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RNA-seq analysis reveals a key role of brassinolide-regulated pathways in NaCl-stressed cotton

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Abstract

Brassinolide (BL) alleviates salt injury in cotton seedlings; however, little is known about the molecular mechanisms of this response. In this study, digital gene expression analysis was performed to better understand the regulatory pathways of BL in NaCl-stressed cotton (*Gossypium hirsutum* L.). Compared with control plants (CK), a total of 1 162 and 7 659 differentially expressed genes (DEGs) were detected in the leaves and roots of NaCl-treated plants, respectively. Most of the DEGs in NaCl-treated plants, compared to CK, were regulated by BL. Moreover, expression patterns of DEGs in BL+NaCl treated plants were similar to those in CK plants; however, the responses of DEGs in the leaves and roots of NaCl-treated plants to BL differed. In the roots, BL-regulated DEGs were involved in protein biosynthesis, whereas in the leaves, BL promoted photosynthesis in NaCl-stressed cotton. BL treatment also significantly increased the overall biomass, chlorophyll $a + b$ content in leaves, and the protein content in roots in NaCl-stressed cotton. The down-regulation of stress-responsive genes in BL+NaCl-stressed leaves was also found. These results suggest that BL can alleviate NaCl injury in cotton plants.

Additional key words: chlorophyll, differentially expressed genes, gene ontology enrichment analysis, photosynthesis, proteins.

Introduction

Brassinosteroids (BRs) are important plant hormones that affect plant growth and development (Wu *et al.* 2008, Divi and Krishna 2009) and ameliorate the effects of biotic and abiotic stresses (Anuradha and Rao 2003, Bajguz and Hayat 2009, Divi and Krishna 2009, Fariduddin *et al.* 2014). The application of brassinolide (BL) changes the activities of antioxidant enzymes in salt-stressed crops (Sharma *et al.* 2013, Shu *et al*. 2015). Furthermore, exogenous application of 24-epibrassinolide in rice alters salt stress responses, which included a decrease in the malondialdehyde content and alterations in the expression of the brassinosteroid receptor (*OsBRI1*) and biosynthesis (*OsDWF4*) genes (Sharma *et al.* 2013). These reports suggest that BRs regulate the growth of salt-stressed plants; however, most of these studies focused on the responses of physiological characteristics.

In recent years, high-throughput DNA sequencing techniques have become a valuable tool dramatically improving the efficiency and speed of gene discovery (Hershkovitz *et al.* 2013). In addition, gene annotations of cotton genome have been recently completed (Wang *et al.* 2012, Li *et al.* 2014, Zhang *et al.* 2015). Several studies have examined the effect of salt stress on cotton at the gene expression level. Yao *et al.* (2011) investigated differentially expressed genes (DEGs) in cotton roots exposed to 100 mM NaCl for 3 h, and Peng *et al.* (2014) generated a global transcription map of genes expressed in salt-stressed leaves. However, few studies have examined the effect of BRs on gene expression of salt-stressed cotton.

Based on previous studies (Shu *et al.* 2015, 2016), the aim of this study was to examine differential gene expression patterns in salt-stressed cotton to determine the regulatory mechanisms of BL-mediated salt tolerance.

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Abbreviations: BL - brassinolide; BRs - brassinosteroids; Chl - chlorophyll; CK - control; DEG - differentially expressed gene; EBR - epibrassinolide; GO - gene ontology; KEGG - Kyoto encyclopedia of genes and genomes; L - leaf; RT-qPCR - reverse transcriptase quantitative polymerase chain reaction; R - root.

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We carefully compared the DEGs among the different treatments, and determined which pathways in salt-stressed cotton were regulated by BL. The findings can improve our understanding of the mechanisms underlying the effect of BL on salt-stressed cotton.

