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Identification of *EXPA4* as a key gene in cotton salt stress adaptation through transcriptomic and coexpression network analysis of root tip protoplasts

Qiankun Liu[1,2†], Pengtao Li[2†], Muhammad Jawad Umer[1†], Mubashir Abbas[1], Yongqing Zhao[2,4], Yu Chen[3], Yanfang Li[2,4], Aiming Zhang[2,4], Yuling Liu[2], Yangyang Wei[2], Quanwei Lu[2], Mengying Yang[5], Yiman Liu[5], Xiaoyan Cai[1], Zhongli Zhou[1], Shuxun Yu[1*], Fang Liu[1,6*] and Renhai Peng[1,2*]

Abstract

Background Salinity stress impairs cotton growth and fiber quality. Protoplasts enable elucidation of early salt-responsive signaling. Elucidating crop tolerance mechanisms that ameliorate these diverse salinity-induced stresses is key for improving agricultural productivity under saline conditions.

Results Herein, we performed transcriptome profiling of *Gossypium arboreum* root tips and root tips-derived protoplasts to uncover salt tolerance genes and mechanisms. Differentially expressed genes (DEGs) were significantly enriched in the plant hormone signal transduction and MAPK signaling pathways. Transcriptome based weighted gene coexpression network analysis (WGCNA) clustered 885 commonly differentially expressed genes into four distinct modules. Black and yellow modules were highly upregulated under salt treatment, containing hub genes integral to signaling and transport, highlighting their importance. Differential expression analysis revealed more dynamic changes in protoplasts, identifying key genes including the *Ga-α-expansin 4* (*GaEXPA4)*. Silencing of the *GaEXPA4* gene through virus-induced gene silencing heightened cotton's sensitivity to salt stress, leading to increased wilting, elevated lipid peroxidation, and impaired antioxidant activity under salt conditions compared to controls.

Conclusion These findings underscore the functional significance of *GaEXPA4* in the salt stress response. Future research should focus on elucidating the precise mechanisms of putative salt tolerance genes like *GaEXPA4* and evaluating the potential of signaling pathways, such as MAPK, for engineering enhanced salt resilience in cotton.

†Qiankun Liu, Pengtao Li and Muhammad Jawad Umer contributed equally to this work.

*Correspondence:
Shuxun Yu
Ysx195311@163.com
Fang Liu
liufcri@163.com
Renhai Peng
aydxprh@163.com

Full list of author information is available at the end of the article

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Integrating multi-omics approaches could further expand the genetic resources available for improving cotton cultivation in saline environments.

Keywords Cotton, Salt stress, Transcriptome, Protoplast dissociation, *EXPA4* gene

Background

Soil salinity, drought, and temperature extremes are key abiotic stresses that sessile plants must cope with. As many crops demonstrate limited salt tolerance [1], salinity poses a major constraint on crop yield and agricultural development. To understand plant responses to salt stress, it is important to consider the primary and secondary stress signals induced by high soil salinity. High salinity imposes both osmotic and ionic stresses on plant cells [1]. The osmotic stress component results from a reduction in the soil water potential, while the ionic stress arises from the accumulation of potentially toxic ions. Additionally, secondary effects of salinity further challenge plant health through oxidative damage to cell structures like membranes, proteins, and nucleic acids. High salinity also disrupts central plant metabolic processes. Taken together, the combination of osmotic, ionic, and secondary stresses makes salinity a complex physiological challenge for crop plants to overcome. Elucidating crop tolerance mechanisms that ameliorate these diverse salinity-induced stresses is key for improving agricultural productivity under saline conditions.

To survive salt stress, plants have evolved mechanisms to maintain cellular ion balance, including reducing Na^+ uptake and increasing K^+ absorption [2]. The plant primary cell wall, composed of cellulose, hemicelluloses like xyloglucans and arabinoxylans, and pectins, provides structural support but is also impacted by salt stress [2]. Salinity induces reactive oxygen species (ROS) accumulation and alters cell wall properties. Specifically, ROS cause cross-linking of wall-bound phenolics and glycoproteins, rigidifying the wall. Separately, salinity can induce expression of cell wall remodeling proteins like expansins and xyloglucan modifying enzymes, further altering the wall [2]. Ca^{2+} levels similarly shift. Ultimately, cell wall properties strongly influence plant stress adaptation. For instance, in *Arabidopsis thaliana*, mutations in pectin biosynthesis genes like *AtCSLD5* confer oxidative, osmotic, drought and salt stress sensitivity, due to impaired cell wall function [3]. Thus, the cell wall is integral for salt stress survival. At present, there have been many transcriptome studies on plant responses to different stress treatments, such as sugarcane response to cold stress [4], poplar response to salt stress [5], rice response to salt stress [6], and the transcriptome study on the increase of Ca^{2+} ions in the germination stage of soybeans [6]. Transcriptome analysis showed that silencing of *GhERF4L* and *GhERF54L* significantly reduced the salt tolerance of cotton seedlings, suggesting that *GhERF54L* regulates the response of cotton to salt stress [7].

Cotton is an economically vital crop grown for its fiber, a key raw material in the textile industry and a staple of clothing and fabrics. As a pioneer salt tolerant species, cotton is cultivated even under increasingly extreme climate conditions and land shortages. However, developing cotton varieties tailored to unique regional environments and saline soils remains crucial. With advances in elucidating cotton gene function, several genes that confer cotton salt tolerance have been uncovered, facilitating targeted breeding efforts. Fundamental salt tolerance mechanisms found in other plants, including ion homeostasis, osmotic adjustment, and reactive oxygen species (ROS) detoxification, are conserved in cotton. Examples include the ion transporters *GhSOS1* [8] and *GhNHX1* [9], which maintain ion balances, and dehydrins like *GhDHN03* [10] and *GhDHN04* [10], which provide osmoprotection. Further mining the cotton genome for novel tolerance alleles will be key to sustaining production under marginal conditions. In addition, transcription factors and protein kinases of cotton play important roles in regulating growth and development, such as *GhERF54L* [7], *GhWRKY17* [11], *GhTOM* [12] and *GhMAPK* [13]. All these genes or proteins have been shown to be salt-tolerant in *G. hirsutum*. At present, a series of salt-tolerant genes have also been screened out in *G. arboreum*. A genome-wide analysis of the MATE (Multidrug and toxic compound extrusion) gene family in *G. arboretum* was conducted, and evaluated its expression levels under salt, cadmium and drought stress. Sixty-eight MATE genes were identified in *G. arboreum*, and the relationship between salt, drought and cadmium stress was revealed by GO annotation. Under drought, salt stress and cadmium stress, the expressions of *GaMATE41* and *GaMATE51* genes are significantly upregulated and can be used as candidate genes for salt tolerance [14].

The *α-expansin 4* (*EXPA4*) gene, a vital player in cell wall loosening proteins, significantly influences plant growth, development, and responses to environmental stress. In tobacco, manipulating *EXPA4* expression impacts susceptibility to tobacco mosaic virus (TMV-GFP), revealing its pivotal role in growth and development. *EXPA4* RNA interference mutants exhibit heightened sensitivity to salt and drought stress, while overexpression enhances tolerance, reduces cell damage, increases fresh weight, and induces elevated soluble sugar and proline accumulation. Moreover, overexpressed lines

show increased sensitivity to the viral pathogen TMV-GFP, emphasizing central role of *EXPA4* in both abiotic stress response and defense against pathogens in tobacco [15].

Protoplast refers to the general name of various structures within the cell wall, and is also a morphological structural unit of the cell, where various metabolic activities in living cells are carried out. At present, high-quality protoplasts have been prepared from the root tip, whole root, stem tip and leaf of model plant *Arabidopsis* [16–22] and other tissue parts, and the selected enzymatic hydrolysates mainly include cellulase, hemicellulase, macerase and pectinase. Different proportions of these dissociating solutions provided some reference value for selecting the proportion of enzymatic hydrolysate in the corresponding tissue parts of other plants. In addition, high-quality protoplasts can also be prepared from rice root tips [19, 23], corn stem tips [24] and maize root tips [25], peanut leaves [26] and poplar xylem [27], and have been applied to single-cell transcriptome sequencing. Protoplasts play a significant role in crop genetic improvement. Due to the existence of distant hybridization incompatibility among different species, protoplast fusion can overcome this characteristic of distant hybridization incompatibility, improve the genetic traits of crops, and provide a new foundation for cultivating crops with excellent economic traits. Protoplasts are also utilized in the study of gene function and subcellular localization. By fusing the target protein gene with a detectable reporter gene, a fusion expression vector is constructed and introduced into protoplasts through PEG-mediated transient transformation of protoplasts. The fusion protein is transiently expressed in protoplasts, and the expression location of the target gene protein is determined through the localization of the reporter gene expression. The commonly used reporter genes are mainly green fluorescent protein (GFP) and other colored fluorescent proteins (such as red fluorescent protein, RFP). After culturing the fluorescent proteins in protoplasts for a period of time, the location of the corresponding fluorescence can be observed under the corresponding wavelength using a laser scanning confocal microscope.

In this investigation, we employed RNA-seq to scrutinize the salt stress response in *G. arboreum* root tip tissue, both before and after dissociation into protoplasts. Employing GO and KEGG analyses of differentially expressed genes, and a weighted gene coexpression network analysis (WGCNA), we pinpointed key genes and signaling pathways implicated in the salt stress response. Notably, under salt stress conditions, the *GaEXPA4* gene exhibited heightened expression levels, as corroborated by both RNA-seq data and RT-qPCR analysis. Furthermore, we utilized virus-induced gene silencing to knock out *GaEXPA4* in cotton, elucidating the underlying mechanisms contributing to salt stress tolerance in this context. This research significantly advances our comprehension of the intricate pathways governing salt responsiveness in cotton.

