Expression profiling of *Chrysanthemum crassum* under salinity stress and the initiation of morphological changes

Zhiyong Guan*, Yitong Feng, Aiping Song, Xiaomeng Shi, Yachao Mao, Sumei Chen, Jiafu Jiang, Lian Ding, Fadi Chen

College of Horticulture, Institution of Nanjing Agricultural University, City of Nanjing, State of Jiangsu Province, Country of China

* guanzhy@njau.edu.cn

Abstract

*Chrysanthemum crassum* is a decaploid species of *Chrysanthemum* with high stress tolerance that allows survival under salinity stress while maintaining a relatively ideal growth rate. We previously recorded morphological changes after salt treatment, such as the expansion of leaf cells. To explore the underlying salinity tolerance mechanisms, we used an Illumina platform and obtained three sequencing libraries from samples collected after 0 h, 12 h and 24 h of salt treatment. Following de novo assembly, 154,944 transcripts were generated, and 97,833 (63.14%) transcripts were annotated, including 55 Gene Ontology (GO) terms and 128 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The expression profile of *C. crassum* was globally altered after salt treatment. We selected functional genes and pathways that may contribute to salinity tolerance and identified some factors involved in the salinity tolerance strategies of *C. crassum*, such as signal transduction, transcription factors and plant hormone regulation, enhancement of energy metabolism, functional proteins and osmolyte synthesis, reactive oxygen species (ROS) scavenging, photosystem protection and recovery, and cell wall protein modifications. Forty-six genes were selected for quantitative real-time polymerase chain reaction detection, and their expression patterns were shown to be consistent with the changes in their transcript abundance determined by RNA sequencing.

Introduction

Salt can diffuse from underground soil, wind and rain can carry salt from the ocean, and human overuse of chemical fertilizers also causes salty soils [1, 2]. These factors have increased the amount of land affected by salt. According to a study by the Food and Agriculture Organization (FAO) in 2011, more than 800 million hectares of land were estimated to be affected by salt around the world.
Salinity can influence the survival and growth of plants. Salinity weakens photosynthesis and enhances the respiration of plants, thereby slowing their growth rate [3]. Moreover, salinity can cause osmotic stress, which disturbs water assimilation by plants [4] and can lead to the accumulation of ROS, which, in excessive levels, damage plant cell membranes [5]. Salinity can also cause Na\(^+\) to accumulate, which disrupts the assimilation of other cations, such as K\(^+\), and disturbs normal plant cell functions [6]. Under salinity stress, plants regulate the expression levels of a series of genes involved in stress adaptation. Some of these genes, including those involved in plant hormone regulation (such as PP2C and MYC2) [7], osmolyte synthesis (TP and P5CS) [8], ROS scavenging (SOD and CAT) [9] and salt exclusion and compartmentalization (HKT, SOS1 and NHX) [6], have been widely studied in response to salinity stress. The tissues of some plants also display morphological changes under salinity stress. For example, the leaves of Populus euphratica become greatly incrassated under salinity stress [10], and some cell wall modification proteins, such as HRGP and XET, are considered to be responsible for these changes [11].

We previously found that most species of the Chrysanthemum genus planted in saline soil display unhealthy phenotypes, such as reduction of the growth rate, tissue wilt, leaf necrosis and abscission, and interference with flowering [12]. Such negative impacts of salinity have an adverse influence on the ornamental quality of chrysanthemums and disturb breeding by researchers. However, the salinity tolerance of chrysanthemums varies among different species, and C. crassum is an elite germplasm that can survive and grow well under moderate salt stress (120 mM NaCl) conditions [13]. In this study, we found that under long-term moderate salt stress, C. crassum could survive and maintain an ideal growth rate; interesting morphological changes also occurred, such as the cells of salt-treated leaves expanded, and the leaves were elongated and incrassated compared with the controls. Additionally, the accumulation of Na\(^+\) in the functional leaves of C. crassum increased slowly and eventually stopped increasing over time. All of changes would be initiated and controlled by genes that respond to salinity stress at an early stage. Therefore, illuminating the mechanisms involved in the response of C. crassum to salinity stress may help to identify the genes that contribute to salinity tolerance in plants, and it is important to determine how salt initiates morphological changes.

Next-generation sequencing (NGS), with its excellent characteristics of cost-effectiveness and high throughput [14], is the most promising method for studying expression profiles in non-model plants that lack whole-genome sequencing data [15]. In this study, we used NGS to examine the changes in transcriptional levels in C. crassum after salt treatment. We also aimed to determine the essential metabolic pathways and key genes that contribute to salinity tolerance.

Given the remarkable phenotypic changes in the leaves of C. crassum, we chose to sequence RNA from the leaves. According to previous studies [8], the salinity tolerance traits of leaves are mainly related to osmotic tolerance and tissue tolerance; the former is instantaneously triggered and related to sensing and signaling mechanisms, while the latter is related to the compartmentalization of salt to intercellular spaces and vacuoles and may be associated with processes involving ion transporters, proton pumps and synthesis of compatible solutes. In this study, we sampled the leaves of C. crassum at 0 h, 12 h and 24 h after salt treatment, and performed RNA-seq. As we determined the transcriptomes of these samples, we analyzed the genes and mechanisms that contributed to salinity tolerance, especially osmotic and tissue tolerance, and summarized the strategies adopted by C. crassum under salinity stress. In addition, responsive genes can be selected for breeding into other species of chrysanthemum to improve their salinity tolerance. Thus, this study presents transgenic experiments aiming to improve salinity tolerance.
Materials and methods

Plant materials and growth conditions

Plants were obtained from the Chrysanthemum Germplasm Resource Preservation Center of Nanjing Agricultural University, China. Shoot cuttings of *C. crassum* were rooted and grown in matrix (vermiculite: perlite: nutritive soil = 1:1:1). Rooted seedlings at the 6–7 leaf stage were selected and then transplanted into plastic containers (volume 20 L) filled with diluted (1:2) Hoagland nutrient solution, with aeration for 24 h•d⁻¹. The plants were maintained in a greenhouse with a normal photoperiod and sunlight, an average temperature of 25˚C and relative humidity of 70%; all solutions were renewed every two days. After one week, one set of plants grown in Hoagland solution alone was kept as a control (CK), and salt treatment (S) was performed by supplementing the nutrient solution with 120 mM NaCl.

Recording of morphological changes after salt treatment and paraffin sectioning

At 0 d, plants that showed similar sizes and shapes were selected from the salt treatment and control groups and photographed. Photographs of these plants were subsequently taken at 5 d, 10 d, and 20 d. At the same time points, the fourth unfolded leaves of the plants were traced on paper without injuring them.

The fourth unfolded leaves numbered from the tops of both salt-treated and control plants were picked (at 0 d, 5 d, 10 d, and 20 d), cut into pieces of 0.3 × 0.3 cm, then immediately fixed in FAA solution. The samples were subsequently dehydrated through an alcohol series, infiltrated with xylene, and embedded in paraffin wax. Transverse sections were cut to a thickness of 25 μm and stained with 0.1% toluidine blue solution (w/v). The sections were finally observed under an Olympus BX41 microscope [16].

Determination of Na⁺ and K⁺ levels

The unfolded leaves numbered from the top of both salt-treated and control plants were collected (approximately 3 g, at 0 d, 5 d, 10 d, 20 d, and 30 d). The samples were then oven-dried at 70˚C for 48 h, after which 50 mg DW was digested in 35% HNO₃. Na⁺ and K⁺ was resuspended in 10 ml of HCl (0.1 N) and the solutions were filtered. Ions were quantified via flame atomic absorption spectrophotometry (VARIAN spectra-300) [17]. Three replicates of each sample were examined.

RNA extraction and quality determination

The prepared samples were collected at 0 h, 12 h, and 24 h, and the leaves were immediately harvested, snap-frozen in liquid nitrogen and stored at −80˚C. A Total RNA Isolation System (TaKaRa Bio, Tokyo, Japan) was employed to extract RNA from the samples according to the manufacturer’s protocol. The quality of the RNA was verified using a 2100 Bioanalyzer RNA Nanochip (Agilent, Santa Clara, CA), and its concentration was determined using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). At least 20 mg of RNA from three replicates were pooled in an equimolar fashion [18].