Materials and methods

Plants and growth conditions: Experiments were conducted at Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu, China. Cotton (*Gossypium hirsutum* L.) cv. Sumian 12 was firstly grown in the greenhouse and then uniformly growing seedlings (at 2-leaf stage) were transplanted into vessels filled with Hoagland nutrient solution. The solution was replaced every two days. At the 3-leaf stage, plants were supplied with Hoagland nutrient solution only (control, CK); nutrient solution with 200 mM NaCl (NaCl), or nutrient solution with 200 mM NaCl and 0.1 mM BL (BL+NaCl). Three vessels with 20 seedlings each were used per treatment and arranged in a completely randomized block design. After 15 d, three plants from each treatment group were collected at 08:00, frozen in liquid nitrogen and stored at -70 °C until RNA extraction. An additional five plants were used for analysis of their growth (dry mass was determined after drying at 70 °C).

RNA isolation and quantification: Total RNA was isolated from frozen root and leaf tissues using the cold acidic phenol method with modified extraction buffer [10 mM Tris-HCl (pH 8.0), 25 mM ethylene diamine tetraacetic acid (EDTA), 0.2 % (m/v) cetyltrimethyl ammonium bromide (CTAB), and 2 % (m/v) polyvinyl pyrrolidone (PVP)]. RNA was then precipitated with ethanol, dissolved in diethypyrocarbonate (DEPC)-treated water and stored at -70 °C until use.

Library preparation and digital gene expression sequencing: To obtain complete gene expression information and reduce sequencing costs, pooled RNA samples were obtained from the roots and leaves of CK or NaCl-, and BL+NaCl-treated plants and used to construct a reference library for *de novo* transcriptome sequencing (*Nevogene,* Beijing, China). Six tested samples (each tested sample was a composite of three independent samples) were also used to construct respective libraries for digital gene expression sequencing. Total RNA (3 μg) from each sample was collected for isolation of poly (A) mRNA using *Oligo* (dT) beads. The mRNA was fragmented using a fragmentation buffer and suitable fragments selected for PCR amplification. Finally, PCR products were purified (*AMPure XP* system) and library quality assessed using the *Agilent Bioanalyzer 2100* system (*Agilent*, Beijing, China).

Mapping and quantification of gene expression: *HTSeq* software (www-huber.embl.de/users/anders/HTSeq/) was used to count the number of reads of DEG sequences mapped to the reference transcripts. Gene expressions of each sample were estimated using RNA-seq by expectation maximization (RSEM; Li and Dewey 2011) as follows: *1*) clean data were mapped back onto the assembled transcriptome; *2*) read counts of each gene were obtained from the mapping results. For all comparisons, the read counts were normalized to the aligned reads per kilobase per million (RPKM; Mortazavi *et al.* 2008) to obtain relative expression.

Differential expression analysis and DEG analyses: Prior to differential gene expression analysis, the read counts of each sequenced library were adjusted using the *edgeR* program package through one scaling normalized factor. Differential expression analysis of two samples was performed using the *DEGseq* (Anders and Huber 2010) *R package*. *P*-values were adjusted using the *q* value (Storey and Tibshirani 2003), and a *q* value < 0.005 and $|\log_2(\text{foldchange})| > 1$ were set as the threshold for significantly different expression. Cluster analysis of all treatments was based on the fragments per kilobase of exon per million fragments mapped (FPKM) values of the DEGs, and Venn diagrams were generated using the function "Venn Diagram in R" based on the gene list of each sample.

Gene ontology (GO) enrichment analysis of the DEGs was carried out using the *GOseq R* package based on Wallenius non-central hyper-geometric distribution (Young *et al.* 2010), which adjusts for gene length bias in DEGs. GO with corrected *P*-values less than 0.05 were considered significantly enriched by DEGs.

The Kyoto encyclopedia of genes and genomes (KEGG; Kanehisa *et al.* 2008) database is used to understand functions and utilities of biological systems from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). KEGG orthology based annotation system (*KOBAS*; Mao *et al.* 2005) software was used to test the statistical enrichment of DEGs in KEGG pathways.

