GarWRKY5, a Member of the WRKY Transcription Factor Gene Family from a Diploid Cotton Species (Gossypium aridum L.), Is Involved in Salt Stress Response

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Abstract

Background Cotton is one of the most economically important crops in the world, and it is exposed to various abiotic stresses during its lifecycle, especially salt stress. However, the molecular mechanisms underlying cotton tolerance to salt stress are still not fully understood due to the complex nature of salt response. Therefore, identification of salt stress-tolerance-related functional genes will help us to understand key components involved in stress response and to provide valuable genes for salt stress tolerance improvement via genetic engineering in cotton. In a previous study, expression of a Group III WRKY gene family member from the diploid cotton species Gossypium aridum, GarWRKY5, was significantly induced in response to salt stress. Results In this present study, virus-induced gene silencing of GarWRKY5 in cotton showed enhanced salt sensitivity compared to wild-type plants under salt stress. Overexpression of GarWRKY5 in Arabidopsis positively regulated salt tolerance at the stages of seed germination and vegetative growth. Additionally, GarWRKY5-overexpressing plants exhibited higher activities of superoxide dismutase (SOD) and peroxidase (POD) under salt stress. The transcriptome sequencing analysis of transgenic Arabidopsis plants and wild-type plants revealed that there was enriched co-expression of genes involved in reactive oxygen species (ROS) scavenging (including glutamine S-transferases (GSTs) and SODs) and altered response to jasmonic acid and salicylic acid in the GarWRKY5-OE lines. Conclusion GarWRKY5 is involved in salt stress response by the jasmonic acid- or salicylic acid-mediated signaling pathway based on overexpression of GarWRKY5 in Arabidopsis and virus-induced gene silencing of GarWRKY5 in cotton.

Introduction

WRKY proteins comprise one of the largest transcription factor families in plants. The conserved WRKY domain contains approximately 60 amino-acid residues. The WRKY domain is defined based on the conserved WRKYGQK hexapeptide sequence is usually followed by a C2H2- or C2HC-type zinc finger motif at N-terminal end. WRKY transcription factors are classified on the basis of both the number of WRKY domains and zinc finger motifs that they contain; WRKY proteins with two WRKY domains belong to group I, whereas Group II and Group III members have only a single WRKY domain, followed by a novel zinc-finger-like motif C2H2 (C-X4–5-C-X22–23-H-X-H) and C2HC (C-X7-CX23-H-X-C), respectively [1]. WRKY proteins play diverse roles in regulating plant defense responses, and developmental and physiological processes of plants. In addition to their role in plant development, WRKY family genes are also important in regulating plant biotic and abiotic stress. For example, pathogen-induced defense pathways, drought, salt stress and others [2–4]. Increasing numbers of studies are reporting that WRKY genes are involved in regulating plant responses to salt stress. Their function has been elucidated using genetic and molecular approaches in different species, such as *AtWRKY25* and *AtWRKY33* in *Arabidopsis [*5], *OsWRKY11* and *OsWRKY45* in rice [2], *GmWRKY13* and *GmWRKY54* in soybean [6], and *TaWRKY10* in wheat [7].

Cotton is one of the most economically important crops in the world, which endures various abiotic stresses during its lifecycle, especially salt stress. However, the molecular mechanisms underlying cotton

tolerance to salt stress are still not fully understood due to the complex nature of this response. With the release of the *Gossypium* genome sequence [8–10], genome-wide identification of WRKY family genes has been conducted in *G. raimondii, G.arboreum* and *G. aridum [*11–14]. Several studies have suggested the importance of specific WRKYs in the transcriptional regulation of salt-related genes in cotton. For example, overexpression of *GhWRKY25* from *G. hirsutum* in *Nicotiana benthamiana* enhanced tolerance to salt stress [15]; *GhWRKY39–1*-overexpressing plants exhibited increased tolerance to salt and oxidative stress and increased transcription of antioxidant enzyme genes [16]. Overexpression of *GhWRKY34* in *Arabidopsis* resulted in a transgenic plant with increased tolerance to salt stress [17]. The ectopic expression of the *GhWRKY6*-like gene significantly increased salt tolerance in *Arabidopsis thaliana* while silencing the *GhWRKY6*-like gene increased the sensitivity of cotton to abiotic stresses [18]. Despite the abovementioned reports, the molecular mechanisms by which WRKY transcription factors (TFs) regulate salt stress still remain largely unclear in cotton.

In a previous study, 109 *GarWRKYs* gene were identified in a salt-tolerant wild cotton species, *Gossypium aridum,* based on transcriptome sequencing data; meanwhile, 28 salt-responsive *GarWRKY* genes were identified from transcriptome data and real-time quantitative PCR analysis [14]. Of the cotton WRKY genes, *GarWRKY5* belongs to Group III of the WRKY family. The open reading frame of *GarWRKY5* (GenBank accession number KM438453) is 921 bp and putatively encodes a 306-amino acid protein. In roots of G. *aridum, GarWRKY5* genes were activated within 3 h of salt treatment, with expression peak during the 72 h. This observation implies a potentially important role for *GarWRKY5* in mediating NaCl stress responses. In the present study, we further confirmed the role of *GarWRKY5* in salt stress response by overexpression of *GarWRKY5* in *Arabidopsis* and by silencing it in upland cotton. The expression profile of *GarWRKY5*-OE lines and wild-type plants was measured to investigate possible mechanisms by which *GarWRKY5* participates in salt stress responses.