Illumina cDNA library preparation and sequencing

Sequencing based on an Illumina platform (San Diego, CA) was performed at the Beijing Genomics Institute (Shenzhen, China). Briefly, poly(A) mRNA was isolated using beads coated with oligo (dT). Fragmentation buffer was added to fragment the mRNA to a size of 100–400
bp. The fragments were used for the synthesis of first-strand cDNA employing random hexamer primers. Second-strand cDNA was synthesized with the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen, Camarillo, CA). The cDNA was then purified using a Qiaquick PCR Extraction Kit (Qiagen, Hilden, Germany) and resolved with EB buffer for end reparation and poly(A) addition. The products were ligated with each other using sequencing adapters, and after agarose gel electrophoresis, a suitable size range of fragments was selected for PCR amplification. The resulting library was sequenced using the Illumina HiSeqTM 2000 system [18].

Data filtering and de novo assembly
The image data output from the sequencing system was transformed into raw reads and stored in FASTQ format. These data were filtered to remove raw reads that included adapter sequences, had more than 5% unknown nucleotides or that were of low quality (the percentage of reads with quality value \( \geq 10 \) was more than 20%). Transcriptome de novo assembly was carried out with Trinity [19]. The resulting Trinity sequences were considered unigenes. After sequence splicing and redundancy removal, gene family clustering was performed, and the unigenes were divided into two classes. In one cluster (with the prefix “CL” followed by the cluster id), there were several unigenes showing similarity of greater than 70%. The others were singletons (which have the prefix “Unigene”). BLASTX [20] alignment between each unigene sequence and those registered in the Nr (non-redundant protein database, NCBI), Nt (non-redundant nucleotide database, NCBI), Swiss-Prot, and GO (gene ontology, http://www.geneontology.org/) databases was performed, and the best alignments for inferring the directionality of each unigene were determined. For the best alignments in which the outcome from the various databases conflicted with one another, the priority order applied was Nr, followed by Swiss-Prot. The software tool ESTScan [21] was used to assign directionality.

Gene annotation and analysis
Functional annotation was assigned using the protein (Nr and Swiss-Prot) and GO databases. BLASTX was employed to identify related sequences in the protein databases based on an E-value of less than \( 10^{-5} \). The annotations acquired from Nr were processed by using the Blast2GO program [22] to obtain the relevant GO terms, which were then analyzed with WEGO software [23] to assign a GO functional classification and to illustrate the distribution of gene functions.

Prediction of unigene coding regions
Unigenes were aligned to protein databases using BLASTX with the following order of priority: Nr, Swiss-Prot, and KEGG. The proteins with highest ranks in the BLAST results were selected to determine the coding region sequences of the unigenes, and the sequences of the coding regions were then translated into amino sequences using the standard codon table. Thus, both the nucleotide sequences (5' -> 3') and amino acid sequences of the Unigene coding region were acquired. Unigenes that could not be aligned to any database were scanned by ESTScan to determine the direction of the nucleotide sequence (5' -> 3') and the amino acid sequence of the predicted coding region [21].

Gene validation and expression analysis
Samples were prepared, and total RNA was extracted using the method indicated above. A total of 16 unigenes that responded to salt stress were chosen for validation. Three independent
biological replicates of each sample were used in the analysis. A set of gene-specific primer pairs was designed using Primer3 software [24]. Reverse transcription was performed with M-MLV reverse transcriptase (TaKaRa). The relative expression of these genes was determined through qRT-PCR analysis using a SYBR® Green reaction kit (TaKaRa), with the elongation factor 1-alpha gene as a reference. Then, a Mastercycler ep realplex device (Eppendorf, Hamburg, Germany) was used to run the qPCR assays. The transcription data were calculated using the $-\Delta\Delta C_t$ method [25].

**Results**

**Morphological changes of *C. crassum* and Na$^+$ accumulation in leaves under salinity stress**

*C. crassum* showed remarkable tolerance under salinity stress, as the salt-treated plants grew almost as fast as the controls (Fig 1A), with only the leaves near the roots wilting, revealing the damage due to salinity stress. As shown in Fig 1A, the leaves treated with salt grew differently from the controls; therefore, we chose salt-treated and control leaves of the same size and followed their growth by tracing the outlines of the leaves without injuring them at 0 d, 5 d, 10 d, and 20 d. The shapes of the leaves are shown in S1 Fig. It was apparent that the leaves that were treated with salt were larger than those of the controls, and the analysis of paraffinic slices of leaves (Fig 1B) showed that the leaves that were salt-treated were thicker than the leaves of the controls. This incrassation was mainly caused by cell expansion and not cell replication, as the number of cell layers was not increased. The morphological changes that occur in *C. crassum* under salinity stress have rarely been studied; however, they may be relevant to the remarkable salinity tolerance of *C. crassum*.

The accumulation of Na$^+$ in the leaves of salt-treated *C. crassum* was slow, requiring days to reach a peak, and a balance was maintained around the peak as time passed (Fig 2A). The K$^+$ content of leaves also increased under salinity stress. These behaviors are beneficial to the survival and growth maintenance of *C. crassum* under salinity stress. The transcriptomes of the three samples helped us to determine the implications of these behaviors and identify which strategies *C. crassum* employs under salinity stress.

**De novo assembly and quantitative assessment of RNA sequences**

To investigate the global gene expression profiles of the *C. crassum* transcriptomes at different stages of salinity stress, we constructed three cDNA libraries using leaves from hydroponic cutting seedlings treated with 120 mM NaCl for 0 h, 12 h, and 24 h.

Sequencing was carried out on an Illumina platform; a total of 29,831,115,240 nt were generated, and 346,363,148 raw reads were obtained. After removing low-quality regions, adapters, and contamination, we obtained a total of 331,456,836 clean reads with Q20 $> 98.34\%$ and a GC percentage between 43.38\% and 43.75\% (Table 1).

In the assembly results, 154,944 unigenes were detected; the total length of the unigenes was 167,656,676 nt; the average length was 1,082 nt; and the N50 was 1,661 nt. We generated 160,301, 164,718, and 174,748 contigs for the samples obtained at 0 h, 12 h, and 24 h after salt treatment, with average lengths of 328, 321, and 318 nt, respectively, and average N50 lengths of 603, 556, and 546 (Table 1).

**Sequence annotation and functional categories of annotated sequences**

For the annotation of functional information in the assembled unigenes, we used several applications, including information on protein sequence similarities, GO and Kyoto Encyclopedia
of Genes and Genomes (KEGG) pathways. All of the sequences were successively subjected to BLAST searches in the NCBI Nr database, the Nt database, and the Swiss-Prot protein database, with E-values < 1e-5. Using the best hits provided by BLAST, the most reasonable functions were assigned to the sequences. After functional annotation analysis, we obtained 93,296, 68,322, 65,127, 60,906, and 55,754 unigenes annotated to the NR, NT, Swiss-Prot, KEGG, and GO databases, respectively, totaling 97,833 annotated unigenes.

Fig 1. Growth conditions of *Chrysanthemum crassum* (A) and the incassation of *C. crassum* leaves (B) under salinity stress. S = Salt, CK = Control; bar = 3 cm (A) and 100 μm (B).

https://doi.org/10.1371/journal.pone.0175972.g001
The E-value distribution of the top hits in the Nr database showed that 50.74% of the sequences were mapped to the known genes in plants with best hits (E-value < 1e-45, mean identity = 53.21%; S2A Fig), and approximately 15.88% of the unigenes hit deposited sequences with a similarity > 80% (S2B Fig). Approximately 72.32% of the annotated unigenes

Fig 2. Changes in the Na⁺ (A) and K⁺ (B) contents of *Chrysanthemum crassum* leaves under salt stress. The Na⁺ and K⁺ contents were measured from the leaves collected at 0 d, 5 d, 10 d, 20 d, and 30 d after salt treatment. (n = 3, vertical bars indicate standard deviation [SD]).

https://doi.org/10.1371/journal.pone.0175972.g002
could be assigned with a best score to deposited sequences from the top seven species: Vitis vinifera (24.82%), Lycopersicon esculentum (15.95%), Amygdalus persica (8.06%), Ricinus communis (7.24%), Populus balsamifera subsp. trichocarpa (7.06%), Fragaria vesca subsp. vesca (4.99%), and Glycine max (4.22%; S2C Fig).

