Identification of potential key genes affecting soybean growth under salt stress *via* transcriptome study

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Abstract

Soybean is one of the most important economic crops in the world. However, the salinization of soil results in the decrease of soybean yield as it is only a moderately salt-tolerant crop. We treated three soybean cultivars with low and high concentrations of NaCl. The differentially expressed genes between the control group and the salt treatment group were identified by mRNA sequencing and analyzed by *gene ontology* and *Kyoto encyclopedia of genes and genomes* annotations. We performed weighted gene co-expression network analysis on all samples and found genes most related to the phenotype. After verifying the results of differentially expressed genes by quantitative PCR, we finally identified *Glyma06G01990*, *Glyma08G22730*, *Glyma019G05140*, and *Glyma06G20160* as key genes affecting the soybean growth under salt stress.

Additional key words: differentially expressed genes, *Glycine max*, RNA-seq, WGCNA.

Introduction

Soybean (*Glycine max* L.) is the most widely planted oil crop in the world, and the global demand for soybean is still increasing (Chaudhary *et al.* 2015). Soybean contains about 40 % of protein and 21 % of oil, and plays an important role in human diet, animal feed, bio-oil production, and raw material for industrial products (Le *et al.* 2011). However, various biotic and abiotic stresses affect the soybean growth and development, leading to the decline of soybean yield and quality. Soil salinization affected the development of many crops around the world and its area is still growing (Sharifi *et al.* 2007). In saline soil, Na$^+$ and Cl$^-$ are the main causes of toxicity (Munns 2002). Na$^+$ is not an essential element for plant growth, and high concentration of Na$^+$ ion is toxic to most plants (Tester and Davenport 2003). High concentration of chloride ion can also inhibit the growth of some plants (Geilfus 2018). Soybean is a moderately salt-tolerant crop. Salt stress inhibits the germination and growth of soybean seeds, and hinder the formation of soybean nodules (Wang *et al.* 1999). When the soil salt content exceeds 5 dS m^{-1}, the yield of soybean would be seriously decreased (Ashraf

and Akram 2009). Therefore, research on salt tolerance of soybean is vital for breeding salt-tolerant cultivars.

Transcriptome is the sum of all RNA transcribed by a certain organism under certain environmental conditions. Transcriptome technology plays an important role in explaining gene function, revealing the molecular composition of cells and tissues, and molecular mechanisms of many processes (Luo *et al.* 2015). Transcriptome sequencing technology can not only accurately determine the expression of known genes, but also detect the sequence and functional classification of unknown genes specific to plants. Several studies have performed transcriptomes sequencing of salt-tolerant plants and screened the salt stress related genes. Long *et al.* (2015) analyzed the transcriptome of NaCl-treated rape roots and found 163 differentially expressed genes (DEGs), some of which were related to proline metabolism, sugar metabolism, saccharide metabolism, and redox processes. Dang *et al.* (2013) obtained 65 340 DEGs by sequencing the transcriptome of salt-stressed halophyte *Reaumuria trigyma*, and put these genes into 29 *Kyoto encyclopedia of genes and genomes* (*KEGG*) pathways, pointing out that the salt-stress response genes were related to ion transport

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and reactive oxygen species scavenging. Liu *et al*. (2018) performed whole transcriptome sequencing on soybean root and leaf, demonstrating that phytohormone signal pathways were triggered and photosynthesis was decreased under salt stress.

In this study, we treated three different soybean cultivars with high and low concentration of NaCl and performed mRNA sequencing. To identify several potential genes affecting soybean growth under salt stress, we combined the results of transcripts assembly and annotation, weighted gene co-expression network analysis (WGCNA), and DEGs.

Materials and methods

Plants and treatments: To study the common mechanism of soybean salt tolerance, we used *Glycine max* (L.) Merr. cultivars: LD (Lindou 10, bred and provided by Linyi Academy of Agricultural Sciences, Shandong, China), QH (Qihuang 34, bred and provided by Crop Research Institute, Shandong Academy of Agricultural Sciences, China), and WD8 (Weidou 8, bred and provided by Weifang Academy of Agricultural Sciences, Shandong, China). Plants were divided into three groups: the control (CK) group, the low concentration (LS) group, and the high concentration (HS) group. Each treatment was repeated three times.