Results

Characterization of GarWRKY5 based on structure, evolution and expression

Based on previous studies, *GarWRKY5* encodes a member of Group III of WRKY. The predicted GarWRKY5 proteins and homologous genes from *G. hirsutum* (Gh_A02G0029/Gh_D02G0043), *G. raimondii* (Gorai.005G003900) and *G. arboreum* (Cotton_A_04316) contain an approximately 60-amino-acid WRKY domain that is composed of the conserved amino acid sequence (WRKYGQK) and a zinc-finger motif (C-X_{4-5}-CX_{22-23}-H-X_1-C) (*Figure 1A)*.. Based on the evolutionary tree, Gorai.005G003900 from the G. *ramondii* genome was close to Gh_D02G0043 from the Dt-subgenome of allotetraploid cotton in evolutionary relationships. The paralogous pairs had ratios of nonsynonymous to synonymous substitutions (Ka/Ks) of more than 1.0 between *GarWRKY5* and the other four genes except Gh_D02G0043, indicating that they had gone through positive selection in the evolutionary process

(*Figure 1B, Supplementary Table S1).*. In terms of expression in vegetative and reproductive organs of G. *aridum, GarWRKY5* had a higher expression level in the root than in other organs *(Figure 1C)*..

Silencing GarWRKY5 in upland cotton line compromises salt tolerance.

To elucidate the role of *GarWRKY5,* the virus-induced gene silencing (VIGS) method was used to knockdown the expression of *GhWRKY5,* a homologous gene of *GarWRKY5* in upland cotton. After growing plants in illumination incubator for one week, we hand-infiltrated *Agrobacterium* cultures carrying the VIGS vector into cotton cotyledons. Approximately 7 d after agroinfiltration, leaves of the *GhCLA1*-silenced plant displayed the photobleaching phenotype as expected which was uniformly distributed on the entire true leaves (*Figure 2A),*, Suggesting that the VIGS system can work well based on our experimental conditions. To investigate the silencing efficiency of *GhWRKY5* in the tested plants, Semi-quantitative RT-PCR was used to determine the expression levels. The results showed that the *GhWRKY5* expression level in the silenced plants was much lower than in the control plants. At least five *GhWRKY5*-silenced plants with four true leaves were treated with 300 mM NaCl solution, the distilled deionized water was used to the control. Ten days later, the tolerance of the TRV::*GhWRKY5* plants decreased significantly compared with TRV::00 (infiltrated with empty vector) plants in two upland cultivars, the growth of TRV::*GarWRKY5* plants from the salt-sensitive cultivar (Su12) being inhibited more severely than was that of TRV::*GhWRKY5* plants from the salt-tolerant cultivar (Miscott 7913–83) (*Figure 2B, 2C).*. VIGS experiments were repeated at least three times with more than ten plants for each construct per repeat.

Overexpression of GarWRKY5 regulates salt tolerance in Arabidopsis.

To further analyze the function of *GarWRKY5* under salt stress conditions, we generated *GarWRKY5*-overexpressing lines in *Arabidopsis* for phenotypic observation and physiological analysis. Three positive transgenic *Arabidopsis* lines (lines 1, 6 and 14) with high expression levels of *GarWRKY5* were selected for further analysis.

The transgenic *Arabidopsis* lines overexpressing the *GarWRKY5* gene were germinated on solid medium containing 0 or 150 mM NaCl. The germination rates and root length showed no significant difference in between WT and transgenic plants under normal growth conditions. However, the germination rates of the three *GarWRKY5*-overexpressing lines (*GarWRKY5*-1,6,14) were significantly improve than that of the WT (40.0%, 49.3% and 44.0% vs 20.0%, respectively) (*Figure 3A).*. When 20-day-old *GarWRKY5*-overexpressing transgenic plants were treated with 150 mM or 200 mM NaCl solution and the distilled deionized water was used to control, four weeks later, the growth of both the *GarWRKY5*–overexpressing transgenic and the WT plants was significantly inhibited while the *GarWRKY5*-overexpressing seedlings

can remain green and continue to grow (*Figure 3B).*. These data indicated that *GarWRKY5* positively regulated salt tolerance at the stage of seed germination and vegetative growth.