Regarding the functional categories of the 154,944 unigenes, a total of 55,754 unigenes were assigned at least one GO term, which are mainly divided into three categories: biological processes, molecular functions, and cellular components. Among the 22 GO terms corresponding to biological processes, the terms, “cellular processes”, “metabolic processes”, “single-organisms”, “response to stimulus”, and “biological regulation” were significantly overrepresented, while “cell”, “cell part”, and “organelle” were significantly overrepresented among the 17 GO groups of cellular components, and “catalytic activity” and “binding” were significantly over-represented among the 16 GO groups of molecular functions (Fig 3).

We obtained KEGG pathway annotation for 97,833 unigenes. A total of 60,906 sequences were assigned to 128 pathways. “Metabolic pathways (ko01100)” represented the largest group (13,741; 22.56%), followed by “biosynthesis of secondary metabolites (ko01110)” (7,136; 11.72%), “plant-pathogen interaction (ko04626)” (3,933; 6.46%), “plant hormone signal transduction (ko04075)” (2,745; 4.51%), “spliceosome (ko03040)” (2,275; 3.74%), and “RNA transport (ko03013)” (2,270; 3.73%; S1 Table).

Additionally, in the prediction of protein coding regions, the number of CDSs that were mapped to the protein database was 92,001, and the number of predicted CDSs was 7,012. The total number of CDSs was 99,013 (S3 Fig).

### Determination and functional categorization of differentially expressed genes (DEGs)

To determine the genes showing altered expressions under salinity stress conditions, DEGs were defined as those with an FDR ≤ 0.001, a log₂ ratio ≥ 1, and an RPKM > 2 in the
compared libraries. We compared the 0 h library with the other two libraries (12 h versus 0 h and 24 h versus 0 h). As shown in Fig 4A, compared with the 0 h library, at the 12 h time point after salt treatment, the number (20,844) of DEGs was much larger than the number (17,811) at the 24 h time point. In the Venn diagram (Fig 4B) of those DEGs, it can be seen that among the genes that were differentially expressed at 12 h, only 48% were still differentially expressed at 24 h. In the figure showing the distribution of gene expression levels, the map corresponding to “12 h versus 0 h” exhibits a wider range than the map for “24 h versus 0 h” (S4 Fig). Therefore, there were many genes that responded to salinity stress in a very early stage, whose levels gradually returned to normal conditions.

All of the DEGs were categorized into 55 functional groups. The GO classification maps of the two comparisons were similar to each other. In these maps, among biological processes, the dominant groups were “cellular process”, “metabolic process”, “single-organism process”, and “response to stimulus”. In the cellular component category, the dominant groups were “cell”, “cell part”, “organelle”, and “membrane”. In the molecular function category, the dominant groups were “catalytic activity” and “binding” (S5 Fig).

The number of KEGG pathways corresponding to “12 h versus 0 h” and “24 h versus 0 h” was 126 in both cases, and the numbers of unigenes that were assigned to KEGG pathways were 9,899, and 7,769, respectively. After multiple testing corrections, we chose pathways with Q values ≤ 0.05 as significantly enriched among the DEGs. Then, we selected the useful significantly enriched pathways according to this criterion, resulting in 67 and 33 pathways, respectively (S2 and S3 Tables). The enriched pathways exhibiting more genes than the other pathways were “metabolic pathways (ko01100)”, “biosynthesis of secondary metabolites (ko01110)”, “ribosome (ko03010)” “Plant hormone signal transduction (ko04075)”, “plant-pathogen interaction (ko04626)”, “endocytosis (ko04144)”, “phenylpropanoid biosynthesis (ko00940)”, “glycerophospholipid metabolism (ko00564)”
“glycolysis/gluconeogenesis (ko00010)”, “ether lipid metabolism (ko00565)”, “starch and sucrose metabolism (ko00500)”, and “oxidative phosphorylation (ko00190)”. These pathways were used as a reference when defining the useful genes under conditions of salinity stress.
Detection of genes related to salinity tolerance

The identified DEGs are more likely to be related to salinity tolerance, and thousands of genes are induced or differentially expressed after salt treatment. Using the above tools in combination with previous reports, we selected some genes associated with salinity tolerance as relevant and divided them into categories. All of these genes are categorically listed in S4 Table. The main categories of the selected genes were as follows: transcription factors such as bHLH (basic helix-loop-helix), DREB1A (dehydration-responsive element binding protein 1A), AP2/ERF (APETALA2/ethylene response factor), NAC (NAM/ATAF/CUC), AREB (ABA responsive elements binding proteins), and MYB2 (myb proto-oncogene protein); signal transduction molecules such as CaM (calmodulin), YWHAs (14-3-3 proteins) and MAPKs (mitogen-activated protein kinases); energy metabolism-related genes such as AAC (ADP, ATP carrier protein), ALDH (aldehyde dehydrogenase), GTP (Phosphoenolpyruvate carboxykinase), GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), and ADHs (zinc-dependent alcohol dehydrogenase); various types of transporters, such as SPs (sugar transporters), AATs (amino acid transporters), NRTs (high-affinity nitrate transporters), and MPTs (mitochondrial phosphate transporters); proteins involved in Na\(^+\) exclusion and compartmentalization, such as HKTs (high-affinity potassium transporters), SOS1 (salt overly sensitive), NHXs (Na\(^+\)/H\(^+\) antiporters), and AVPs (H\(^+\) pyrophosphatases); aquaporins such as plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), and NOD26-like intrinsic proteins (NIPs); key enzymes involved in osmolyte synthesis, such as TPS (trehalose-6-phosphate synthase), TTP (trehalose-6-phosphate phosphatase), mt1D (mannitol-1-phosphate dehydrogenases), MIP (L-myo-Inositol-1-phosphate synthase), BADH (Betaine aldehyde dehydrogenase), CHDH (Choline dehydrogenase), and PCS (delta-1-pyruvyl-5-carboxylate synthetase); proteins related to water transportability and retention, such as AQPs (aquaporins), LEAs (late embryogenesis-abundant proteins) and DHNs (dehydrin); enzymes that cleave ROS such as SOD (superoxide dismutases), MDHAR (monodehydroascorbate reductases); cell wall proteins such as HRGPs (hydroxyproline-rich glycoproteins), XETs (xyloglucan endotransglycosylases), AGPs (arabinogalactan proteins), PRPs (proline-rich proteins) and GRPs (glycine-rich proteins); and photosynthesis-related proteins, such as AdSS2 (adenylosuccinate synthetase 2), KARI (ketol-acid reductoisomerase), NQO (quinone oxidoreductase), RPS4ε (small subunit ribosomal protein S4ε), LHCA4 (chlorophyll a/b binding protein 4), psbA (photosystem II protein D1), psbO (photosystem II oxygen-evolving enhancer protein 1), psaD (photosystem I subunit II), psaH (photosystem I subunit VI), petA (apocytochrome f), and FAD (ferredoxin-NADP\(^+\) reductase) (S4 Table). These genes will be discussed later.

Verification of differential gene expression through qRT-PCR

To validate the results obtained from the Illumina sequencing data, 46 relevant unigenes were selected for quantitative real-time PCR (qRT-PCR) analysis with samples that were treated with 120 mM NaCl for 0 h, 12 h, and 24 h. The expression trends of the unigenes from the qRT-PCR and RNA sequencing analyses were consistent (S6 Fig). Ten of these Unigenes are shown in the text (Fig 5a), and their transcript abundances at 0 h, 1 h, 6 h, 12 h, 24 h and 48 h were also measured by qRT-PCR, demonstrating that these unigenes respond strongly to salt (Fig 5b). These results demonstrated that the transcriptomic profiling data accurately reflected the responses of C. crassum to salt stress.
Discussion

Global patterns of transcription in response to salt treatment

To improve the salinity tolerance of plants, researchers worldwide have performed myriad studies to elucidate how plants respond to salinity stress. However, salinity tolerance mechanisms are too complex for researchers to have achieved a full understanding as yet. Despite being limited by technologies and knowledge, studies on elite salinity-tolerant germplasms are helpful for improving the salinity tolerance of other germplasms. For example, over-expression of the C. crassum plasma membrane Na⁺/H⁺ antiporter gene CcSOS1 improved the salinity tolerance of chrysanthemum ‘Jinba’ [12]. Therefore, functional genes from C. crassum could be used to improve the salinity tolerance of other species of chrysanthemums.

