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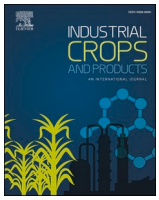

Sea-island cotton (*Gossypium barbadense* L.) GbTCP5 improves plant adaptation to drought and salt stress by directly activating *GbERD7*, *GbUBC19*, and *GbGOLS2* expression

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ABSTRACT

Cotton is one of the most important cash crops in the world. Most of its production areas are in arid or semiarid regions, and drought and salinity seriously affect cotton production. Plant teosinte branched1/cycloidea/proliferating cell factor 1 (TCP) transcription factors play a pivotal role in abiotic stress responses. However, the role of TCPs in the abiotic stress response in cotton has not been fully elucidated. Here, the function of the GbTCP5 transcription factor in the responses to drought and salt stresses was identified. The expression of *GbTCP5* was induced by drought, salt, and abscisic acid (ABA) treatments. The exposure of 35S:*GbTCP5* transgenic *Arabidopsis* to drought stress increased its survival rate and ABA content and decreased its leaf water loss rate and malondialdehyde (MDA) content compared with those of the wild type (WT). In addition, the expression levels of the stress response genes *AtCOR15*, *AtCOR414*, *AtRD29A*, *AtLTP4*, and *AtPUB22* and the ABA biosynthesis-related gene *AtNCED3* were significantly higher in 35S:*GbTCP5* transgenic lines than in the WT. The exposure of 35S:*GbTCP5* transgenic *Arabidopsis* to salt stress accelerated its germination rate, increased its root length, survival rate and chlorophyll content, and decreased the MDA content compared with those of the WT. Under drought or salt stress, cotton with virus-induced gene silencing of *GbTCP5* showed a decreased survival rate, a shorter root length, a smaller root area, a reduced root volume and a higher MDA content compared with pTRV2 cotton. Transcriptome sequencing results confirmed that *GbTCP5* was involved in several abiotic stress-related metabolic pathways. The results confirmed that GbTCP5 protein activated the expression of *GbGLOS2*, *GbUBC19*, and *GbERD7* and then participated in the drought and salt tolerance of cotton by binding to the TCP *cis*-acting element in the promoter regions of these genes. All the above results prove that *GbTCP5* plays a positive role in plant responses to drought and salt stress.

1. Introduction

Abiotic stresses severely affect plant growth and survival and reduce crop yields (Gupta et al., 2020). Salt and drought are the two most common abiotic stresses for crops, especially in arid and semiarid areas, and cause significant damage to many crops (Zhu, 2016). Cotton, the main source of natural fibers, is also a very economically important crop (Yang et al., 2020). However, drought and salt stresses limit both the cotton yields and growing areas, and breeders are constantly working to develop new varieties adapted to local environmental conditions (Zhu, 2016). The main challenge in improving the drought and salt tolerance of cotton is the complexity of this trait (Gupta et al., 2020). To withstand drought and salt stresses, plants have evolved coping strategies such as signal transduction, gene expression regulation, and physiological and biochemical reactions (Gupta et al., 2020). During signal transduction, many genes regulating the stress response, including different transcription factor families, are involved (Rohit et al., 2016).

Transcription factors are the most important regulatory factors in plants. These DNA-binding proteins mainly bind to specific motifs in the promoter region of downstream genes and play an important role in regulating the growth, development and stress responses of plant (Mrinalini et al., 2020). In plants, the TCP family is a unique family of

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transcription factors with an atypical basic helix-loop-helix (bHLH) structure, and its members are classified into two groups, I and II, based on differences in the domain amino acid sequences (Selahattin, 2016). The basic region of class I TCPs contains 4 fewer amino acid residues than that of class II TCPs, which belong to the PCF subfamily, and the binding sequence of the encoded protein is GGNCCCAC. According to differences in the basic region and bHLH domain, class II TCPs are further divided into CIN TCPs and CYC/TB1-like TCPs, which encode the protein binding sequence G(T/C)GGNCCC (Selahattin, 2016). TCP transcription factors are associated with various life activities in plants, including flower formation, leaf development, control of plant branching, seed germination, transduction of hormonal signals, and fiber development (Manassero et al., 2013). Furthermore, environmental stimuli affect the expression of TCP transcription factors in plants (Manassero et al., 2013).

TCP gene family members have been identified in numerous plants due to the ongoing advancement of whole-genome sequencing and transcriptome sequencing technology, and some *TCP* gene family members reportedly respond to drought and salt stresses, such as in rice (*Oryza sativa*) (Guan et al., 2022), corn (*Zea mays*) (Ding et al., 2019), soybeans (*Glycine max*), kidney beans (*Phaseolus vulgaris*), chickpeas (*Cicer arietinum*), Tribulus alfalfa (*Medicago truncatula*), hundred-vein root (*Lotus japonicus*) (Ling et al., 2020), alfalfa (*Medicago sativa*) (Wei et al., 2022), cassava (*Manihot esculenta Crantz*) (Lei et al., 2017), strawberries (*Fragaria ananassa*) (Wei et al., 2016), grapes (*Vitis vinifera*) (Leng et al., 2019), rapeseed (*Brassica napus*) (Wen et al., 2021), sweet potatoes (*Dioscorea esculenta*) (Ren et al., 2022), millet (*Setaria italica*) (Xiong et al., 2022), switchgrass (*Panicum virgatum*) (Huo et al., 2019) and apple (*Malus domestica*) (Xu et al., 2014). Subsequently, genetic, molecular, and biochemical methods have revealed the functions and mechanisms of some *TCP* genes in response to drought and salt stresses. For example, overexpression of the *OsTCP19* gene in rice can reduce water loss, induce the expression of genes in multiple signaling pathways, including abscisic acid (ABA)-related genes, and improve the tolerance to salt and mannitol (Mukhopadhyay and Tyagi, 2015). Drought stress induces upregulated expression of the *PeTCP10* gene, which improves drought resistance in *Arabidopsis* by increasing the number of lateral roots and reducing the number of stomatal openings (Liu et al., 2020). Upon exposure to drought stress, *Arabidopsis* overexpressing *ZmTCP42* shows increased survival compared with the WT (Ding et al., 2019). Under drought stress, sea buckthorn (*Hippophae rhamnoides*) overexpressing *HrTCP20* shows higher superoxide dismutase activity, increased polyphenol oxidase activity, and a higher chlorophyll content than the WT, whereas *HrTCP20*-silenced plants have a lower relative water content and higher relative electrical conductivity (REC) than WT plants, indicating that *HrTCP20* improves plant drought resistance (Yao et al., 2022). Under drought and salt stresses, *VuTCP9* overexpression in cowpea (*Vigna unguiculata*) improves plant tolerance (Mishra et al., 2022). The overexpression of *AtTCP13* in *Arabidopsis* slows its leaf water loss rate and increase its dehydration tolerance (Urano et al., 2022). In rice, *PCF2* regulates plant salt tolerance by directly targeting *NHX1* (Almeida et al., 2016). In contrast, the overexpression of *MdTCP46* decreases ABA sensitivity and drought resistance (Liu et al., 2022).

Previous studies have identified 38, 36, 74, and 75 TCP transcription factors in Raymond cotton (*Gossypium raimondii*) (Ma et al., 2014), Asian cotton (*G. arboreum*) (Ma et al., 2016), upland cotton (*G. hirsutum*) (Li et al., 2017), and sea-island cotton (*G. barbadense*) (Zheng et al., 2018), respectively, and have shown that some cotton *TCP* genes regulate flowering and branch, root and epidermal hair and fiber development. For example, the heterologous overexpression of *GhTCP14* advances and prolongs the development of root and epidermal hairs in *Arabidopsis* (Wang et al., 2013). *GhBRC1*, *GhBRC2*, and *GhTCP13* restore part of the *brc1–2* phenotype in the mutant and reduces the number of *brc1–2* branches (Diao et al., 2019). The overexpression of *GhTCP4* results in short fibers of varying lengths with thickened cell walls, whereas the downregulation of *GhTCP4* has the opposite result (Cao et al., 2020). The overexpression of *GhTCP62* reduces the branches of *Arabidopsis* rosette and stem leaves (Liu et al., 2021). *GbTCP* (GenBank accession no. DQ912941) overexpression advances, prolongs and enhances the branching of root hairs in *Arabidopsis*, whereas silencing of the *GbTCP* gene results in shorter fibers and reductions in the lint percentage and fiber quality in cotton (Hao et al., 2012). The overexpression of *GbTCP4* in *Arabidopsis* improves the root hair length, root hair number, and number of stem epidermal hairs (Wang et al., 2019). *GrTCP11* reduces the number of root hairs and delays flowering in *Arabidopsis* (Hao et al., 2021). However, only 2 cases of *TCP* genes being involved in the drought and salt tolerance of cotton have been reported. A transcriptome analysis confirmed that 41 genes in the *GhTCP* family of upland cotton are associated with the reaction to saltiness, temperature, and water stress (Yin et al., 2018). Transgenic *Arabidopsis* overexpressing *GbTCP4* shows increased plant drought and salt stress resistance, whereas the silencing of *GbTCP4* in cotton yields the opposite results (Wang et al., 2022). Research on the drought and salt tolerance of cotton is limited compared with that on the role of TCPs in cotton development; thus, it is necessary to study the effects of *TCP* genes on drought and salt tolerance in cotton.

Previous studies revealed that heterologous overexpression of *GbTCP5* in *Arabidopsis* increased the length and number of root hairs, the number of epidermal hairs, and the content of stem lignin compared with those of the WT (Wang et al., 2020). This research mainly identified the function of *GbTCP5* in the drought and salt tolerance of plants. A quantitative real-time polymerase chain reaction (qPCR) analysis showed that expression of the *GbTCP5* gene was induced by NaCl, ABA, and polyethylene glycol (PEG). Transgenic *Arabidopsis* overexpressing *GbTCP5* showed increased drought and salt stress resistance, whereas virus-induced gene silencing (VIGS) of *GbTCP5* in cotton reduced the drought and salt tolerance. A transcriptome analysis demonstrated that *GbTCP5* affects multiple metabolic pathways related to abiotic stress. Yeast one-hybrid and luciferase (LUC) activity assays revealed that GbTCP5 enhances the ability to resist drought and salt by directly activating the expression of *GbERD7*, *GbUBC19*, and *GbGOLS2*. These results suggest that the responses of sea-island cotton to drought and salt stresses are positively regulated by *GbTCP5*.