To study the physiological response of *GarWRKY5*-overexpressing *Arabidopsis* plants to salt stress, the three transgenic lines (*GarWRKY5*-1, 6, 14) were selected to further analyze the activities of SOD and POD in leaves of WT and transgenicplants. The SOD activity of transgenic plants was significantly higher than that of WT plants with or without salt stress. In particular, the SOD activity of the three transgenic lines, relative to the wild-type plants under salt stress, increased 5.1 times (*GarWRKY5*-1), 5.5 times (*GarWRKY5*-6) and 5.0 times (*GarWRKY5*-14) (*Figure 3C).*. The POD activity of transgenic plants was significantly improving in the WT plants under salt stress. The activity of POD in the three transgenic lines can increase 5.8 times (*GarWRKY5–1),*, 7.0 times (*GarWRKY5–6)* and 6.6 times (*GarWRKY5–14)* (*Figure 3D).*. Plants have complex antioxidative defense systems to maintain reactive oxygen species (ROS)-scavenging ability and control intracellular (ROS) homeostasis [19, 20]. These data demonstrated that overexpression of *GarWRKY5* in transgenic *Arabidopsis* plants resulted in increased activities of antioxidative enzymes, which was associated with the increased salt tolerance of the transgenic *Arabidopsis* plants.

The GarWRKY5 regulatory network in salt stress

To identify potential target genes of *GarWRKY5,* we then performed RNA-sequencing (RNA-Seq) analysis of 35S:*GarWRKY5* and wild-type plants grown under salt stress for 0 d or 3 d. The SOD and POD activities were assayed in the three independent *GarWRKY5*-overexpressed transgenic lines (*GarWRKY5–1, GarWRKY5–6* and *GarWRKY5–14),*, with three independent biological replicates of each line. In total, expression of 398 genes was significantly changed in the *GarWRKY5*-OE lines compared with the wild-type plants under normal growth conditions (0 d NaCl). Among them, 253 differentially expressed genes (DEGs) were upregulated in the OE lines whereas 145 DEGs were down-regulated (OE 0d vs WT 0d; *Supplementary Table S2).*. These genes represented the candidate downstream genes regulated directly or indirectly by *GarWRKY5.*

GO enrichment analysis was performed on the 253 upregulated DEGs and 145 downregulated genes, respectively. For the upregulated DEGs, under “biological process,” oxidant detoxification, response to jasmonic acid and salicylic acid, response to salt stress and osmotic stress were significantly enriched, like glucosyltransferase activity and calcium ion binding in “molecular function,” and vacuole and protein-containing complex in “cellular component.” For downregulated DEGs, response to ethylene, oxidative stress and abiotic stimulus were significantly enriched in “biological process,” but no categories were significantly enriched with respect to “molecular function” and “cellular component” (*Supplementary Table S3).*.

GO enrichment analysis showed there were 19 DEGs involved in salt stress and the osmotic stress process. These 19 genes were grouped into two main categories, namely ROS scavenging and response to hormone. The “ROS-scavenging group” included glutathione S-transferases (GSTs) (AT1G02920 and

AT1G02930) and superoxide dismutase (SOD) (AT1G08830 and AT2G28190). The “response of hormone group” included jasmonic-acid-response genes (AT1G43160, AT1G56650, AT3G16470, AT4G23600 and AT5G24770) and salicylic-acid-response genes (AT1G43160 and AT2G33380) (*Table 1)*..

Because *GarWRKY5* was homologous to *AtWRKY70* [14], we performed network analysis for these 19 DEGs and *AtWRKY70* using the STRING database, version 11.0 (https://string-db.org/). The result showed that *AtWRKY70* could regulate AT1G02930 (glutathione S-transferase F6, GSTF6) and AT5G24770 (acid phosphatase VSP2) (*Supplementary Figure S1)*.. In addition, we analysed the promoters (1-kb upstream of the translation start sites) of the 19 DEGs using the JASPAR database [21]. Promoter region screening showed that all 19 DEGs had three to ten W-box motifs in their promoter regions, a DNA-sequence motif (T)TGAC(C/T) which could bind to a WRKY transcription factor (*Table 1)*.. Based on the data presented in this study, we hypothesize that *GarWRKY5* may be a positive transcription regulator involved in plant response to high salinity stress through the ROS-scavenging system such as by activating expression of GST and SOD genes by jasmonic acid-mediated or salicylic acid-mediated signaling pathways.

Discussion

WRKY TFs are key regulators of many plant processes, including responses to biotic and abiotic stresses, senescence, seed dormancy and seed germination [22]. Recent studies have broadened our knowledge of the WRKY TF family and its functions in salt stress responses in cultivated cotton [18, 23, 24]. However, wild relatives of crops represent potentially valuable gene pools and are primary source of important genes. In the previous study [14], by using a D-genome diploid species (G. *aridum)* from the Pacific coastal states of Mexico, which shows remarkable tolerance to salt stress, we set out to perform transcriptome analysis and identified the response of 28 WRKY TFs in *G. aridum* to salt stress conditions. Based on overexpression of *GarWRKY17* and *GarWRKY104* in *Arabidopsis,* functional analysis indicated that these two genes could positively regulate salt tolerance in different developmental stages of transgenic *Arabidopsis* [14]. In the present study, we have provided evidence that *GarWRKY5* positively regulates salt stress by overexpressing *GarWRKY5* in *Arabidopsis* and silencing it in upland cotton. Together with the findings from the previous study, we have concluded that *GarWRKYs* from *G. aridum* might play a significant role in modulation of salt-stress response and could potentially be utilized to improve salt tolerance in cultivated cotton and other crops.