Reverse transcription qPCR analysis: First-strand cDNA was synthesized using a *PrimeScript* RT reagent kit with a gDNA eraser (*Takara*, Dalian, China) according to the manufacturer's instructions. Primers are listed in Table 1 Suppl. The RT-qPCR was performed on an *ABI PRISM 7500* real-time PCR system. The reaction mixture (20 mm^3) contained 10 mm^3 of *SYBR Green*

Premix Ex Taq (*Takara*), 1 ng of cDNA sample, 0.2 μM of each gene-specific primer, and 0.4 mm^3 of *ROX Reference Dye II* (50×). The RT-qPCR was carried out with three biological replicates. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with the cotton *actin* gene (AY305733) as an internal reference gene (Kosmas *et al.* 2006).

Determination of soluble protein and chlorophyll content: Frozen root segments (0.5 g) were crushed into a fine powder using a mortar and pestle in an ice bath. Samples were extracted with 5 cm^3 of 0.05 M phosphate buffer (pH 7.8) containing 1 % (m/v) polyvinylpyrrolidone. The homogenates were then centrifuged at 15 000 *g* and 4 °C for 15 min, and the supernatant was used to measure the soluble protein content according to Sharma *et al*. (2013).

Chlorophyll *a*+*b* (Chl) content was determined using the rapid extraction method with an ethanol-acetone mixture (1:1 by volume). Fresh leaves were cut into pieces, and then 0.2-g samples were placed in a 25-cm^3 colorimetric tube for extraction for 18 - 24 h in the dark. The tubes were shaken once every hour. Absorbance was measured with a UV spectrophotometer (*UV-1700, Meixi,* Nanjing, China) and Chl content was calculated according to Liu *et al*. (2014).

Results

To conduct a broad survey of the genes associated with the effect of BL on NaCl-stressed cotton, a mixed RNA sample was prepared from all tested samples and sequenced using the *Illumina* sequencing platform. After cleaning and quality checks, approximately 50.5 million clean reads were obtained from the mixed library. Gaps in the scaffolds were then filled using paired-end reads, giving 93 356 unigenes with a mean size of 730 bp and 20 442 (21.9 %) were greater than 1 000 bp in length (Table 2 Suppl.). Functional annotation of the transcripts was performed based on *BLASTX* homology searches against various public protein databases (Table 3 Suppl.). From the 93 356 of non-redundant unigenes, 58 537 (62.7 %) showed significant similarity to known proteins in the *NCBI* non-redundant protein sequences (NR) database, while 38 490 (41.22 %) had significant hits in the *SWISS-PROT* database. GO (Fig. 1 Suppl.) and KEGG (Fig. 2 Suppl.) analyses helped further our understanding of the biological functions of these genes and their interactions.

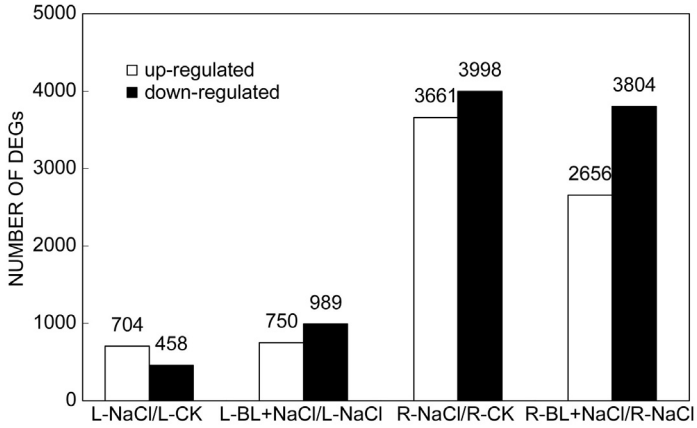

Fig. 1. Analysis of differentially expressed genes (DEGs) in cotton leaves and roots under different treatments. Number of up- and down-regulated DEGs in leaves of NaCl treated plants compared to control plants (L-NaCl/L-CK); in leaves of BL+NaCl treated plants compared to NaCl treated plants (L-BL+NaCl/L-NaCl); in roots of NaCl treated plants compared to CK plants (R-NaCl/R-CK); and in roots of BL+NaCl treated plants compared to NaCl treated plants (R-BL+NaCl/R-NaCl).

