulated microRNAs from roots of *Medicago sativa* and *Medicago truncatula*. *Physiologia Plantarum*, **154**, 13–27.
- Ma X, Liang W, Gu P, Huang Z. 2016. Salt tolerance function of the novel C₂H₂-type zinc finger protein TaZNF in wheat. *Plant Physiology and Biochemistry*, **106**, 129–140.
- Mohammadi-Nejad G, Singh R K, Arzani A, Rezaie A M, Sabouri H, Gregorio G B. 2010. Evaluation of salinity tolerance in rice genotypes. *International Journal of Plant Production*, **4**, 199–207.
- Moulin M, Deleu C, Larher F. 2000. L-Lysine catabolism is osmo-regulated at the level of lysine-ketoglutarate reductase and saccharopine dehydrogenase in rapeseed leaf discs. *Plant Physiology and Biochemistry*, **38**, 577–585.
- Munns R, Tester M. 2008. Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, **59**, 651–681.
- Nagaraja Reddy R, Madhusudhana R, Murali Mohan S, Chakravarthi D V N, Seetharama N. 2011. Characterization, development and mapping of unigene-derived microsatellite markers in sorghum [*Sorghum bicolor* (L.) Moench]. *Molecular Breeding*, **29**, 543–564.
- Paul S, Kundu A, Pal A. 2011. Identification and validation of conserved microRNAs along with their differential expression in roots of *Vigna unguiculata* grown under salt stress. *Plant Cell Tissue and Organ Culture*, **105**, 233–242.
- Postnikova O A, Shao J, Nemchinov L G. 2013. Analysis of the alfalfa root transcriptome in response to salinity stress. *Plant & Cell Physiology*, **54**, 1041–1055.
- Reddy V S, Shlykov M A, Castillo R, Sun E I, Saier Jr M H. 2012. The major facilitator superfamily (MFS) revisited. *FEBS Journal*, **279**, 2022–2035.
- Robinson M D, McCarthy D J, Smyth G K. 2010. edgeR: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*, **26**, 139–140.
- Sekhwil M K, Sharma V, Sarin R. 2013. Identification of MFS proteins in sorghum using semantic similarity. *Theory in Biosciences*, **132**, 105–113.
- Shabala S, Cui T A. 2008. Potassium transport and plant salt tolerance. *Physiologia Plantarum*, **133**, 651–669.
- Shi H, Jiang C, Ye T, Tan D X, Reiter R J, Zhang H, Liu R, Chan Z. 2015. Comparative physiological, metabolomic, and transcriptomic analyses reveal mechanisms of improved abiotic stress resistance in bermudagrass [*Cynodon dactylon* (L.) Pers.] by exogenous melatonin. *Journal of Experimental Botany*, **66**, 681–694.
- Storey J D, Tibshirani R. 2003. Statistical significance for genomewide studies. *Proceedings of the National Academy of Sciences of the United States of America*, **100**, 9440–9445.
- Sun Y, Li M L, Mao P S. 2011. Study of standard germination method in *Carex rigescens*. *Northern Horticulture*, **19**, 68–70. (in Chinese)
- Tang L, Cai H, Ji W, Luo X, Wang Z, Wu J, Wang X, Cui L, Wang Y, Zhu Y, Bai X. 2013. Overexpression of *GsZFP1* enhances salt and drought tolerance in transgenic alfalfa (*Medicago sativa* L.). *Plant Physiology and Biochemistry*, **71**, 22–30.
- NSSO (The National Soil Survey Office of China). 1998. *Soil of China*. China Agriculture Press, China. (in Chinese)
- Wang C T, Shao J M. 2012. Characterization of the *ZmCK1* gene encoding a calcium-dependent protein kinase responsive to multiple abiotic stresses in maize. *Plant Molecular Biology Reporter*, **31**, 222–230.
- Wang J, Li B, Meng Y, Ma X, Lai Y, Si E, Yang K, Ren P, Shang X, Wang H. 2015. Transcriptomic profiling of the salt-stress response in the halophyte *Halogeton glomeratus*. *BMC Genomics*, **16**, 169.
- Wang L K, Feng Z X, Wang X, Wang X W, Zhang X G.

2010. DEGseq: An R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics*, **26**, 136–138.
- Wang X, Han H, Yan J, Chen F, Wei W. 2015. A new AP2/ERF transcription factor from the oil plant *Jatropha curcas* confers salt and drought tolerance to transgenic tobacco. *Applied Biochemistry and Biotechnology*, **176**, 582–597.
- Wang Y. 1997. Advances in research of heat shock factors. *Journal of China Three Gorges University*, **2**, 110–114. (in Chinese)
- Weber H, Hellmann H. 2009. *Arabidopsis thaliana* BTB/POZ-MATH proteins interact with members of the ERF/AP2 transcription factor family. *FEBS Journal*, **276**, 6624–6635.
- Xie Q, Niu J, Xu X, Xu L, Zhang Y, Fan B, Liang X, Zhang L, Yin S, Han L. 2015. *De novo* assembly of the Japanese lawngrass (*Zoysia japonica* Steud.) root transcriptome and identification of candidate unigenes related to early responses under salt stress. *Frontiers in Plant Science*, **6**, 610.
- Xu Z S, Chen M, Li L C, Ma Y Z. 2011. Functions and application of the AP2/ERF transcription factor family in crop improvement. *Journal of Integrative Plant Biology*, **53**, 570–585.
- Yamamoto N, Takano O, Tanakay K, Ishige T, Terashima S, Endo C, Kurusu T, Yajima S, Yano K, Tada Y. 2015. Comprehensive analysis of transcriptome response to salinity stress in the halophytic turf grass *Sporobolus virginicus*. *Frontiers in Plant Science*, **6**, 00241.
- Yang Z, Xie T, Liu Q. 2014. Physiological responses of *Phragmites australis* to the combined effects of water and salinity stress. *Ecohydrology*, **7**, 420–426.
- Zhang F, Li L, Jiao Z, Chen Y, Liu H, Chen X, Fu J, Wang G, Zheng J. 2016. Characterization of the *calcineurin B-Like* (CBL) gene family in maize and functional analysis of *ZmCBL9* under abscisic acid and abiotic stress treatments. *Plant Science*, **253**, 118–129.
- Zhang K, Li M N, Cao S H, Sun Y. 2017. Response of *Carex rigescens* to different NaCl concentrations and its salinity threshold calculation. *Pratacultural science*, **34**, 479–487. (in Chinese)

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