In the salt stress, the plants can accumulate ROSs and enhance the expression of ROS-scavenging enzymes. Alleviation of oxidative damage by scavenging ROSs is an important strategy by which plants can tolerate stress [25]. Transgenic plants overexpressing ROS-scavenging enzymes such as SOD, glutathione S-transferase/glutathione peroxidase (GST/GPX), and ascorbate peroxidase (APX) have shown increased tolerance to osmotic, oxidative stress, and temperature [26–28]. In the current study, the activities of POD and SOD were higher in the *GarWRKY5*-OE lines than in the wild-type plants and contributed to the increased salt tolerance of the transgenic *Arabidopsis* plants. Meanwhile, based on GO enrichment and prediction of the W-box motif, ROS-scavenging genes were enriched, including

glutathione S-transferases (GSTs) (AT1G02920 and AT1G02930), and superoxide dismutase (SODs) (AT1G08830 and AT2G28190) in the *GarWRKY5*-OE lines. They contained 6–10 W-box motifs at their promoter region. GSTs and SODs could be regarded as candidate target genes to which *GarWRKY5* binds. Taken together, we hypothesize that *GarWRKY5* may be a positive transcription regulator in response to high salinity stress through the ROS-scavenging system by activating expression of GST genes, highlighting the importance of *GarWRKY5 as* a metabolic engineering tool for improvement of salt stress in cotton.

GarWRKY5 shows sequence homology with *OsWRKY45* in rice and *AtWRKY70* in *Arabidopsis* [14]. The expression of rice *WRKY45* (*OsWRKY45)* was markedly induced in response to the stress-related hormone abscisic acid (ABA) and various stress factors, e.g. application of NaCl, polyethylene glycol (PEG), mannitol or dehydration. Constitutive over-expression of *OsWRKY45* conferred a number of properties to transgenic plants, including increased resistance to the bacterial pathogen, and increased tolerance to salt and drought stresses in *Arabidopsis* [29]. GST and cytochrome P450 genes are regulated by *WRKY45* in rice [30]. In this study, network analysis for *AtWRKY70* andthe 19 DEGs enriched with respect to the salt stress and osmotic stress processes by the STRING database showed that *AtWRKY70* could regulate AT1G02930 (glutathione S-transferase F6, GSTF6). All these observations showed that *GarWRKY5* might have regulatory mechanisms similar to those of *OsWRKY45* in rice and *AtWRKY70* in *Arabidopsis.*

Previous studies have demonstrated that the Group III WRKY members may play prominent roles under biotic and abiotic stress responses. For example, overexpression of a grape Group III WRKY transcription factor gene, *VlWRKY48,* in *A. thaliana* increased disease resistance and drought stress tolerance [31]. Another Group III member, *AtWRKY46,* functioned in both basal resistance against pathogens and tolerance to oxidative stress and aluminium toxicity to be induced by drought, salt and oxidative stresses [32]. Overexpressed *OsWRKY45* in *Arabidopsis* increased pathogen defense, drought and salt resistance [29]. Overexpression of *AtWRKY70* led to upregulation of PR genes and downregulation of PDF1.2, leading to enhanced resistance against biotrophic pathogens and enhanced susceptibility to necrotrophic pathogens. *AtWRKY70,* as a repressor of JA-responsive genes and an activator of SA-induced genes, integrating signals from these mutually antagonistic pathways [33]. The function of Group III WRKY members may be a node of convergence that integrates biotic and abiotic stress signals, so they have great potential for increased stress tolerance [34]. Encoding a member of the Group III WRKY family, the potential role of *GarWRKY5* in mediating response to multiple stress factors needs to be further investigated.

Conclusions

Based on the data presented in this study, we hypothesize that *GarWRKY5* may be a positive transcription regulator in plant response to high salinity stress through the ROS-scavenging system, such as activating expression of GST and SOD genes by the jasmonic acid- or salicylic acid-mediated signaling pathway.

Materials And Methods

Plant materials and treatment conditions

The National Wild Cotton Plantation in Hainan Island, China, kindly supplied seeds from the wild *Gossypium* species G. *aridum.* The same treatment procedure was used as described by Xu et al. (2013) [35]. The G. *aridum* seeds were germinated in distilled deionized water, the growth conditions were 60% humidity, the day and night temperature were 28℃ and 23℃ respectively, photoperiod of 12h light/12h dark in the growth chamber. The germinated seeds were planted into nutritional soil and cultured in the plant growth chamber with the same set conditions. The uniform cotton seedlings with about 20cm in height and four true leaves were transferred into paper cup with 1×Hoagland's nutrient solution. After three days, the uniform cotton seedlings were treated for 200 mM NaCl for 0, 1, 3, 6, 12, 24 and 72 h, and untreated seedlings were used for the control. Root and leaf tissues were collected respectively at each stage under salt stress treatment. All samples were immediately frozen in liquid nitrogen and stored at –70℃.