Solexa/Illumina digital gene expression analysis was performed to identify the genes involved in BL regulation in the leaves and roots of NaCl-stressed cotton. Six DEG libraries were constructed and sequenced to include the following categories: L-CK (leaves of control plants), L-NaCl (leaves of NaCl-stressed plants), L-BL+NaCl (leaves of BL+NaCl treated plants), R-CK (roots of control plants), R-NaCl (roots of NaCl-stressed plants), and R-BL+NaCl (roots of BL+NaCl treated plants). The number of obtained clean tags was approximately 12 million from each of the six libraries, and 90 % matched to our transcriptome reference database.

To identify the genes in NaCl-stressed cotton that undergo significant changes in response to BL, differentially-expressed tags were analyzed by comparing the NaCl and CK libraries, and BL+NaCl and NaCl libraries. A total of 1 162 and 7 659 DEGs were detected after NaCl treatment in the leaves and roots, respectively (Fig. 1). Moreover, compared to NaCl treatment, a total of 1 739 and 6 460 DEGs were detected in BL+NaCl

treatment, respectively (Fig. 1). These results suggest that the number of root DEGs exceeded that of leaf DEGs. Moreover, the number of down-regulated DEGs in leaves and roots of BL+NaCl-treated plants compared to NaCl (BL+NaCl/NaCl) was higher than the number of up-regulated DEGs.

Cluster analysis of the DEGs revealed similar expression patterns between BL+NaCl treatment and CK (Fig. 3 Suppl.), suggesting that BL had a significant effect on global gene expression profiles in cotton leaves and roots under NaCl stress. However, the expression patterns of the DEGs differed between the leaves and roots.

To understand the functions of the DEGs, all genes were mapped to GO terms in the GO database, to identify significantly enriched terms compared to the reference gene background. Comparisons revealed similar enriched GO terms of the DEGs between NaCl/CK and

