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Original Article

Improvement of plant tolerance to drought stress by cotton tubby-like protein 30 through stomatal movement regulation

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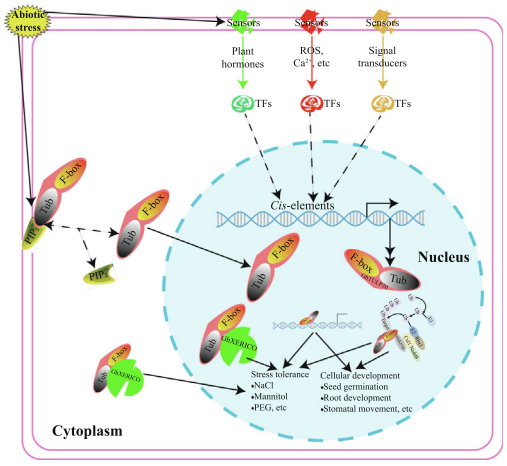
HIGHLIGHTS

- *GhTULP30* transcription in cotton was induced by drought and salt stress.
- Ectopic *GhTULP30* expression significantly improved yeast cell tolerance to drought and salt.
- *GhTULP30* overexpression increased the tolerance of *Arabidopsis* to drought and salt stress.
- Silencing *GhTULP30* affected stomatal movement and decreased tolerance to drought stress.
- Protein experiments exhibited the interaction of GhTULP30 with GhSKP1B and GhXERICO.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Cotton is a vital industrial crop that is gradually shifting to planting in arid areas. However, tubby-like proteins (TULPs) involved in plant response to various stresses are rarely reported in cotton. The present study exhibited that *GhTULP30* transcription in cotton was induced by drought stress.
Objective: The present study demonstrated the improvement of plant tolerance to drought stress by *GhTULP30* through regulation of stomatal movement.
Methods: *GhTULP30* response to drought and salt stress was preliminarily confirmed by qRT-PCR and yeast stress experiments. Ectopic expression in *Arabidopsis* and endogenous gene silencing in cotton were used to determine stomatal movement. Yeast two-hybrid and spilt-luciferase were used to screen the interacting proteins.
Results: Ectopic expression of *GhTULP30* in yeast markedly improved yeast cell tolerance to salt and drought. Overexpression of *GhTULP30* made *Arabidopsis* seeds more resistant to drought and salt stress during seed germination and increased the stomata closing speed of the plant under drought stress conditions. Silencing of *GhTULP30* in cotton by virus-induced gene silencing (VIGS) technology slowed down the closure speed of stomata under drought stress and decreased the length and width of the stomata.

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The trypan blue and diaminobenzidine staining exhibited the severity of leaf cell necrosis of *GhTULP30*-silenced plants. Additionally, the contents of proline, malondialdehyde, and catalase of *GhTULP30*-silenced plants exhibited significant variations, with obvious leaf wilting. Protein interaction experiments exhibited the interaction of GhTULP30 with GhSKP1B and GhXERICO.
Conclusion: *GhTULP30* participates in plant response to drought stress. The present study provides a reference and direction for further exploration of *TULP* functions in cotton plants.

Introduction

Crops growing in a complex natural environment are faced with several unfavorable factors such as drought and salinity, which adversely affect crop growth and hinder sustainable agricultural development [1]. Plants subjected to environmental stress such as drought and salt resist stress damage through a series of physiological and biochemical mechanisms, for example, by changing their morphology and metabolism [2]. Drought stress leads to produce ABA, inhibits stomatal opening and decreases leaf photosynthesis, which reduce dry matter production via oxidative stress and reduction in photosynthetic characteristics [3–5]. The morpho-anatomical traits of plant leaves and the cell membranes stability have important effects on water retention [6]. Water deficit affects stomatal opening and conductance, resulting in reduced carbon import and net photosynthesis [3,7], which ultimately lead to a substantial loss of crop productivity [8,9]. Therefore, the genes that respond to environmental stress such as tubby-like proteins should be identified.