Materials and methods

Plant materials, growth conditions and salt stress treatment

In this study, the *G. arboreum* L. accession Shixiya1 (SXY1) was used for transcriptome sequencing. Tissue culture was used to obtain young cotton root tip tissues and root tip protoplasts. Healthy seeds of *G. arboreum* L. were selected, sterilized with 0.1% mercury for 10 min, and then washed with sterilized water for 5 times, 3 min each time. Next, the sterilized and cleaned seeds were placed on MS solid medium for growth under the conditions of 28℃, 16 h light culture /26℃, 8 h dark culture. After the seedlings grew on MS medium for one day and one night, the roots of the seedlings were inserted into the medium on the ultra-clean table, and the seedlings were allowed to grow for 5 days before salt stress treatment. Tissue culture seedlings were randomly divided into six groups, among which one group was selected as the non-salt stress control group, and the other two groups were treated with 60mL MS liquid medium containing 100 mM NaCl for 0.5 h and 1 h, respectively. Then the lateral root tips (0.5–1 cm) of the control group and the treatment group were selected and put in liquid nitrogen for further RNA extraction. The other three groups were treated in the same way as the first three groups. The difference was that protoplasts were extracted from the root tip tissues after treatment. The enzymolysis, counting and viability of protoplasts (1.5% cellulase R10, 1% pectolyase, 0.4 M mannitol, 0.1 M KCl, 0.08 M MES, 0.02 M $CaCl_2$ and 0.1% BSA) and the extraction was performed as described in our previous study [28].

In the VIGS experiment, the *G. arboretum* L. accession Shixiya1 (SXY1) obtained from Chinese Academy of Agricultural Sciences (Anyang, Henan, China) was also used as an experimental material, and the carrier was TRV2. The seeds were soaked overnight, sown in sand for germination, and allowed to germinate for six days before being transplanted into hydroponic tanks containing Hoagland solution. Seedlings growth conditions were 28℃ in daytime and 25℃ at night, with light/dark cycles of 16 h/8 h and relative humidity of 60–70%. At the three true leaves stage, the plants were moved to Hoagland nutrient solution with 200 mM NaCl for salt stress treatment, and sampling was performed at 0 and 48 h after salt stress treatment. Immediately after sampling, leaf samples were put in liquid nitrogen and stored in a − 80℃ freezer till further use.

RNA extraction, cDNA library construction and sequencing

Due to the relatively smaller number of axial roots, the 0.5–1 cm region of lateral root tips on the 5th day were collected for protoplast isolation. The RNAprep Plant kit is provided by TianGen Biotechnology and performed RNA extraction according to the instructions.

The quality of RNA samples was evaluated with a NanoDrop 2000 spectrophotometer. The RNA samples that fulfilled the quality criteria ($OD_{260/230} \geq 2.0$, $OD_{260/280}$ = 1.8–2.1, RIN ≥ 7.0) were used to construct libraries and then sequencing [29]. Sequencing was completed at Shanghai OE Biotech (Shanghai, China). Finally, Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) sequencing platform was utilized for RNA-seq on each cDNA library [30].

Identification and analysis of differentially expresses genes (DEGs)

The raw data was processed by Trimmomatic (version 0.36). After removing low quality sequences, the clean reads were mapped to the *G. arboreum* reference genome [31] using HISAT2 version 2.2.1.0 [32]. The fragments per kb per million reads (FPKM) was used to normalize and represent the gene expression. The differentially expressed genes (DEGs) were certified by the cufflinks (version 2.2.1) [33–36]. Finally, enrichment analyses were carried on identifying the DEGs which was aimed at investigating the key functional genes or signal pathways regulating cotton root response to salt stress. The gene function annotation was analyzed via Gene Ontology analysis (GO, http://geneontology.org/) [37, 38] and Kyoto Encyclopedia of Genes and Genomes analysis (KEGG, https://www.genome.jp/kegg/) [39, 40]. The raw RNA-seq data were deposited in the National Center for Biotechnology Information (NCBI).

Expression dynamics via quantitative real-time PCR (qRT-PCR)

Collected root samples were used for RNA extraction and then were reverse-transcribed into cDNA by TransScript-All-in-One First-Strand cDNA Synthesis SuperMix (TransGen Biotech kit, Beijing, China) and ChamQ Universal SYBR qPCR Master Mix (Vazyne, Nanjing, China) for RT-qPCR. An ABI 7500 qRT-PCR system (Applied Biosystems, Foster City, CA, USA) was used for the reaction. The cotton *Ubiquitin7* gene (UB7, GenBank: DQ116441) was used as a reference gene [41]. The relative expression level of targeted genes were evaluated by the $2^{-\triangle\triangle Ct}$ method [42]. Three biological and technical replicates were collected for qRT-PCR.

Bioinformatics analysis of *GaEXPA4*

The conserved domains of the *GaEXPA4* protein were predicted by NCBI-CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd) and InterPro (https://www.ebi.ac.uk/interpro/), and the motifs within *GaEXPA4* were predicted using MEME. The analysis of the gene structure of *GaEXPA4* and the visualization of the results were processed by TBtools (TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data.). The physicochemical properties and secondary structure of *GaEXPA4* were analyzed using ProtParam (http://web.expasy.org/protparam/) and SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl), respectively. SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict signal peptides. The transmembrane structure of *GaEXPA4* was analyzed using TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). SWISS-MODEL (http://swissmodel.expasy.org/) was employed to predict the tertiary structure of the protein. BLAST tools from Phytozome (https://phytozome-next.jgi.doe.gov/) and TAIR (https://www.arabidopsis.org/) were used to obtain homologous sequences from other species, and Jalview was utilized for multiple sequence alignment and visualization. Finally, the phylogenetic tree was constructed using MEGA11 software.

Validation of *GaEXPA4* by virus inducted gene silencing in cotton under salt stress

Using cDNA as a template, *GaEXPA4* was amplified according to specific primers (forward: GTGAGTAAG GTTACCGAATTCAATCGCCGAGTACCGTGC and reverse: CGTGAGCTCGGTACCGGATCCGCCACGT TCAAGGAAGTG). The VIGS vector construction was made by the double enzyme cutting method and its sites was *Bam*HI and *Eco*RI [43]. The required bacterial solutions were TRV:*GaEXPA4*, TRV: PDS and empty TRV:00. Cotton seeds were grown on filter paper for three days, transferred to 1/2 Hoagland nutrient solution for hydroponics, and prepared to be injected with Agrobacterium when the cotyledon was horizontally spread. The Agrobacterium solution OD600 concentration is about 1.5. After injecting the cotyledon, the cotton was cultured under dark conditions for 24 h. Later, cotton was exposed to normal growth conditions [43]. After gene silencing, plants were growth to three leaves for treatment, using 1/2 Hoagland nutrient solution containing 200 mM NaCl to simulate salt treatment. As for the data processing part, firstly, the gene expression level was analyzed, and three experimental replicates and three technical replicates were performed respectively in the control group and the silent group. Secondly, the phenotype changes and the degree of leaf wilting were photographed after salt stress treatment, and physiological and biochemical indices such as chlorophyll content, SOD (superoxide dismutase) and MDA (malondialdehyde content) of leaves were measured. Three biological replicates were

collected for measurements and the experiment was repeated three times.

Results

Isolation of root tip protoplasts and viability assessment under salt stress in cotton

Previous studies have demonstrated that the duration of salt treatment on cotton root tips can significantly affect the viability of protoplasts obtained through dissociation [28]. This study compared protoplast yield and viability after 0, 0.5 h, and 1 h of 100 mM NaCl treatment (Additional file 1: Fig. S1A). The control group received no salt treatment. Protoplasts were obtained by enzymatic dissociation of root tips and viability was assessed (Additional file 1: Fig. S1B and S1C). Statistical analysis found that protoplast yield was similar across groups, but viability decreased with longer salt treatments (Additional file 1: Fig. S1D and S1E). Specifically, control protoplasts had 89% viability and 2.26×10^6/mL yield (Additional file 1: Fig. S1D). After 0.5 h and 1 h of treatment, viability dropped to 82.79% and 80.48%, respectively, while yield remained at 2.2×10^6/mL. Since protoplast viability from 0.5 h to 1 h treatments met transcriptome sequencing requirements, these conditions were selected for further study. In summary, 0.5 h and 1 h of 100 mM NaCl treatment reduced cotton root tip protoplast viability while maintaining adequate yield for transcriptomics.

Comprehensive transcriptome profiling of cotton roots under salt stress

To determine the molecular response of *G. arboretum* to salt stress and differences before and after protoplast isolation, transcriptomes were sequenced from 18 libraries with 3 biological replicates at 3 time points (0, 0.5, and 1 h after salt treatment). In total, 890.77 million raw reads were generated by RNA-seq, averaging 49.49 million reads per library (Additional file 2: Table S1) with 44.29–47.26% GC content. After filtering, 871.41 million clean reads remained, averaging 48.41 million per library, with > 91.85% of bases having Q30 quality, indicating reliable data.

Mapped reads in fresh roots and protoplasts under normal conditions reached 98.27–98.37% and 87.79–95.36% alignment rates, respectively. Under salt stress, these alignment rates were 96.83–98.62% in roots and 80.74–96.57% in protoplasts. Both quantity and quality of RNA-seq data were higher in non-salt treated versus salt-treated samples. Similarly, data quality was better before versus after protoplast isolation. This suggests that both salt stress and protoplast isolation negatively impact *G. arboretum* gene expression and growth.

Identification of differentially expressed genes (DEGs) between roots and protoplasts

To further confirm the reproducibility among biological replicates, effectively identify the relationships between key genes and samples, and ensure the reproducibility of experimental operations and the reliability of experimental results, PCA (Principal Component Analysis) and Spearman correlation plots were generated for the samples. The results further demonstrated good reproducibility of the samples (Additional file 1: Fig. S2). To investigate dynamic changes between roots and protoplasts under salt treatment versus control conditions, differentially expressed genes (DEGs) were identified (fold change ≥ 2) under various comparisons: A_0.5-vs-A: Protoplasts after 0.5 h salt vs 1 h salt, B_0.5-vs-B: Roots after 0.5 h salt vs 1 h salt, A_1-vs-A: Protoplasts after 1 h salt vs 1 h control, B_1-vs-B: Roots after 1 h salt vs 1 h control, A_1-vs- A_0.5: Protoplasts after 1 h vs 0.5 h salt, B_1-vs-B_0.5: Roots after 1 h vs 0.5 h salt.