2. Materials and methods

2.1. Plant materials and treatments

GbTCP5-transgenic homozygous T_3-generation *Arabidopsis* obtained as in previous research was used in this study (Wang et al., 2020).

For both the VIGS transient transformation receptor and qPCR analyses, the sea-island cotton variety XH14 was utilized. The seeds were soaked in 50% hydrogen peroxide for 6 h (h), sown in nutrient soil (nutrient soil: plantarstone, 1:1), and cultured at 28 °C under a 16-h light/8-h dark cycle. For hydroponic treatment, cotton at the 3-leaf stage with consistent and healthy growth was transferred to hydroponic culture. After 2 days (d) of recovery, the cotton roots were immersed in solutions containing 15% PEG6000, 100 μM ABA, or 350 mM NaCl (Liu et al., 2018), and samples were collected after 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h of treatment.

2.2. qPCR

Total RNA extraction was performed with a plant total RNA extraction kit (BIO Flux, Germany). First-strand cDNA was synthesized using a reverse transcription kit according to the manufacturer's instructions (Thermo, Shanghai, China). SYBR and MixTaq (TransGen Biotech, Beijing, China) reagents were used, and qPCR was performed using an ABI 7500 instrument with a total volume of 20 μL. Cotton *GbUBQ7* and *Arabidopsis AtUBQ3* were used as internal reference genes. The qPCR primers are shown in Table S1.

2.3. Arabidopsis stress treatment

Seeds of 35S:*GbTCP5* transgenic and Col-0 plants were sterilized with 0.5% sodium hypochlorite and planted in Murashige and Skoog (MS) medium containing different stress solutions (400 mM mannitol, 150 mM NaCl, and 2 μM ABA) (Yu et al., 2020; Zhao et al., 2019) at 25 °C under a 16-h light/8-h dark cycle. The germination rate was measured for 7 consecutive days.

For determination of the root length, seeds of 35S:*GbTCP5* transgenic and Col-0 plants were planted on MS medium containing different stress solutions (no added stress solution, 400 mM mannitol, 150 mM NaCl, and 2 μM ABA) and cultured vertically for 7 d, and the root length was measured.

For the drought test, after 13 d of cultivation under a normal water supply, watering was stopped, water cultivation was resumed for 5 d, and the survival rate was calculated (Yu et al., 2015).

For the salt stress treatment, Col-0 and 35S:*GbTCP5* were cultured under a normal water supply for 4 weeks, watering was stopped for 1 week, and the plants were then bottom-watered with 250 mM NaCl solution (Yang et al., 2019). Once the soil was saturated, the NaCl solution was removed, the plants were cultured normally for 6 d, and the survival rate was calculated.

Each of the aforementioned experiments was repeated at least three times, and all replicates included at least 45 *Arabidopsis* plants.

The leaf water loss rate, chlorophyll content (soil plant analysis development, SPAD), and ABA content were determined according to Yu et al. (2020). The MDA content was determined as described by Wang et al. (2022).

2.4. VIGS of GbTCP5

The 291-bp 3′ untranslated region of *GbTCP5* was double-digested with the viral vector pTRV2 and then connected to obtain pTRV2-*GbTCP5*, which was transferred into *Agrobacterium* GV3101 by the freeze—thaw method. The injection of cotton was performed as described by Zhang et al. (2019), and cotton injected with the *GbCLA1* gene served as a positive control. The silencing efficiency of pTRV2-*GbTCP5* cotton was analyzed by qPCR. Each of the aforementioned experiments was repeated at least three times, and each replicate included at least 30 cotton plants. The PCR and qPCR primers are displayed in Table S1.

2.5. Cotton stress treatment

Natural drought and PEG stresses were applied according to previously reported methods (Liu et al., 2018). Salt stress treatments can be divided into two types: hydroponics using NaCl and direct watering of soil with salt water (Zhang et al., 2019). The cotton survival rate, MDA content, leaf water loss rate, and SPAD value were determined based on previously described approaches (Hu et al., 2022).

An Epson root scanner was used to scan the cotton roots subjected to the aforementioned hydroponic stress treatments (NaCl and PEG), and WinRHIZO software was used to analyze the scanned images.

Each of the aforementioned experiments was repeated at least three times, and all replicates included at least 30 cotton plants.

2.6. RNA-sequencing analysis

The samples used for transcriptome sequencing were leaves of the pTRV2-*GbTCP5* and pTRV2 cotton plants grown without any treatment, and three biological replicates of each sample were included. Bamark Biomarker Technology (Qingdao, China) performed the transcriptome sequencing, RNA-seq library construction, and RNA extraction tasks. The differentially expressed genes (DEGs) between the pTRV2-*GbTCP5* and pTRV2 samples were screened based on the following criteria: false discovery rate < 0.01 and fold change≥ 2. The Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Swiss-Prot databases were used for DEG annotation. DEGs were randomly selected, and the transcriptome sequencing results were verified by qPCR. The qPCR primers are shown in Table S1.

2.7. Yeast one-hybrid assay

pGADT7-GbTCP5 was obtained as described previously (Wang et al., 2020). The PLACE online prediction tool (https://www.dna.affrc.go.jp/PLACE/) was used to examine the *cis*-acting elements of the promoter. After being cloned from the DNA genome of sea-island cotton, the full-length promoter sequences of *GbEDR7*, *GbUBC19*, and *GbGOLS2* and the mutant TCP *cis*-acting elements of *GbEDR7*, *GbUBC19*, and *GbGOLS2* were ligated with the pHIS2 vector to produce pHIS2-*GbEDR7*/m*GbEDR7*, pHIS2-*GbUBC19*/m*GbUBC19*, and pHIS2-*GbGOLS2*/m*GbGOLS2*. The aforementioned sequence and pGADT7-*GbTCP5* were introduced into Y187 yeast cells and tested in SD-Trp-Leu-His+ 5 mM 3-amino-1,2,4-triazole (3-AT) selection medium (Wang et al., 2022).

2.8. Dual-luciferase activity detection

The full-length open reading frame of the *GbTCP5* gene was ligated with the pGreen-62-SK vector to obtain pGreen-62-SK-*GbTCP5*. The full-length promoter sequences of *GbEDR7*, *GbUBC19*, and *GbGOLS2* were connected to the pGreen-0800-LUC vector, which yielded pGreen-0800-LUC-*GbERD7*, pGreen-0800-LUC-*GbUBC19*, and pGreen-0800-LUC-*GbGOLS2*, respectively. A Promega (Beijing, China) kit was used to measure luciferase activity, and tobacco injection was conducted in accordance with previously reported procedures (Mary et al., 2011). The leaves were observed under a low-light cooled charge-coupled device imaging apparatus (Lumazone_1300B, Roper Bioscience), and photographs were then taken.

2.9. Statistical analysis

SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) were utilized for graphing, and the data were analyzed by Student's t test and analysis of variance (ANOVA).

3. Results

3.1. GbTCP5 is affected by a variety of abiotic stresses

The transcription levels of *GbTCP5* under ABA, NaCl, and PEG treatments were analyzed by qPCR. The experimental results showed that *GbTCP5* showed differential expression at different times under the abovementioned three stress treatments. The highest transcript levels of *GbTCP5* were obtained after treatment with ABA for 2 h (117.8 times) (Fig. 1A), treatment with NaCl for 6 h (12.3 times) (Fig. 1B), and treatment with PEG for 4 h (59.8 times) (Fig. 1C). Moreover, several *cis*-acting elements related to drought and ABA responses, such as ABREs and MBSs, were found in the *GbTCP5* gene promoter sequence (Table S2). ABA, NaCl, and PEG induced *GbTCP5* expression, as demonstrated by the above findings.

3.2. Under ABA or NaCl stress, Arabidopsis seed germination and root length are enhanced by GbTCP5 gene overexpression

GbTCP5 was overexpressed in *Arabidopsis* to explore its function in plants, and semiquantitative RT—PCR results showed that *GbTCP5* was expressed in 3 homozygous lines but not in Col-0 (Fig. S1A). No difference in the seed germination rate, root length, and root hair length was found between the 35S:*GbTCP5* and Col-0 plants under mannitol treatment (Fig. S1B-E; Fig. S2). The 35S:*GbTCP5* and Col-0 seed germination rates under ABA or NaCl treatments were compared. The findings