The tubby-like protein family, also known as *TULP* or *TLP*, was first identified in a delayed obese mouse with retinal degeneration and neurosensory hearing loss [10]{Kleyn, 1996 #4}{Kleyn, 1996 #4}{Kleyn, 1996 #4}. Subsequently, *TULP*s were reported in numerous plants such as rice [11], *Arabidopsis* [12], apple [13], and wheat [14]. Although TULPs exhibit a conserved Tub domain at the carboxyl terminus, the amino terminus varies greatly among members. The three-dimensional Tub domain structure comprises 12 antiparallel chains that form a closed β-barrel, surrounding a central hydrophobic α helix at the carboxyl terminus of the protein. The Tub domain selectively binds to a specific membrane phosphatidylinositol (PIP_2) [15].

As transcription factors, *TULP*s are crucial in the plant response to abiotic stresses such as drought and salt. After *AtTLP3* and *AtTLP9* gene mutation, *Arabidopsis* increased tolerance to NaCl, mannitol, and abscisic acid (ABA) during seed germination and seedling growth, with the double mutant being more tolerant than the single mutant [12,16]. *CaTLP1* overexpression increased root and leaf development; net photosynthesis; biomass; and tolerance to dehydration, salinity, and oxidative stress in transgenic plants [17]. Overexpression of the apple gene *MdTLP7* markedly improved NaCl and extreme temperature tolerance in *Escherichia coli* and *Arabidopsis* [18–20]. Interaction of GhTULP34 with GhSKP1A protein caused a significant reduction in the germination rate of seeds overexpressing *GhTULP34* under salt stress, and inhibited root development and stomatal closure under drought stress [21]. *CsTLP8* probably activates the ABA signaling pathway and inhibits seed germination and seedling growth under drought and salt stress by affecting the antioxidant enzyme activity [22]. The tomato *TULP* gene *SlTLFP8* affects the epidermal cell size, thereby changing stomatal density, reducing water loss, and improving water efficiency [23].

*TULP*s are also involved in facilitating the development of male gametophytes and tissue, in response to biological stress. *OsTLP2*, the expression of which is induced by pathogen inoculation, binds to the PRE4 element in the promoter of *OsWRKY13* to regulate the expression of *OsWRKY13* and promote the defense response of rice against pathogen invasion [24]. AtTLP2 translocates from the plasma membrane to the nucleus by interacting with NF-YC3 and participates in the regulation of homogalacturonan biosynthesis in seed mucus [25]. AtTLP7 interacts with ASK1, a protein essential for male meiosis, to influence male gametophyte development [16]. Additionally, *attlp6* and *attlp7* mutant plants exhibited pollen abortion phenomenon to some extent [26].

Cotton is a vital industrial and economic crop because cotton fiber and oil are used as renewable energy sources. Although *TULP*s are involved in a few vital processes in response to abiotic stress in some plants, research on the role of these genes in cotton is still lacking. The transcriptome and quantitative real-time polymerase chain reaction (qRT-PCR) data exhibited the remarkable upregulation of *GhTULP30* expression under drought and salt stress [21]. Therefore, *GhTULP30*, a member of the *TULP*s in cotton, was selected as a candidate gene for research. Ectopic expression of *GhTULP30* in yeast increased yeast tolerance to NaCl and mannitol. Under drought and salt stress conditions, *Arabidopsis* seeds overexpressing *GhTULP30* exhibited a high germination rate and rapid closure of the plant stomata. Silencing of *GhTULP30* in cotton through the virus-induced gene silencing (VIGS) was found to inhibit stomatal closure and cause leaf wilting, cell necrosis, remarkable change in the expression of drought-responsive marker genes and metabolites, as well as a significant reduction in drought tolerance of silenced plants. Additionally, GhTULP30 interacted with GhSKP1B and GhXERICO at the protein level, and some stress-related genes were co-expressed with *GhTULP30*, indicating the positive regulatory role of *GhTULP30* in response of cotton to drought stress. Thus, this study provides a theoretical reference for cotton resistance breeding.