The number of DEGs differed under various salt conditions (Fig. 1A). Overall, more DEGs were identified from protoplasts than intact roots after salt treatment. Specifically, 10,875, 9,584, and 375 DEGs were found for A_0.5-vs-A, A_1-vs-A, and A_1-vs-A_0.5, respectively, while only 2,678, 3,882, and 1,440 DEGs were found for the respective root comparisons (Fig. 1A). With longer salt treatment of protoplasts, the number of up-regulated DEGs increased while down-regulated DEGs decreased over time. A total of 7,811 DEGs overlapped between A_0.5-vs-A and A_1-vs-A, while 1,814 DEGs overlapped between the respective root comparisons (Fig. 1B). These results indicate more dynamic gene expression changes in protoplasts than intact roots after salt stress. The number of common differential genes in the four combinations was 885 (Fig. 1B).

To further analyze the expression patterns of DEGs from a global perspective, volcano plot and heatmaps were generated using the common DEGs from each comparison (Additional file 1: Fig. S2 and S3). The heatmaps showed distinct expression profiles of DEGs between salt-treated and control tissue groups, validating the DEG screening approach. GO enrichment analysis was performed to characterize the functions of DEGs, by tallying DEGs mapped to each GO term and calculating enrichment significance using a hypergeometric test. Analysis was done for the common DEGs between: (1) different salt duration comparisons within each tissue type; and (2) same salt duration comparisons between tissue types (Fig. 1C and D).

In cellular components, enriched DEGs mapped to nucleosome, nucleolus, small ribosomal subunit, and nuclear chromatin (Additional file 2: Table S2 and S3). For molecular function, enriched terms included ubiquitin transferase activity, protein kinase activity (Additional

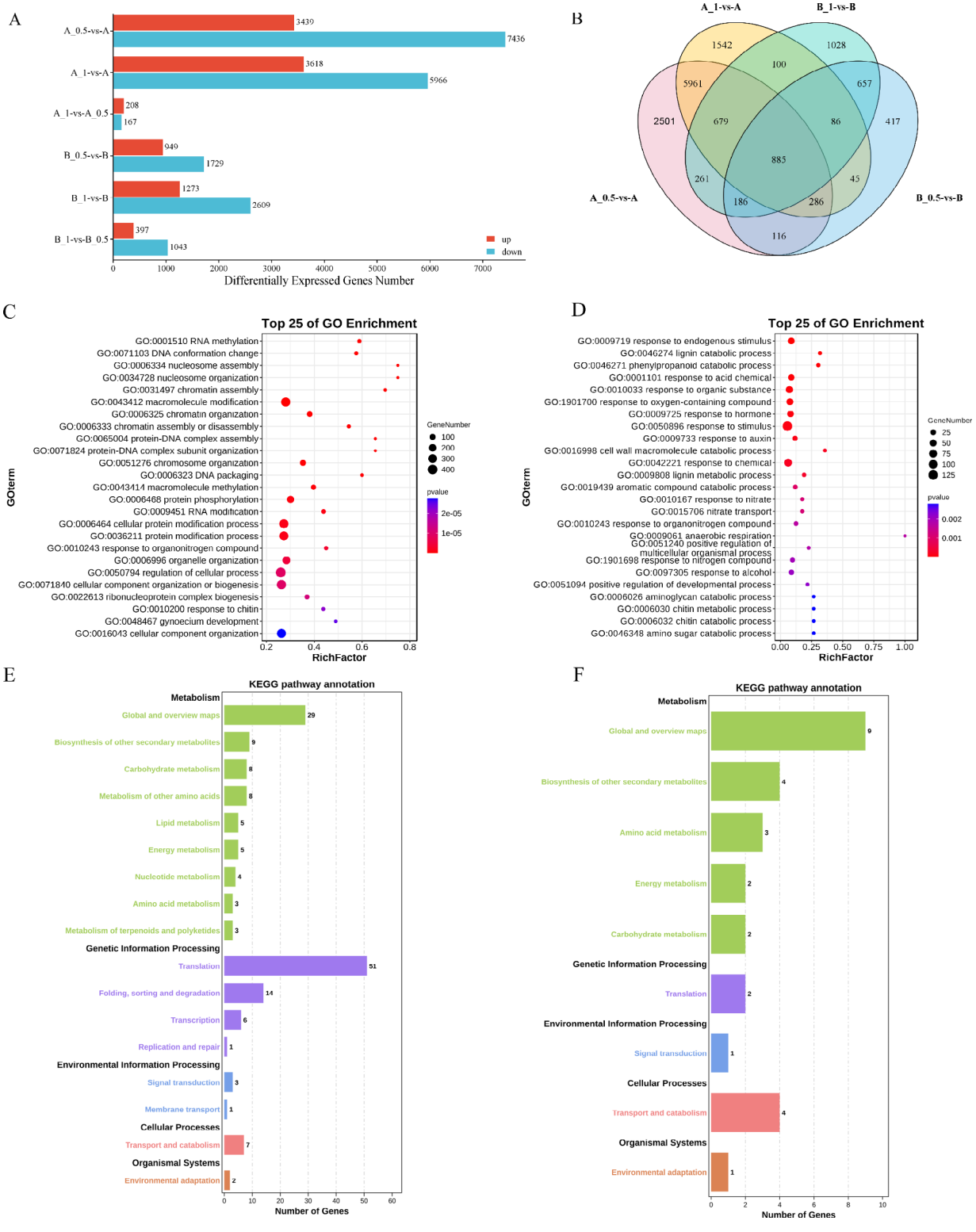

Fig. 1 Gene expression analysis, Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway analysis of differentially expressed genes (DEGs) and enrichment results in common differentially expressed genes of various comparison groups. (**A**) Number of up- and down-regulated differentially expressed genes (DEGs) in different comparison groups: A_0.5-vs-A, B_0.5-vs-B, A_1-vs-A, B_1-vs-B, A_1-vs-A_0.5 and B_1-vs-B_0.5; (**B**) Venn diagram illustrating the overlap of DEGs in the comparison group of A_0.5-vs-A, A_1-vs-A, B_0.5-vs-B and B_1-vs-B; GO level secondary gene annotation for the common DEGs in the comparison group A_0.5-vs-A and A_1-vs-A (**C**), B_0.5-vs-B and B_1-vs-B (**D**); KEGG pathways for the common DEGs in the comparison group A_0.5-vs-A and A_1-vs-A (**E**), B_0.5-vs-B and B_1-vs-B (**F**)

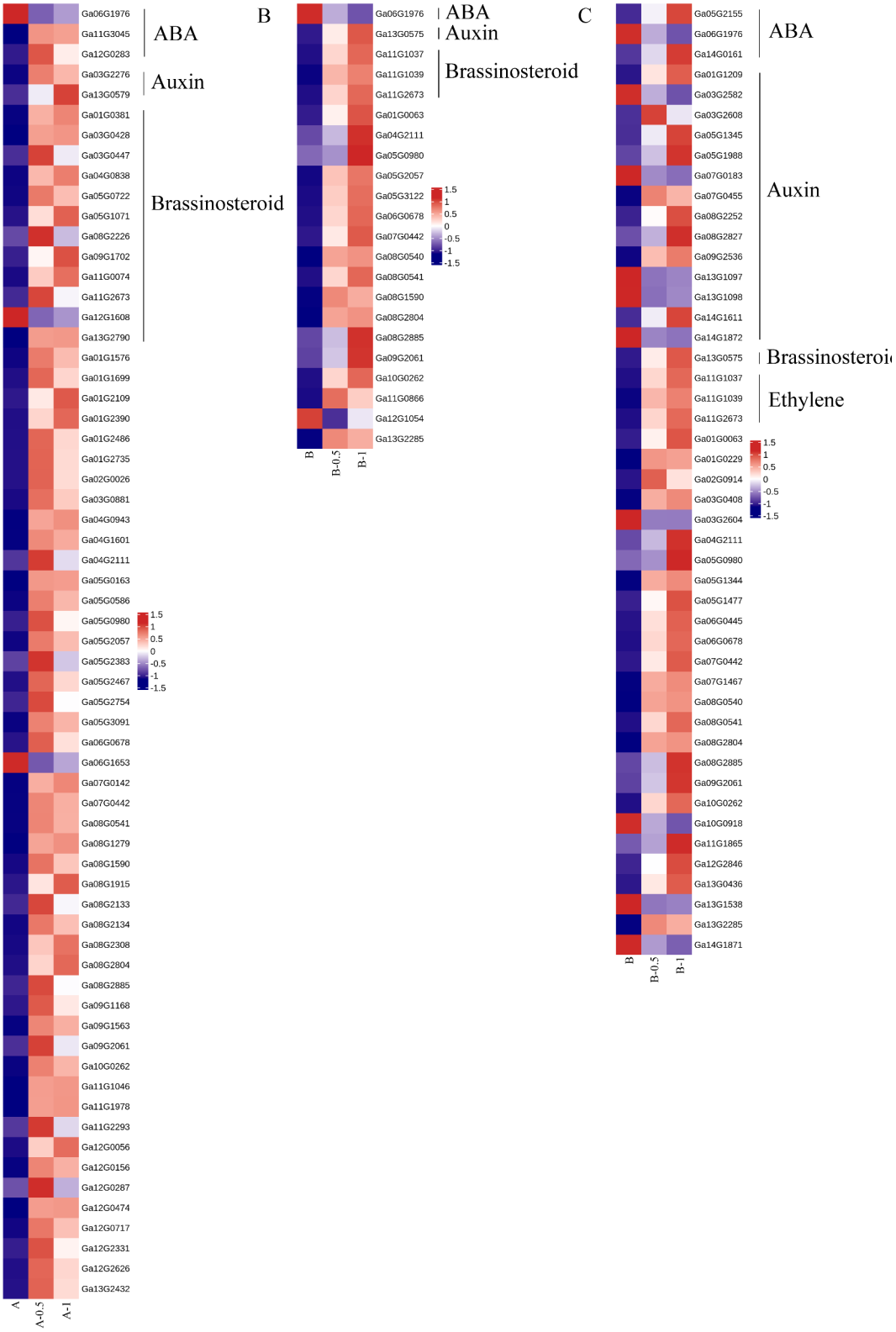

Fig. 2 (See legend on next page.)