Twenty seven seeds (nine for each cultivar) were individually cultivated in a pot with soil. After the first true leaf unfolding, the seeds were moved into a 1/4 Hoagland nutrient solution and grown under day/night temperatures of 27/23 °C, an air humidity of 50 %, a light intensity of 30 000 lx, and a 17-h photoperiod. After 20 d, the three groups were treated with 1 dm³ of 1/4 Hoagland nutrient solution with 0 mM NaCl (CK), 75 mM NaCl (LS), and 150 mM NaCl (HS) solution, respectively. Three roots from each treatment were collected as three replicates at the time point of 6 h.

Isolation of RNA, cDNA library preparation, and sequencing: The total RNA was extracted using *TRIzol* reagent (*Thermo Fisher Scientific*, Waltham, USA) according to the manufacture's protocol. The quantity of RNA was detected by *Nanodrop2000* (*Thermo Fisher Scientific*) and the purity was detected by agarose gel electrophoresis. The *Agilent2100* (*Agilent Technologies*, Palo Alto, CA, USA) was used to measure the RNA integrity number. The total amount of RNA was 1 μg and the absorbance A_{260}/A_{280} value ranged from 1.8 to 2.2. The mRNA was isolated from the total RNA using *Oligo* (dT) magnetic beads paired with poly(A) and randomly cleaved into small fragments by fragmentation buffer (*Agilent Technologies*). The cDNA library was built with the *Truseq*™ RNA sample prep kit (*Illumina*, San Diego, CA, USA) according to the manufacture's instruction. The sequencing was performed with the *HiSeq 2500* system (*Illumina*).

Analysis of RNA sequencing data and alignment: The *SepPrep* software (https://github.com/jstjohn/SeqPrep)

was used to control the quality of the original reads, and the screening criteria are as follows: *1*) removed the adaptor sequence reads and the sequences without fragments insertion due to adaptor self-connection; *2*) trimmed the base whose Q-score was < 20 at the 3'end of the sequence. The remaining sequence with base whose Q-score was still < 1 0 would be also deleted; *3*) removed the N-containing reads; *4*) discarded the sequences less than 30 bp in length after trimming. The trimmed reads were mapped to the reference genome (NCBI Glycine_max_v2.1 GCF_000004515.5) using the *HISAT2* software (Kim *et al.* 2015).

The transcript assembly and annotation: The mapped reads were assembled to a transcriptome using *Cufflinks* software (http: //cole-trapnell-lab.github.io/cufflinks/), and compared with known transcripts to obtain transcripts without annotation. Functional annotation of potential new transcripts allows us to discover new genes and transcripts, or new exon regions of known genes. *Gene ontology* (*GO*) was used to annotate genes and their products according to their participation in biological process (BP), molecular function (MF), and cellular component (CC) to get general understanding of functional classification. Comparing the genes with the Kyoto encyclopedia of genes and genomes (*KEGG*) database revealed the specific biological pathways that a gene or its transcripts might participate in, and helped to understand the biological functions of genes at the system level.

Differential expression analysis: Read counts of each gene and transcript were obtained with the *RSEM 1.3.1* software (Li and Dewey 2011) by using the alignment results and genome annotation files and then performed fragments per kilobase of exon model per million mapped fragments and transcripts per kilobase of exon model per million mapped reads transformation to obtain the standardized gene/transcript expression level. The *DESeq2 1.24* package (http://www.bioconductor.org/packages/DESeq2) in *R Bioconductor 3.6* (http://www.bioconductor.org/) was used to analyze the differential expression among different groups. The gene expression of each sample was as input matrix, and the cultivars and salt stress treatments were as phenotypic information. *WGCNA 1.67* package (Langfelder and Horvath 2008) was used to discover the modules related to different apparent characteristics. The visualization analysis was performed using *Cytoscape 3.7.1* software (Shannon *et al.* 2003).

Real-time quantitative PCR analysis: The tissue samples were added into the mortar containing liquid nitrogen and ground to fine powder with a pestle. The total RNA was isolated with *TRIzol* reagent (*Invitrogen,* Carlsbad, CA, USA). The cDNA was synthesized using reverse transcription kit (*Invitrogen*), and then amplified using *SYBR Green* PCR mix (*Invitrogen*) and the *ABI Prism 7300* sequence detection system (*Applied Biosystems*, Foster City, USA) according to the manufacturer's instructions. The quantitative PCR data were normalized to *actin* and analyzed with *GenEX* software (*MultiD Analyses AB*, Goteborg, Sweden). The primer pairs used

for amplification are shown in Table 1 Suppl.