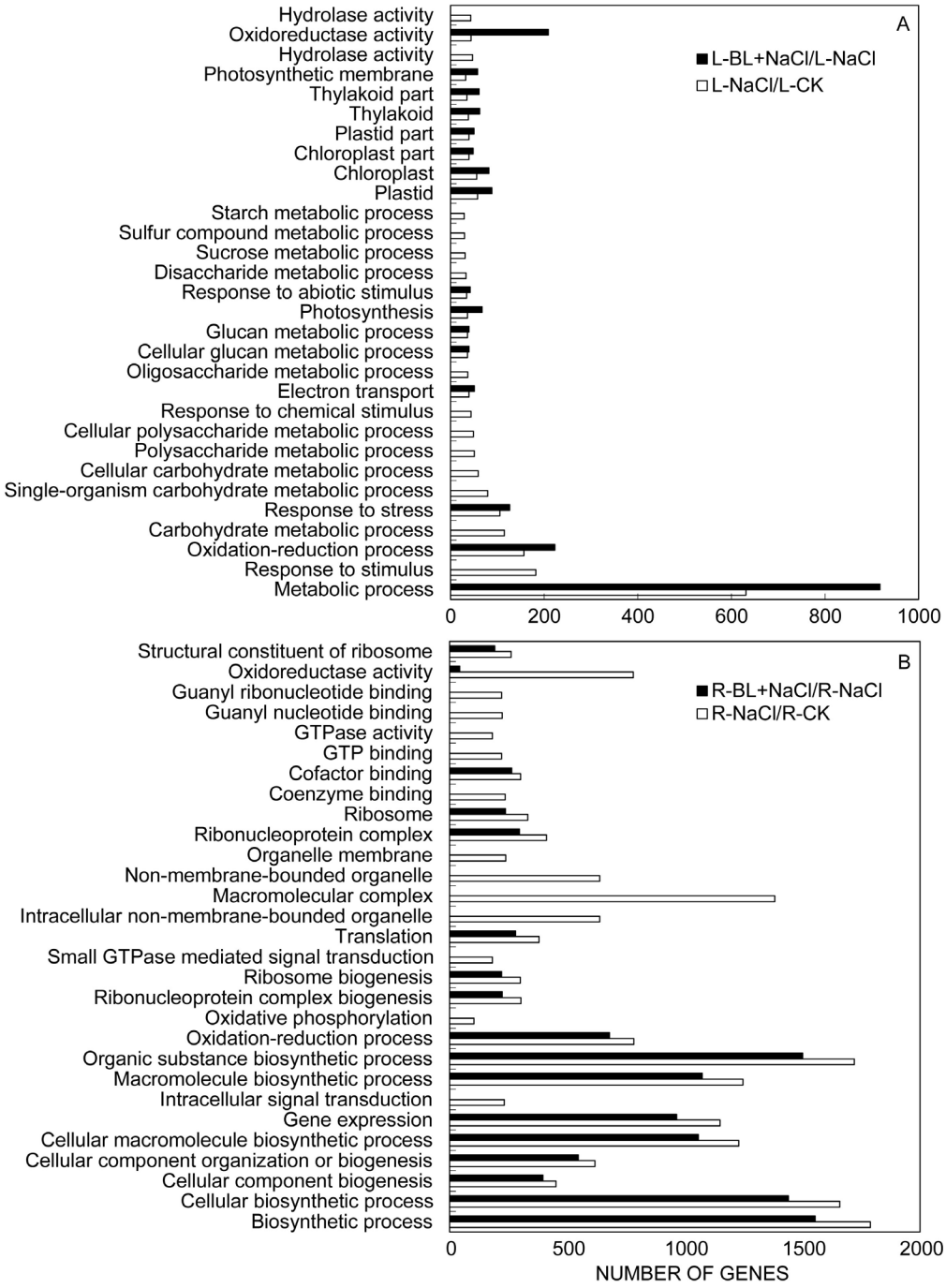

Fig. 2. The enriched gene ontology (GO) terms of differentially expressed genes (DEGs) between BL+ NaCl/NaCl and NaCl/CK in leaves (*A*) and roots (*B*).

BL+NaCl/NaCl. However, the enriched GO terms differed between roots and leaves (Fig. 2). Of the top 30 GO categories of NaCl/CK DEGs in the leaves and roots, more than a half appeared in the enriched GO terms of BL+NaCl/NaCl DEGs, respectively. These findings suggest that the changes in gene expression under NaCl stress might be the result of BL application.

According to KEGG classification, the enriched clusters in the leaves of L-NaCl/L-CK and L-BL+NaCl/L-NaCl were similar, and included "flavonoid biosynthesis", "photosynthesis", and "photosynthesis-antenna proteins". In the roots, the enriched clusters of R-NaCl/R-CK and R-BL+NaCl/R-NaCl included "MAPK signaling pathway-yeast", "proteasome" and "oxidative phosphorylation". These results further suggest that the BL response pathways differ between NaCl-stressed cotton leaves and roots.