Phylogenetic analysis of GarWRKY5 genes

A phylogenetic tree was constructed using ClustalW alignment and the Neighbor-Joining (NJ) method in MEGA 7.0 software [36] (https://www.megasoftware.net/), with 1,000 replicates bootstrap test. The ratios of nonsynonymous to synonymous substitutions (Ka/Ks) between the paralogous pairs were analyzed with DnaSP v6.0 software [37].

Fluorescence real-time qPCR

The RNA sequencing samples that were also isolated used to perform real-time quantitative (qPCR) analysis. Total RNA samples with 2μg per reaction were reverse transcribed into cDNA by using M-MLV Reverse Transcriptase (Promega, USA). qPCR specific primers were designed based on the candidate gene sequences close to the 3' end using Beacon Designer 7.0 software from Premier Biosoft International, Palo Alto, CA, USA. The *Histone3* (GenBank NO: AF024716) was used as reference gene. The light cycler carried out using IQ SYBR Green Supermix (Bio-Rad, USA) based on the manufacturer's instructions and the qPCR products were quantified using the ABI 7500 fast (Applied Biosystems, USA). The amplification reactions conditions for PCR were performed as follows: 94°C for 3 min, followed by 40 cycles at 94°C for 15 s, 60°C for 15 s and 72°C for 30 s. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with three biological replicates and three experimental replicates [38].

Analysis of salt tolerance in transgenic Arabidopsis plants

For the salt tolerance of *GarWRKY5* transgenic *Arabidopsis* plants during the seed germination stage, 50 seeds of T_2 generation transgenic lines (three lines for *GarWRKY5)* were surface sterilized and sown on Murashige & Skoog (MS) medium with and without 150 mM NaCl, respectively. The wild type (WT) was

used to control. After ten days, the germination rate of seeds was calculated. The experiment was repeated at least three biological replicates. For further verification overexpression of *GarWRKY5* could enhance tolerance to salt stress during vegetative growth, sterilized seeds of WT and T_2 transgenic *Arabidopsis* were sown in soil. After 20 days, the seedlings were grown in a pot supplemented with 150 mL NaCl solution (150 mM/L) and the distilled deionized water was used for control. The phenotype of seedlings was observed after four weeks. For the determination of antioxidant enzymes activity, three-week-old seedlings from WT and T_2 generation of three *GarWRKY5*–overexpressing transgenic lines (*GarWRKY5–1, GarWRKY5–6* and *GarWRKY5–14)* were soaked in 150 mM/L NaCl solution for 24 h. Leaves of at least ten seedlings were collected from the wild type and three transgenic lines, respectively. The activity of peroxidase (POD) and superoxide dismutase (SOD) was determined based on the procedure described by Liu et al. (2008)[39]. It is one unit of SOD activity that the mount of enzyme required to cause 50% inhibition of nitro blue tetrazolium (NBT) reduction. The SOD was measured at 560 nm by the ultraviolet spectrophotometer. The activity of Peroxidase (POD) was analyzed at 470nm using guaiacol as a substrate by the ultraviolet spectrophotometer. The experiment was performed in 50 mmol/L phosphate buffer, 50 mmol/L guaiacol and 2% H_2O_2 and 2 μl of enzyme extract were added. The data was recorded after adding 2.0 ml 20% chloroacetic acid. All the above procedures of enzyme extraction were carried out at 0–4 ℃. The enzyme assays were performed in three biological replicates.

Virus-induced gene silencing (VIGS) assays

In order to knockdown the expression of the *GhWRKY5* gene, a 389-bp fragment of the *GhWRKY5* cDNA from TM–1 was amplified using the VIGS primers. The resulting PCR product with double digested (XbaI and KpnI) was recombined into XbaI-KpnI- digested pTRV2 in order to produce pTRV2::*GhWRKY5.* The pTRV2::*GhWRKY5* vector was introduced into the*Agrobacterium* strain GV3101 by means of electroporation (Bio-Rad, Hercules, CA, USA). For the VIGS assay, the GV3101 containing pTRV1, pTRV2 (mock-treated controls), pTRV2::*GhWRKY5* and pTRV2::*GhCLA1* respectively were used for VIGS experiments. The strains were grown overnight at 28°C with shaking at 150 rpm in LB broth containing two antibiotics kanamycin and rifampicin in concentrations of 50 mg/L each. The *Agrobacterium* were harvested by centrifugation for 5 min at 5,000 rpm, and resuspended in infiltration buffer (10 mM MES, 10 mM $MgCl_2$ and 200 mM acetosyringone) to a final OD_{600} of 2.0. The *Agrobacterium* strains with the TRV1 or TRV2 vectors were mixed by equal volume and incubated for three hours at 28℃. Seedlings with mature cotyledons but no visible true leaf (about one-week post-emergence) were infiltrated by inserting the *Agrobacterium* suspension into the cotyledons surface via lightly pricking with a syringe. The plants were grown at 23°C (day/night) in an illumination incubator with a 16 h light/8 h dark cycle and at a relative humidity of 60% for one week [40–42]. VIGS experiments were repeated at least three times with more than five plants for each construct per repeat.