Materials and methods

RNA extraction and qRT-PCR

The upland cotton variety CCRI36 (upland cotton with high tolerance to drought and salt stress [27,28]) was grown in a greenhouse at a temperature of 25℃, with 16/8h of light/dark cycle. When the seedlings grew to the three-leaf stage, they were treated with 200 mM NaCl and 15% PEG6000. The control plants were treated with the corresponding volume of ddH_2O. The young plant leaves were collected at 0, 1, 3, 6, and 12 h after the treatment, placed in liquid nitrogen, and refrigerated at − 80℃ for RNA extraction. The RNAprep Pure Plant kit (Tiangen, Beijing, China) was used to separate and extract RNA. The PrimerScript 1st Strand cDNA synthesis kit (TaKaRa, Dalian, China) was used to reverse transcribe RNA into cDNA. The SYBR Premix Ex Taq Kit (TaKaRa) and ABI 7500 real-time PCR System (Applied Biosystems, Foster City, CA, United States) were used for qRT-PCR analysis. The primers are listed in Table S1. The results were standardized using cotton ubiquitin 7 (*UBQ7*) as the internal reference gene, and the relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Three biological replicates were prepared using the selected 30 plants under the same growth conditions, whereas each biological replicate was used to prepare three technical replicates.

Analysis of gene structure characteristics

*TULP*s of other species such as *Gossypium barbadense*, *Gossypium arboretum*, *Theobroma cacao*, *Arabidopsis*, tomato, cucumber and chickpea were obtained from the NCBI database [29]. Amino acid sequence alignment and mapping were analyzed using the default parameters of CLUSTALW [30] and ESPript 3.0 [31], respectively. The phylogenetic tree with *p*-distance model and 1,000 bootstrap replications was constructed using the maximum likelihood and JTT methods in MEGA 7.0 [32]. MEME and TBtools programs were used for motif analysis and drawing, respectively [33,34]. The secondary and tertiary protein structures were predicted and analyzed using the Phyre^2 database [35]. The PlantCARE database [36] was used to analyze *cis*-acting elements in a 2000-bp region located upstream of *GhTULP30*.

Yeast ectopic expression analysis

The coding sequence (CDS) of *GhTULP30* in CCRI36 was cloned from mixed cDNA and subcloned between the *Not*I and *Bam*HI restriction sites of pYES2 plasmid (primers listed in Table S1). Then, the pYES2-*GhTULP30* and empty pYES2 plasmids were transformed into yeast (INVSc1). The empty plasmid control and positive transformants were diluted to $OD_{600} \approx 0.01$ and transferred to the liquid SD-Ura induction medium for culture at 30℃. Then, they were cultured in the liquid SD-Ura propagation medium until $OD_{600} \approx 1$. OD_{600} detection was performed using the ultraviolet (UV) spectrophotometer at 6, 22, 28, 32, and 36 h after culture. Three biological replicates were prepared for each treatment, and three technical replicates were prepared for each sample. The transformant $OD_{600} \approx 1$ was simultaneously transferred to the solid SD-Ura induction medium and subjected to 1:10 serial dilution for four times. The SD-Ura induction medium contained stress-inducing chemicals, namely 2 mM mannitol and 300 mM NaCl.

Genetic transformation and stress treatment in Arabidopsis

The open reading frame of *GhTULP30* was subcloned into the pBI121 plasmid, driven by the cauliflower mosaic virus 35S promoter, to construct the 35S:*GhTULP30* overexpression vector (Table S1). Subsequently, the *Agrobacterium*-mediated dipping method was used to transform *Arabidopsis* [37]. The transgenic-positive *Arabidopsis* plants were screened on 1/2 MS medium containing 50 ng/μL of kanamycin. RNA was extracted, and the *GhTULP30* transcription level was determined through qRT-PCR. Wild-type and transgenic *Arabidopsis* seeds harvested under the same conditions were sterilized and uniformly arranged on MS media plates of the same composition containing 0, 150 mM NaCl, and 15% PEG6000. The tolerance of seed germination to NaCl and PEG6000 was determined on MS medium containing 0, 150 mM NaCl, and 15% PEG6000. Wild-type and transgenic *Arabidopsis* seedlings growing uniformly on a normal MS plate were transferred to the soil for normal culture for 1 month and treated with 15% PEG6000. The stomatal opening and closing were observed, and the size of the stomata was measured under a scanning electron microscope (JCM-7000). Each experiment was performed with three biological replicates, and three technical replicates were prepared for each biological replicate.