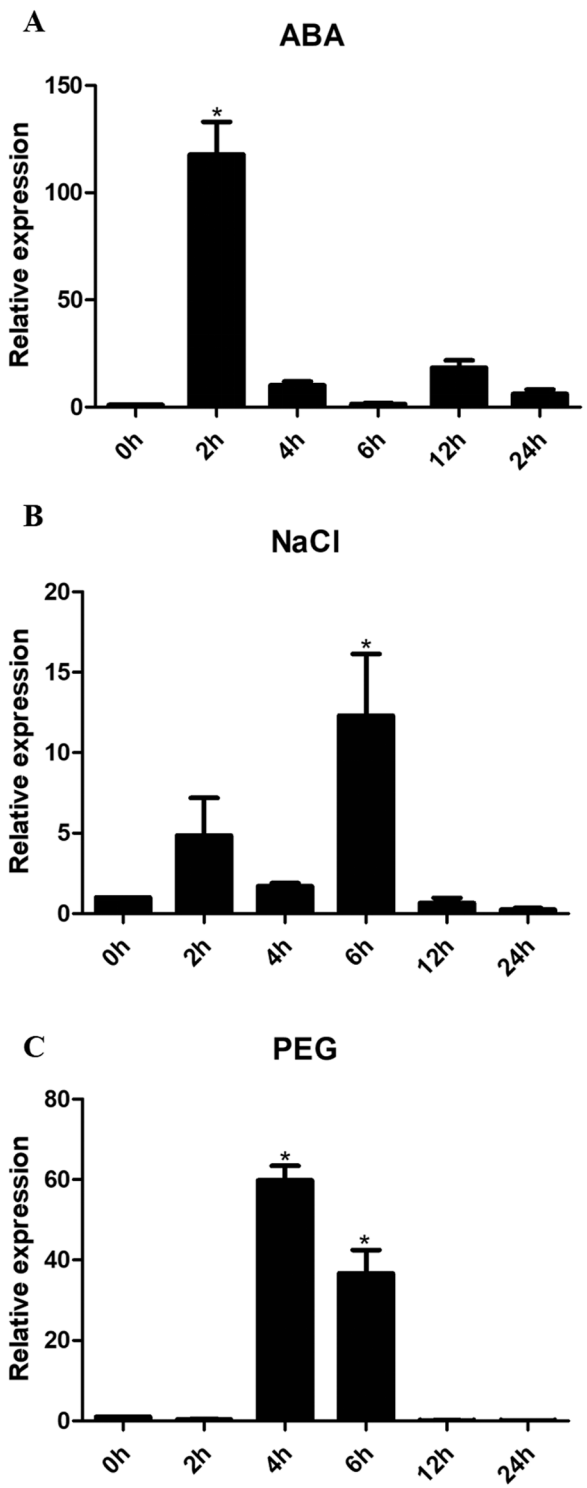

Fig. 1. Expression levels of *GbTCP5* after different treatments. (A) Analysis of the expression of *GbTCP5* during treatment with 100 μM abscisic acid (ABA). (B) Analysis of the expression of *GbTCP5* during treatment with 350 mM NaCl. (C) Analysis of the expression of *GbTCP5* during treatment with 15% polyethylene glycol (PEG) 6000. The vertical bars represent the means ± SDs; a significant difference compared with the control (0 h) is indicated by *P < 0.05.

demonstrated that the germination rates of the three 35S:*GbTCP5* lines were comparable to those of Col-0 when grown in MS medium with or without supplementation either 0.5 μM or 1 μM ABA solution (Fig. 2 A, D; Fig. S3). The germination rates of the 35S:*GbTCP5* and Col-0 seeds on MS medium containing 50 mM NaCl solutions were comparable (Fig. S4). However, the seed germination of three 35S:*GbTCP5* lines on medium with 2 μM ABA or 75 mM or 150 mM NaCl was significantly faster than that of Col-0 (Fig. 2B-C, E-F; Fig. S4). Similarly, the 35S:*GbTCP5* roots were longer than the Col-0 roots in MS, 2 μM ABA or 150 mM NaCl medium (Fig. 2G-K). These findings suggest that the seed germination and root length are enhanced by ABA or NaCl treatments in *GbTCP5*-overexpressing *Arabidopsis*.

3.3. *Arabidopsis* drought resistance is enhanced by GbTCP5 overexpression

Arabidopsis seedlings that had grown normally for 2 weeks were subjected to drought stress, and phenotypic differences appeared after 13 d of stress. The seedlings were then rehydrated. After 5 d of rehydration, some plants of the three 35S:*GbTCP5* lines showed recovery of growth, and the survival rates were 47.93%, 62.68%, and 71.67% (Fig. 3A, B). In contrast, the Col-0 plants were severely withered, most of them died, and the survival rate was only 25.56% (Fig. 3A, B). The three 35S:*GbTCP5* lines exhibited leaf water loss rates that were significantly lower than that of Col-0 (Fig. 3C). The MDA and ABA levels of the 35S:*GbTCP5* plants under normal growth conditions and drought stress were determined. The results showed that the MDA contents of 35S:*GbTCP5* prior to and following drought stress were lower than those of Col-0 (Fig. 3D). The 35S:*GbTCP5* plants had higher ABA contents prior to and following drought stress compared with the Col-0 plants (Fig. 3E). The 3000-bp upstream promoter of *AtNCED3* contained TCP *cis*-acting elements, as demonstrated by a *cis*-acting element analysis (Table S3). qPCR was then performed to examine the transcription levels of *AtNCED3* in 35S:*GbTCP5* and Col-0 plants under normal and drought stress conditions. The findings indicated that under normal or drought stress conditions, the transcription levels of *AtNCED3* in the three 35S:*GbTCP5* lines were significantly higher than those in Col-0 (Fig. 3F).

Because drought resistance in *Arabidopsis* is enhanced by overexpression of the *GbTCP5* gene, the changes in drought stress-related gene expression were examined to ascertain the molecular mechanism of *GbTCP5*. The qPCR results indicated that the 35S:*GbTCP5* lines had higher levels of *AtCOR15*, *AtCOR414*, *AtRD29A*, *AtLTP4*, and *AtPUB22* expression than Col-0 (Fig. 4A-E).

The abovementioned results demonstrate that the overexpression of *GbTCP5* increases resistance to drought in *Arabidopsis*, which may be achieved by reducing the MDA content, increasing the ABA content, and increasing the transcription levels of genes related to drought resistance.

3.4. *GbTCP5* overexpression increases *Arabidopsis* salt tolerance

To analyze the function of *GbTCP5* in response to salt stress, *Arabidopsis* was irrigated with 250 mM NaCl solution for 4 weeks. On day 5 after irrigation, the Col-0 plants showed more yellowing and wilting of leaves than the 35S:*GbTCP5* plants (Fig. 5A). The Col-0 plants had a survival rate of 36.11%, whereas the three 35S:*GbTCP5* lines had survival rates of 70.83%, 69.44%, and 70.14% (Fig. 5B). The abovementioned results indicated that the 35S:*GbTCP5* lines had better salt tolerance than the WT. The MDA content and SPAD value of the 35S:*GbTCP5* and Col-0 plants under normal and salt stress conditions were determined. The findings revealed that the 35S:*GbTCP5* and Col-0 plants had similar SPAD values under normal conditions (Fig. 5C). Both the 35S:*GbTCP5* and Col-0 plants had lower SPAD values after salt stress than before salt stress, but the 35S:*GbTCP5* plants had a higher SPAD value than the Col-0 plants (Fig. 5C). Before and after salt stress treatment, the MDA content of the 35S:*GbTCP5* lines was significantly lower than that of the Col-0 plants (Fig. 5D). Moreover, the transcription levels of the stress-related genes *AtCOR15*, *AtCOR414*, *AtRD29A*, *AtLTP4*, *AtPUB22*, and *AtKIN2* were significantly higher in the 35S:*GbTCP5* lines than in the Col-0 plants under salt stress (Fig. S5). According to the abovementioned findings, *GbTCP5* overexpression increases the salt tolerance of *Arabidopsis*, which may result from a reduction in the MDA

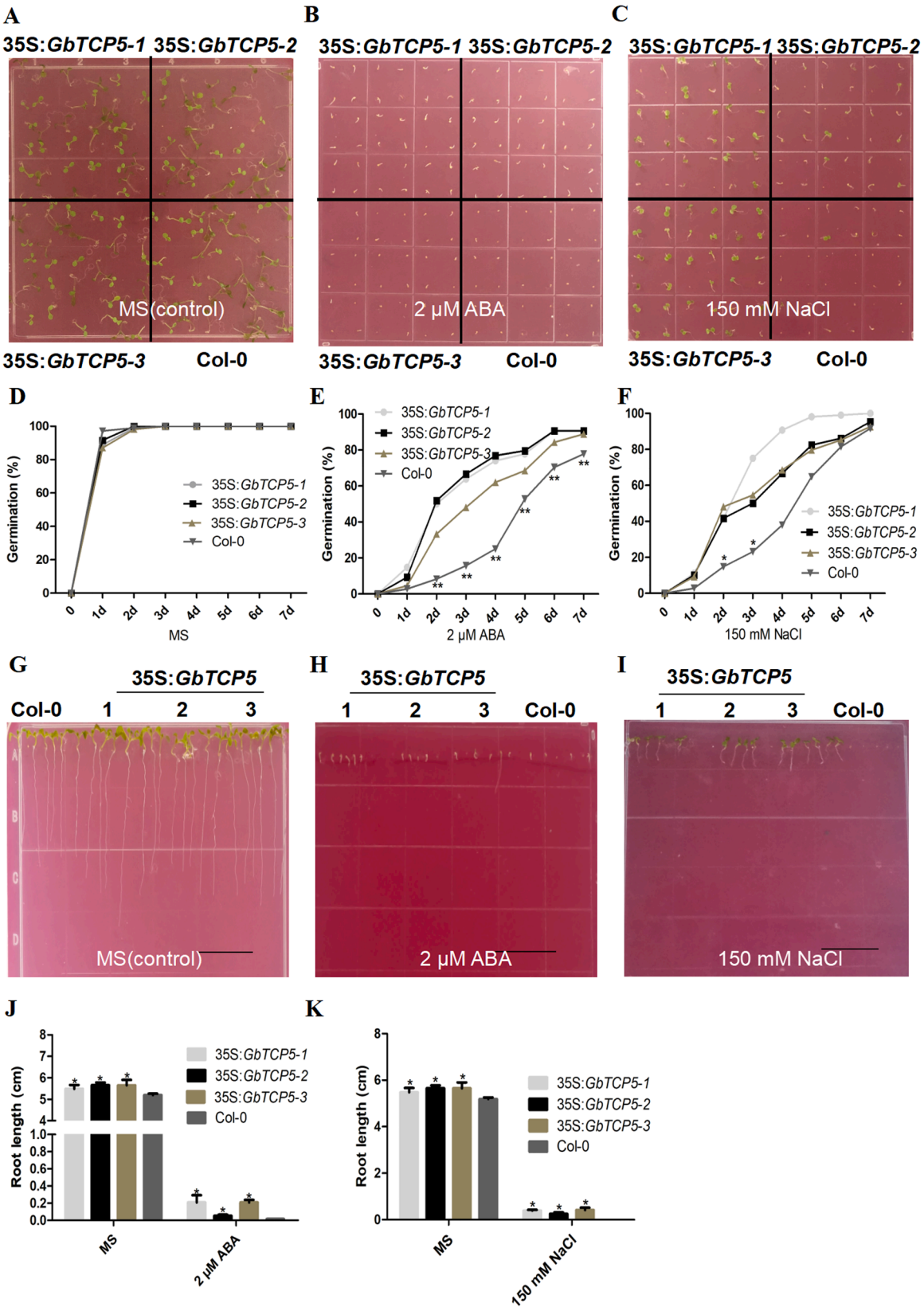

Fig. 2. 35S:*GbTCP5* transgenic *Arabidopsis* outperforms Col-0 in terms of germination and root length during exposure to 150 mM NaCl stress and 2 μM abscisic acid (ABA). (A) Seeds germinated in the absence of stress. (B) Seed germination following treatment with 2 μM ABA. (C) Seed germination after treatment with 150 mM NaCl. (D-F) Seed germination rate. (G) Root length in the absence of stress. (H) Root length phenotype after treatment with 2 μM ABA. (I) Root length phenotype after treatment with 150 mM NaCl. (J, K) Root length statistics. The vertical bars represent the means ± SDs of 3 biological replicates consisting of 45 seeds each. Significant differences compared with Col-0 are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