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Fig. 2 Gene expression patterns in MAPK signaling pathways and plant hormone signal transduction. (**A**) Gene expression patterns in MAPK signaling pathways for the comparison groups A_0.5-vs-A and A_1-vs-A. (**B**) Gene expression patterns in MAPK signaling pathways for the comparison groups B_0.5-vs-B and B_1-vs-B. (**C**) Gene expression patterns in plant hormone signal transduction for the comparison groups B_0.5-vs-B and B_1-vs-B. In panels (**A**) and (**B**), the expression levels of genes associated with MAPK signaling pathways are presented for different treatment conditions. Panel (**C**) focuses on gene expression related to plant hormone signal transduction. The differential expression patterns provide insights into the regulation of these vital biological processes under specific treatment conditions. Data represent gene expression analysis based on identified DEGs, and the analyses are derived from biological triplicates

file 2: Table S2), DNA-binding transcription factor activity, and transcription regulation (Additional file 2: Table S3).

In biological process, common protoplast DEGs (A_0.5-vs-A, A_1-vs-A) were mainly mapped to chromosome organization, macromolecule modification, protein modification process and regulation of cellular process (Fig. 1C and Table S2). Common root DEGs (B_0.5-vs-B, B_1-vs-B) were enriched in responses to stimulus, hormone, chemical and organic substance (Fig. 1D and Additional file 2: Table S3). In roots but not protoplasts, the number of stress-related DEGs increased over salt duration (Additional file 2: Table S4). These provides an important framework for further analyzing the salt stress response in *G. arboreum*.

KEGG pathway analysis was performed to further understand the biological functions of DEGs before/after salt treatment and between tissue types. Pathway enrichment calculates significance of DEGs mapping to each pathway using a hypergeometric test. The top 30 enriched pathways are shown for each key comparison: A_0.5-vs-A (protoplasts, 0.5 h vs 1 h salt) and A_1-vs-A (protoplasts, 1 h salt vs control): Significantly enriched pathways ($P \leq 0.05$) included translation, signal transduction, MAPK signaling (ko04016), plant hormone signaling (ko04075), alpha-linolenic acid metabolism (ko00592), and alanine/aspartate/glutamate metabolism (ko00250) (Fig. 1E, Additional file 2: Table S5 and Additional file 1: Fig. S5A) B_0.5-vs-B (roots, 0.5 h vs 1 h salt) and B_1-vs-B (roots, 1 h salt vs control): Significant pathways included global and overview maps, biosynthesis of other secondary metabolites, transport and catabolism, plant hormone signaling (ko04075), MAPK signaling (ko04016), plant-pathogen interaction (ko04626), Phenylpropanoid biosynthesis (ko00940) and Carotenoid biosynthesis (ko00906) (Fig. 1F, Additional file 2: Table S6 and Additional file 1: Fig. S5B).

Gene interaction network analysis was also performed to visualize connections between enriched KEGG pathways. The results showed highly interconnected differential gene enrichment mainly across plant hormone signaling, alanine/aspartate/glutamate metabolism, pentose/glucuronate interconversions, and phenylpropanoid biosynthesis pathways (Additional file 1: Fig. S6). Therefore, further analysis of differential genes in phytohormone pathways could help verify key salt tolerance genes.

In summary, KEGG pathway analysis combined with prior evidence on plant salt stress responses indicate that plant hormone and MAPK signaling cascades play crucial roles in the cotton root response to salt exposure. Follow-up studies are warranted to identify specific gene candidates and elucidate associated genetic mechanisms conferring salt tolerance.

Identification of key signaling and hormonal pathways involved in cotton salt tolerance

Pathway analysis revealed that many differentially expressed genes (DEGs) were highly enriched in the MAPK signaling pathway when comparing root and protoplast samples before and after salt treatment. Specifically, 64 and 22 MAPK-related DEGs were found in the A_0.5-vs-A and A_1-vs-A protoplast comparisons, and the B_0.5-vs-B and B_1-vs-B root comparisons, respectively (Fig. 2A-B). These included DEGs involved in ABA signaling (3 and 1 DEGs), brassinosteroids signaling (2 and 1 DEGs), and ethylene signaling (12 and 3 DEGs) (Additional file 2: Table S7 and S8). The majority were upregulated in both protoplasts (57 of 64) and roots (20 of 22) after salt treatment (Fig. 2A-B, and Additional file 2: Table S7 and S8).

Plant hormone pathways play critical roles in biotic and abiotic stress responses over plant growth. Comparing protoplast samples (A_0.5-vs-A and A_1-vs-A), 127 DEGs were identified in plant hormone signal transduction, including 116 upregulated and 11 downregulated (Additional file 2: Table S9 and Additional file 1: Fig. S7). These were involved in auxin (17 genes), ABA (8 genes), ethylene (10 genes), and brassinosteroid (2 genes) pathways. In the root comparisons (B_0.5-vs-B and B_1-vs-B), 47 plant hormone signaling DEGs were found, with 37 upregulated and 10 downregulated. These genes participated specifically in ABA (3 genes), auxin (14 genes), brassinosteroid (1 gene), and ethylene (3 genes) pathways (Fig. 2C and Additional file 2: Table S10). Most DEGs showed increased expression in both roots and protoplasts after salt treatment (Fig. 2C and Additional file 2: Table S6).

Identification of key gene networks in response to salt stress in *Gossypium arboreum* via WGCNA

Weighted gene coexpression network analysis (WGCNA) was performed on the 885 commonly differentially expressed genes across all salt treatment comparisons

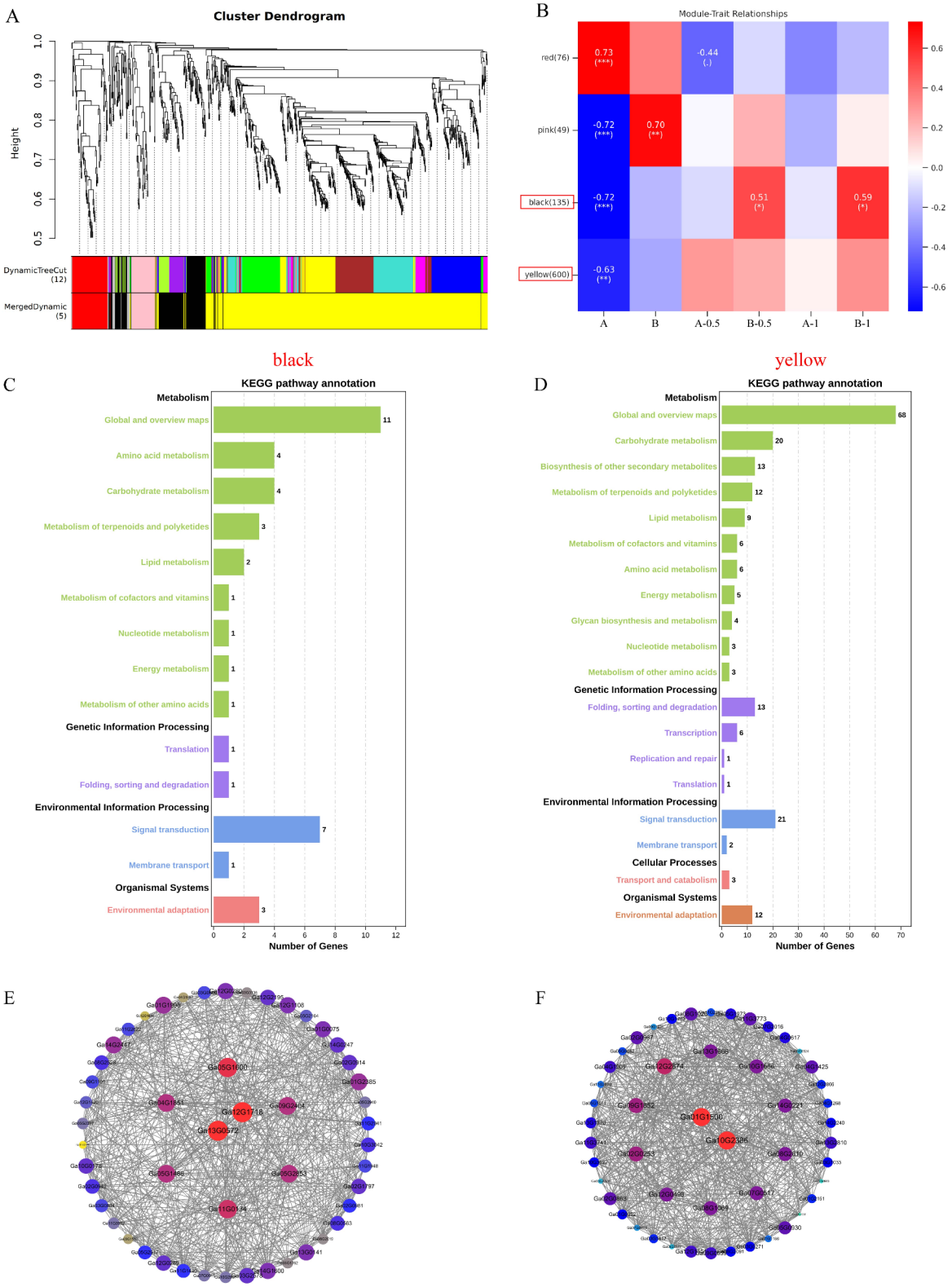

Fig. 3 (See legend on next page.)