Cotton VIGS and stress treatment

The 475 bp reverse cDNA fragment of *GhTULP30* was subcloned between the *Eco*RI and *Xma*I restriction sites of the pTRV plasmid (primers listed in Table S1). The four vectors, namely TRV:*GhTLUP30*, TRV:*CLA1* (*Cloroplastos alterados 1* as positive control), TRV:*00*, and TRVB, were transformed into the *A. tumefaciens* strain GV3101. The expanded positive monoclonal bacterial solution was diluted with the impregnation buffer containing 10 mM $MgCl_2$, 10 mM MES, and 200 μM acetosyringone to $OD_{600} \approx 1.5$. The resulting solution was mixed with TRV:*GhTLUP30* and TRVB, TRV:*00* and TRVB, and TRV:*CLA1* and TRVB at a ratio of 1:1 and injected into the fully unfolded CCRI36 cotyledon [38]. After 24 h of dark treatment, all plants were transferred to the cotton greenhouse (25℃; 16/8h light/dark photoperiod) for cultivation. TRV:*GhTLUP30* and TRV:*00* plants were irrigated with 15% PEG6000 when TRV:*CLA1* plants appeared albino. Subsequently, the analysis of stomatal opening and closing and measurement of stomatal size were performed under a scanning electron microscope (JCM-7000). RNA was extracted from TRV:*GhTLUP30* and TRV:*00* plants to determine the expression level of *GhTLUP30* and stress-related genes. Each experiment involved three biological replicates and three technical replicates.

Cell death analysis and substance content determination

The leaves of VIGS and control plants treated with drought stress were stained with trypan blue and diaminobenzidine (DAB) to observe cell death [39,40]. The leaves of VIGS and control plants were collected after wilting of these plants to determine the contents of proline, malondialdehyde (MDA), and catalase (CAT). The colorimetric PRO Kit (Nanjing Jiancheng Institute of Bioengineering, China) and UV spectrophotometer were used to determine the proline content, and the wavelength was set at 520 nm. The MDA and CAT contents were determined using the MDA Assay Kit and Micro CAT Assay Kit (Solarbio, Beijing, China), respectively. Three biological and technical replicates were set up for each experiment.

Protein interaction and co-expression analysis

The CDS of *GhTULP30* was constructed into the yeast interaction vector pGBKT7 (BD, between the *Bam*HI and *Not*I restriction sites) for yeast screening library and point-to-point interaction verification. The interaction protein gene was constructed into the yeast interaction vector pGAKT7 (AD, between the *Bam*HI and *Sac*I restriction sites). The positive transformants were screened on the SD/-Trp/-Leu medium, and protein interaction was verified on the SD/-Trp/-Leu/-Ade/-His medium. *GhTULP30* and the interaction gene were constructed into the nLUC vector between the *Sac*I and *Sal*I restriction sites and into the cLUC vector between the *Kpn*I and *Sal*I restriction sites. Then, the vectors were transformed into the *Agrobacterium* strain GV3101 (pSoup-p19). The bacterial liquid was mixed in a ratio of 1:1 and injected into the back of tobacco leaves. After 12 h of dark treatment, they were transferred to light and incubated for 48 h. The leaves were removed and coated with fluorescein (100 mm), treated in the dark for 10 min, and placed in a low-light cooled charge-coupled device imaging apparatus Lumazone_1300B (Roper Bioscience) for observation and photography. The co-expression network data for *GhTULP30* were obtained and analyzed using the default parameters of the ccNET database [41].

Results

GhTULP30 structural characteristics

SlTLP8, *CsTLP8*, *MdTLP7*, and *CaTLP1* are involved in the drought and salt stress response; however, the underlying molecular mechanism remains unclear [17,18,22,23]. *GhTULP34* is vital for the plant response to NaCl and mannitol stress [21]. Additionally,

GhTULP30 is a crucial member of the TULP family, and it participates in response to abiotic stress [21]. Therefore, multiple sequence alignment of the amino acid sequence of GhTULP30 and other plant TULPs was performed (Fig. 1A). GhTULP30 contains an F-Box domain at the N-terminus, indicating that GhTULP30 may function as a subunit of the SCF-type protein complex. All of TULPs contain a conserved Tub domain following the F-Box domain. Additionally, a highly conserved phosphatidylinositol 4, 5-bisphosphate (PIP_2) binding site (K_{175}/R_{177}) is present in the Tub domain, which is crucial for the localization of TULPs on the plasma membrane [42]. Therefore, *GhTULP30* may be localized on the plasma membrane.