Transcriptome sequencing and DEGs analysis

Approximately a total of 8 μg RNA per sample was used. The RNA purity and quality were assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Three biological replicates RNA samples were prepared for library construction and sequencing. Based on the manufacturer's guide protocol of kit (Illumina® TruSeq™ RNA Sample Preparation Kit (Illumina Inc. San Diego, CA, USA)), the cDNA libraries were prepared. The cDNA libraries were sequenced based on an Illumina HiSeq™ 2000 with 100bp single end reads each. The basis of gene expression analysis was as follows the number of unambiguous clean tags for each gene was calculated and then normalized to TPM (number of transcripts per million clean tags) [43, 44].

The reads from RNA-Seq were aligned to the reference genome (TAIR10 data) using Tophat v.2.0.11, which was compatible with Bowtie2 v2.2.1[45]. All reads were allowed only one nucleotide mismatch. Clean reads mapping to reference sequences from multiple genes were filtered out. For differentially expressed genes (DEGs) analysis, we adopted a conservative criterion by choosing consistent results of cuffdiff (ref), with $|\log_2 (\text{fold change})| \geq 1$ and significant expression with FDR < 0.05 and genes FPKM value ≥ 1.

Abbreviations

DEGs: Differentially Expressed Genes

GO: Gene Ontology

POD: Peroxidase

SOD: Superoxide dismutase

TF: Transcription Factor

VIGS: Virus-induced Gene Silencing

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All raw transcriptomics reads have been deposited in NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra). The BioProject and SRA accession are PRJNA529955.

Competing Interests

The authors have declared that no competing interests exist.

Authors' contributions

XLS designed the experiments. QG and LZ conceived the experiments and analyzed the results. XQF carried out all computational analyses. PX, ZZX and XGZ participated in part of experiments. QG and XLS drafted the manuscript and XLS revised the manuscript. All authors read and approved the final draft of the manuscript for submission.

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Table

Table 1 Nineteen DEGs involved in salt stress and osmotic stress process based on GO enrichment analysis

Gene ID	Gene Annotation	$log_2^{(OE\ 0d\ vs\ WT\ 0d)}$	padj	W-box
AT1G02920	glutathione S-transferase F7	1.50	4.706E-15	6
AT1G02930	glutathione S-transferase F6	1.25	1.1499E-20	8
AT1G08830	superoxide dismutase [Cu-Zn]	1.16	9.3988E-31	6
AT1G27730	zinc finger protein STZ/ZAT10	1.19	4.2955E-07	5
AT1G43160	ethylene-responsive transcription factor RAP2-6	1.59	8.063E-08	8
AT1G52400	beta glucosidase 18	1.51	2.5569E-18	3
AT1G56650	transcription factor MYB75	2.50	9.7036E-37	3
AT1G65690	late embryogenesis abundant (LEA) hydroxyproline-rich glycoprotein	1.29	6.2703E-06	7
AT2G28190	copper/zinc superoxide dismutase 2	1.44	5.1156E-56	10
AT2G33380	caleosin 3	1.63	1.2821E-33	4
AT2G38750	annexin D4	1.29	3.9269E-23	4
AT2G38760	annexin D3	1.25	1.6239E-12	6
AT3G16470	JA-responsive protein 1	1.07	6.0879E-12	4
AT3G49580	protein RESPONSE TO LOW SULFUR 1	1.41	3.7982E-06	5
AT4G23600	cystine lyase CORI3	1.06	0.00040635	5
AT4G30650	putative low temperature and salt responsive protein	1.19	6.0605E-18	10
AT5G24660	protein RESPONSE TO LOW SULFUR 2	1.80	2.4011E-10	6
AT5G24770	acid phosphatase VSP2	1.31	1.2086E-06	5
AT5G59820	high light responsive zinc finger protein ZAT12	1.24	1.4181E-05	8