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Fig. 3 Weighted gene coexpression network analysis (WGCNA) of common differentially expressed genes (DEGs) among different groups (A_0.5-vs-A, A_1-vs-A, B_0.5-vs-B, and B_1-vs-B). (**A**) Hierarchical cluster tree illustrating coexpression modules identified by WGCNA. Each leaf in the tree represents one gene. (**B**) Module-sample group association analysis. Each row corresponds to a module as in panel (**A**), and each column corresponds to a sample group. The color of each cell at the row-column intersection indicates the correlation coefficient between the module and the sample group. The numbers next to each module represent the count of genes contained in that module. WGCNA provides a systems biology approach to explore coexpression patterns among DEGs across different control groups. Panel (**A**) visually represents the hierarchical clustering of genes into co-expression modules, while panel (**B**) showcases the association between these modules and sample groups, revealing potential functional relationships within the identified modules. Data represent results from WGCNA analysis based on identified DEGs, and the analyses are derived from biological triplicates; KEGG pathways for the black module **(C)** and yellow module (**D**); (**E**) Correlation networks within the black module. (**F**) Correlation networks within the yellow module. In panel (**E**), the correlation networks within the black module are depicted, illustrating the coexpression relationships among genes belonging to this module. Similarly, panel (**F**) shows the correlation networks within the yellow module, providing insights into the coexpression patterns of genes within that module. The edges in the network represent the strength and direction of the correlation between genes. Nodes represent individual genes, and the visualization offers a comprehensive view of the coexpression relationships within each key module. Data represent results from coexpression network analysis based on identified modules from Weighted gene coexpression network analysis (WGCNA), and the analyses are derived from biological triplicates

to comprehensively understand their distribution in roots and protoplasts and identify salt-associated genes. WGCNA clusters genes into modules with coordinated expression changes, indicating common functionality.

The 885 DEGs clustered into 4 modules, represented by branch colors, with each leaf representing a gene (Fig. 3A). Two modules (highlighted red) contained genes highly expressed under salt treatment, suggesting importance in the salt response: black module (135 genes) - highly correlated with salt-treated root samples B_0.5 and B_1, Yellow module (600 genes) - highly correlated with all salt-treated protoplast (A_0.5, A_1) and root (B_0.5, B_1) samples, increasing over salt duration. This demonstrates these modules' key roles in the response to salt during cotton root tip protoplast isolation (Fig. 3B). KEGG analysis showed the black module significantly enriches signal transduction and global/overview pathways, while the yellow module enriches carbohydrate metabolism, global/overview, and signal transduction pathways (Fig. 3C and D). These coordinated gene set likely mediate early cotton root developmental adjustments to ensure seedling survival upon salt exposure. The top 50 genes from each module were selected by module membership (kME) to visualize networks. Significantly coexpressed genes were included, sized by node degree centrality. This identifies key network hubs - genes with the most neighbors. From the key black and yellow modules, four "hub" genes were identified as candidate regulators of the cotton salt response: *Ga12G1718* (*Aspartate tRNA ligase*), *Ga13G0572* (*Sulfate transporter*), *Ga05G1600* (WRKY transcription factor), *Ga01G1500* (*Syntaxin vesicle trafficking gene*) and *Ga10G2386* (ABC transporter). These central, highly interconnected genes likely play pivotal roles governing salt tolerance in Asian cotton (Fig. 3E and F).

Identification of key salt-responsive transcription factors

Transcription factors (TFs) are key molecular regulators of downstream stress-response genes. Their importance in plant salt tolerance is established. To investigate their roles in salt-stressed cotton, differential TF analysis was performed on the 885 DEGs identified from comparisons of A_0.5-vs-A, A_1-vs-A, B_0.5-vs-B and B_1-vs-B.

In total, 91 differentially expressed TFs were identified, spanning 26 families including AP2/ERF, MYB, NAC, bHLH and WRKY - known mediators of plant salt tolerance (Fig. 4A). The AP2/ERF family contained the most differentially expressed TFs (18), followed by WRKY (14 TFs), MYB (9), and NAC (5) (Fig. 4B, C, D, E and F). This suggests AP2/ERF TFs feature prominently in the cotton root salt response.

Expansins are also linked to environmental stress tolerance in plants. Wheat and Arabidopsis expansin overexpression enhances drought, oxidation, osmotic, and salt tolerance. To further understand expansin involvement in the *G. arboreum* salt response, 14 expansin genes were found among the 885 DEGs – 10 in protoplasts and 4 in roots (Fig. 4G and H). The expression of Expansins-related genes in root tips increased with the duration of salt stress. The function of the DEG *Ga09G1764* (*GaEXPA4*) was assessed via follow-up experiments to validate reliability of the transcriptomes and probe *GaEXPA4* functional significance.

Expression dynamics of candidate genes in cotton roots and root derived protoplasts in response to salt stress

To ensure the reliability and accuracy of the RNA-seq data and differential expression analysis, quantitative reverse transcription PCR (qRT-PCR) was employed to validate the expression profiles of five differentially expressed genes (DEGs) from the total set of 885 identified. The selected genes included *GOBAR_AA30878*, encoding a hypothetical protein (*Ga01G0063*); *Indole-3-acetic acid-amido synthetase GH3.6-like protein* (*Ga03G2153*); *α-expansin 4* (*Ga07G0142*); *abscisic acid receptor PYL6* (*Ga06G1976*); and *protein phosphatase 2 C 77* (*Ga09G1764*). Gene-specific primers were meticulously designed for each DEG, as listed in Additional file 2: Table S11. The qRT-PCR experiments measured the expression changes of these genes in both

cotton root tissues and root-derived protoplasts, and the observed trends closely aligned with those obtained from the RNA-seq analysis (Fig. 5 and Additional file 2: Table S12). The concordance between qRT-PCR and RNA-seq results underscores the high quality and robustness of the transcriptomic data. This validation reinforces confidence in the differential expression findings, providing a reliable foundation for further investigation into the biological functions and regulatory pathways involving these genes.

Gene structure, chromosomal distributions and physicochemical analysis of *GaEXPA4* gene in cotton

Current results suggests that *GaEXPA4* is located on chromosome Chr09, spanning the region from 75,504,939 to 75,505,104 bp, with three exons. Prediction of the conserved domain of *GaEXPA4* revealed the presence of a typical PLN00050 domain, which is the primary domain of Expansin A (Fig. 6A), indicating that *GaEXPA4* belongs to the Expansin A family. Physicochemical analysis of the protein showed that *GaEXPA4* has a theoretical relative molecular mass of 28.34529 kDa and a theoretical isoelectric point of 9.55. There is a total of 9 negatively charged residues and 21 positively charged residues in *GaEXPA4*, with a molecular formula of $C_{1266}H_{1927}N_{355}O_{359}S_{15}$. The aliphatic index of *GaEXPA4* is 71.70, suggesting thermal stability, and its grand average of hydropathicity (GRAVY) is 0.033, indicating that it is a hydrophobic protein (Fig. 6B).

Signal peptide prediction indicated that *GaEXPA4* contains a signal peptide (Signal Peptide (Sec/SPI)), suggesting it may be a secretory protein. Transmembrane structure analysis predicted that *GaEXPA4* possesses a transmembrane structure, with an expected number of amino acids in transmembrane helices (Exp number of AAs in TMHs) of 23.54973 (greater than 18), further confirming the presence of transmembrane helices in *GaEXPA4*. Secondary structure prediction of *GaEXPA4* showed the highest proportion of random coil (59.09%), followed by extended strand (21.59%) and alpha helix (19.32%) (Fig. 6B). The three-dimensional structure of *GaEXPA4* was predicted using SWISS-MODEL homology modeling, which also showed a dominant random coil structure (Fig. 6B), consistent with the predicted secondary structure.

Multiple sequence alignment analysis of *GaEXPA4* from *Gossypium arboreum*, *Gossypium raimondii*, *Gossypium barbadense*, *Gossypium hirsutum*, *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays* revealed highly conserved regions in these species (Fig. 6C). Homology analysis found that the *GaEXPA4* gene shares high homology with these species, indicating the high conservation of the EXPA4 gene across different species. Based on the phylogenetic tree, *GaEXPA4* exhibited strong homology with EXPA4 genes from the other three cotton species (Fig. 6D).

Silencing of *GaEXPA4* down-regulates the sensitivity of cotton to salt stress

To investigate the role of the *GaEXPA4* gene in cotton salt tolerance, the gene was silenced using virus-induced gene silencing (VIGS). Agroinfiltration of cotyledons successfully induced silencing, as evidenced by the characteristic bleached leaf phenotype observed at 10 days post-infiltration, which persisted for 20 days (Fig. 7A). Quantitative reverse transcription PCR (RT-qPCR) confirmed reduced expression levels of *GaEXPA4*. While the TRV:00 control plants exhibited slightly lower *GaEXPA4* expression compared to wild-type (WT) plants, the expression was markedly suppressed in TRV:*GaEXPA4* plants, verifying effective gene silencing (Fig. 7B). Under normal growth conditions, WT plants exhibited more robust growth, whereas both TRV:00 and TRV:*GaEXPA4* plants displayed stunted development (Fig. 7C). Following 77 h of salt stress, TRV:*GaEXPA4* plants exhibited more pronounced leaf wilting compared to WT and TRV:00 plants (Fig. 7D). Reactive oxygen species (ROS) accumulation, as indicated by DAB staining, was minimal across all plant groups under non-stress conditions, with no significant brown discoloration observed in the leaves. However, after exposure to 200 mM NaCl, leaves of TRV:*GaEXPA4* plants developed extensive brown regions, signifying severe oxidative damage, whereas WT and TRV:00 plants showed comparatively less browning (Fig. 7E). These findings underscore the pivotal role of *GaEXPA4* in mediating salt tolerance in cotton. Silencing this gene compromises the plant's ability to withstand saline conditions, as evidenced by enhanced wilting, elevated ROS-induced damage, and reduced overall vigor under salt stress.