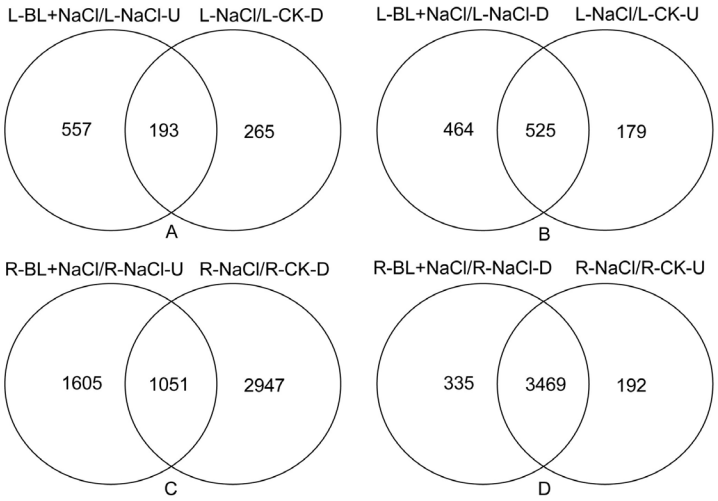

Fig. 3. Venn diagrams of differentially expressed genes (DEGs) in cotton leaves and roots among different treatments. Number of up- (U) and down-regulated (D) genes in leaves and roots of BL+NaCl treated plants compared to NaCl treated plants (L-/R-BL+NaCl/NaCl-U/D) and in NaCl treated plants compared to the CK (L-/R-NaCl/CK-U/D).

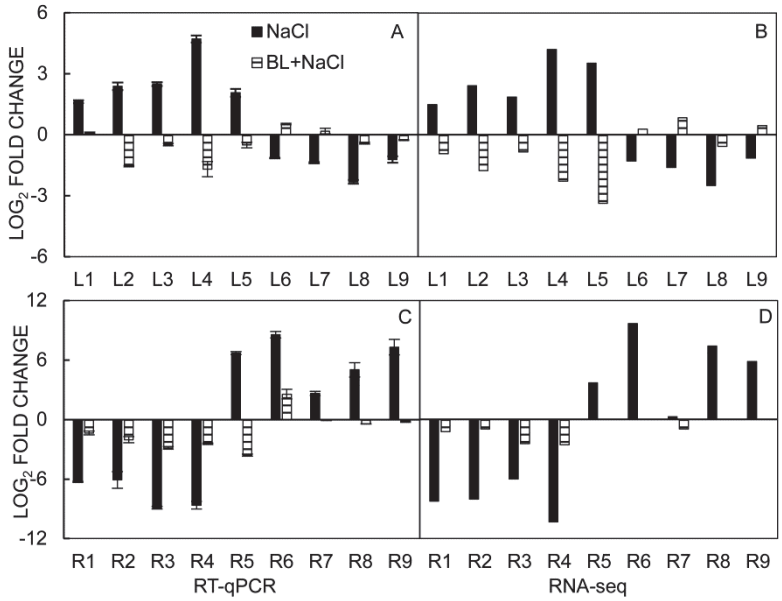

Fig. 4. Validation of expression patterns of nine representative unigenes observed in cotton leaves L1 - L9 (*A,B*) and roots R1 - R9 (*C,D*) by RT-qPCR. Relative expressions were calculated using *actin* as an internal control. Log_2 fold changes were calculated by comparing expression in NaCl or BL+NaCl treated plants with control plants. Means ± SDs, $n = 3$.

Specific DEG responses to exogenous BL in the leaves and roots under NaCl stress were presented using Venn diagrams (Fig. 3). In root and leaf tissues, a large number of DEGs overlapped between NaCl/CK and BL+NaCl/NaCl. In the roots, the number of overlapping genes between up-regulated genes of R-BL+NaCl/

R-NaCl and down-regulated genes of R-NaCl/R-CK was 1 051 (about 26 % of the 3 998 down-regulated genes of R-NaCl/R-CK). The most represented GO terms of these 1 051 genes were "cellular component biogenesis", "ribonucleoprotein complex", "structural molecule activity", "translation" and "ribosome" (Table 4 Suppl.). In addition, of the 3 661 up-regulated genes of R-NaCl/R-CK, 3 469 (95 %) overlapped with the down-regulated genes of R-BL+NaCl/R-NaCl, and the most represented GO terms were "ribonucleoprotein complex", "structural molecule activity", "translation", "ribosome" and "ribonucleoprotein complex biogenesis" (Table 4 Suppl.).