The three-dimensional model exhibited that ChTULP30 contains a conserved tubby domain comprising a barrel formed by 12 positive and antiparallel β-sheets, with an α helix in the center of the barrel (Fig. 1B). Subsequently, motif and evolutionary analyses were performed on the amino acid sequences of TULPs from different plants (Fig. 1C), which indicated that GhTULP30 and TULPs from other plants contain the same motifs. The closeness of the evolutionary relationship indicated relatively similar functions. To further understand the regulatory relationship of *GhTULP30* at the transcriptional level, a 2000-bp upstream sequence of *GhTULP30* was obtained, and a *cis*-element analysis was performed (Fig. 1D), which indicated that the *GhTULP30* promoter region contains many hormones and stress-related elements such as drought response element (MBS), temperature response element (LTR), oxygenase stress element (STRE), anaerobic induction element (ARE), MeJA response elements (TGACG/CGTCA-motif), salicylic acid (SA) response element (TCA-element), and ABA response element (ABRE). Thus, *GhTULP30* may respond to stresses such as drought, salt, and hormones and participate in the stress response.

GhTULP30 responds to drought and salt stress in cotton and yeast

The *GhTULP30* transcription levels in CCRI36 under NaCl and PEG6000 conditions were analyzed to further explore the *GhTULP30* function under drought and salt stress. *GhTULP30* exhibited similar transcription patterns under NaCl and PEG6000 treatments (Fig. 2A and 2B). The *GhTULP30* expression levels began increasing after 1 h of treatment and were notably higher than that of the control, reaching the highest level at 6 h and then gradually decreasing, indicating that *GhTULP30* expression was induced by drought and salt stress.

The CDS of *GhTULP30* from CCRI36 of upland cotton was cloned to further explore *GhTULP30* functions under drought and salt stress conditions. The expression vector pYES2-*GhTULP30* was constructed and transformed into yeast (INVSc1). The yeast of pYES2-*GhTULP30* was found to grow better than the control empty vector under normal culture conditions, and a significant difference in their growth pattern was reached gradually (Fig. 2C and D). Under the stress conditions of NaCl and mannitol, pYES2-*GhTULP30* growth was inhibited; however, pYES2-*GhTULP30* grew normally despite reaching a remarkable level compared with the normal medium. Especially under the mannitol stress condition, the pYES2-*GhTULP30* growth rate at 36 h was the same as that under normal conditions (Fig. 2C and D). However, the growth of the empty plasmid control in the stress liquid medium was completely blocked. When pYES2 and pYES2-*GhTULP30* yeasts were transferred to solid medium for culture, the growth rates of pYES2 and pYES2-*GhTULP30* on normal solid medium were similar. However, the pYES2-*GhTULP30* growth rate was markedly faster than that of pYES2 on the medium containing NaCl and mannitol. Therefore, *GhTULP30* was highly resistant to NaCl and mannitol stresses in yeast.

GhTULP30 affects Arabidopsis seed germination and stomatal movement

To verify the hypothesis that *GhTULP30* responds to drought and salt stress, the CDS of *GhTULP30* was concatenated with the 35S promoter and constructed into the pBI121 overexpression vector, transformed *Arabidopsis* by using the *Agrobacterium*-mediated method, and obtained the transgenic plants overexpressing *GhTULP30* (Fig. S3). The seed germination rate of the obtained homozygous transgenic *Arabidopsis* offspring overexpressing *GhTULP30* on the normal MS medium was similar to that of the wild type (Fig. 3A and E). The growth of the overexpression plants on the normal medium was better than that of the wild type. The seed germination of both 35S:*GhTULP30* and WT were inhibited in the medium containing 150 mM NaCl. However, the germination rate of 35S:*GhTULP30* was observably higher than that of the control (Fig. 3B and E). Additionally, 35S:*GhTULP30* grew better than the control after germination. The germination experiment was also conducted on the medium containing 15% PEG6000, with the germination rate similar to that under NaCl stress (Fig. 3C and E). Therefore, *Overexpression of GhTULP30* improved seed germination and the survival rate of *Arabidopsis* under NaCl and PEG6000 stress.