Data analysis: The statistical analysis of quantitative PCR data was performed by *t*-test with the *GraphPad Prism v. 5.0* software for *Windows* (*GraphPad*, San Diego, CA, USA). The minimum significance level was set at $\alpha = 0.05$.

Results

The statistical results of sequencing each sample are shown in Table 2 Suppl. Taking the control group of the LD cultivar as an example, the distribution of base error and A/T/C/G base content are displayed in Fig. 1. The sequencing error rate increased with the length of sequenced reads due to the consumption of reagents in the

sequencing process, which is known as common feature of *Illumina* high-throughput sequencing platform. The 6 bp random primers used in reverse transcription would induce a preference of the first few nucleotides (Fig. 1).

Taking the control group of the LD cultivar (LDCK_1) as an example, we analyzed the sequencing saturation, sequencing coverage, and reads distribution in different genomic regions (Fig. 1). Most of the genes with reads per kilobase per million mapped reads value > 3.5 were nearly saturated when aligned with 40 % of the sequence reads, indicating that the sequencing could cover most of the expressed genes. Fig. 1 Suppl. showed the coverage of all genes and there was no obvious bias peak, indicating that the sequencing has even distribution. The percentage of reads mapped to genome regions was also shown in Fig. 2 Suppl.
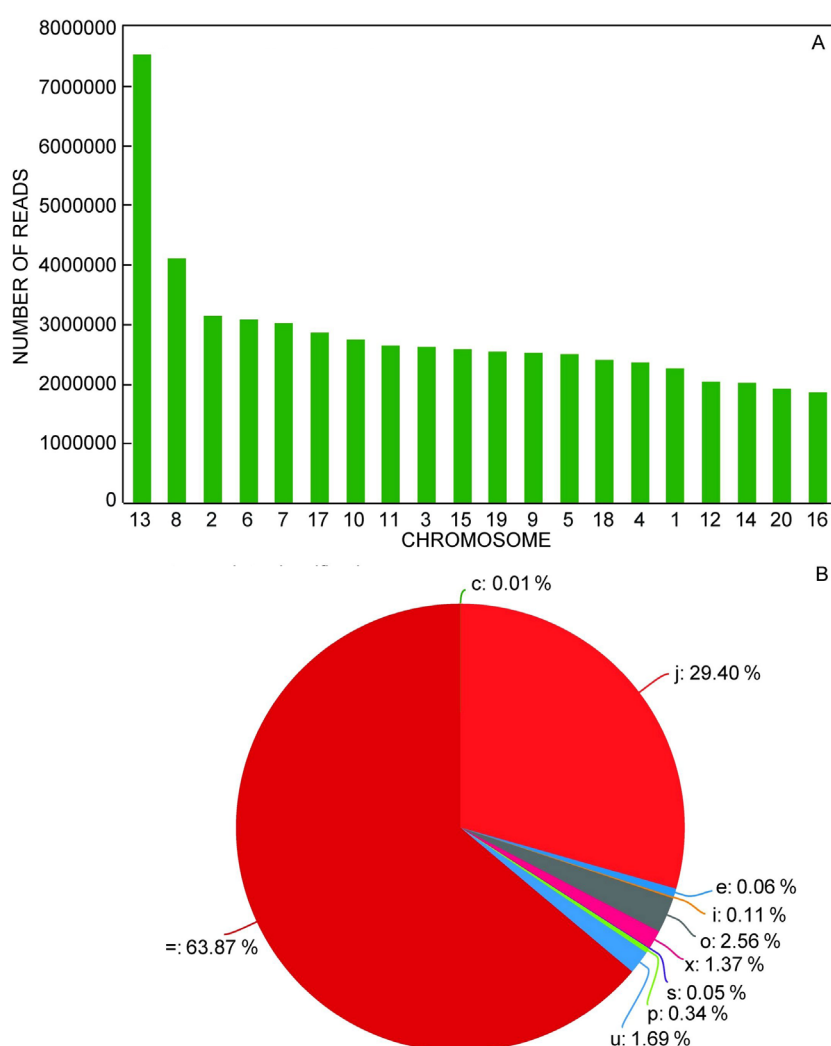