In the leaves, the number of overlapping genes between the up-regulated genes of L-BL+NaCl/L-NaCl and down-regulated genes of L-NaCl/L-CK was 193 (about 42 % of the 458 down-regulated genes of L-NaCl/L-CK). The most represented GO terms of these 193 genes were "metabolic process", "thylakoid part" "thylakoid", "photosynthetic membrane", and "photosynthesis". Of the 704 up-regulated genes of L-NaCl/L-CK, 525 (75 %) overlapped with the down-regulated genes of L-BL+NaCl/NaCl, and the most represented GO terms were "response to stimulus", "response to stress", "response to abiotic stimulus", "response to oxygen-containing compound", and "response to inorganic substance".

To confirm the reliability of the RNA-Seq data, RT-qPCR was performed with the same RNA pools used for next-generation sequencing. A total of 18 genes, nine from the leaves and nine from the roots, were selected for RT-qPCR analysis. Representative genes were over-lapping genes regulated by NaCl and BL. The expression profiles observed in RT-qPCR matched well with those in the RNA-Seq data (Fig. 4).

Table 1. Dry matter of whole plants [g], chlorophyll (Chl *a*+*b*) content of leaves [mg g^{-1}(f.m.)], and protein content of roots [mg g^{-1}(f.m.)] in control plants (CK), plants treated with 200 mM NaCl, or 200 mM NaCl + 0.1 mM brassinolide (BL). Means ± SDs, n = 3; different letters indicate significant differences at $P < 0.05$.

Treatments	Dry matter	Chl *a*+*b*	Protein content
CK	0.423±0.014a	1.24±0.07a	5.32±0.24a
NaCl	0.187±0.011c	0.77±0.01c	2.63±0.22c
BL+NaCl	0.343±0.009b	1.05±0.05b	4.20±0.26b

BL treatment significantly increased biomass accumulation in NaCl-stressed cotton plants compared to NaCl treatment alone. Moreover, BL+NaCl plants showed an increase in dry mass by 83 %, although this was lower than that of the CK plants (Table 1). BL also resulted in a significant increase in Chl *a*+*b* content under NaCl treatment (Table 1). Furthermore, compared to NaCl treatment, BL+NaCl treatment resulted in a 91 % increase in the protein content in the roots (Table 1).

Discussion

Salt stress affects the growth and development of cotton plants. Previous studies on salt stress have focused on the transcriptomes of cotton leaves and roots, or mixed leaf and root responses. Peng *et al.* (2014) identified DEGs in cotton leaves at 4 and 24 h after 200 mM salt stress, and revealed enriched GO terms including "response to stress", "regulation of biological process", and "transcription factor activity". Yao *et al.* (2011) reported enriched GO terms of DEGs in cotton roots exposed to 100 mM NaCl for 3 h. They included "response to water stress", "hormone metabolism" and "signal transduction". The enriched GO terms differ between leaves and roots of NaCl-stressed cotton. So, we studied the response of cotton roots and leaves to salt stress, and found some significant differences between them. A higher number of distinct and clean tags were observed in the roots compared to the leaves. Moreover, whereas enriched GO terms of DEGs in the leaves included "metabolism process", "response to stimulus" and "oxidation reduction process", in the roots they included "biosynthetic process", "cellular biosynthetic process" and "organic substance biosynthetic process". These findings confirmed the different responses of cotton roots and leaves to salt stress. However, the DEGs of NaCl/CK observed in this study were not associated with those described in previous studies (Yao *et al.* 2011, Xu *et al.* 2013, Peng *et al.* 2014). The overall response to salt stress in plants involves stress sensing, signaling, recovery, and adaptation. Xu *et al.* (2013) suggested that the genes identified 6 d after salt stress exposure were involved in acclimation or stress resistance in the steady-state. In this study, the enriched GO terms of DEGs indicated that the leaves and roots of salt-stressed cotton may be in different response processes; the leaves were in recovery, while the roots might be in the adaptation state.