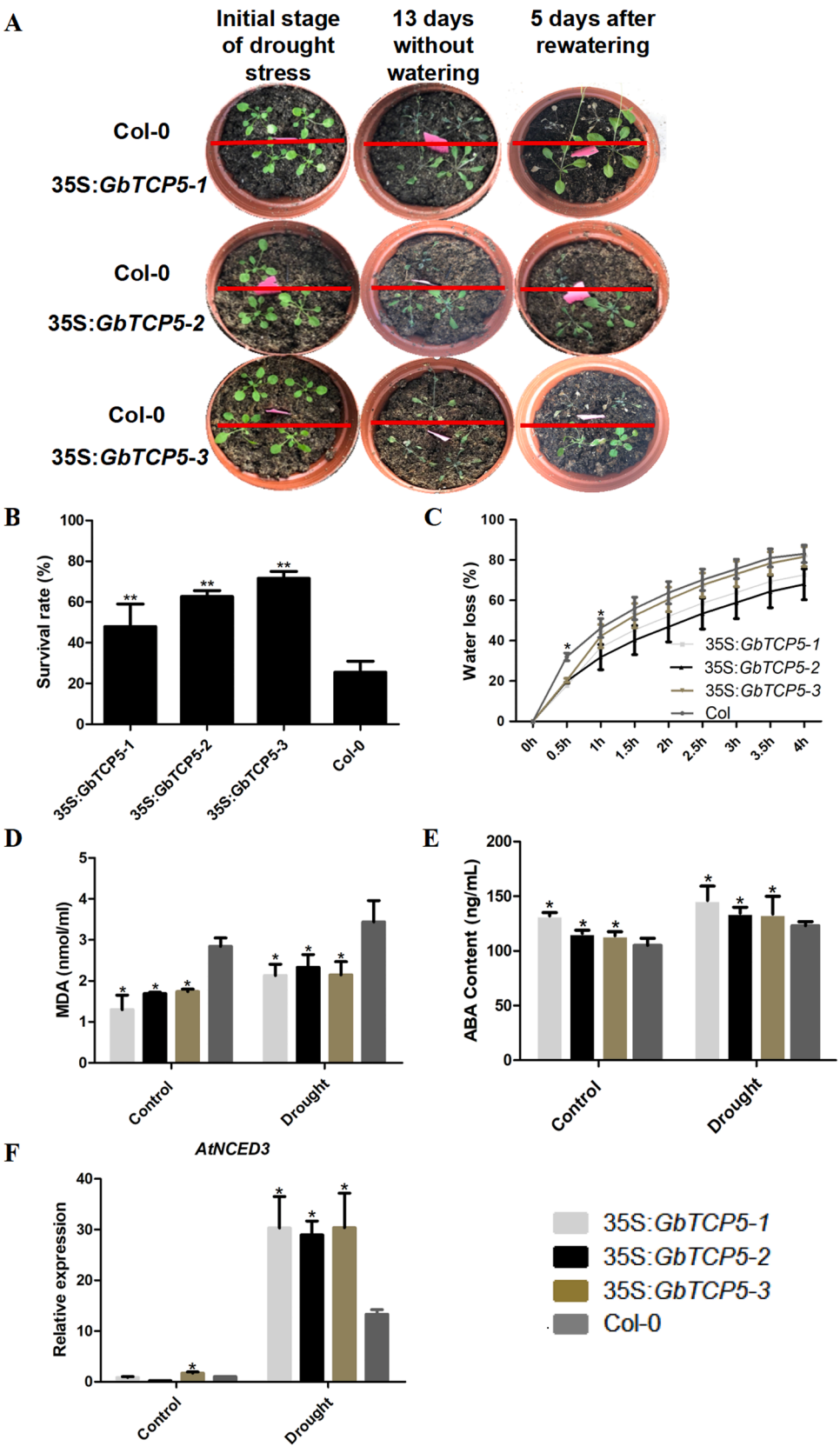

Fig. 3. 35S:*GbTCP5* transgenic *Arabidopsis* is more drought tolerant than *Arabidopsis* Col-0. (A) Identification of the drought resistance phenotype. (B) Survival rate under drought stress. (C) Rate of water loss in isolated leaves. (D) MDA content before and after drought stress. (E) Abscisic acid (ABA) content before and after drought stress. (F) *AtNCED3* transcript levels before and after drought stress. *AtUBQ3* served as the reference gene. The vertical bars represent the means ± SDs of 3 biological replicates consisting of 45 seeds each. Significant differences compared with Col-0 are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

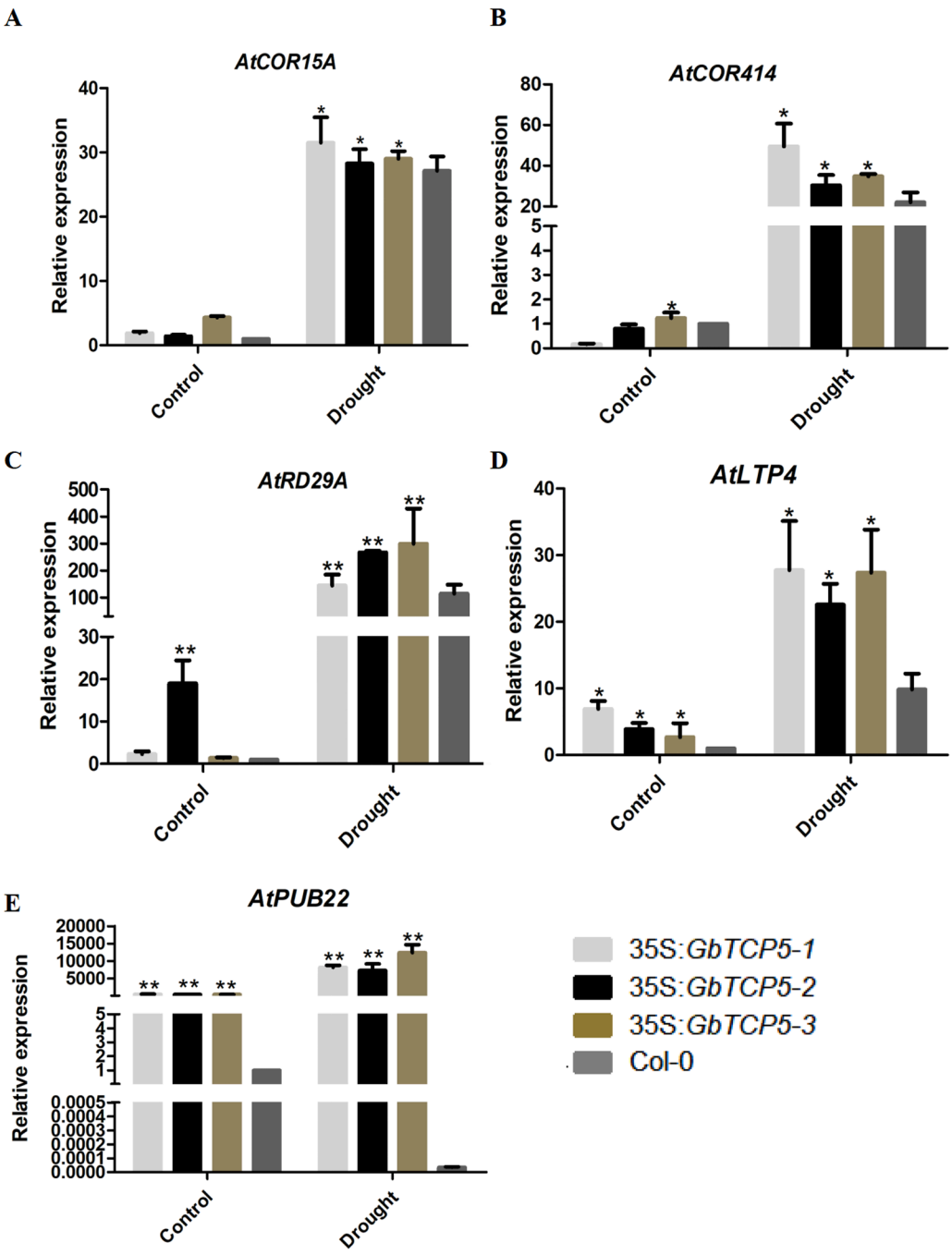

Fig. 4. 35S:*GbTCP5* transgenic *Arabidopsis* showed expression of more stress-related genes than *Arabidopsis* Col-0 under drought stress. (A) *AtCOR15A* expression (AT2G42450). (B) *AtCOR414* expression (AT1G29395). (C) *AtRD29A* expression (AT5G52310). (D) *AtLTP4* expression (AT2G42450). (E) *AtPUB22* expression (AT2G42450). *AtUBQ3* served as the reference gene. The vertical bars represent the means ± SDs of 3 biological replicates. Significant differences compared with Col-0 are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

content, an increase in the SPAD value, and the activation of stress response-related gene expression.