There have been many studies on the salinity tolerance of model plants, such as Arabidopsis and Nicotiana tabacum [26], and on some valuable crops with known genomes, such as Oryza sativa and Zea mays [27]. These studies have provided instructional theories and established components related to plant salinity tolerance, such as transcription factors, plant hormones, photosynthesis, osmolyte accumulation, ion elimination and toxicity alleviation. In cases where whole-genome sequencing data are lacking, NGS technology provides a powerful tool for transcriptome analysis of plants, and the de novo assembly of transcript sequences offers a rapid approach for determining expressed gene catalogs of non-model plants [28].
In this study, we performed sequencing analysis of *C. crassum* in response to salinity stress using the Illumina platform and *de novo* transcriptome assembly. Many DEGs were identified after salt treatment (Fig 4A). To better understand the function of the DEGs in leaves of *C. crassum* under salinity stress, the DEGs were categorized into 52 functional groups based on GO classification terms (S5 Fig). The ten most-enriched, dominant GO terms were “cell”, “cell part”, “metabolic process”, “cellular process”, “catalytic activity”, “organelle”, “binding”, “single-organism process”, “response to stimulus”, and “membrane”. These categories are also dominant in the transcriptomes of *Haloclaton glomeratus* [29] and *Reaumuria soongorica* [30]. This result shows a similarity to other salt-resistant plants and emphasizes the remarkable salinity tolerance of *C. crassum*.

The DEGs determined in the two comparisons were also mapped to 128 pathways with KEGG annotations. By analyzing the obtained maps, we could not only identify which metabolic functions responded to salinity stress but also determined the particular genes in those metabolic pathways that were up- or downregulated. This provided us with conventional references for determining the functional genes and metabolic pathways involved in responses to salinity stress. For example, key enzymes involved in energy metabolism pathways (such as “gluconeogenesis (ko00010)”, “citrate cycle (ko00020)”, and “oxidative phosphorylation (ko00190)”; amino acid synthesis pathways (such as “alanine, aspartate and glutamate metabolism (ko00250)”, and “valine, leucine and isoleucine biosynthesis (ko00290)”; and DNA-, RNA- and protein-related pathways (such as “DNA replication (ko03030)”, “RNA transport (ko03013)”, “ribosome (ko03013)” and “protein export (ko03030)”) were upregulated; the genes encoding these enzymes were regarded as functional genes that respond to salinity. Using these tools, we selected functional genes and drew conclusions about strategies employed by *C. crassum* under salinity stress.

Earliest-induced genes under salinity stress, such as transcription factors and signal molecules

Genes encoding transcription factors are some of the first genes to be induced under stress conditions [8]. Transcription factors are considered to have the greatest effect on crop salinity tolerance because these key transcription factors may regulate the induction or repression of a range of salinity tolerance genes. In the transcriptomes obtained in the present study, many reported transcription factors responding to salinity stress were found, such as *bHLHs* (Unigene40838, Unigene37206, and Unigene40839), *DREB1A* (CL6489.Contig1 and CL6489.Contig2), *AP2/ERF* (Unigene38328 and CL12828.Contig2), *NAC* (CL4758.Contig1, CL14612.Contig1, and Unigene60684), *AREB* (Unigene41165), *WRKY* (Unigene40047 and CL15510.Contig1), *GTL* (CL10054.Contig1) and *MYB2* (CL1046.Contig3 and CL1046.Contig1). All of these genes were upregulated at 12 h and returned to a low level at 24 h, and these genes have been reported to respond to stresses and regulate the growth conditions of plants under stress [31–33].

Additionally, some signal transduction molecules were found to be upregulated, such as *CaMs* (Unigene69398, Unigene70126, and Unigene70474), *YWHAs* (Unigene60260 and Unigene8553), and MAPKs (CL7742.Contig3, Unigene71806, and Unigene46385). These molecules have been reported to play a regulatory role in signal transduction [34]. These earliest-induced genes under salinity stress trigger a series of reactions related to stress endurance.

Genes related to plant hormone transduction pathways were differentially altered under salinity stress

In the obtained transcriptomes, genes involved in hormone transduction pathways were differentially expressed. The four key enzymes in the abscisic acid transduction pathway (*PYL*...
[Unigene29811], PP2C [Unigene69780], SnRK2 [CL1333.Contig3], and ABF [Unigene53263]) [7] were upregulated, as were key enzymes in the jasmonic acid transduction pathway (COI1 [Unigene21774], JAZ [CL18161.Contig17], and MYC2 [CL12169.Contig2]) [35] and key enzymes in the salicylic acid transduction pathway (TGA [Unigene21774], and PR-1 [Unigene33280]) [36]. These three well-studied hormones respond to stresses, can trigger many reactions, and may react with growth-related hormones, resulting in morphological changes in plants [37].

Some of the identified genes in hormone signal transduction pathways were considered to present functions regulating the morphological changes observed in C. crassum. For example, key enzymes in the auxin transduction pathway (TIR1 [Unigene46121], AUX [CL1780.Contig1], ARF [Unigene53204], and SAUR [CL16292.Contig2]) [38] were upregulated. Auxin plays an important role in coping with salinity and could help to maintain an ideal growth rate [39]. In the cytokinin transduction pathway [40], A-ARR (CL9399.Contig1) was downregulated, which could affect cell division [41], and leaf cells did not divide under salinity stress (Fig 1B). In the brassinosteroid transduction pathway [42], TCH4 (Unigene38906, Unigene13917, and Unigene54097) was upregulated, which could contribute to cell elongation [43], while CYCD3 (CL15069.Contig1, and Unigene61981), a cyclin that may contribute to cell division [44], was downregulated. These findings suggested that these three hormones played an important role in leaf incrassation and succulence in C. crassum.

Genes that contribute to overcoming osmotic stress caused by salinity in C. crassum

Osmotic stress must accompany salinity stress [8]. In the transcriptomes of C. crassum, many water retention-related genes were identified. For example, aquaporins can regulate water transport and may have an important function (i.e., the detection of osmotic and turgor pressure gradients) [45]. There are many types of aquaporins in the plasma membrane and vacuolar membrane of plants, such as PIPs, TIPs, and NIPs. These aquaporins present distinct cell type- and tissue-specific expression patterns, and some of them respond to stress. However, their expression profiles vary under salinity stress, and some posttranscriptional mechanisms regulate aquaporin trafficking to the plasma membrane [46]. In our data, PIPs (Unigene72479, CL11177.Contig14, and CL392.Contig3) and NIPs (Unigene43528, CL6774.Contig4, and Unigene54541) were upregulated after salt treatment, while TIPs (CL1377.Contig1 and CL6342.Contig4) were downregulated after salt treatment. This means that different aquaporins showed different expression profiles under salinity stress in C. crassum and contribute to the regulation of water transport.

In addition, LEAs (Unigene52031, CL16208.Contig1, Unigene69354, Unigene70057, Unigene70137, and Unigene69772) and DHNs (CL3765.Contig11, Unigene44626, and Unigene33157) were significantly upregulated and were widely expressed after salt treatment. These LEAs and DHNs have been reported to be abundant proteins in plants that are highly hydrophilic and provide protection to plants under osmotic stress [47].

To improve osmotic pressure, some osmolytes must be synthesized to endure osmotic stress [8]. In our transcriptomes, a number of osmolyte genes were upregulated after salt treatment, such as TPS (Unigene42563, and Unigene14695), TPP (CL14450.Contig3, and Unigene45055), mt1D (CL11217.Contig1), MIP (Unigene55075, Unigene69121, and CL1330.Contig1), BADH (Unigene71886), CHDH (Unigene70102, Unigene69590, Unigene70406, and Unigene71308), and P5CS (Unigene44661, and Unigene41949). Additional enzymes participating in the synthesis of amino acids (such as GPT [Unigene70282], GFPT [Unigene4584] and ABAT [Unigene70080]) [48] and monosaccharides (such as HK [Unigene71355], ALDO [Unigene69763]
and GMDS [CL9344.Contig1] [49] were also upregulated. Amino acids and monosaccharide are types of osmolytes [50]. We also found that some enzymes that participate in the synthesis of ascorbate (including GME [CL9645.Contig2], VTC4 [Unigene72075], and D-threo-aldose 1-dehydrogenase [Unigene71897]) [51] were upregulated after salt treatment. Ascorbate is a soluble carbohydrate that is abundant in leaves, and its accumulation would protect photosynthesis and improve the osmotic pressure of cells. Moreover, ascorbate is a cofactor for a large number of key enzymes and can influence both cell wall synthesis and hormone transduction [52]. Through the expression of the above genes, the plants were prevented from lacking water, and the ability of water to be taken up and retained was improved.