Studies have shown that TULPs affect stomatal movement in plants [22,23]. Therefore, the effect of *GhTULP30* on stomatal opening and closing in *Arabidopsis* was also investigated. Under normal growth conditions, the leaf stomatal opening rate of plants overexpressing *GhTULP30* grown for one month was the same as that of WT plants (Fig. 3D and F). When 15% PEG6000 was used to simulate drought treatment, many stomata on the leaves of 35S:*GhTULP30* and WT plants were closed. Particularly, the 35S:*GhTULP30* plants exhibited more closed stomata, reaching a remarkable level compared with the control, which indicated that *GhTULP30* promoted stomatal closure and increased plant tolerance to drought stress.

GhTULP30 silencing affects stomatal movement in cotton plants

Stomatal movement and characteristics are vital factors that affect plant response to drought stress [1]. Therefore, the stomatal characteristics and movement of cotton leaves after *GhTULP30* silencing were further investigated. Under normal growth conditions, the leaf abaxial stomata of the control and TRV:*GhTULP30* plants were normally opened (Fig. 4A and C). After drought treatment, stomatal closure was observed in both control and TRV:*GhTULP30* plants. However, the TRV:*GhTULP30* plants exhibited fewer closed leaf abaxial stomata compared with controls, and the difference was significant (Fig. 4A and C). Under normal circumstances, the measurements of the leaf abaxial stomatal size exhibited no difference in the length and width between TRV:*00* and TRV:*GhTULP30* plants. However, when subjected to drought stress, the length and width of the stomata of TRV:*GhTULP30* plants were 1.4-fold and 1.5-fold that of TRV:*00* plants, respectively, with both reaching a remarkable difference (Fig. 4B and D). Therefore, *GhTULP30* silencing inhibited the closure of stomata in cotton plants.

The expression levels of marker genes in response to abiotic stress were further determined as *GhTULP30* silencing reduced the tolerance of cotton to drought stress. The *PXG1*, *RAB18*, *CPN60B2* and *UBI3* transcription levels in TRV:*GhTULP30* and TRV:*00* plants were quantitated by qRT-PCR under drought conditions (Fig. 4F-I). Thus, the *PXG1*, *RAB18* and *CPN60B2* expression levels were observably lower in TRV:*GhTULP30* plants than in TRV:*00* plants. Therefore, *GhTULP30* might play a positive regulatory role in cotton response to drought stress.