3.5. *GbTCP5* gene silencing decreases the tolerance of cotton to drought and salt

The expression level of *GbTCP5* in XH14 was silenced by the VIGS method. At approximately 2 weeks, cotton leaves injected with the positive control gene *GbCLA1* started to show bleaching (Fig. S6), which suggested that *GbTCP5* is also silenced in sea-island cotton, and this finding was further demonstrated by qPCR (Fig. 6A). The qPCR results showed that the relative expression level of *GbTCP5* in pTRV2-*GbTCP5*-silenced cotton was significantly lower than that in pTRV2 cotton (Fig. 6A). This finding indicated that *GbTCP5*-silenced cotton was obtained. Phenotypic differences in cotton plants appeared after 10 d of drought stress. Plants with pTRV2 showed less wilting than those with pTRV2-*GbTCP5* (Fig. 6B), the pTRV2-*GbTCP5* plants had a survival rate of 15.09% (Fig. 6C), and the survival rate of the pTRV2 plants was 73.15% (Fig. 6C). In addition, detached leaves of the pTRV2 plants exhibited reduced water lost than those of the pTRV2-*GbTCP5* plants (Fig. 6D). The determination of the MDA contents under normal growth and drought stress conditions showed that the MDA content of pTRV2 was lower than that of pTRV2-*GbTCP5* both before and after stress (Fig. 6E). The above-described results prove that silencing of the *GbTCP5* gene decreases the drought resistance of cotton because it increases the MDA content in leaves and reduces the water loss rate.

Salt stress was applied to cotton plants, and phenotypic differences began to be observed after 8 d of stress. The chlorosis and wilting of pTRV2 were more severe than those of pTRV2-*GbTCP5* (Fig. 7A). The survival rate of pTRV2-*GbTCP5* was 52.86% (Fig. 7B), and that of pTRV2

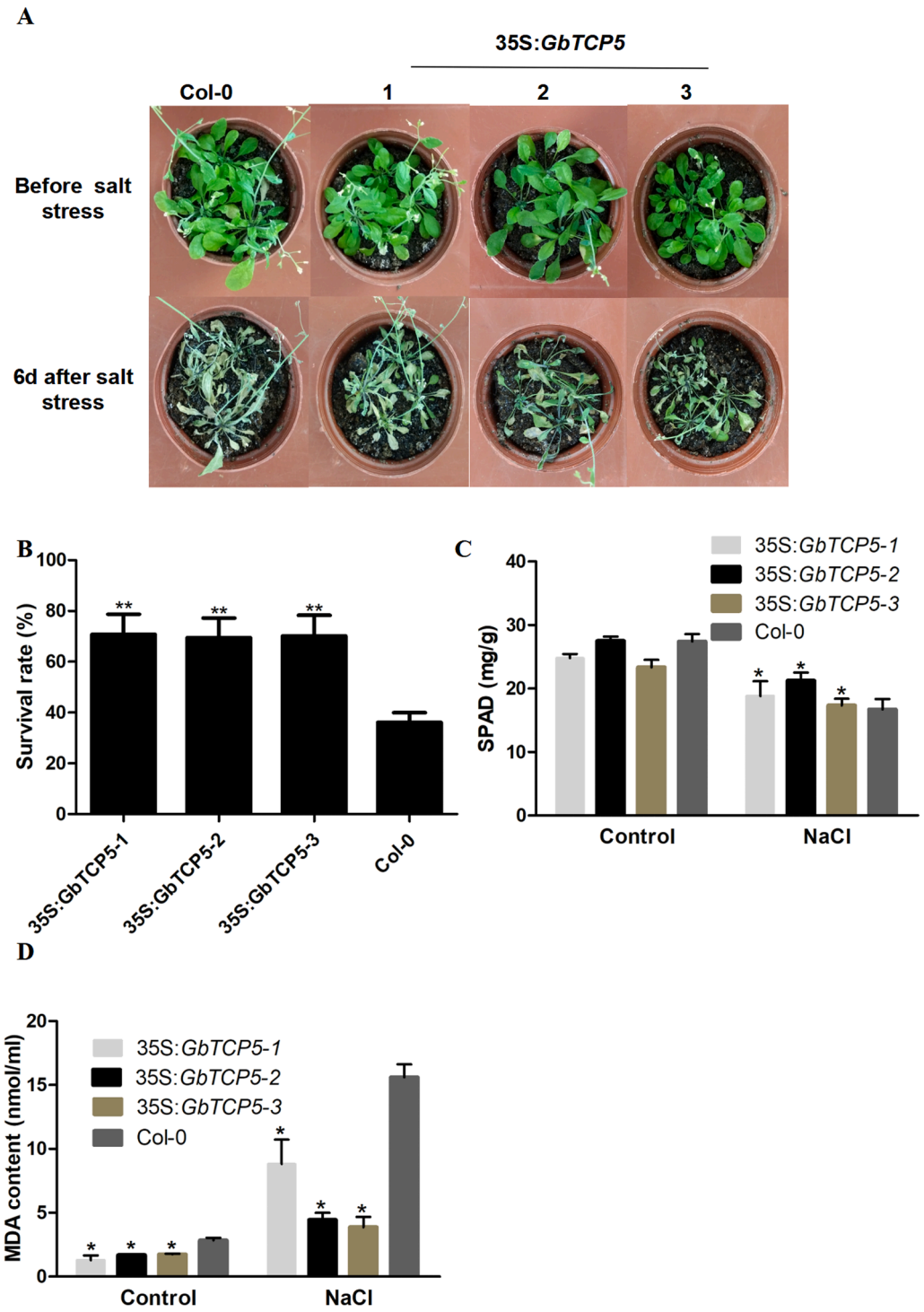

Fig. 5. 35S:*GbTCP5* transgenic *Arabidopsis* has a higher salt tolerance than Col-0. (A) Identification of the salt tolerance phenotype. (B) Survival statistics. (C) SPAD value. (D) Malonaldehyde (MDA) content. The vertical bars represent the means ± SDs of 3 biological replicates consisting of 45 seeds each. Significant differences compared with Col-0 are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

was 98.15% (Fig. 7B). The SPAD value and MDA content of the plants during normal growth and under salt stress were measured. The pTRV2 plants had higher SPAD values after exposure to salt stress than the pTRV2-*GbTCP5* plants (Fig. 7C), whereas the pTRV2 plants had lower MDA contents than the pTRV2-*GbTCP5* plants both before and after salt stress (Fig. 7D). These findings demonstrate that silencing of the *GbTCP5* gene in cotton reduced the SPAD value and increased the water loss rate and MDA content in leaves, which resulted in a reduction in the salt tolerance.

The root length, root area, and root volume of the pTRV2 plants were greater than those of the pTRV2-*GbTCP5* plants after 24 h of PEG and NaCl treatment (Fig. 8). The aforementioned results demonstrate that silencing of the *GbTCP5* gene in cotton reduced and shortened the root system, which resulted in decreases in the drought resistance and salt tolerance of the plants.

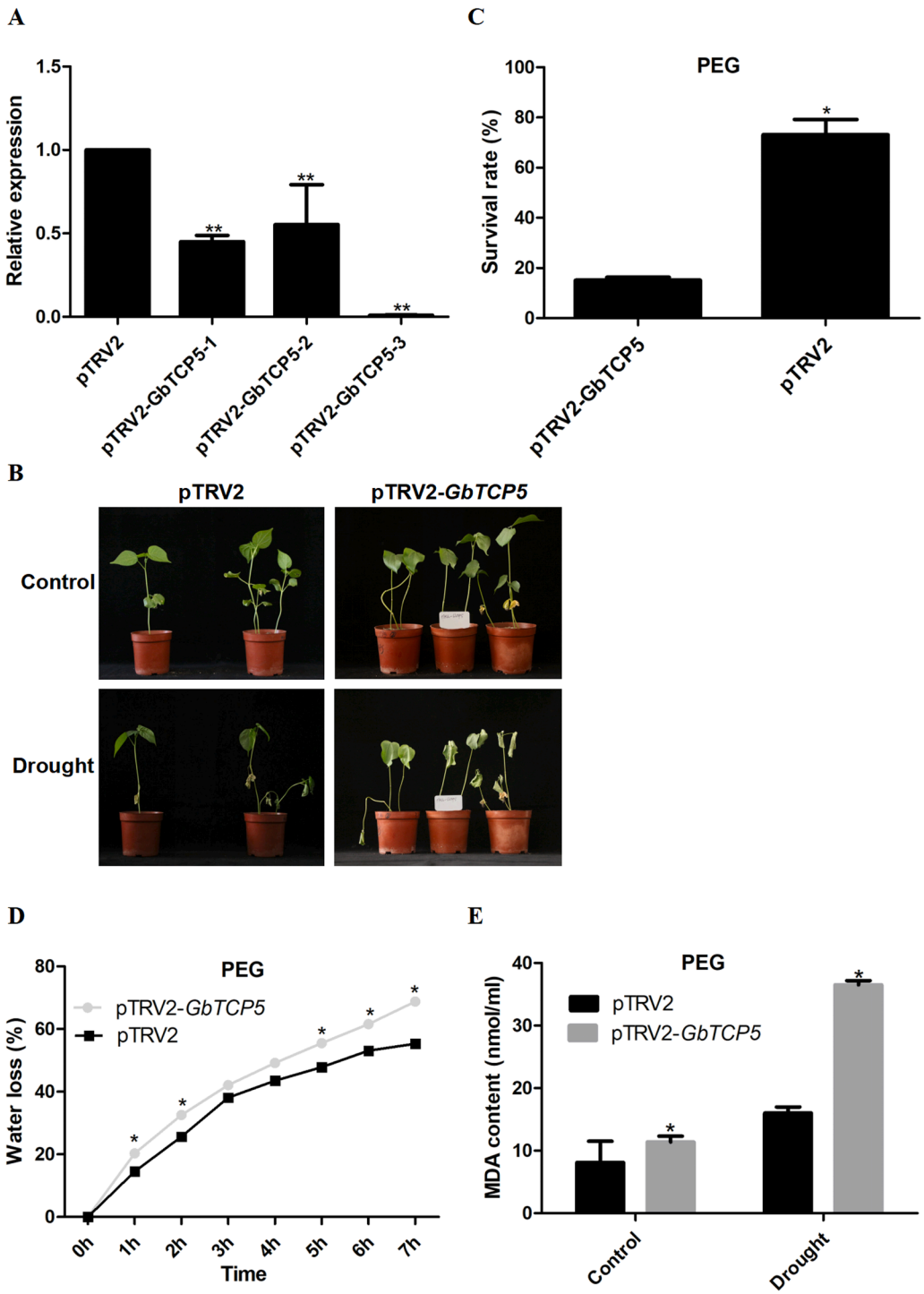

Fig. 6. *GbTCP5* silencing reduces the drought tolerance of cotton. (A) Relative expression of *GbTCP5* in pTRV2- and pTRV2-*GbTCP5*-silenced cotton. *GbUBQ7* served as the reference gene. (B) Identification of the drought resistance phenotypes of cotton. (C) Statistics of drought stress survival rates. (D) Water loss rate. (E) Amount of MDA before and after drought stress. The vertical bars represent the means ± SDs, and significant differences compared with pTRV2 are indicated by *P < 0.05 and **P < 0.01.