Genes that contribute to the exclusion and compartmentalization of Na⁺

To prevent ion toxicity caused by salt, Na⁺ must be kept from accumulating to excessive levels in protoplasts. The results in Fig 2 show that there must be some metabolic pathways that control the exclusion of Na⁺ and the passive assimilation of K⁺. In our transcriptomes, F-type H⁺-transporting ATPases (Unigene65029, Unigene69416, Unigene69674, CL11916.Contig1 and Unigene69648) and V-type H⁺-transporting ATPases (Unigene69565, Unigene70189, Unigene70388, Unigene70530, and Unigene71306) were significantly upregulated after salt treatment. These H⁺-transporting ATPases establish an electrochemical H⁺ gradient across membranes to energize the membranes and participate in the synthesis of ATP [53, 54], thereby providing energy and electric potential for Na⁺ exclusion. Some ion transporters that have been frequently reported were identified in our transcriptomes, such as HKTs (CL15304.Contig2, and CL15304.Contig1), SOS1 (Unigene38015), NHXs (Unigene12739, CL15766.Contig3, and Unigene12656), and AVPs (Unigene61687, Unigene61689, and Unigene61692). They were upregulated after salt treatment and shown to contribute to the reduction of the Na⁺ concentration in the cytoplasm [6].

Reactive oxygen species (ROS) scavengers in the transcriptomes

Salinity also causes the production of ROS, which are noxious to plants [9], and C. crassum must control ROS to a concentration that does not do harm to plants [55]. SOD (Unigene40004, Unigene69671, Unigene70615, Unigene70870, Unigene64998, and Unigene70976), MDHAR Unigene69762, Unigene71436, and CL11284.Contig1), and CAT (Unigene70090, Unigene69612, Unigene69512, Unigene70251, and Unigene70939), which were significantly upregulated after salt treatment, provide effective protection to cells and ensure that many reactions in cells proceed normally.

Genes contributing to the supply of energy and materials to the reactions triggered by salinity

Salinity causes many types of reactions in cells, and these reactions require energy and materials to function properly. Searching our transcriptomes revealed many energy metabolism-related genes, such as AAC (CL17351.Contig1, Unigene69746, and Unigene68723), ALDH (Unigene68789, Unigene69865, Unigene69866, and CL4198.Contig4), GTP (Unigene69854, Unigene56731, and Unigene68499), GAPDH (CL2444.Contig1, CL370.Contig1, CL2444.Contig2,and Unigene68703), and ADHs (CL14096.Contig2, Unigene71447, and Unigene69264), that were significantly upregulated after salt treatment and are helpful in supplying energy to other metabolic pathways to endure salinity stress. This is a common reaction of plants under stress [56, 57].

Some transporters, such as SPs (CL2828.Contig1, Unigene70725, and Unigene49470), AABs (Unigene69901, Unigene69792, Unigene69745 and CL14913.Contig1), NRTs
(Unigene69733 and Unigene67760), and MPTs (Unigene64947, Unigene69759, and Unigene70273), were upregulated after salt treatment. These transporters supply materials to other metabolic pathways to endure salinity stress [58, 59].

The above genes were significantly upregulated at 12 h and returned to a low level at 24 h. Thus, mechanisms that scavenge genes that have performed their functions and have no need to remain active are expected to function at this stage. We found that some proteasome subunits (Unigene71130, Unigene70519, Unigene70385, and Unigene71486) and some ubiquitin-conjugating enzymes (Unigene69417, Unigene30464, and CL15637.Contig1) were upregulated after salt treatment, which work as “cleaners” to digest unwanted mRNA and proteins [60] and return the expression of certain genes to normal levels (Fig 4 and S4 Fig).

Behavior of the photosynthetic systems under salinity stress

The growth of plants must be supported by photosynthetic systems. Generally, in plants with less salinity tolerance, there is more damage to the photosynthetic systems under salinity stress [3]. After salt treatment, various parts of the photosynthetic systems of C. crassum showed different expression profiles. We found that components such as RPS4e (Unigene1713 and Unigene41867) and LHCA4 (CL12376.Contig1 and CL13565.Contig1) of the “LHC (light-harvesting chlorophyll protein complex)”, which is responsible for trapping and transporting light energy to “photosystem I” and “photosystem II”[61], were downregulated, whereas components of “photosystem I” and “photosystem II” (such as psbA [CL6343.Contig11], psbO [CL6874.Contig2], psaD [CL5804.Contig1 and CL5804.Contig2], and psaH [Unigene58654]), which are responsible for oxidizing H2O to H+ and O2 [62], were downregulated. These changes may have been caused by the lack of water under osmotic stress. However, components of “cytochrome b6f complex”, “photosynthetic electron transport”, and “F-type ATPase” (such as petA [Unigene26782], FAD [CL14326.Contig2 and CL14326.Contig1], and F-type H+ transporting ATPase subunit gamma [Unigene43756]), which are responsible for mediating electron transport between PSII and PSI and converting the redox energy into part of the proton gradient used for ATP formation [62], were upregulated, as were key enzymes of the “carbon fixation in photosynthetic organisms” pathway (such as FBA [Unigene71038, CL13604.Contig2, Unigene69763, and Unigene35247], PK [Unigene71285 and Unigene62599], and RPIA [Unigene69514]) [63]. Thus, because of the difficulty in water uptake under salt shock, some components responsible for oxidizing H2O in the photosystem of C. crassum were downregulated, whereas other components were stimulated by the stress. At 24 h, all of these genes showed a tendency to recover to control levels. The previously mentioned ROS-scavenging enzymes provide protection to photosynthetic systems [55] and aid in the recovery of the photosynthetic systems of C. crassum. The recovery of the photosynthetic systems provides nutrition for the survival and growth of C. crassum under salinity stress.

Explanation of the morphological changes in C. crassum based on transcriptome analysis

The growth condition under salinity stress is considered a main norm for judging the salinity tolerance of a plant, with a better growth condition indicating better salinity tolerance [39]. The growth rate of C. crassum (Fig 1A) after salt treatment showed no obvious decrease, indicating that this species exhibits remarkable salinity tolerance. Incrassation and succulence of salt-treated leaves that are often observed in halophytes such as Salicornia europaea [64] and Populus euphratica [10]. The leaves of C3 plants such as Arabidopsis have also been reported to become slightly succulent after salt treatment [39]. As shown in Fig 1B, the leaves of C. crassum
became obviously succulent after salt treatment, which mainly due to the expansion of each cell. It is common for plants to show some morphological changes under salinity stress, which will improve the adaptation of plants to stress. The morphological changes displayed in *Chrysanthemum crassum* are rarely observed in chrysanthemums, and it would be very novel and interesting to elucidate how these changes were initiated. Here, we discuss how the morphological changes observed in *Chrysanthemum crassum* are initiated based on transcriptome analysis. The genes contributing to morphological changes are listed in S5 Table.

As previously discussed, these morphological changes are mainly regulated by hormones such as auxins, cytokinins, and brassinosteroids (genes such as *TIR1, AUX, ARF, A-ARR, TCH4, CYCD3* and *SAUR* are hypothesized to be responsible). Additionally, the appropriate functioning of photosynthetic systems provides nutritional preconditions for the ideal growth of *Chrysanthemum crassum* (functional genes are *SOD, MDHAR, CAT, LHCs, photosystem proteins, FBA, and RPIA*). Additionally, stress-induced hormones trigger certain components of metabolism, such as energy metabolism and transporters, to disrupt growth by depleting nutritional resources [65].