Fig. 1. Assembly of expressed transcripts. *A* - distribution map of transcription length; *B* - statistical map of new transcript classification, = - known transcripts consistent with reference annotations, c - assembled transcripts contained in known transcripts, e - fragments of potential RNA precursors, i - transcripts fragments completely overlapping introns of reference transcripts, j - potential new transcripts or transcriptional fragments with at least one junction site consistent with reference transcripts, o - overlapped with the exon of reference transcripts, p - transcriptional fragments that might be produced by polymerase, s - introns of transcriptional fragments overlapped with the antisense chain of reference transcript introns, u - unknown transcripts of intergenic regions, x - overlapped with the antisense chain of reference transcript exons).
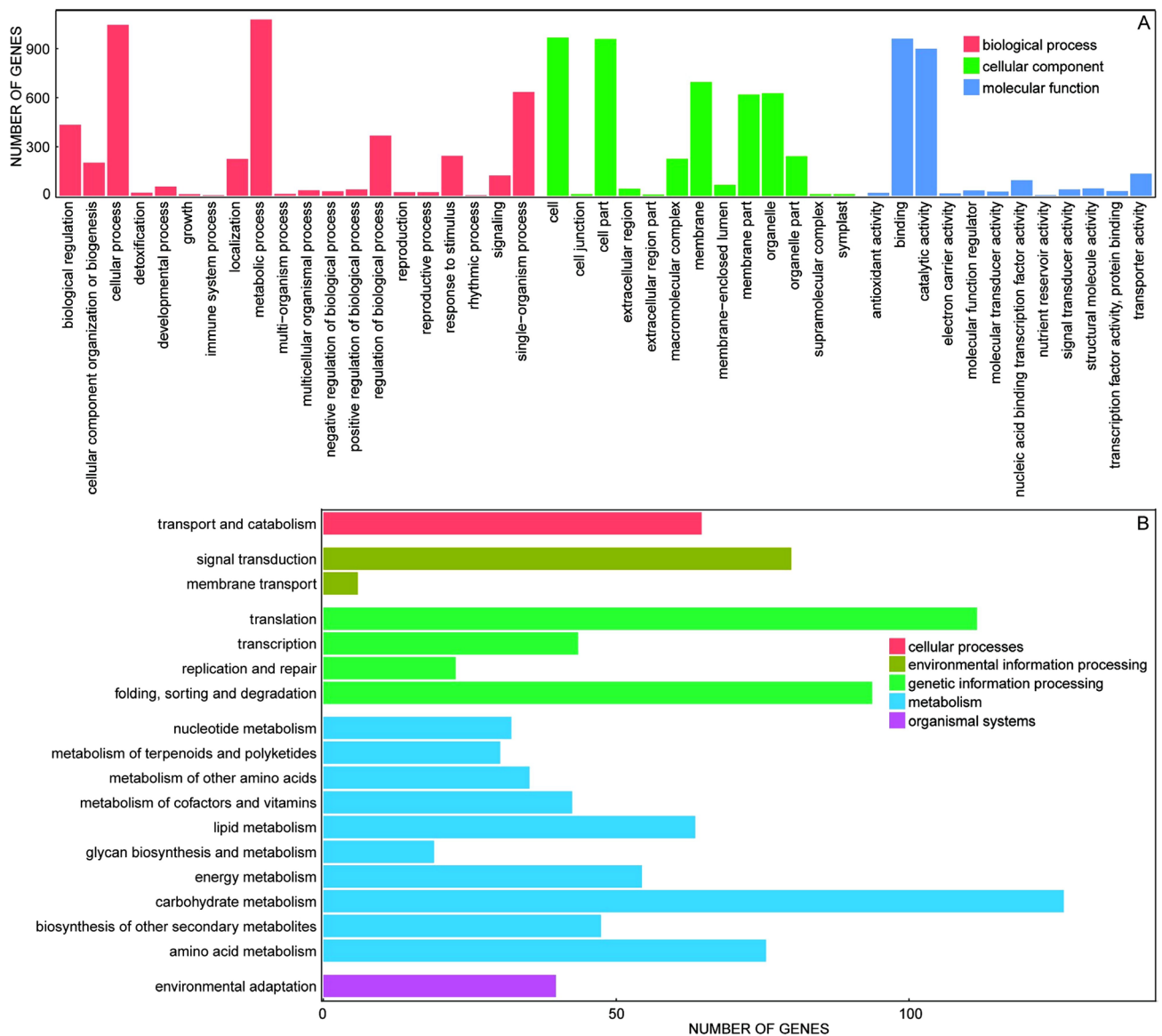
Fig. 2. Classification of the defferentially expressed gene biological functions. *A* - analysis based on the *gene ontology* database. *B* - analysis based on the *Kyoto encyclopedia of genes and genomes* database.