3.6. Transcriptome analysis of GbTCP5-silenced cotton

Reductions in drought and salt tolerance were found in cotton after silencing of the *GbTCP5* gene. pTRV2-*GbTCP5* and pTRV2 plants were subjected to RNA-seq to identify DEGs. A total of 489 DEGs, which included 234 upregulated and 255 downregulated DEGs, were identified (Fig. 9A, Table S4). The top ten metabolic pathways revealed by KEGG enrichment analysis were zeatin biosynthesis, galactose metabolism, carotenoid biosynthesis, linoleic acid metabolism, isoflavonoid biosynthesis, fatty acid elongation, diterpenoid biosynthesis, ABC transporters, and valine, leucine, and isoleucine degradation (Fig. 9B). The genes of the following three pathways were analyzed: ubiquitin-mediated proteolysis, endocytosis, and galactose metabolism. Six genes involved in endocytosis and *early responsive to dehydration* (*ERD*) were identified (Fig. 9C). Eight genes involved in galactose metabolism were found: *GOLS2/-1/-2/-3/-4* encode inositol galactosidases, and *RFS2/5/5–1* encode raffinose series oligosaccharides (Fig. 9D). Nine genes were found to be involved in the ubiquitin degradation pathway, and among these, *PUB30*, *PUB5*, and *PUB30-D* belong to the E3 ligase family, and *UBC19* is a ubiquitin-conjugating enzyme (Fig. 9E). *GbERD7* (Gbar_D12G003510), *GbGOLS2* (Gbar_A13G012650), and *GbUBC19* (Gbar_A05G029480), which showed downregulated expression in pTRV2-*GbTCP5* compared with pTRV2, were found to be related to drought stress (Fig. 9C-E).

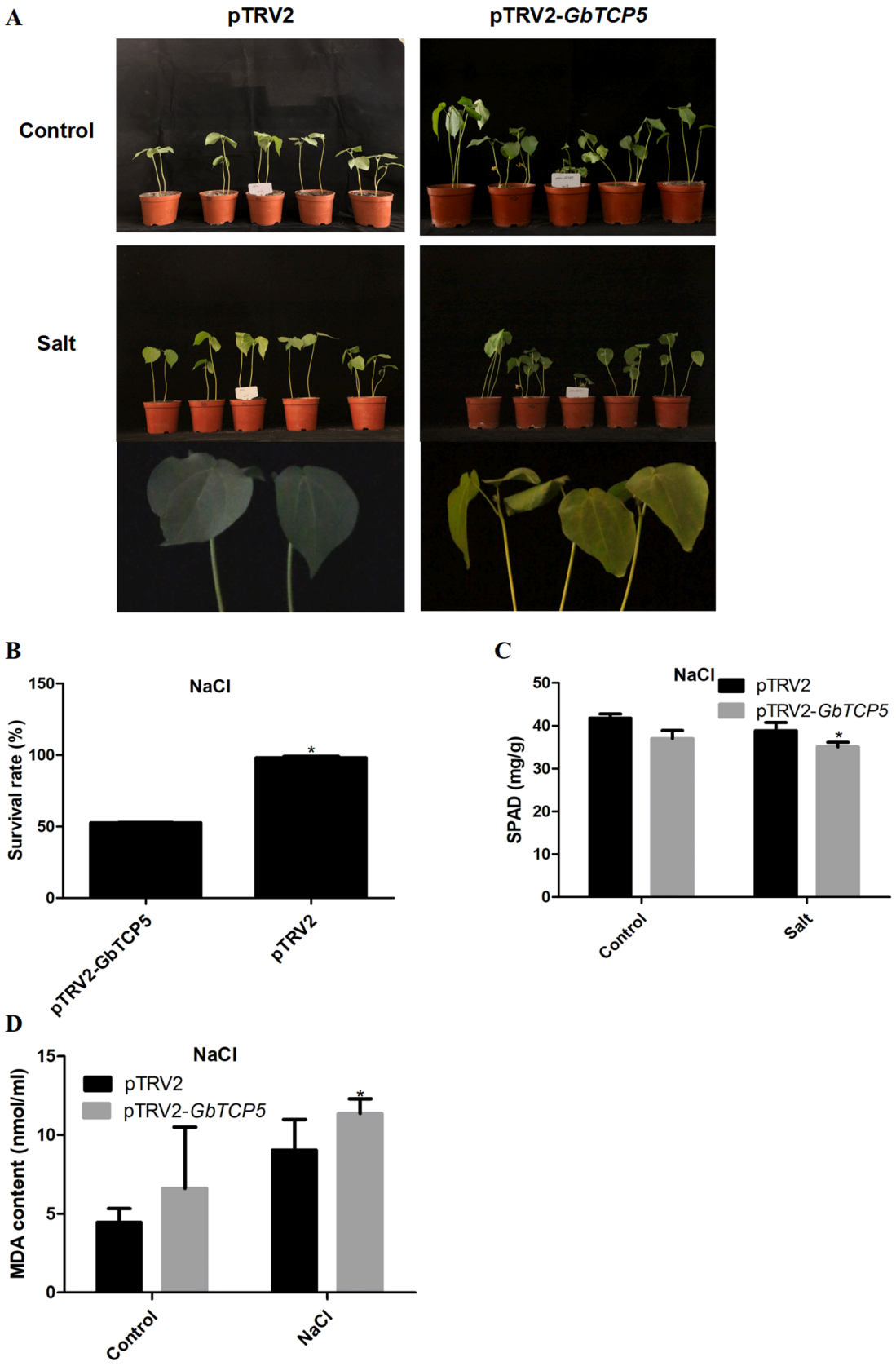

Fig. 7. The silencing of *GbTCP5* reduces the salt tolerance of cotton. (A) Identification of the cotton salt tolerance phenotype. (B) Survival rate under salt stress. (C) SPAD value. (D) Amount of MDA before and after salt stress. The vertical bars represent the means ± SDs, and significant differences compared with pTRV2 are indicated by *P < 0.05.

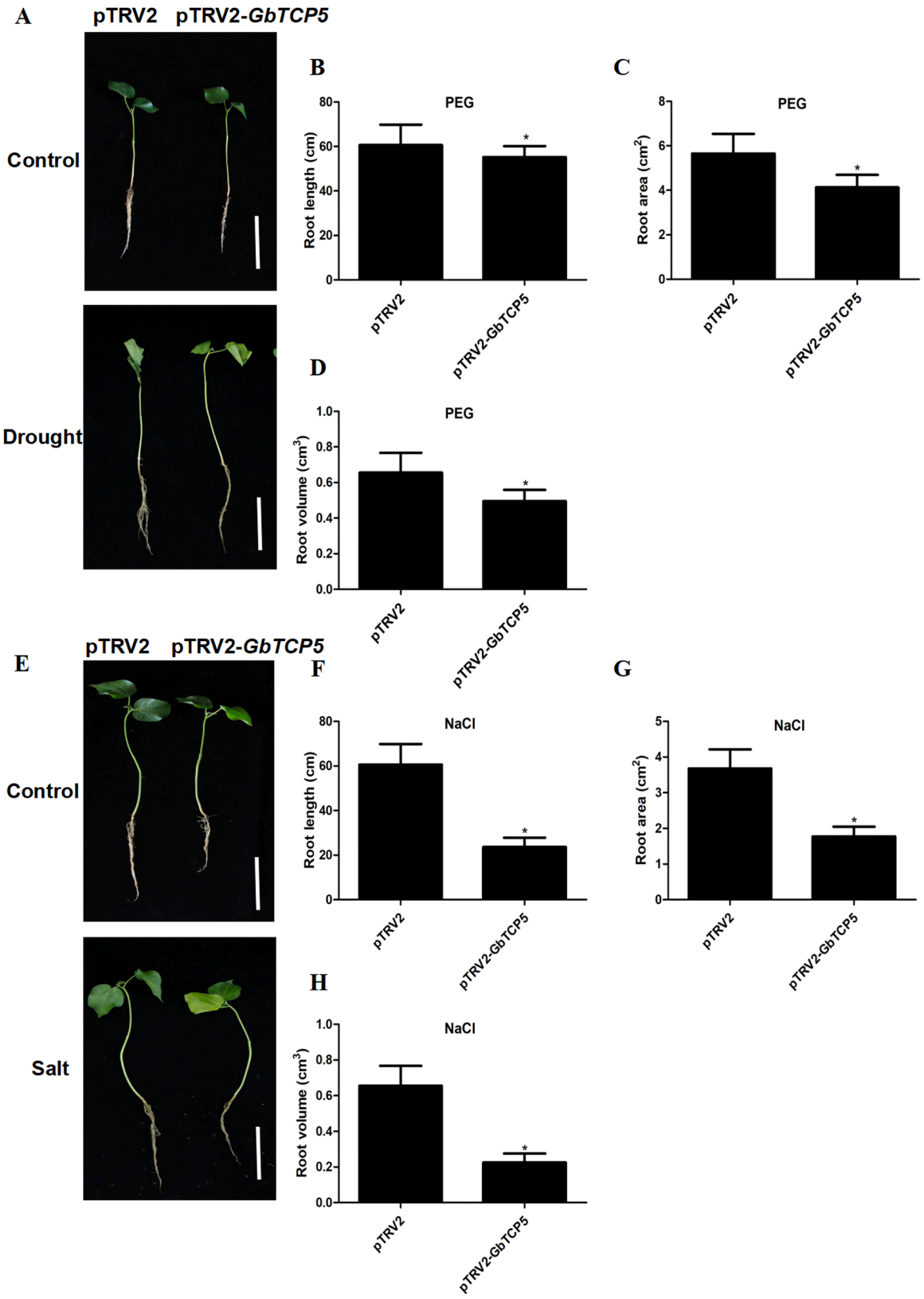

Fig. 8. The silencing of *GbTCP5* shortens and reduces cotton root systems under drought or salt stress. (A) Cotton root phenotype under drought stress. (B-D) Length, area, and volume of the roots under drought stress. (E) Cotton root phenotype under salt stress. (F-G) Length, area, and volume of the roots under salt stress. The vertical bars represent the means ± SDs, and significant differences compared with pTRV2 are indicated by *P < 0.05.