Plant cell expansion is often characterized as being the product of the opposing forces of intracellular turgor pressure (growth-promoting) and the turgor resistance of the cell wall (growth-inhibiting) [66]. As previously mentioned, we found that osmolytes accumulated to high levels and water absorbability and retention were improved in *Chrysanthemum crassum*. In this part, the mentioned genes are *TIPs, NIPs, PIPs, TPS, TPP, mt1D, MIP, BADH, CHDH and P5CS*. Therefore, as time went by, the “intracellular turgor pressure” became stronger.

We next consider the cell wall to examine “turgor-resisting forces”. The plant cell wall, and especially the cell wall proteins, undergoes adjustments under stress, as reported previously [67, 68]. The cell wall proteins exhibit functions involved in the morphogenesis and signal transduction of plants, and changes in the cell wall are a sign of cell differentiation [11]. In our transcriptomes, many types of cell wall proteins were found to be upregulated after salt treatment, such as *HRGPs* (Unigene69380, CL1283.Contig2, Unigene68945, and Unigene70662), which have an expansion-like domain and show a strong correlation with final cell length [69]. These expansions cause the cellulose microfibrils to slide apart and are considered to be the primary determinants of wall elongation [70], together with a group of enzymes known as *XETs* (CL10820.Contig2, CL10820.Contig3, and Unigene33390) [71], which were also upregulated after salt treatment. *PRPs* (Unigene69599, Unigene70083, Unigene70589, and Unigene70081) were also upregulated after salt treatment, which are extension proteins involved in the response to stress [72]. The adjustment of cell wall proteins of *Chrysanthemum crassum* under salinity stress showed a signal interaction and served as a marker for cell morphological changes. Furthermore, the expression of many extension proteins weakened “turgor-resisting forces”, making it easier for mesophyll cells to expand. Additionally, the cell wall proteins called *AGPs* (Unigene69390, Unigene68964, Unigene70408, and Unigene72883) were also upregulated after salt treatment; these proteins do not have structural function, but instead act as cell positional markers or as messengers in cell–cell interactions [73] Other upregulated genes included *GRPs* (Unigene68520, Unigene39763, and CL6740.Contig11), which play important roles in the development of vascular tissues and response to stresses [74].

With the expansion of cells, the membranes of the cells must also expand. In this context, the *LTPs* (Unigene70157, CL1283.Contig3, CL16785.Contig3, CL16785.Contig4, CL14640.Contig2, Unigene41619, and CL9481.Contig2) were also significantly upregulated and highly expressed after salt treatment, accounting for as much as 4% of the total soluble proteins of higher plants [75]. *LTPs* are thought to participate in membrane biogenesis, regulation of the intracellular fatty acid pools, cutin synthesis and responses to various stresses [76].
abundance of this type of protein would benefit C. crassum in maintaining membrane stabilization and facilitating fatty acid-related pathways that would be helpful in stress tolerance.

Genes responding to salinity without annotation

There were many genes with unknown functions without annotation in the databases that were significantly upregulated after salt treatment (such as Unigene43394, Unigene35778, Unigene69835, Unigene48169, Unigene69368, Unigene69480, Unigene51684, Unigene69811, Unigene48542, Unigene69615, Unigene69400, and Unigene68832) or were significantly downregulated (such as Unigene65383, Unigene65380, Unigene55783, Unigene65381, Unigene64461, CL12561.Contig1, CL16010.Contig4 and Unigene3931). The functions of these genes require further study.

Conclusions

Using the Illumina platform, we surveyed three transcriptomes of C. crassum leaves. We aimed to list the functional genes and pathways that contributed to salinity tolerance. Combined with the morphological changes observed under salinity stress, the results of the present study allowed us to infer and list the strategies adopted by C. crassum under salinity stress (Fig 6). Briefly, salinity stress first caused osmotic stress, which led to a lack of water. To maintain the water status of cells and cell functions effectively in C. crassum, large amounts of highly
hydrophilic proteins, such as LEAs and DHNs were expressed, along with a reduction of water waste and regulation of the expression of aquaporins to sense turgor pressure and regulate water transportation. *C. crassum* also synthesized osmolytes to improve osmotic pressure. Osmotic stress was overcome by these strategies. Salinity also caused toxicity and perturbed the normal function of cell reactions. This toxicity mainly consisted of the accumulation of Na$^+$ and the production of ROS. Accordingly, *C. crassum* expressed ion transporters to exclude and compartmentalize Na$^+$ and expressed ROS-scavenging enzymes to remove ROS. The damage due to salinity was controlled by these strategies. The applied salinity stress triggered some transcription factors and plant hormones (especially some stress-induced hormones), including abscisic acid, jasmonic acid, and salicylic acid. *C. crassum* improved the activity of energy metabolism and transporters to supply energy and materials to other reactions to endure salinity stress. Additionally, *C. crassum* activated cleaners, such as proteasomes and ubiquitin, to remove unwanted mRNA and proteins. As time passed, the plants exhibited morphological changes to adapt to salinity stress, and these changes were initiated during an early stage of the stress treatment. The salinity treatment initiated changes in some transcription factors and plant hormones, including growth-related hormones such as auxin, cytokinins, and brassinosteroids. *C. crassum* expressed many types of cell wall proteins to expand the cell wall, and osmolyte accumulation and water storage increased turgor pressure. Therefore, the mesophyll cells of *C. crassum* expanded over time under salinity stress. To maintain normal growth, the photosynthesis systems of *C. crassum* were protected and recovered well under salinity stress. Through these strategies, *C. crassum* adapted to salinity and grew well.

**Supporting information**

**S1 Fig. Growth conditions of Chrysanthemum crassum leaves under salinity stress.** Salt: a, 0 d; b, 5 d; c, 10 d; d, 20 d; Control: e, 0 d; f, 5 d; g, 10 d; h, 20 d. Bar = 1 cm.

(TIF)

**S2 Fig. Characteristics of similarity searches of unigenes against Nr databases.** E-value distribution of Nr annotation results (A). Similarity distribution of Nr annotation results (B). Species distribution of Nr annotation results (C).

(TIF)

**S3 Fig. All unigene CDS predictions.**

(DOC)

**S4 Fig. Distribution of differentially expressed genes in the two comparisons.** The genes were classified into three categories. Red genes are upregulated, in that gene expression is higher in the right sample than the left sample. Green genes are downregulated, in that gene expression is higher in the left sample than the right sample. Blue genes are not differentially expressed. The horizontal coordinates are the expression levels of the right sample, and the vertical coordinates are the expression levels of the left sample.

(TIF)

**S5 Fig. Overview of GO function classification.**

(DOC)

**S6 Fig. qRT-PCR analyses of unigene responses to salinity stress.** The expression patterns of selected genes were analyzed at 12 h and 24 h and compared with those at 0 h. RNA-seq values are the log2 values of the RPKMs of two libraries; the qRT-PCR values were determined via qPCR using the $-\Delta\Delta$Ct values.

(TIF)
S1 Table. KEGG analysis of all unigenes.
(XLS)

S2 Table. KEGG analysis of DEGs for 0 h vs. 12 h.
(XLS)

S3 Table. KEGG analysis of DEGs for 0 h vs. 24 h.
(XLS)

S4 Table. Selected genes that responded to salinity stress.
(XLS)

S5 Table. Selected genes that related to the initiation of morphological changes.
(XLS)

Acknowledgments

This work was supported by grants from the 948 Project of the Ministry of Agriculture (2016-X18), the Fund for Independent Innovation of Agricultural Sciences in Jiangsu Province [CX (16) 1025], and the Fundamental Research Funds for the Central Universities (KYCYL201501).

Author Contributions

Conceptualization: ZG SC JJ FC.
Data curation: YF XS.
Formal analysis: JJ SC.
Funding acquisition: ZG.
Investigation: YF YM XS.
Project administration: FC.
Resources: FC.
Software: LD.
Supervision: ZG FC.
Validation: AS.
Visualization: YF AS.
Writing – original draft: YF AS.
Writing – review & editing: YF AS.