Silencing *GaEXPA4* resulted in increased water loss, providing further evidence of reduced salt tolerance upon gene suppression. Under normal conditions, the relative leaf water loss (RLWL), chlorophyll content, and excised leaf water loss (ELWL) of wild-type (WT), TRV:00, and TRV:*GaEXPA4* plants showed no significant differences. However, after exposure to salt stress, these physiological parameters decreased across all groups compared to non-stress conditions. Importantly, the values for TRV:*GaEXPA4* plants were significantly lower than those for WT and TRV:00 controls (Fig. 7H, I, and J). This indicates that silencing the *GaEXPA4* gene exacerbates cell membrane damage and diminishes salt tolerance in upland cotton. Antioxidant activity was evaluated by measuring superoxide dismutase (SOD) activity and malondialdehyde (MDA) content. Following salt treatment, TRV:*GaEXPA4* plants exhibited lower SOD activity compared to WT and TRV:00 controls, reflecting a

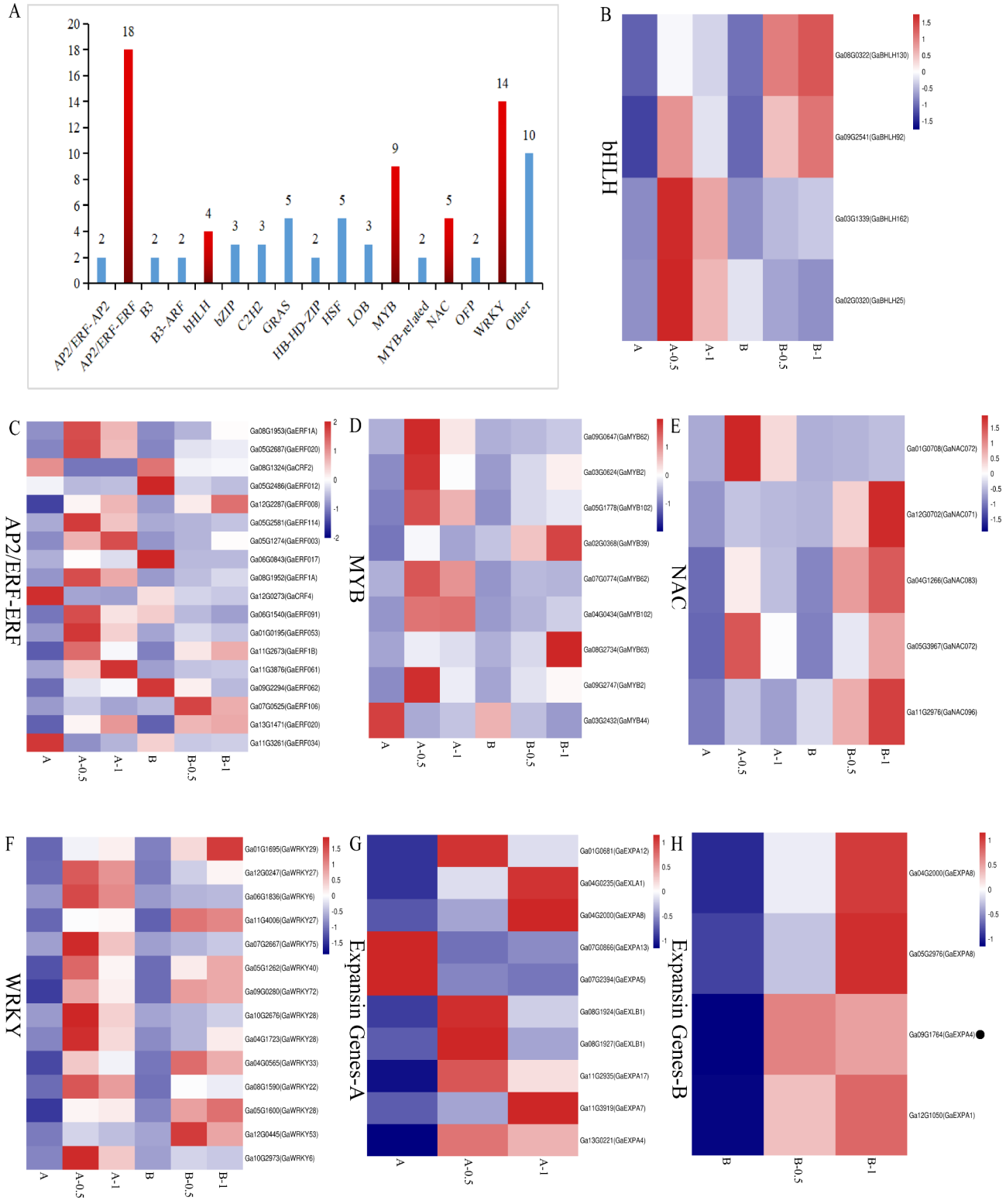

Fig. 4 Transcription factor (TF) family distribution and expression comparison heat maps. (**A**) Distribution of TF families in common differentially expressed genes across four control groups (A_0.5-vs-A, A_1-vs-A, B_0.5-vs-B, and B_1-vs-B). (B-G) Heat maps indicating expression comparisons of specific TF families among six comparison sets: (**B**) Heat shock transcription factor (bHLH). (**C**) AP2/ERF-ERF. (**D**) MYB. (**E**) NAC. (**F**) WRKY. (**G**) Expansin genes in A_0.5-vs-A and A_1-vs-A. (**H**) Heat map for Expansin genes in B_0.5-vs-B and B_1-vs-B. In panel (**A**), the distribution of TF families provides an overview of the transcriptional regulatory landscape. Panels (**B**-**H**) feature heat maps illustrating the expression profiles of specific TF families in different comparison sets. The color intensity represents expression levels, enabling the visual comparison of TF expression patterns across conditions. Data represent results from comprehensive transcriptional analysis based on identified TFs and DEGs, with analyses conducted using biological triplicates

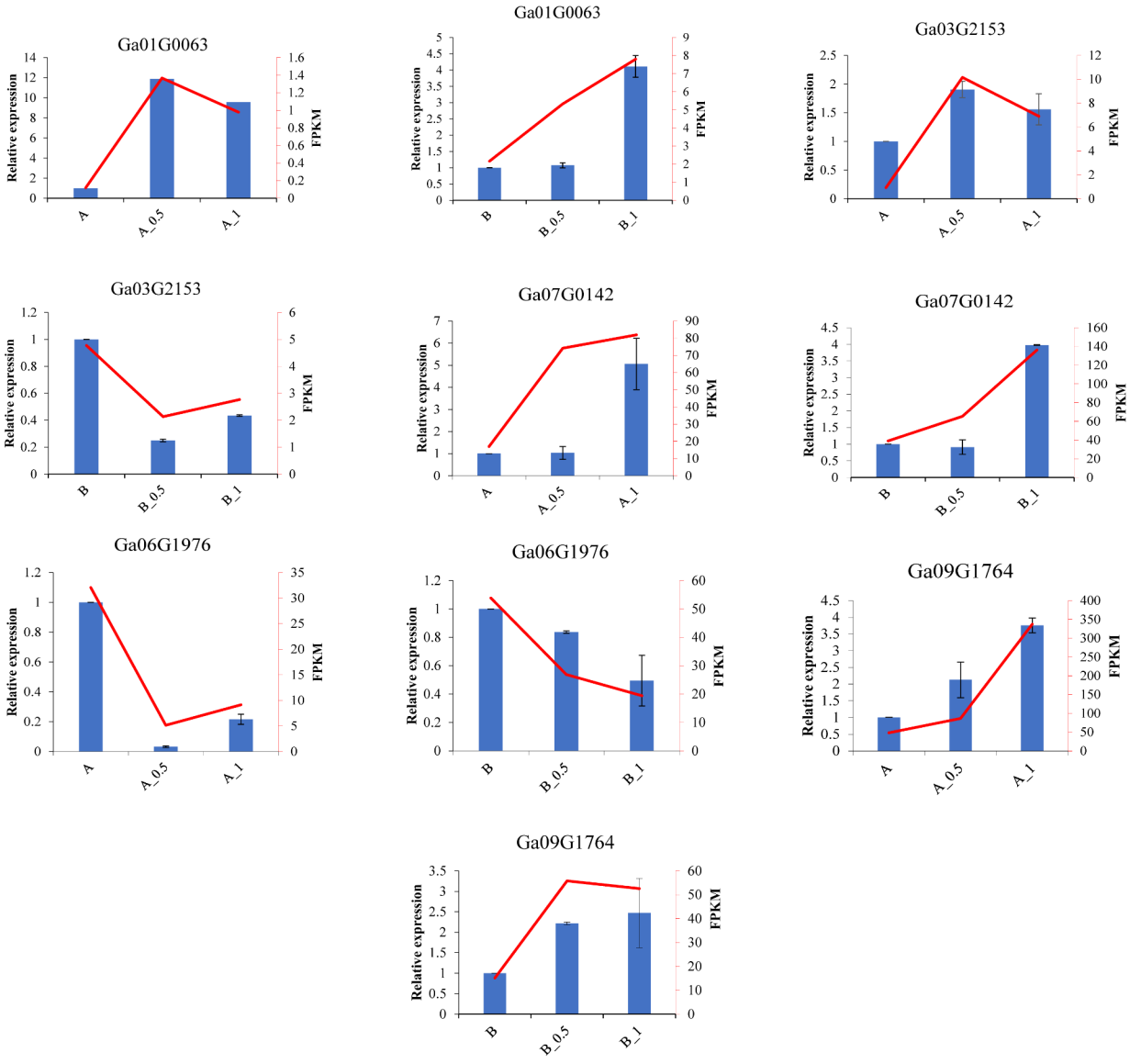

Fig. 5 Expression dynamics of hub genes and validations of RNA-seq data by qRT-PCR. The results of each gene are based on three biological and three technical replicates. The error bars indicate standard errors

reduced capacity for reactive oxygen species (ROS) scavenging. Concurrently, these plants displayed elevated MDA levels, indicative of heightened lipid peroxidation and oxidative stress (Fig. 7F and G). Collectively, these physiological and biochemical assays highlight the critical role of *GaEXPA4* in maintaining salt tolerance in cotton. Silencing this gene increases water loss, impairs ROS scavenging, and intensifies cellular damage under saline conditions, thereby confirming its protective function against salt-induced stress.