Cufflinks software was used to assemble the results of alignment with the reference genomes, to identify pair end fragments from different spliceosomes, and to search for the overlapping fragments. Transcripts were assembled according to each fragment. The differences between the sequence and the original annotations were obtained by comparing them with the reference transcripts. Fig. 1*A* showed the distribution of transcript length in chromosomes. Fig. 1*B* showed the classification statistics of new transcripts. The majority transcripts (63.87 %) were consistent with reference annotations, and classes of x, i, j, u, and o were defined as new transcripts, while genes in the class of u was defined as new genes.

Then we analyzed the differentially expressed genes (DEGs) between the control group and the high NaCl concentration group of the three cultivars. 18 804 DEGs were found between CK group and HS group in cv. QH,

18 488 DEGs were found between CK group and HS group in cv. WD, and 20 172 DEGs were found between CK group and HS group in cv. LD (Fig. 2 Suppl.). In order to show the common genes among the different cultivars, we cross-compared the DEGs and obtained 8 383 genes that were commonly expressed in these three cultivars (Fig. 2 Suppl. and https://pan.baidu.com/s/1_ zUz0sCTo9GWq6eVEPkn3g, password "0000").

To further understand the potential biological functions of the DEGs and the possible pathways they participate in, we annotated them using *GO* and *KEGG* database. According to *GO* analysis, the transcripts were classified into three groups: 20 in biological processes, 13 in cellular components, and 12 in molecular functions. In biological processes, the two most abundant subcategories were metabolic process and cellular process. For cellular components, cell and cell part were the most dominant
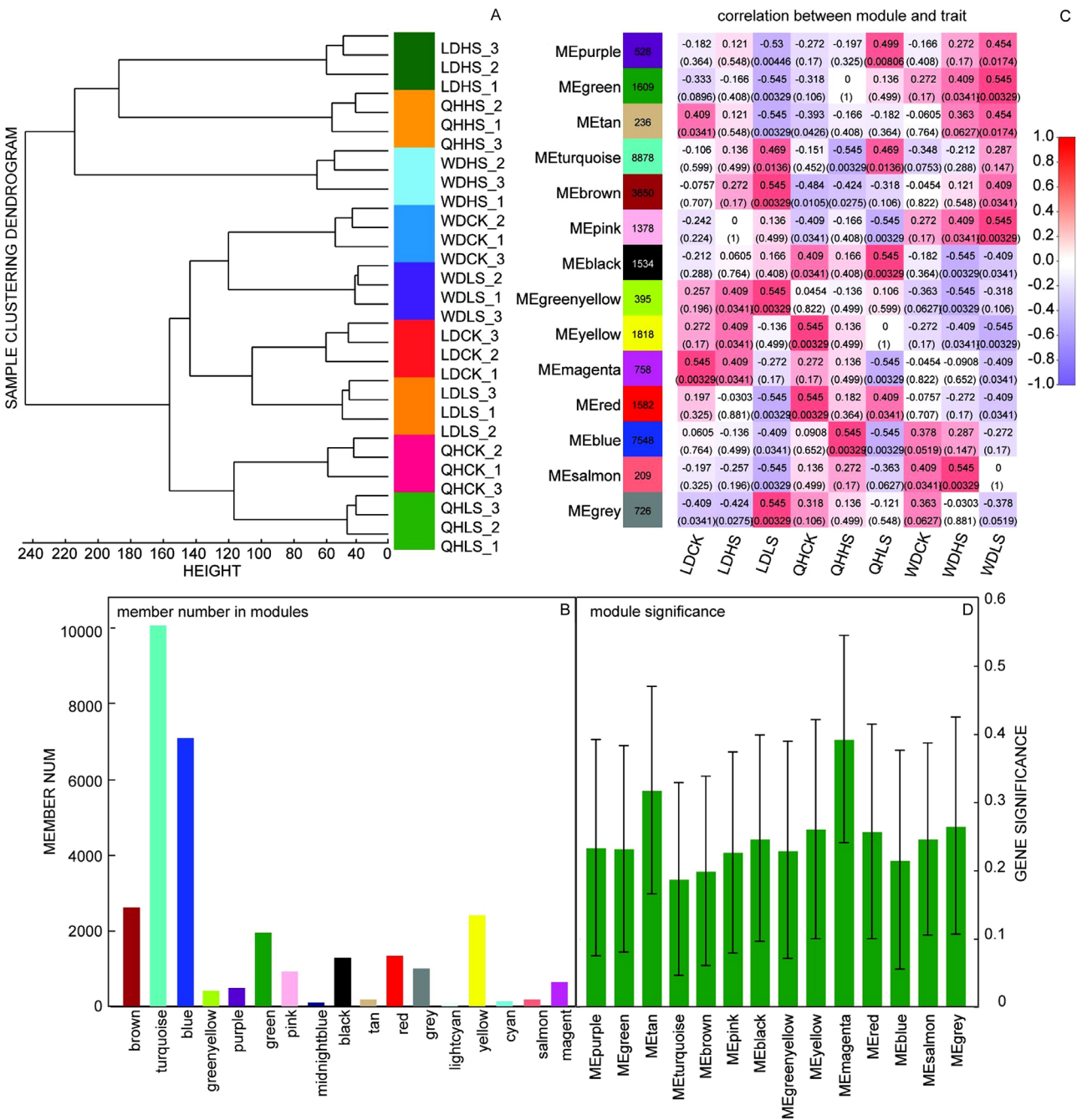
695