Figures

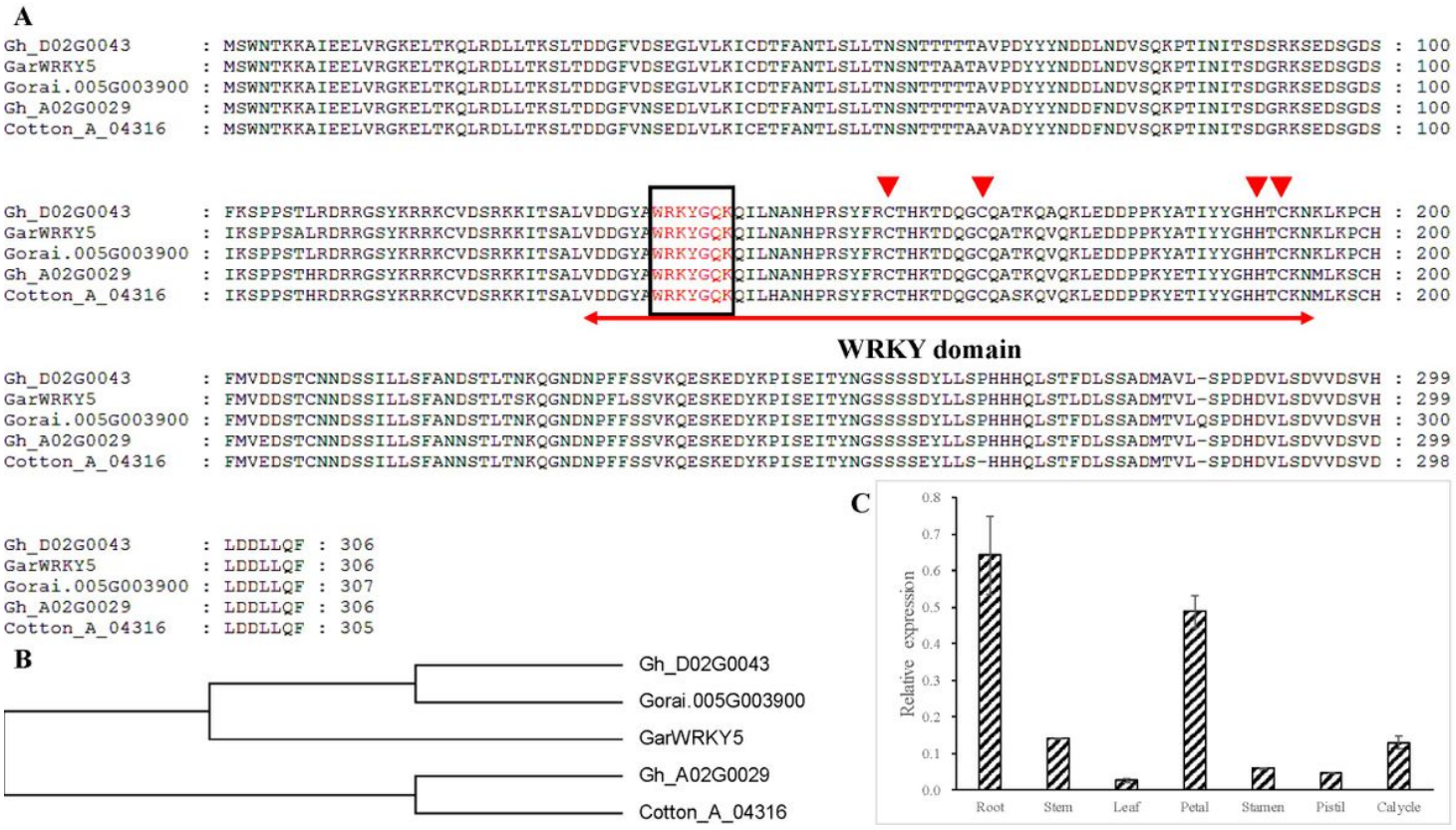

Figure 1

Structure, evolution and expression of GarWRKY5. A: Identification of the WRKY domain. The approximately 60-amino-acid WRKY domain and C-X7-CX23-HXC-type zinc-finger-like motif. B: Phylogenetic analysis of GarWRKY5 between homologous genes from G. hirsutum (Gh_A02G0029/Gh_D02G0043), G. raimondii (Gorai.005G003900) and G. arboreum (Cotton_A_04316). C: The expression of GarWRKY5 in different tissues and organs from G. aridum.