References

1. Pichu R. Soil processes affecting crop production in salt-affected soils. Funct Plant Biol. 2010; 37 (7):613–20.
2. Savc S. Investigation of Effect of Chemical Fertilizers on Environment. Apcbee Procedia. 2012; 1:287–92.
3. Zahra J, Nazim H, Cai S, Han Y, Wu D, Zhang B, et al. The influence of salinity on cell ultrastructures and photosynthetic apparatus of barley genotypes differing in salt stress tolerance. Acta Physiol Plant. 2014; 36(5):1261–9.
4. Moghaieb REA, Saneoka H, Fujita K. Effect of Salinity on Osmotic Adjustment, Glycinebetaine Accumulation and the Betaine Aldehyde Dehydrogenase Gene Expression in Two Halophytic Plants, Salicornia europaea and Suaeda maritime. Plant Sci. 2004; 166((5)):1345–9.

5. Wang Y, Shen W, Chan Z, Wu Y. Endogenous Cytokinin Overproduction Modulates ROS Homeostasis and Decreases Salt Stress Resistance in Arabidopsis Thaliana. Front Plant Sci. 2015; 6(8).

6. Munns R, Tester M. Mechanisms of salinity tolerance. Annu Rev Plant Biol. 2008; 59:651–81. https://doi.org/10.1146/annurev.arplant.59.032607.092911 PMID: 18444910

7. Zhao Y, Chan Z, Xing L, Liu X, Hou YJ, Chinnusamy V, et al. The unique mode of action of a divergent member of the ABA-receptor protein family in ABA and stress signaling. Cell Res. 2013; 23(12):1380. https://doi.org/10.1038/cr.2013.149 PMID: 24189045

8. Roy SJ, Negrao S, Tester M. Salt resistant crop plants. Curr Opin Biotech. 2014; 26:115–24. https://doi.org/10.1016/j.copbio.2013.12.004 PMID: 24679267

9. Apel K, Hirt H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol. 2004; 55:373–99. https://doi.org/10.1146/annurev.arplant.55.031903.141701 PMID: 15377225

10. Ottow EA, Brinker M, Teichmann T, Fritz E, Kaiser W, Brosche M, et al. Populus euphratica displays apoplastic sodium accumulation, osmotic adjustment by decreases in calcium and soluble carbohydrates, and develops leaf succulence under salt stress. Plant physiol. 2005; 139(4):1762–72. https://doi.org/10.1104/pp.105.069971 PMID: 16299175

11. Caffall KH, Mohnen D. The structure, function, and biosynthesis of plant cell wall pectic polysaccharides. Carbohyd Res. 2009; 344(14):1879–900.

12. An J, Song A, Guan Z, Jiang J, Chen F, Lou W, et al. The over-expression of Chrysanthemum crassum CcSOS1 improves the salinity tolerance of chrysanthemum. Mol Biol Rep. 2014; 41(6):4155–62. https://doi.org/10.1007/s11033-014-3287-2 PMID: 24566689

13. Song A, Lu J, Jiang J, Chen S, Guan Z, Fang W, et al. Isolation and characterisation of Chrysanthemum crassum SOS1, encoding a putative plasma membrane Na+/H+ antiporter. Plant Biol. 2012; 14(5):706–13. https://doi.org/10.1111/j.1438-8677.2011.00560.x PMID: 22404736

14. Wilhelm BT, Samuel M, Stephen W, Falk S, Valerie W, Ian G, et al. Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. Nature. 2008; 453(7199):1239–43. https://doi.org/10.1038/nature07002 PMID: 18488015

15. Likun W, Zhixing F, Xi W, Xiaowo W, Xuegong Z. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. Bioinformatics. 2010; 26(1):136–8. https://doi.org/10.1093/bioinformatics/btp612 PMID: 19855105

16. Fei J, Tan S, Zhang F, Hua L, Liao Y, Fang W, et al. Morphological and physiological differences between dehiscent and indehiscent anthers of Chrysanthemum montifolium. J Plant Res. 2016.

17. Quinet M, Ndayiragije A, Lefèvre I, Lambillotte B, Dupont-Gillaud CC, Lutts S. Putrescine differently influences the effect of salt stress on polyamine metabolism and ethylene synthesis in rice cultivars differing in salt resistance. J Exp Bot. 2010; 61(10):2719–33(15). https://doi.org/10.1093/jxb/erq118 PMID: 20472577

18. Wang H, Jiang J, Chen S, Qi X, Peng H, Li P, et al. Next-Generation Sequencing of the Chrysanthemum nankingense (Asteraceae) Transcriptome Permits Large-Scale Unigene Assembly and SSR Marker Discovery. PLoS ONE. 2013; 8(4):e62293–e. https://doi.org/10.1371/journal.pone.0062293 PMID: 23626799

19. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat biotechnol. 2011; 29(7):644–52. https://doi.org/10.1038/nbt.1883 PMID: 21572440

20. Cameron M, Williams HE, Cannane A. Improved Gapped Alignment in BLAST. IEEE Acm T Comput Bi. 2004; 1(3):116–29.

21. Iseli C, Jongeneel CV, Bucher P. ESTScan: A Program for Detecting, Evaluating, and Reconstructing Potential Coding Regions in EST Sequences. Proceedings of the Seventh International Conference on Intelligent Systems for Molecular Biology. 660818: AAAI Press; 1999. p. 138–58.

22. Ana C, Stefan GT, Juan Miguel GG, Javier T, Manuel T, Montserrat R, Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics. 2005; 21(18):3674–6. https://doi.org/10.1093/bioinformatics/bti610 PMID: 16081474

23. Jia Y, Lin F, Hongkun Z, Yong Z, Jie C, Zengjin Z, et al. WEGO: a web tool for plotting GO annotations. Nucleic acids res. 2006; 34(2):W293–W7.

24. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. Meth Mol Biol. 2000; 132(3):365–86.
25. Fan Q, Song A, Jiang J, Zhang T, Sun H, Wang Y, et al. CmWRKY1 Enhances the Dehydration Tolerance of Chrysanthemum through the Regulation of ABA-Associated Genes. PLoS ONE. 2016; 11(3): e0150572. https://doi.org/10.1371/journal.pone.0150572 PMID: 26938878

26. Fahad S, Hussain S, Matloob A, Khan FA, Khalilq A, Saud S, et al. Phytohormones and plant responses to salinity stress: a review. Plant Growth Regu. 2014; 75(2):391–404.

27. Yadav S, Irfan M, Ahmad A, Hayat S. Causes of salinity and plant manifestations to salt stress: a review. J Environ Biol. 2011; 32(5):667–85. PMID: 22319886

28. Strickler SR, Bombarely A, Mueller LA. Designing a transcriptome next-generation sequencing project for a nonmodel plant species. Am J Bot. 2012; 99(2):257–66. https://doi.org/10.3732/ajb.1100292 PMID: 22268224

29. Wang J, Li B, Meng Y, Ma X, Lai Y, Si E, et al. Transcriptomic profiling of the salt-stress response in the halophyte Haloclonon glomeratus. BMC Genomics. 2015; 16(1):169.

30. Shi Y, Yan X, Zhao P, Yin H, Zhao X, Xiao H, et al. Correction: Transcriptomic Analysis of a Tertiary Relict Plant, Extreme Xerophyte Reaumuria soongorica to Identify Genes Related to Drought Adaptation. Revista Española De Derecho Int. 2013; 59(3):págs. 681–96.

31. Gao C, Li P, Song A, Wang H, Wang Y, Ren L, et al. Isolation and characterization of six AP2/ERF transcription factor genes in Chrysanthenum nankingense. Int J Mol Sci. 2015; 16(1):2052–65. https://doi.org/10.3390/ijms16012052 PMID: 25607731

32. Song A, Li P, Jiang J, Chen S, Li H, Zeng J, et al. Phylogenetic and transcription analysis of chrysanthemum WRKY transcription factors. Int J Mol Sci. 2014; 15(8):14442–55. https://doi.org/10.3390/ijms15081442 PMID: 25196345

33. Song A, Wu D, Fan Q, Tian C, Chen S, Guan Z, et al. Transcriptome-Wide Identification and Expression Profiling Analysis of Chrysanthemum Trihelix Transcription Factors. Plant Physiol Bioch. 2016; 102(2):10–6.