Fig. 3. Weighted gene co-expression network analysis. *A* - clustering analysis on all samples; *B* - gene dendrogram and module colors. A height was set to 0.25. *Grey* represents genes that could not cluster to any other modules; *C* - correlation between modules and phenotypes. *Red* represents a positive correlation, and *blue* represents a negative correlation. The *numbers* in the graph are correlation coefficients, and the *values in parentheses* indicate the significance of correlation coefficients; *D* - module significance.

subcategories. With regards to molecular function, the dominant terms were binding and catalytic activity (Fig. 2*A*). As shown in Fig. 2*B*, the DEGs were annotated to five processes based on the *KEGG* database, including cellular processes, environment information processing, genetic information processing, human diseases, metabolism, and organism systems. And the DEGs were significantly enriched in saccharide metabolism, translation, and folding, sorting, and degradation.

Cluster analysis was performed on all samples. As shown in Fig. 3*A*, there were no outliers, and thus the samples were suitable for further analysis. The expression matrix was transformed into adjacency matrix, then transformed into topological matrix. We used average-linkage hierarchical clustering method to cluster genes based on transfer occurrence matrix and set the minimum number of bases for each gene network module according to the standard of dynamic tree cut algorithms (Langfelder
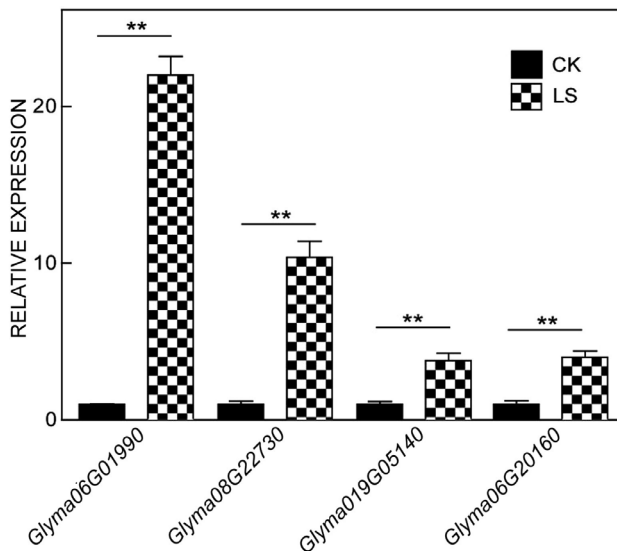
Fig. 4. Verification of gene expression of representative genes by quantitative PCR. Expressions of *Glyma06G01990, Glyma08G22730, Glyma019G05140*, and *Glyma06G20160* of the soybean cultivars treated with 1 dm³ of 0 mM NaCl (CK) and 75 mM NaCl (LS) for 6 h were analyzed by quantitative PCR; the gene expressions were normalized to *actin*. Means ± SDs, *n* = 3, ** - significant differences at *P* < 0.01 determined by Student's *t*-test.

et al. 2008). After the gene modules were determined, we calculated the eigengenes of each module in turn and performed clustering analysis, which merged the closer modules into new modules. Seventeen modules were obtained (Fig. 3*B*). According to the eigenvectors of each module, we calculated the correlation between these modules and the sample phenotypes (Fig. 3*C*). We also calculated the significance of each module and found the *Magenta* module in Fig 3*B* had the most significant correlation with phenotype (Fig. 3*D*). To study the gene interaction in this module, we performed visualization analysis (Fig. 3 Suppl.) and screened top 60 genes with strong connection. By cross-comparing these 60 genes with 8 383 DEGs of the three cultivars, 35 genes were obtained (Table 3 Suppl.), and we speculated they might relate to the soybean growth under salt stress.