Cotton plants treated with BL applied to roots undergo an increase in physiological functions to improve biomass (Shu *et al.* 2015). BL treatment has also been shown to increase the expression of genes involved in various physiological responses in species such as *Arabidopsis thaliana* (Clouse and Sasse 1998), *Brassica napus* (Dhaubhadel and Krishna 2008), and *Solanum lycopersicum* (Goetz *et al.* 2000). Studies of BL-induced gene expression under salt-stress conditions will therefore provide further insight into how BL mediates stress tolerance. The results of DEG analysis indicated similar expressions in BL+NaCl and CK plants, with most up- and down-regulated genes from NaCl/CK being

regulated by BL. This finding suggests that BL regulates gene expression in salt-stressed cotton plants.

To determine which pathways were regulated by BL, functional annotations of the DEGs were performed using the GO and KEGG databases. DEGs of NaCl/CK in the roots were found to be regulated by BL and the top five enriched GO terms of up- and down-regulated genes were similar. KEGG results (data not shown) indicated that BL up-regulated DEGs of the ribosome pathway in NaCl-stressed cotton roots, and down-regulated those of the proteasome pathway. The ribosome is a large complex molecular machine found in all living cells, serving as the site of biological protein synthesis (translation). Therefore, the findings suggest that BL enhanced protein biosynthesis in salt-stressed cotton roots by altering the expression of genes involved in GO terms "ribonucleo-protein complex", "translation", and "ribosome". Meanwhile, the main function of the proteasome is to degrade unneeded or damaged proteins. Therefore, the results further suggest that BL decreases the numbers of damaged proteins in salt-stressed cotton roots. In addition, the enriched GO terms "cellular component biogenesis" and "structural molecule activity" suggest that BL alleviates NaCl injury to cell structure in cotton roots. Roots supply water and inorganic nutrients to the plant, and are the first organ to contact with salinity. Enhanced adaptation of cotton roots to salt stress might therefore be the reason for the increased biomass after BL treatment.

Approximately 75 % of the 704 upregulated genes of L-NaCl/L-CK were reduced by BL, and the most representative GO terms were related to stress response pathways. These findings suggest that BL regulated the expression of stress-responsive genes in salt-stressed cotton leaves, indicating one possible reason for BL-induced alleviation of NaCl injury in cotton (Shu *et al.* 2015). In the present study, the most representative GO terms of the 193 BL-mediated down-regulated genes of NaCl/CK in the leaves were related to "thylakoid function", "photosynthetic membranes", and "photosynthesis". The thylakoid membrane is a highly dynamic system that reacts with environmental factors and it is involved in adaptation, thereby playing an intrinsic role in plant plasticity. BRs control thylakoid architecture, and an optimal BR content is required for the maintenance of thylakoid structure and function (Krumova *et al.* 2013). Dobrikova *et al.* (2014) revealed alterations in the structural organization of the thylakoid membranes after EBR treatment, which in turn increased the effective quantum yield of photosystem II and photochemical quenching. The EBR-induced changes in the photosynthetic membranes are most probably involved in stress tolerance in plants. In this study, BL treatment also resulted in an increase in the Chl *a+b* content in salt-stressed cotton leaves, reflecting an increase in photosynthetic capacity. These results suggest that BL regulates photosynthesis in salt-stressed cotton *via* changes in expression of genes related to GO terms "thylakoid part", "thylakoid", "photosynthetic membrane", and "photosynthesis". These results are also consistent with previous studies (Janeczko *et al.* 2011, Sharma *et al.* 2013), where BRs alleviate the effects of various stresses on plant growth by improving photosynthesis in the leaves.

Overall, our RNA-seq data show that BL alleviated NaCl injury in cotton, which in turn is attributable to changes in expression of genes related to protein synthesis in the roots and photosynthesis and stress-responsive processes in the leaves (Fig. 4 Suppl.). These findings will contribute to our understanding of BL-regulated responses in NaCl-stressed cotton.

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