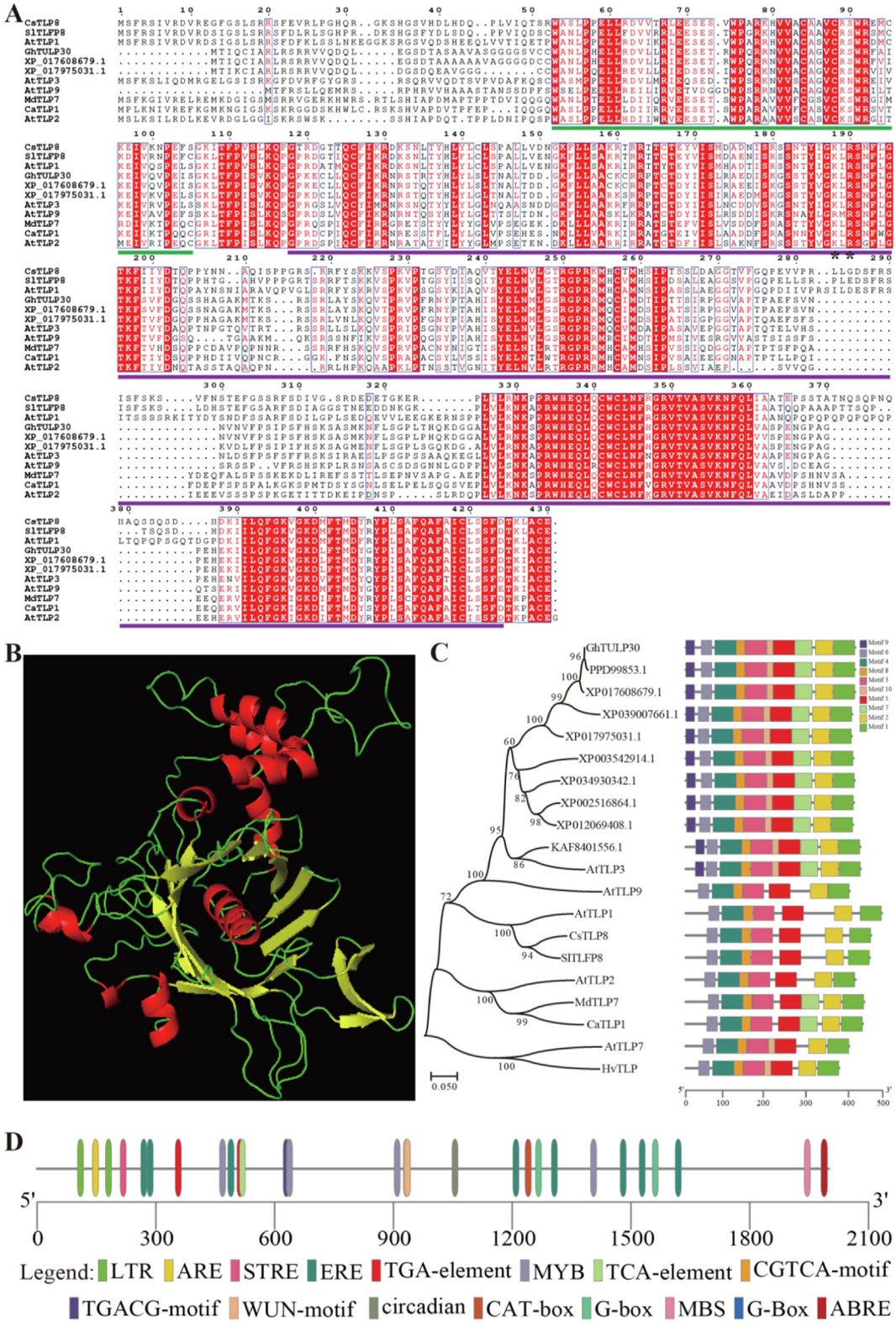

Fig. 1. Structural characteristics of GhTLUP30. (A) Alignment of GhTLUP30 and TULP sequences from other plants. F-box and Tub domains are marked with solid green and purple lines. Black asterisks represent conserved PIP_2 binding sites. (B) Three-dimensional GhTLUP30 model. (C) Phylogenetic tree and motif analysis of GhTLUP30, AtTLPs, SlTLFP8, CsTLP8, MdTLP7, and CaTLP1. (D) *Cis*-acting element analysis of *GhTLUP30* promoter region.

GhTULP30 silencing reduces drought tolerance in cotton

Drought and salt stress treatment markedly increased the *GhTULP30* transcription level. *GhTULP30* improved the tolerance of yeast and *Arabidopsis* to salt and drought stress, suggesting that *GhTULP30* played a positive regulatory role in the response of cotton to drought and salt stress. VIGS was used to reduce the *GhTULP30* transcription level in CCRI36, thereby confirming the