From the 35 genes, 10 of the top differently expressed genes were selected for verification. Among them, the expression of *Glyma06G01990, Glyma08G22730, Glyma019G05140*, and *Glyma06G20160* in LS group were 22.02, 10.38, 3.78, and 4.01 times to that in CK group (Fig. 4). The differences in expression of these genes were consistent with the analysis results mentioned above.

Discussion

Salinity is one of the most important environmental factors controlling plant growth and development and affecting the crop yield and quality. Plants were damaged by salt stress through osmotic stress, ionic injury and other processes (Yadav *et al.* 2011). The salt tolerance mechanism of soybean is very complex, belonging to

quantitative traits controlled by multiple genes. However, molecular mechanism of salt tolerance in soybean remains not to be completely clear and many key factors have not been discovered yet. It is particularly important to obtain key salt-tolerant genes for bioengineering research. To this purpose, we studied the gene expression in soybean at transcriptome level and analyzed the DEGs under salt stress.

Different organs of the same plant can be selected for salt-tolerant transcriptome sequencing. Most sampling sites are root (Gu *et al.* 2018), leaf (Yuan *et al.* 2016), and whole seedling (Cui *et al.* 2018). Root system, absorbing water and mineral nutrition from soil, is the first organ exposed to salt stress thus becomes an ideal material for transcriptome analysis in most plant salt tolerance studies (Yu *et al.* 2016). Therefore, we also chose root of soybean as the study object in this experiment. The differential gene analysis of soybean root showed there were 8 383 overlapping DEGs between control group and high NaCl concentration group among the three cultivars. The pathway enrichment analysis of these DEGs revealed that saccharide metabolism was significantly enriched. Saccharides, the product of photosynthesis and the substrate for respiration, provides carbon skeleton and energy for plant growth, and ability to enhance stress resistance (Fettke and Fernie 2015). The synthesis and decomposition of saccharides affect osmotic regulation, which relates to the ability of plants to resist stress environment and helps the membrane maintain its integrity and function (Da Silva and Arrabaca 2004). Several studies have investigated the association between salt stress and saccharide metabolism. The activities of acid and neutral invertase, and especially sucrose synthase, were enhanced under salt stress in tomato seedlings of salt-tolerant cultivars (Khelil *et al.* 2007). Dubey and Singh (1999) found that the activities of sucrose phosphate synthase in salt-sensitive and salt-tolerant rice cultivars were both enhanced under salt stress. In many plants, salt stress increases the content of soluble sugars such as glucose, sucrose, and starch in leaves, and the negative feedback inhibition of the increasing saccharide content is one of the reasons for the decrease of photosynthesis (Badr *et al.* 2004). Our findings in this study provided evidence that genes involved in saccharide metabolism had strong association with salt tolerance. Since soluble sugars are signal molecules for plants to adapt to the environment, this may be an adaptation ability of plants to salt tolerance.

According to the WGCNA analysis results, a total of 35 genes were differentially expressed among the three cultivars and most closely related to phenotype. The most significant DEGs (based on the value of log2fc), *Glyma06G01990*, annotated as "glycosyl transferase family 2", was strongly induced under salt stress in this study. Glycosyl transferase plays a vital role in maintaining cell homeostasis. Photoassimilates need to be catalyzed by glycosyl transferases to convert into disaccharides, oligosaccharides and polysaccharides (Lim *et al.* 2014). Plants produce a large number of secondary metabolites, which are usually modified by glycosylation. Glycosyl transferase participates in plant hypersensitive reaction by