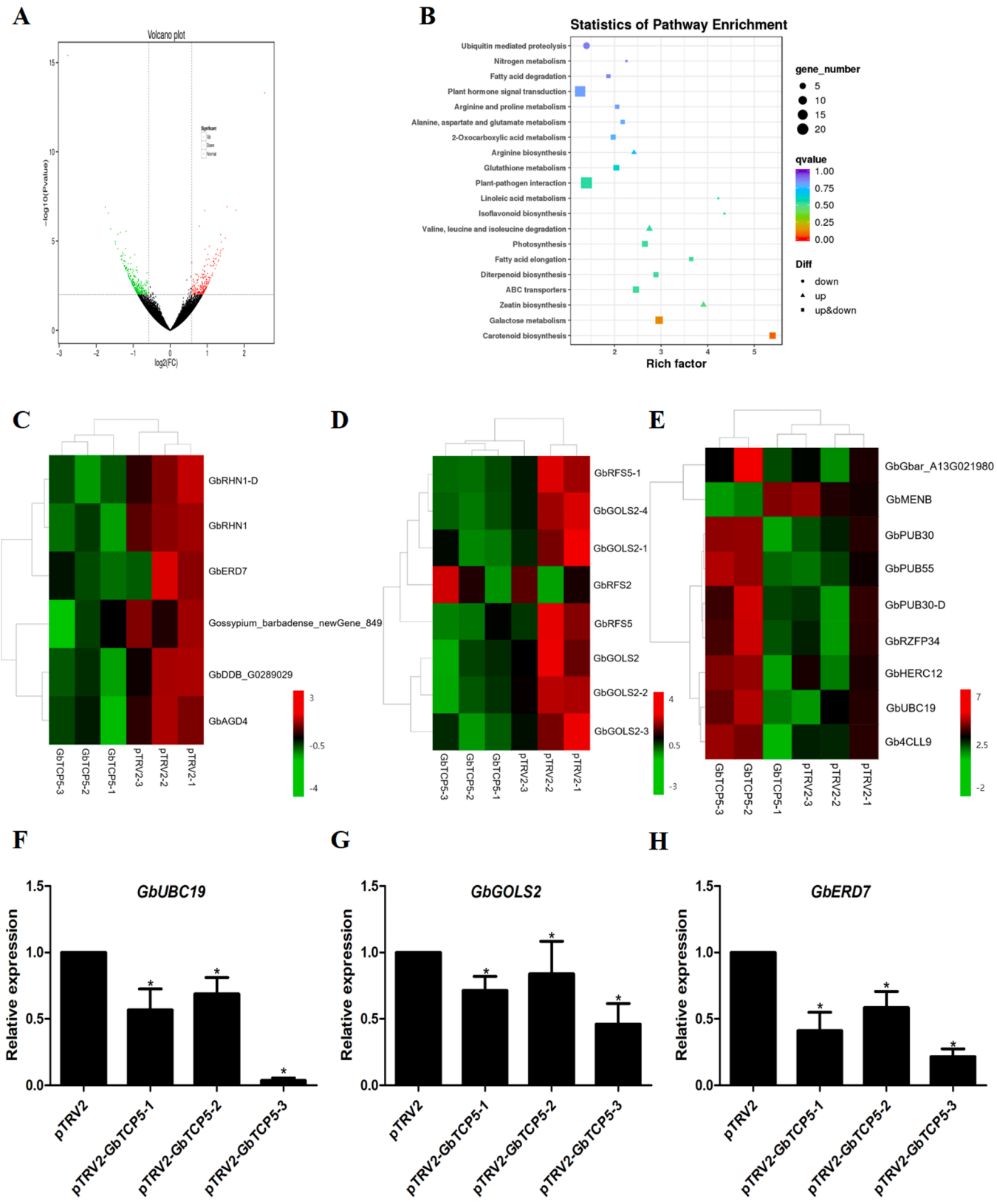

Fig. 9. Transcriptome analysis. (A) Volcano plots showing that pTRV2-*GbTCP5* and the control pTRV2 have DEGs (the red dots, black dots, and green dots indicate upregulated genes, unchanged genes and downregulated genes, respectively). (B) Metabolic pathways coenriched by DEGs upregulated and downregulated in pTRV2-*GbTCP5* compared with pTRV2. (C-E) Heatmap analysis of DEGs in pTRV2-GbTCP5 versus the control pTRV2 that are involved in ubiquitin-mediated proteolysis, endocytosis, and galactose metabolism. (F-H) Transcription levels of genes related to drought stress (*GbUBC19*, *GbGOLS2*, and *GbERD7*) in pTRV2-*GbTCP5* and the control pTRV2 as detected by qPCR. *GbUBQ7* served as the reference gene. The vertical bars represent the means ± SDs, and significant differences compared with pTRV2 are indicated by *P < 0.05.

Similar to the transcriptome sequencing results, the expression levels of the three aforementioned genes were significantly lower in the pTRV2-*GbTCP5* plants than in the pTRV2 plants, which was confirmed by qPCR (Fig. 9F-H). According to these findings, the transcription level of cotton drought stress-related genes is impacted by *GbTCP5* gene silencing.

3.7. *GbERD7, GbUBC19, and GbGOLS2 are the direct target genes of GbTCP5*

The promoter sequences of the *GbERD7*, *GbUBC19*, and *GbGOLS2* genes were analyzed, and the results showed the upstream 3000-bp promoter sequence contains TCP *cis*-acting elements (Table S3). Yeast one-hybrid experiments showed that the TCP *cis*-acting elements in these three promoters are bound by GbTCP5 (Fig. 10A). However, GbTCP5 cannot bind to the *GbERD7*, *GbUBC19*, and *GbGOLS2* promoters that mutate TCP *cis*-acting elements (Fig. 10A). LUC experiments also

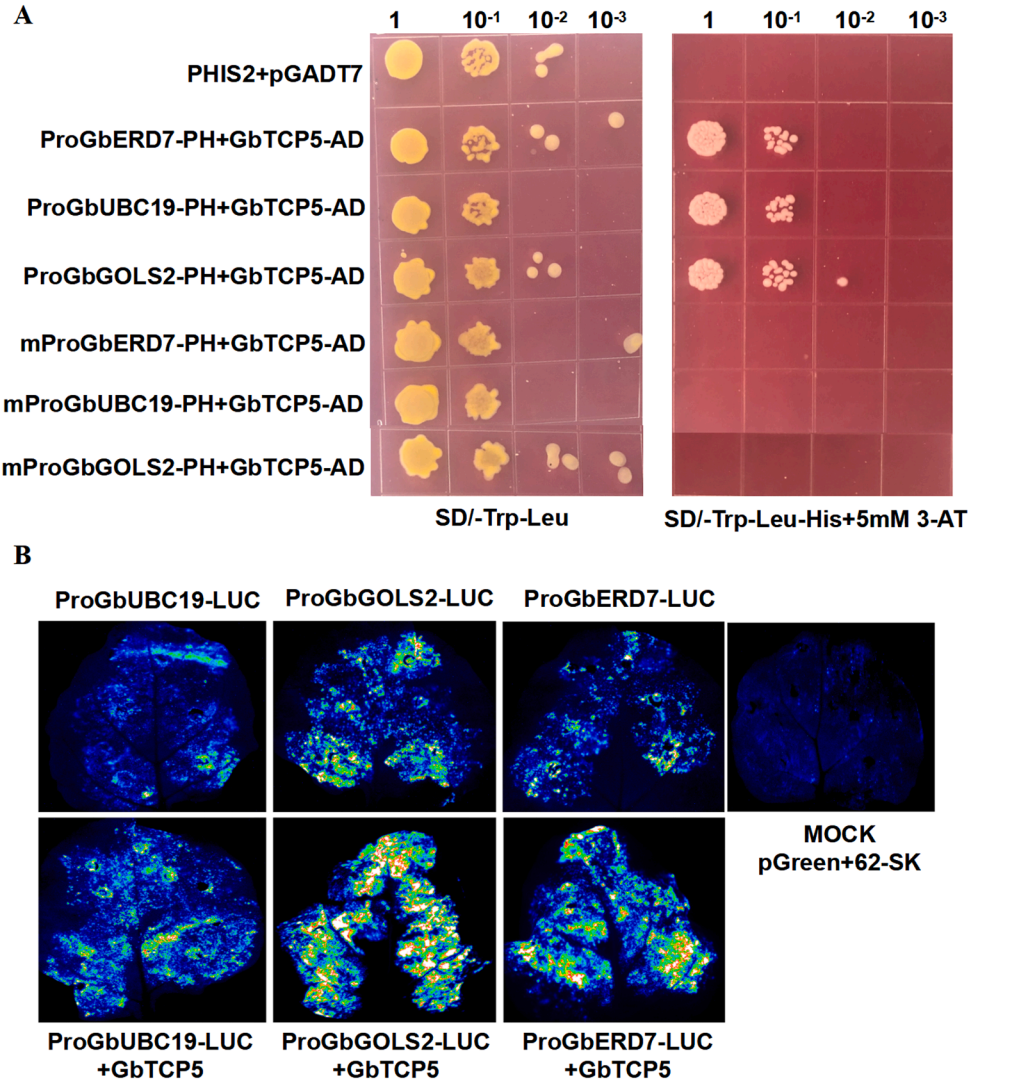

Fig. 10. Analyses of the binding of GbTCP5 to the TCP-binding *cis*-element in the *GbERD7/GbUBC19/GbGOLS2* promoter. (A) Yeast one-hybrid analysis. (B) GbTCP5 activates *GbERD7/GbUBC19/GbGOLS2* promoter activity, as revealed by the GbERD7/GbUBC19/GbGOLS2 promoter-LUC activity in the tobacco leaf transient assay. (C) LUC experiment. The LUC/REN ratio represents the LUC expression level of the extracted protein in leaves. The vertical bars represent the means ± SDs, and significant differences compared with SK are indicated by *P < 0.05.

showed that GbTCP5 strongly bound to *GbERD7*, *GbGOLS2*, and *GbUBC19* (Fig. 10B, C). These results indicate that plant drought resistance and salt tolerance are enhanced by GbTCP5 binding to TCP *cis*-acting elements in the *GbERD7*, *GbUBC19*, and *GbGOLS2* promoters.