Discussion

Cotton (*Gossypium spp.*) is a key crop with intrinsic saline-alkaline tolerance, cultivated in suboptimal soils to enhance soil quality and supply essential fiber and edible oil for the textile and food industries, respectively [28, 44]. However, global climate changes, coupled with unsustainable irrigation and fertilization practices, have intensified soil salinization. This poses significant threats to crop yields, quality, land-use efficiency, and the broader ecological environment, becoming a critical challenge in recent years [28, 45]. Addressing the conflict between food and fiber production requires enhancing the saline-alkaline resistance of cotton cultivars. Such improvements are grounded in genetic modification and

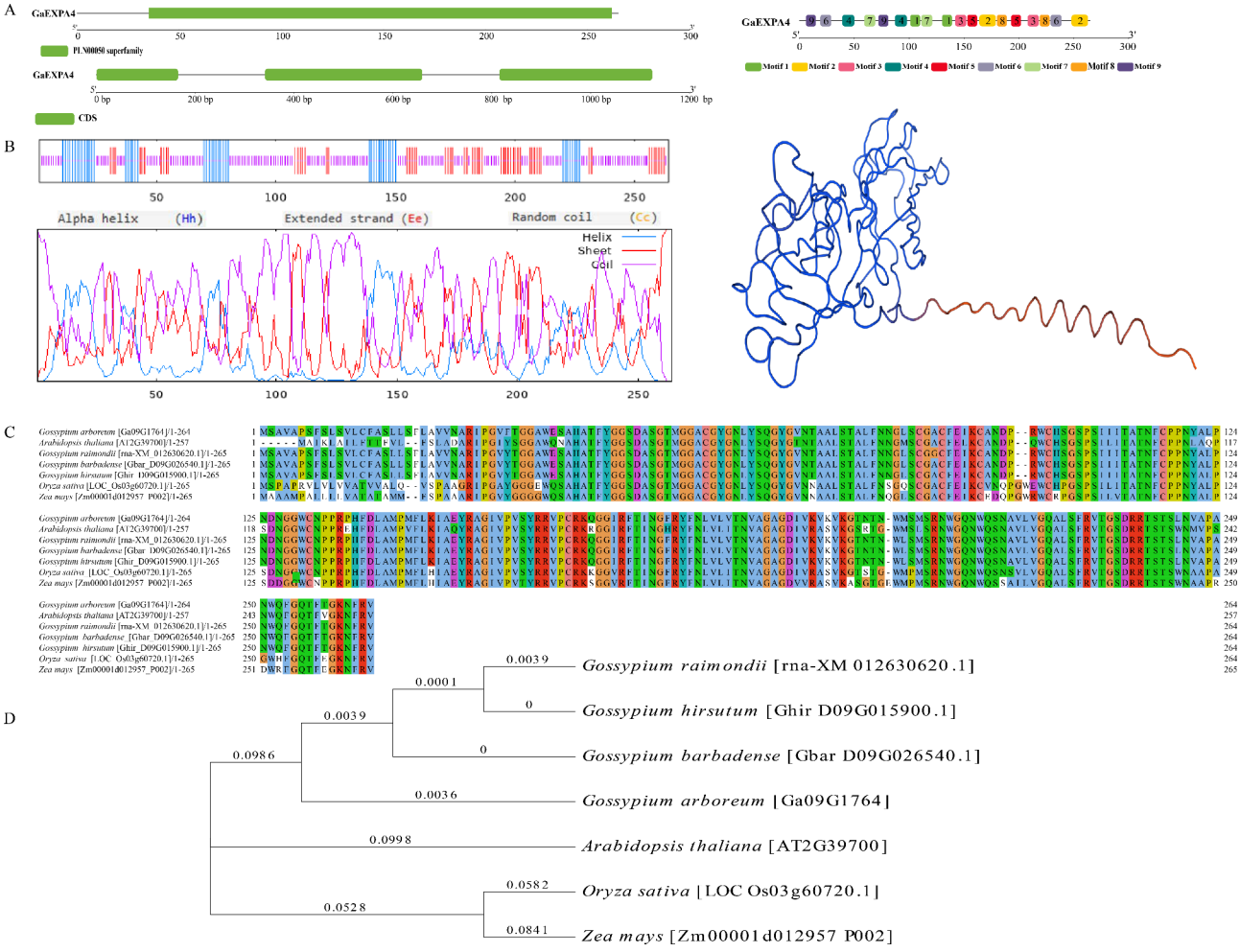

Fig. 6 Bioinformatics analysis of *GaEXPA4* (Ga09G1764) in *G. arboreum* L. (**A**) Gene structure analysis and conserved domain analysis of *GaEXPA4*. (**B**) Secondary and tertiary structure prediction. (**C**) Protein sequence alignment of *GaEXPA4* homologs in different species. (**D**) Phylogenetic tree analysis of *GaEXPA4*

a thorough understanding of the molecular mechanisms underlying stress responses. The limited genetic diversity in cotton complicates the development of transgenic lines, extending the duration needed to obtain viable seedlings. While protoplast-based systems present an alternative, offering flexibility in genetic transformation, they also facilitate studies on subcellular localization and gene interactions [28, 46, 47]. This study utilized salt-tolerant *G. arboreum* SXY-1 to conduct transcriptomic analyses of roots and protoplasts under varying NaCl treatments, aiming to identify key differentially expressed genes (DEGs) and signaling pathways involved in saline tolerance. Notably, protoplasts exhibited more dynamic gene expression changes, potentially reflecting the role of cell wall integrity in mitigating stress effects [28, 48].

Pairwise comparisons across 18 samples identified thousands of DEGs across six comparison groups (Fig. 1A), with a marked increase in up-regulated genes during salt exposure. This indicates that salt stress elicits strong adaptive responses in cotton as reported earlier [28, 49]. Among the 885 common DEGs identified across treatments, significant functional enrichment differed between root and protoplast samples. In protoplasts, DEGs were associated with chromosome organization, macromolecular modifications, and regulation of cellular processes, while in roots, responses were linked to hormone signaling, chemical responses, and environmental stimuli. These findings align with previous studies demonstrating the enrichment of genes associated with osmotic stress, hormone responses, and other stress-related pathways [28, 50]. KEGG pathway enrichment highlighted significant associations with MAPK signaling and plant hormone transduction pathways, underscoring their roles in regulating stress responses [28, 51, 52]. Moreover, phytohormone signaling, as central to the gene interaction network, along with glycometabolism, amino acid metabolism, and phenylpropanoid

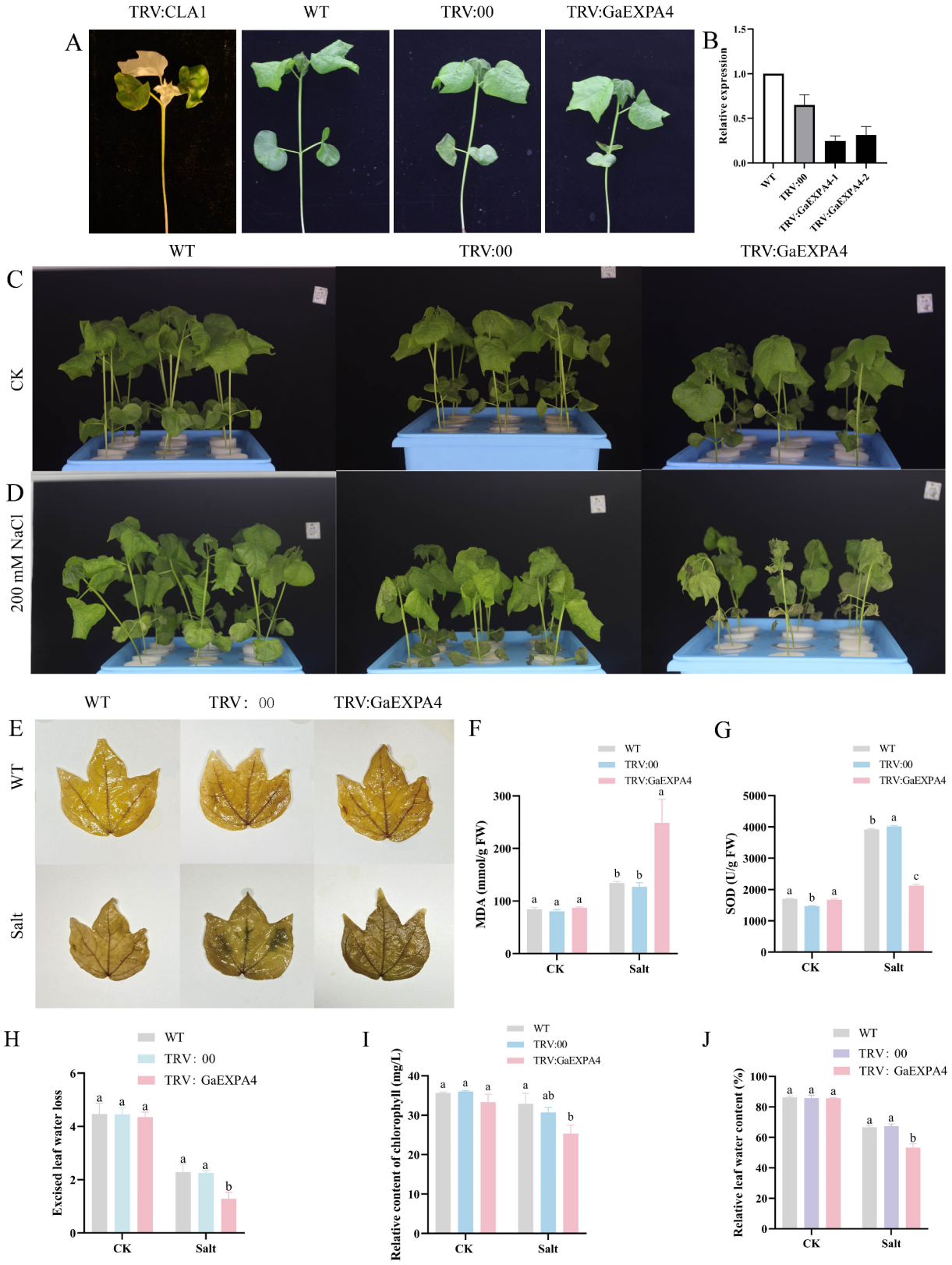

Fig. 7 (See legend on next page.)