synthesizing some resistant products such as scopoletin glucoside, scopoletin, and betacyanins under environmental stresses (Lim *et al.* 2003). Our finding is consistent with the report that glycosyl transferases genes (*UGT79B2* and *UGT79B3*) were found highly induced by abiotic stresses, including salt, drought and cold stresses (Li *et al.* 2017). *Glyma08G22730*, annotated as "desaturase", was up-regulated under salt stress in this study. Hydrophobic lipids, which act as cell barriers, can prevent many ions and macromolecules from entering cells. Salt tolerance of plants depends on their inherent unsaturated fatty acids and their ability to maintain or adjust unsaturated fatty acids, while salt-sensitive plants showed a decrease in oleic acid under salt stress (Im *et al.* 2002). Maintaining the fluidity of membrane lipids by regulating the activity of desaturase and changing the degree of unsaturation of fatty acids is a characteristic of plant adaptation to stress (Kachroo *et al.* 2001). Our finding suggested that when soybean is exposed to salt stress, the increasing activity of desaturase might have a role in maintaining the integrity and fluidity of the membrane under salinity. The expression of *Glyma03G42140* was induced in response to salt stress. *Glyma03G42140* identified in this study had acyl groups transferase activity, which had been reported to be associated with fertility, oil content, and stress resistance in plant (Gidda *et al.* 2009). Acyltransferase participates in the synthesis of unsaturated fatty acids, which can increase salt tolerance of plants (Gupta *et al.* 2013). Li *et al.* (2007) reported that the permeability of young roots and seed coats in *Arabidopsis* acyltransferase (*GPAT5*) mutants is highly increased, resulting in water loss under high salinity. Our finding provided evidence that the increased expression of acyltransferases might help enhance salt tolerance when plants were exposed to salt stress. *Glyma07G34560,* which was annotated as "cytochrome P_{450}", was upregulated in this study. Similarly, studies have shown that cytochrome P_{450} family genes in *Populus euphratica* and barley were also up-regulated under salt stress (Ueda *et al.* 2002, Gu *et al.* 2004). Cytochrome P_{450} is a multifunctional oxidase widely expressed in plants. It has been reported that selective splicing of genes can be induced by adversity stress, and the expression of gene encoding P_{450} can be regulated in different splicing forms by a variety of endogenous and exogenous inducers (Shi *et al.* 2002). Therefore, the salt tolerance of soybean may be improved by using inducers to elevate the expression of cytochrome P_{450}. *Glyma19G05140*, annotated as "calcium-transporting ATPase", was also highly expressed in response to salt stress. Calcium is not only an essential nutrient element for plants, but also a second messenger that couples extracellular signals with intracellular physiological responses (Wang *et al.* 2012). Studies have shown that the concentration of Ca^{2+} in cells fluctuated, resulting in a series of protective physiological reactions thus alleviating the damage by environmental stress (Yang *et al.* 2010, Wang *et al.* 2019). Plant anabolism is always weakened and catabolism is strengthened under stress conditions. This is because the amount of required energy for growth and development becomes insufficient, resulting in various physiological processes involving Ca^{2+} are affected and

hydrolysis of macromolecules in plants is enhanced (Gao *et al.* 2019). Our results combined with these findings suggest that exogenous calcium could be added to salinization soils to restore the balance of saccharide metabolism and alleviate salt damage. *Glyma06G20160*, which was up-regulated in this study, is annotated with nucleic acid phosphodiester bond hydrolysis activity. The DNA is damaged under various exogenous and endogenous factors such as ionizing radiation, high salt, and cell metabolites, which leads to cell cycle stagnation and apoptosis (Dizdaroglu *et al.* 2002, Chen *et al.* 2018). High concentration of salts could induce DNA double strand breaks in *Arabidopsis* and delayed repair of DNA damage (Cools and De Veylder 2009). In our study, salt stress induced the up-regulation of *Glyma06G20160*, which might cause DNA damage through its endonuclease activity. Therefore, the future study of plant DNA double strand break repair under various stresses will be helpful to enhance the resistance of soybeans.

In summary, three cultivars of soybean were used in this experiment to study DEGs between growth under optimum conditions and salt stress conditions. Based on the gene annotation and pathways enrichment analysis, six genes were considered to be the critical genes involved in the response to salt stress and the saccharide metabolism was the most affected pathway in soybean. This experiment and other studies on salt tolerance of soybean mainly focused on the seedling stage of soybean. However, it has been reported that the salt tolerance mechanism of soybean at different growth stages differs (Chen *et al.* 2018), and salt tolerance in bud stage is also the key to ensure the soybean yield in practice. Moreover, a number of salt-tolerant plants have evolved effective mechanism and corresponding genes in the long-term process of resistance to salt stress, which may be applied to soybean breeding by genetic engineering transformation.

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