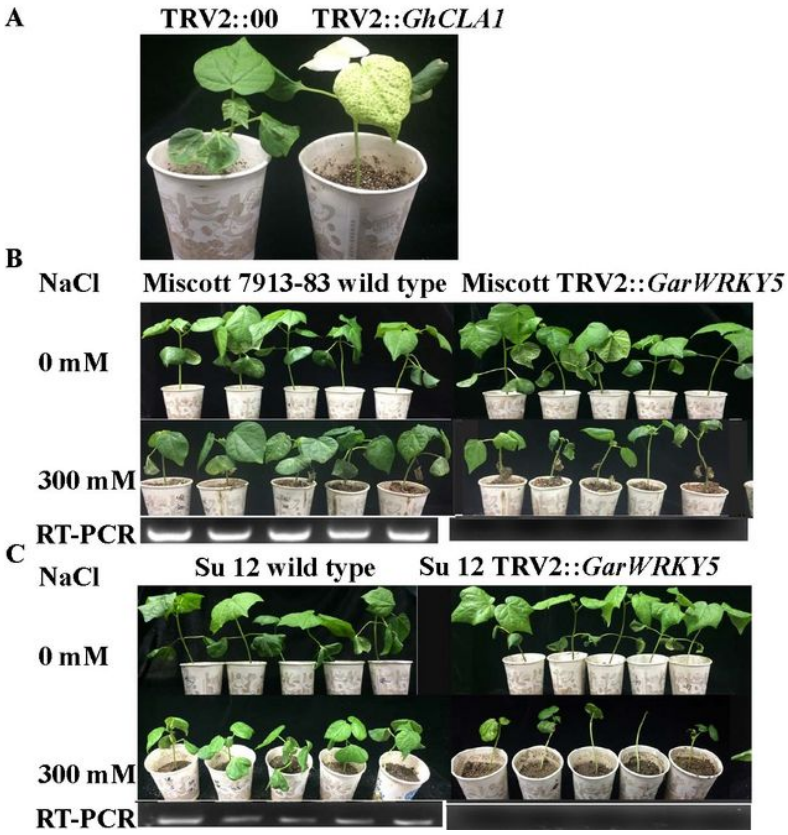

Figure 2

The identification of salt tolerance in the GhWRKY5-silenced plants by virus-induced gene silencing. A: The leaves of TM-1 turned white after TRV2::GhCLA1 gene silencing, and empty vector (TRV2:00) leaves remained as green as the wild-type TM-1. B: The leaves of ‘Miscott,’ a salt-tolerant cultivar, withered and new leaves grew slowly. C: The leaves of ‘Su12,’ a salt-sensitive cultivar, withered, fell off and new leaves grew slowly.

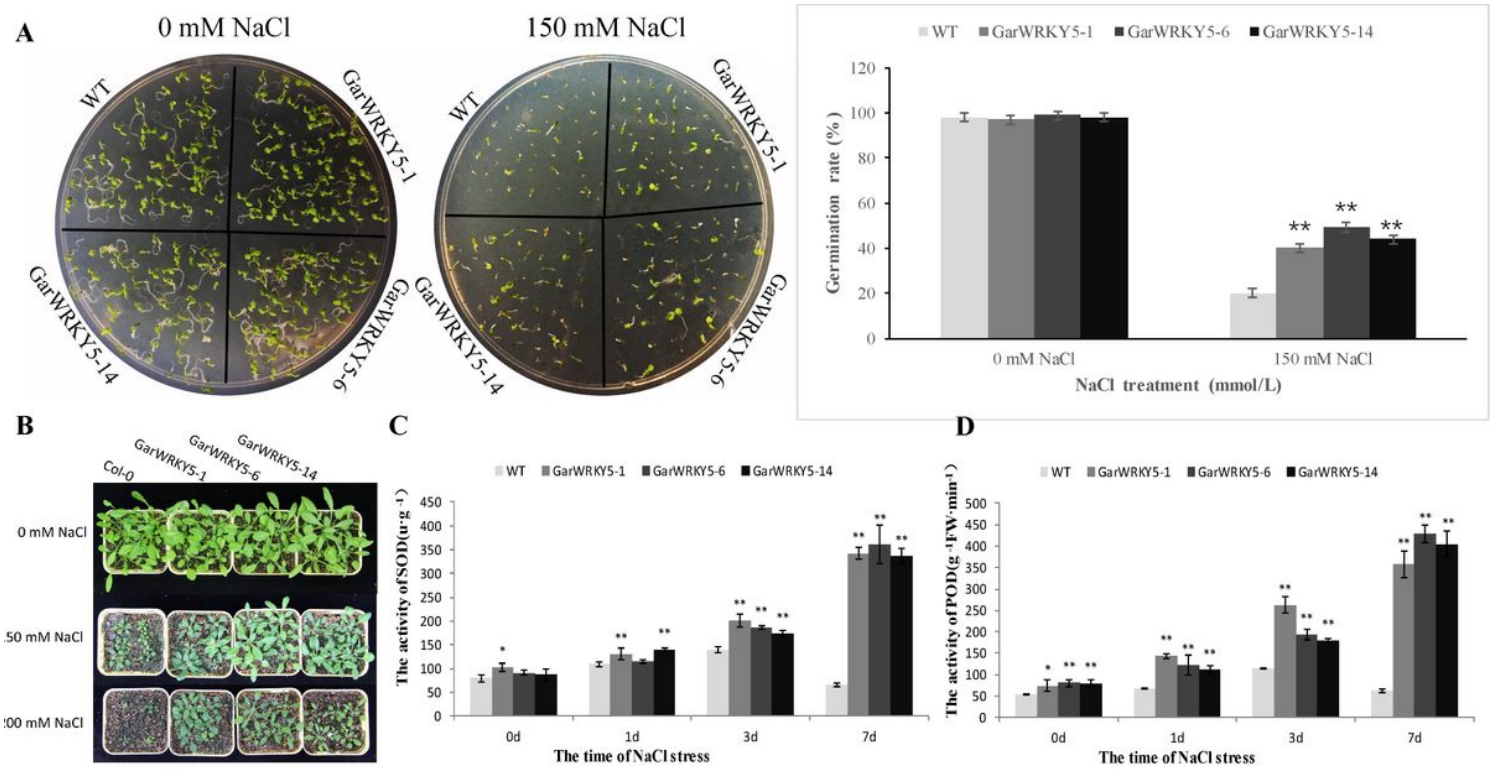

Figure 3

Overexpression of GarWRKY5 regulates salt tolerance in Arabidopsis. A: The germination rate of transgenic Arabidopsis lines overexpressing GarWRKY5 gene. B: GarWRKY5-OE lines were growth on soil medium containing 0mM, 150mM and 200mM NaCl. C: The activity of SOD. D: The activity of POD. Student's t-test: **P<0.01.

Supplementary Files

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- TableS3GOenrichmentofDEGsbetweenOE0dandWT0d.xlsx
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