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Fig. 7 Evaluation of salt stress tolerance phenotype after silencing *GaEXPA4* gene in cotton. (**A**) Phenotype representation of *TRV: CLA1*, wild-type (WT), empty vector (TRV:00), and *GaEXPA4* under normal conditions. (**B**) Validation of VIGS interference by detecting *GaEXPA4* gene expression levels. (**C**) Phenotype representation of WT, empty vector, and *GaEXPA4* under normal conditions. (**D**) Phenotype representation of WT, empty vector, and *GaEXPA4* under salt stress conditions. (**E**) DAB staining; (**F**) Measurement of malondialdehyde (MDA) content as an indicator of oxidative stress. (**G**) Determination of superoxide dismutase (SOD) activities as part of the antioxidant enzyme analysis. (**H**) Excised leaf water loss (ELWL). (**I**) Determination of chlorophyll content in the tested samples. (**J**) Relative leaf water content (RLWL). Three biological replicates were taken for each experiment. Student's t-test was used to determine the mean comparison with ± SD at $p < 0.05$. Means with different letters indicate a significant difference. The results collectively demonstrate the impact of silencing the *GaEXPA4* gene on the physiological traits, gene expression, and stress tolerance phenotype in cotton under both normal and salt stress conditions

biosynthesis, were found to be crucial for cotton's defense mechanisms against salt stress [28, 53–55].

Constructing the weighted gene co-expression network analysis identified five hub DEGs across two modules, with functional annotations suggesting their involvement in stress resistance. Notably, *Ga12G1718* and *Ga10G2386*, annotated as Aspartate tRNA ligase and ABC transporter, respectively, have documented roles in saline stress responses [28, 56, 57]. Additionally, the sulfate transporter family, including *SULTR3;4*, has been linked to enhanced salt tolerance in plants [28, 58, 59]. The WRKY transcription factor family also emerged as a key player, with multiple WRKY genes (*WRKY33*, *WRKY17*, *WRKY5*, *WRKY6*-like, *WRKY41*) contributing to the adaptive responses against salt stress [11, 28, 60–63]. In transcriptomic analysis of wild type salt-tolerant cotton varieties, 109 *GarWRKY* genes were identified [60]. Overexpression of WRKY gene *GhWRKY25* in cotton enhanced the salt tolerance of *Nicotiana benthamiana* [64]. Similarly, tobacco plants overexpressing *GhWRKY39-1* showed higher tolerance to salt and oxidative stress. In addition, overexpression of *GhWRKY39-1* can increase the transcription level of antioxidase-related genes [65]. In addition, overexpression of *GhWRKY6*-like, another cotton WRKY gene, significantly enhanced salt tolerance in Arabidopsis. On the other hand, VIGS technology was used to silence *GHWRKY6*-like genes in cotton, which increased the sensitivity of cotton plants to drought and salt stress [61]. Although direct evidence connecting the Syntaxin vesicle trafficking gene (*SYP121*) to salt tolerance remains limited, its involvement in cadmium stress tolerance through enhanced vesicle trafficking suggests a potential role in abiotic stress resilience [28, 63].

The *α-Expansin EXPA4* gene (*Ga09G1764*) demonstrated significant expression changes in response to salt treatment in both root and protoplast samples. Previous studies have established its involvement in stress responses [15, 28]. In this study, virus-induced gene silencing (VIGS) experiments showed that *EXPA4*-silenced plants exhibited more pronounced stress phenotypes under salt conditions, with physiological indicators confirming increased salt-induced damage. Our results demonstrate that *GaEXPA4* plays a crucial role in salt stress regulation by influencing both cellular water retention and oxidative stress responses. Under salt stress conditions, *GaEXPA4* expression was significantly elevated, suggesting its involvement in maintaining cell wall integrity and osmotic balance. Upon silencing *GaEXPA4*, TRV:*GaEXPA4* plants exhibited a marked increase in water loss, indicating compromised membrane stability and reduced salt tolerance. This was evidenced by a significant decrease in relative leaf water content (RLWL) and chlorophyll content, alongside increased excised leaf water loss (ELWL), compared to WT and TRV:00 controls (Fig. 7H, I, J). Moreover, silencing *GaEXPA4* impaired the plant's antioxidant defense system, as shown by reduced superoxide dismutase (SOD) activity and elevated malondialdehyde (MDA) levels under salt stress (Fig. 7F, G). These results indicate that *GaEXPA4* contributes to reactive oxygen species (ROS) scavenging, preventing lipid peroxidation and subsequent cellular damage. Collectively, these findings highlight *GaEXPA4*'s dual role in modulating water retention and oxidative stress responses, reinforcing its critical function in enhancing salt tolerance in upland cotton.

It has been reported previously that transgenic Arabidopsis plants with a higher expression level of the rose expansin gene *RhEXPA4* exhibited increased tolerance to drought and salt stress compared with the wild-type [66]. These results suggest that *GaEXPA4* may play a pivotal role in enhancing cotton's salt tolerance, warranting further investigation through genetic transformation and protein interaction studies.

Our RNA-seq analysis revealed a larger number of differentially expressed genes (DEGs) in protoplasts compared to root tissues, suggesting that salt stress induces more dynamic and immediate transcriptional changes in isolated cells. The expression dynamics of candidate genes were notably higher in protoplasts, reflecting their role in early stress signaling and rapid response mechanisms. In contrast, root tissues showed fewer DEGs and more stable expression patterns, likely due to systemic regulatory processes and tissue-level buffering effects. These findings underscore the distinct yet complementary roles of protoplasts and root tissues in salt stress responses, with protoplasts capturing the initial cellular responses and roots reflecting long-term adaptive strategies.

Current findings provide a comprehensive molecular perspective on the mechanisms underlying salt tolerance in *G. arboreum*. The identified DEGs and enriched pathways offer valuable targets for breeding and genetic engineering aimed at developing more resilient cotton cultivars. Further functional analyses will be crucial to confirm the roles of key genes in stress resistance and to explore their potential applications in improving crop tolerance to salinity.

Conclusions

This study offers critical insights into the molecular mechanisms underlying salt stress responses in *Gossypium arboreum*, providing valuable information for researchers studying abiotic stress in cotton. By utilizing transcriptome sequencing on protoplasts from root tip tissues before and after salt stress, we identified that differentially expressed genes (DEGs) were significantly enriched in key stress-related pathways, particularly in plant hormone signal transduction and the MAPK signaling pathways. Additionally, transcription factors from the AP2/ERF-AP2, WRKY, MYB, and bHLH families were found to be highly represented, indicating their central roles in regulating the plant's response to salinity stress. The identification and functional validation of *Ga09G1764* (*GaEXPA4*) through virus-induced gene silencing (VIGS) further highlight its importance as a positive regulator of salt tolerance. This gene could serve as a potential target for breeding or biotechnological interventions aimed at improving cotton's resilience to salt stress. Overall, the results presented in this study indicated that *GaEXPA4* has an essential role in the tolerance of cotton to salt stress. Our findings not only contribute to a deeper understanding of the genetic regulatory networks involved in cotton's abiotic stress responses but also provide a foundation for future research aimed at enhancing cotton cultivars with improved salt tolerance traits. This work is thus pivotal for advancing cotton breeding programs focused on stress resilience and sustainability in challenging environments.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05958-w.

Supplementary Material 1: Additional file 1: Figure S1. Protoplast yield and viability in G. arboreum lateral root tips under 100 mM NaCl treatment. Figure S2. PCA Plot, heat map of correlation coefficient between samples and volcano plot of DEGs. Figure S3. Heat map analysis of common DEGs in different groups with different salt stress treatment time. Figure S4. The circle diagram of the top 30 GO terms of differentially expressed genes. Figure S5. The circle diagram of the top 30 enrichment pathways in KEGG pathways of differentially expressed genes. Figure S6. Network Pathway Maps of KEGG Pathways in Differentially Expressed Genes (DEGs). Figure S7. The expression patterns of differentially expressed genes involved in plant hormone signal transduction in A_0.5-vs-A and A_1-vs-A. Additional file 2: Table S1. RNA-seq analysis of cotton under salt-stress. Table S2. The top 25 GO terms of differentially expressed genes in A_0.5-vs-A and A_1-vs-A. Table S3. The top 25 GO terms of differentially expressed genes in B_0.5-vs-B and B_1-vs-B. Table S4. GO terms of DEGs in the same salt concentration group. Table S5. The top 30 enrichment pathways in KEGG pathways of differentially expressed genes in A_0.5-vs-A and A_1-vs-A. Table S6. The top 30 enrichment pathways in KEGG pathways of differentially expressed genes in B_0.5-vs-B and B_1-vs-B. Table S7. The expression of common DEGs (FPKM) involved in MAPK signaling pathways in A_0.5-vs-A and A_1-vs-A. Table S8. The expression of common DEGs (FPKM) involved in MAPK signaling pathways in B_0.5-vs-B and B_1-vs-B. Table S9. The expression of common DEGs (FPKM) involved in hormone signal transduction pathways in A_0.5-vs-A and A_1-vs-A. Table S10. The expression of common DEGs (FPKM) involved in hormone signal transduction pathways in B_0.5-vs-B and B_1-vs-B. Table 11. Primers used for qRT-PCR. Table S12. FPKM values of related genes for qRT-PCR validation.

Author contributions

Qiankun Liu, Yongqing Zhao, Yu Chen, Yanfang Li and Aiming Zhang performed the experiments and Qiankun Liu, Pengtao Li, Muhammad Jawad Umer and Mubashir Abbas wrote the manuscript. Qiankun Liu, Mengying Yang and Yiman Liu analysed the data and discussed the outcomes. Yangyang Wei, Quanwei Lu, Xiaoyan Cai, Zhongli Zhou, Shuxun Yu, Fang Liu and Renhai Peng conceived the idea, discussed data, corrected manuscript and provided logistic support. All authors read and approved the final version of the manuscript.

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Data availability

Sequence data that support the findings of this study have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2022), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA019126).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]National Key Laboratory of Cotton Bio-breeding and Integrated Utilization, Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, Henan 455000, China
[2]College of Biology and Food Engineering, Anyang Institute of Technology, Anyang, Henan 455000, China
[3]State Key Laboratory of Crop Stress Adaptation and Improvement, School of Life Sciences, Henan University, Kaifeng 475004, China
[4]College of Life Science, Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin of Xinjiang Production & Construction Corps, Tarim University, Xinjiang 843300, China

[5]Zhengzhou Research Base, State Key Laboratory of Cotton Biology, School of Agricultural Sciences, Zhengzhou University, Zhengzhou 450001, China
[6]National Nanfan Research Institute (Sanya), Chinese Academy of Agricultural Sciences, Sanya 572024, China

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