4. Discussion

The growth, development, and yield of crops are adversely affected by soil drought and salinization, and drought and salt stresses trigger transcriptional responses in some *TCP* genes. For example, the transcription levels of *CaTCPs* (Ling et al., 2020), *LjTCPs* (Ling et al., 2020), *MtTCPs* (Ling et al., 2020), *PvTCPs* (Ling et al., 2020), *SiTCPs* (Xiong et al., 2022), *MeTCPs* (Lei et al., 2017), and *MdTCPs* (Xu et al., 2014) are significantly changed under drought or salt stress. Similarly, drought, salt stress, and ABA increased *GbTCP5* expression in this study (Fig. 1). In upland cotton, abiotic stress did not induce expression of the homologous *GbTCP5* gene (Yin et al., 2018), which may be due to the functional differentiation of the *TCP5* gene during the evolution of sea-island cotton and upland cotton.

Under drought and salt stresses, class II TCP transcription factors can play both positive and negative regulatory roles, as revealed in previous research. For example, the survival rate, chlorophyll content, and proline content of plants overexpressing *MdTCP46* were decreased under drought stress, whereas the REC increased. This finding suggests that *MdTCP46* acts as a negative regulator under drought stress (Liu et al., 2022). Conversely, an increase in the survival of *ZmTCP42*-overexpressing plants under drought conditions has been observed (Ding et al., 2019). Similarly, the germination and root length of *GbTCP4*-overexpressing plants were increased significantly under mannitol treatment, and the survival and water loss rates of the plants increased and decreased under drought treatment, respectively, indicating that plants may be more tolerant of drought stress during the germination and maturity stages when *GbTCP4* is overexpressed (Wang et al., 2022). In contrast to the results found for plants with *GbTCP4* expression, the seed germination, root length, and root hair length of the 35S:*GbTCP5* plants under mannitol treatment did not differ significantly from those of the WT at the seedling stage (Fig. S1B-E; Fig. 2). However, at the mature stage, the 35S:*GbTCP5* plants had a higher survival rate and less water loss in isolated leaves, and drought-stressed plants had a lower MDA content than the WT plants (Fig. 3A-D). This finding may have been obtained due to the inconsistent effects of *GbTCP5* at the seedling and mature stages, and the specific mechanism needs to be further studied. In addition, consistent with the findings found for *GbTCP4*, *GbTCP5*-silenced cotton plants showed significantly increased wilting, reduced survival, increased leaf water loss, an increased MDA content (Fig. 6) and reductions in the root length, root area, and root volume under drought stress (Fig. 8). Under salt stress, *GbTCP5*-silenced cotton plants exhibited reduced survival and SPAD values and an increased MDA content (Fig. 7), and their root lengths, root areas, and root volumes were decreased (Fig. 8). Whether these two class II TCP transcription factors are functionally redundant in the response of sea-island cotton to drought and salt stress needs to be further explored.

AtRD29A, *AtCOR15*, *AtCOR414*, *AtPUB22*, and *AtLTP4* were identified as *Arabidopsis* abiotic stress response markers in previous research (Zhang et al., 2019). This study showed that the *AtRD29A*, *AtCOR15*, *AtCOR414*, *AtPUB22*, and *AtLTP4* expression levels were significantly higher in 35S:*GbTCP5* plants than in Col-0 plants after drought or salt stress (Fig. 4; Fig.S5). The *AtRD29A* gene transcript levels are upregulated in *ZmTCP42*-overexpressing plants under drought stress, similar to the findings of this study (Ding et al., 2019). In plants overexpressing *GbTCP4*, the transcript levels of the *AtRD29A*, *AtCOR414*, and *AtCOR15A* genes are upregulated (Wang et al., 2022). These findings suggest that the expression of stress-responsive genes triggered by these class II TCP transcription factors may increase the drought and salt tolerance of transgenic *Arabidopsis*.

According to previous research, an ABA-dependent pathway may be used by TCP transcription factors to regulate the responses to salt stress and drought. The results of the current study support this view. In this study, *cis*-acting elements ABREs and MBSs responsive to ABA were found in the 3000-bp region of the *GbTCP5* promoter (Table S2). ABA treatment changed the transcript level of *GbTCP5* (Fig. 1A). In addition, 35S:*GbTCP5* plants showed significant increases in their germination and root length compared with Col-0 plants, indicating that these plants are not sensitive to ABA (Fig. 2B, E, H, J). However, the ABA content of these plants was significantly increased at maturity prior to and following drought stress (Fig. 3E), and the *AtNCED3* gene expression level in 35S:*GbTCP5* plants was significantly higher than that in Col-0 under drought stress (Fig. 3 F). Our results are consistent with the previously reported function of the *GbTCP4* gene (Wang et al., 2022). Numerous TCP transcription factors regulate changes in the ABA sensitivity and content in transgenic plants that are inconsistent with the functions of GbTCP5. The seed germination and root length of *PeTCP10* transgenic *Arabidopsis* plants are significantly increased by treatment with exogenous ABA, but the ABA content remained unchanged (Liu et al., 2020). The overexpression of *Arabidopsis ZmTCP42* makes plant germination more sensitive to ABA (Ding et al., 2019). *MdTCP46*-overexpressing apple calli are not sensitive to ABA, and drought stress reduces their ABA content (Liu et al., 2022). The reason for this inconsistency may be that different TCP transcription factors play distinct roles in ABA-mediated germination and ABA synthesis.

The expression of numerous stress-responsive genes can be influenced downstream by transcription factors and contribute significantly to the abiotic stress response (Rohit et al., 2016). The plant hormone signal transduction, MAPK signaling, and ABC transport pathways are primarily enriched in the upregulated DEGs in *Arabidopsis* overexpressing the *PeTCP10* gene compared with the WT (Liu et al., 2020). DEGs associated with transport and hormone signal transduction are more abundant in *VuTCP9*-overexpressing cowpea than in the WT (Mishra et al., 2022). The environmental stress response is associated with the biological metabolic pathways of plant hormone signal transduction, MAPK signaling, and ABC transport, and these pathways are involved in salt stress and drought responses, as revealed in previous research (Liu et al., 2020; Zhu, 2016). Consistent with the results found for *PeTCP10* and *VuTCP9*, a transcriptome sequencing of pTRV2-*GbTCP5* and pTRV2 cotton leaves revealed that the expression of genes in the abovementioned metabolic pathways (phytohormone signaling, MAPK signaling, and ABC transport) was also affected (Fig. 9B; Table S4).

From these DEGs, we selected 3 previously studied genes associated with abiotic stress responses for qPCR validation (Fig. 9F-H). pTRV2-*GbTCP5* cotton had lower expression levels of *GbERD7*, *GbGOLS2*, and *GbUBC19* (Fig. 9F-H), and their promoter regions contained TCP-binding elements (Table S3). Subsequently, yeast one-hybrid assays showed that yeast cotransformed with the GbTCP5 and *GbERD7*, *GbGOLS2*, and *GbUBC19* promoters grew normally on the three-deficiency medium (Fig. 10A). However, yeast cotransformed with GbTCP5 and the promoters of *GbERD7*, *GbGOLS2*, and *GbUBC19* with mutated TCP *cis*-acting elements could not grow normally (Fig. 10A), indicating that GbTCP5 specifically binds to the TCP *cis*-acting elements of the *GbERD7*, *GbGOLS2*, and *GbUBC19* promoters. Furthermore, LUC assays showed that GbTCP5 can activate the activity of the *GbERD7*, *GbGOLS2*, and *GbUBC19* promoters (Fig. 10B, C). *Arabidopsis* has sixteen *ERD* genes that enhance drought resistance by acting in various metabolic pathways (Kiyosue et al., 1994). Under drought stress, transgenic rice yield increased after heterologous expression of *AtGOLS2,* which is an inositol galactosidase (Selvaraj et al., 2017). UBC is a ubiquitin-conjugating enzyme, and the *AtUBC32*, *AtUBC33*, and *AtUBC34* genes in *Arabidopsis* can mediate ABA participation in stomatal opening and closing, which affects how well plants can handle drought stress (Yong et al., 2018). The results obtained in this study indicate that *GbERD7*, *GbGOLS2*, and *GbUBC19* are directly downstream genes of the GbTCP5 transcription factor. However, the functions of the *GbERD7*,

GbGOLS2, and *GbUBC19* genes in the response of cotton to drought and salt stress still need to be verified using genetic methods.

5. Conclusion

Exposure to ABA, PEG, and NaCl stresses induced *GbTCP5* transcription. Analyses of the phenotypes and physiological indicators of *GbTCP5*-overexpressing *Arabidopsis* and *GbTCP5*-silenced cotton demonstrated that *GbTCP5* is a positive regulator of drought and salt stress responses in plants. GbTCP5 improves plant drought and salt stress tolerance by directly activating the expression of *GbERD7*, *GbUBC19*, and *GbGOLS2*. These results suggest that *GbTCP5* is a potential candidate gene for increasing the tolerance of cotton to salt and drought stresses.

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CRediT authorship contribution statement

Yi Wang: Writing – original draft preparation, Writing – review & editing, Investigation, Data curation. **Yuehua Yu**: Writing – original draft preparation, Writing – review & editing, Supervision. **Huina Wan**: Formal analysis, Investigation. **Zhiyong Ni**: Writing – original draft preparation, Writing – review & editing, Funding acquisition, Project administration, Supervision. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2023.117209.

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