34. Yang L, Ji W, Zhu Y, Gao P, Li Y, Cai H, et al. GsCBRLK, a calcium/calmodulin-binding receptor-like kinase, is a positive regulator of plant tolerance to salt and ABA stress. J Exp Bot. 2010; 61(9):2519–33. https://doi.org/10.1093/jxb/erq084 PMID: 20400529

35. Katsir L, Chung HS, Koo AJ, Howe GA. Jasmonate signaling: a conserved mechanism of hormone sensing. Curr Opin Plant Biol. 2008; 11(4):428–35. https://doi.org/10.1016/j.pbi.2008.05.004 PMID: 18583180

36. Johnson C. Salicylic Acid and NPR1 Induce the Recruitment of Trans-Activating TG2 Factors to a Defense Gene Promotor in Arabidopsis. Plant Cell. 2003; 15(8):1846–58. https://doi.org/10.1105/tpc.012211 PMID: 12897257

37. Wang Y, Mopper S, Hasenstein KH. Effects of Salinity on Endogenous Aba, Iaa, Ja, and Sa in Iris hexagona. J Chem Ecol. 2001; 27(2):327–42. PMID: 14768818

38. Chapman EJ, Estelle M. Mechanism of auxin-regulated gene expression in plants. Annu Rev Genet. 2009; 43:265–85. https://doi.org/10.1146/annurev-genet-102108-134148 PMID: 19686081

39. Li Q, Yin M, Li Y, Fan C, Yang Q, Wu J, et al. Expression of Brassica napus TIG2, a regulator of trichome development, increases plant sensitivity to salt stress by suppressing the expression of auxin biosynthesis genes. J Exp Bot. 2015; 66(19):5821–36. https://doi.org/10.1093/jxb/erv287 PMID: 26071533

40. Brenner WG, Romanov GA, Koller I, Burke L, Schmulling T. Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin-sensitive processes and suggest cytokinin action through transcriptional cascades. Plant J. 2005; 44(2):314–33. https://doi.org/10.1111/j.1365-313X.2005.02530.x PMID: 16212609

41. Shi Y, Yang S. Ethylene signaling negatively regulates freezing tolerance by repressing expression of CBF and type-A ARR genes in Arabidopsis. Plant Cell. 2012; 24(6):2578–95. https://doi.org/10.1105/tpc.1211205 PMID: 22706288

42. Kim TW, Wang ZY. Brassinosteroid signal transduction from receptor kinases to transcription factors. Annu Rev Plant Biol. 2010; 61:681–704. https://doi.org/10.1146/annurev-plant.043008.092057 PMID: 20192752

43. Xu W, Purugganan MM, Polisensky DH, Antosiewicz DM, Fry SC, Braam J. Arabidopsis TCH4 regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. Plant Cell. 1995; 7(10):1555–67. https://doi.org/10.1105/tpc.7.10.1555 PMID: 7580251

44. Hu Y, Bao F, Li J. Promotive effect of brassinosteroids on cell division involves a distinct CycD3 -induction pathway in Arabidopsis. Plant J. 2000; 24(5):693–701. PMID: 11123807

45. Hill AE, Shachar-Hill B, Shachar-Hill Y. What are aquaporins for? J Membrane Biol. 2004; 197(1):1–32.

46. Muries B, Faize M, Carvajal M, Martinez-Ballesta Mdel C. Identification and differential induction of the expression of aquaporins by salinity in broccoli plants. Mol Biosyst. 2011; 7(4):1322–35. https://doi.org/10.1039/c0mb00285b PMID: 21321750
47. Dimitri T, Michel J, Cécile M, Catherine P, Patrick S, Stephen M, et al. Structure and function of a mitochondrial late embryogenesis abundant protein are revealed by desiccation. Plant Cell. 2007; 19(5):1580–9. https://doi.org/10.1105/tpc.107.050104 PMID: 17526751
48. Wu G. Intestinal mucosal amino acid catabolism. J Nutr. 1998; 128(8):1249–52. PMID: 9687539
49. Müller R, Morant M, Jarmer H, Nilsson L, Nielsen TH. Genomic-wide Analysis of the Arabidopsis Leaf Transcriptome Reveals Interaction of Phosphate and Sugar Metabolism. Plant physiol. 2007; 143(1):156–71. https://doi.org/10.1104/pp.106.090167 PMID: 17085508
50. Kunte HJ, Galinski EA, Trüper HG. A modified FMOC-method for the detection of amino acid-type osmolytes and tetrahydropyrimidines (ectoines). J Microbiol Meth. 1993; 17(2):129–36.
51. Davey MW, Gilot C, Persiau G, Pastori GM. Leaf Vitamin C Contents Modulate Plant Defense Transcripts and Regulate Genes That
52. Nelson N, Yocum CF. Structure and function of photosystems I and II. Annu Rev Plant Biol. 2006; 57(9):1610–8. https://doi.org/10.1105/tpc.107.050104 PMID: 17965273
53. Fillingame RH. Membrane sectors of F- and V-type H+-transporting ATPases. Curr Opin Struct Biol. 1996; 6(4):491–8.
54. Wang B, Lütge U, Ratajczak R. Effects of salt treatment and osmotic stress on V-ATPase and V-PPase in leaves of the halophyte. Am J Med Genet. 1993; 48(48):40–6.
55. Foyer CH, Noctor G. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant Cell Environ. 2005; 28(8):1056–71.
56. Ihalainen JA, Klimmek F, Ganeteg U, Stokkum IHMV, Grondelle RV, Jansson S, et al. Excitation energy trapping in photosystem I complexes depleted in Lhca1 and Lhca4. FEBS Lett. 2005; 579(21):4787–91. https://doi.org/10.1105/tpc.107.050104 PMID: 16989711
57. Yao J, Shi WM, Xu WF. Effects of salt stress on expression of nitrate transporter and assimilation-related genes in tomato roots. Russ J Plant Physiol. 2011; 55(2):232–40.
58. Z Wei, Miao Q, S Dan, Yang G, Wu C, Huang J, et al. The Mitochondrial Phosphate Transporters Modulate Plant Responses to Salt Stress via Affecting ATP and Gibberellin Metabolism in Arabidopsis thaliana. PLoS ONE. 2012; 7(8):e0116217. https://doi.org/10.1371/journal.pone.0116217 PMID: 25679513
59. Long W, Zou X, Zhang X. Transcriptome analysis of canola (Brassica napus) under salt stress at the germination stage. PLoS ONE. 2015; 10(2):e0116217. https://doi.org/10.1371/journal.pone.0116217 PMID: 25679513
60. Zheng Q, Liu L, Liu Z, Chen J, Zhao G. Comparison of the response of ion distribution in the tissues and cells of the succulent plants Aloe vera and Salicornia europaea to saline stress. J Plant Nutr Soil Sci. 2009; 172(6):875–83.
61. Porto BN, Alves JD, Magalhães PC, Castro EM, Campos NA, Souza KR, et al. Calcium-Dependent Tolerant Response of Cell Wall in Maize Mesocotyl Under Flooding Stress. J Agron Crop Sci. 2013; 199(2):134–43.
62. Cassab GI, Varner JE. Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. J Cell Biol. 1987; 105(1):2581–8.
70. Mellon JE, Helgeson JP. Interaction of a hydroxyproline-rich glycoprotein from tobacco callus with potential pathogens. Plant physiol. 1982; 70(2):401–5. PMID: 16662504

71. Smith RC, Fry SC. Endotransglycosylation of xyloglucans in plant cell suspension cultures. Biochem J. 1991; 279 (Pt 2)(1):529–35.

72. Luck G, Liao H, Murray NJ, Grimmer HR, Warinski EE, Williamson MP, et al. Polyphenols, astringency and proline-rich proteins. Phytochemistry. 1994; 37(2):357–71. PMID: 7765619

73. Fincher GB, And S BA, Clarke AE. Arabinogalactan-Proteins: Structure, Biosynthesis, and Function. Inorg Chem. 2003; 34(1):47–70.

74. Ryser U., Schorderet M., Zhao GF, Studer D., Ruel K., Hauf G., et al. Structural cell-wall proteins in protoxylem development: evidence for a repair process mediated by a glycine-rich protein. Plant J. 1997; 12(1):97–111. PMID: 9263454

75. Kader JC. LIPID-TRANSFER PROTEINS IN PLANTS [Review]. Annu Rev Plant Physiol Plant Mol Biol. 1996; 47(1):627–54.

76. White AJ, Dunn MA, Brown K, Hughes MA. Comparative analysis of genomic sequence and expression of a lipid transfer protein gene family in winter barley. J Exp Bot. 1994; 45(12):1885–92.