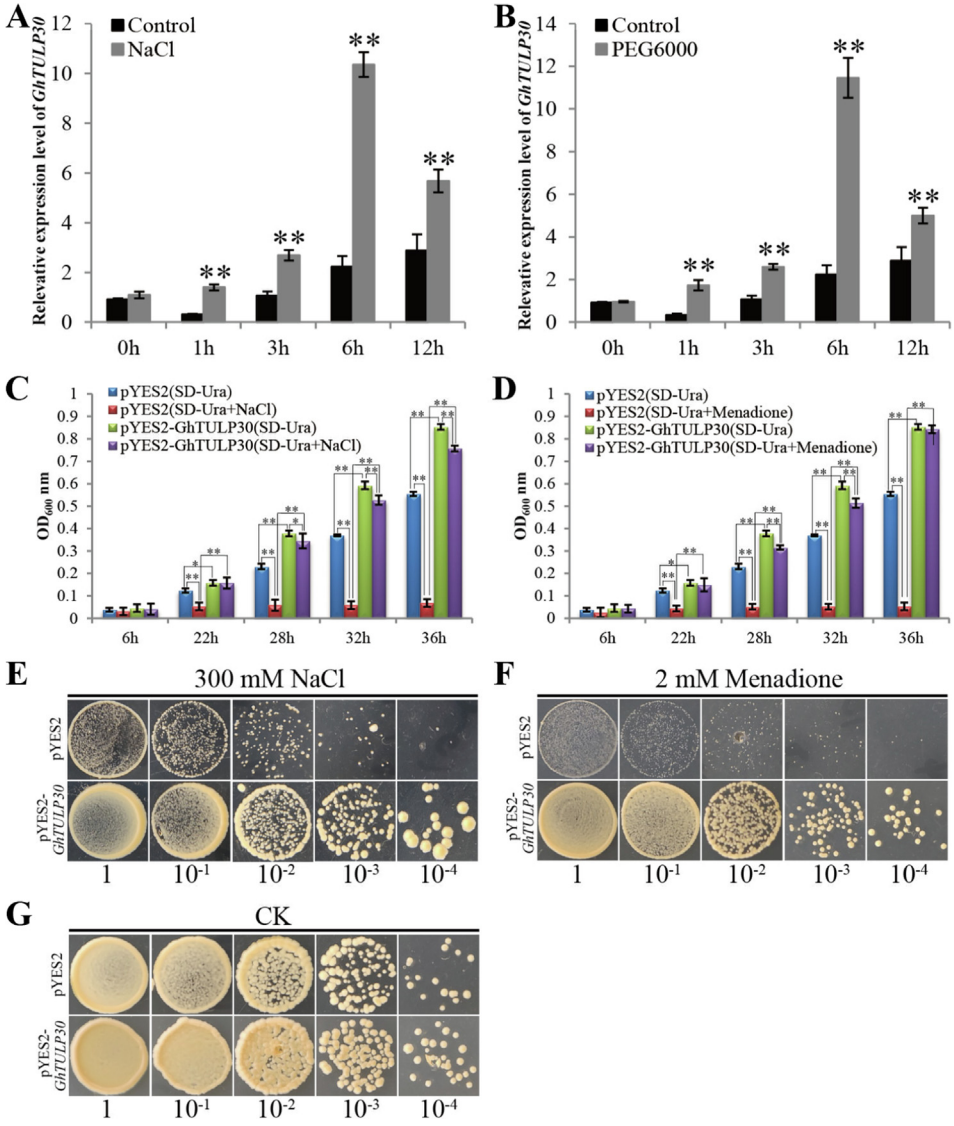

Fig. 2. *GhTULP30* responds to drought and salt stress in cotton and yeast. (A and B) qRT-PCR analysis of the *GhTULP30* expression level after treatment of CCRI36 with NaCl and PEG6000. (C and D) Growth curves of yeast cells in liquid medium containing NaCl and mannitol after ectopic expression of GhTULP30. Values represent the mean ± SD from three biological replicates. *UBQ7* gene was used as a control. (E, F and G) After ectopic expression of GhTULP30, the growth of yeast cells in a control, solid medium containing NaCl and mannitol. 1, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} indicate the initial concentration of $OD_{600} \approx 1$ yeast diluted at 1:10 for 4 times, respectively. * and ** denote $p \leq 0.05$ and 0.01, respectively.

function of *GhTULP30* in response to drought and salt stress in cotton. After the albino phenotype appeared in the true leaves of TRV:*CLA1* positive control plants, the *GhTLUP30* transcription level was measured in TRV:*00* and TRV:*GhTLUP30* plants, indicating that the *GhTLUP30* expression level in VIGS-treated plants decreased by more than 70% (Fig. 5B). To simulate drought and salt stress, 15% PEG6000 was used to irrigate the control and silenced plants. After 1 week, the VIGS plants exhibited an obvious wilting phenotype, whereas the control plants demonstrated relatively better growth (Fig. 5A). Trypan blue staining was performed on true leaves of the same parts of the control and VIGS plants to determine cell mortality and the area of leaf cells after drought treatment (Fig. 5C). The blue spots on the VIGS plant leaves were denser and larger than those of the control plants, indicating higher cell death in TRV:*GhTULP30* plant leaves. DAB staining was used to determine peroxidase accumulation in leaf cells of VIGS plants subjected to drought stress. The results were consistent with those of placental blue staining, and the golden yellow precipitate was more obvious on TRV:*GhTULP30* plant leaves (Fig. 5C). The contents of proline, MDA, and CAT, which are markers of response to drought, were also determined. The proline and CAT contents in the VIGS plant leaves were found to be significantly reduced, whereas the MDA content was found to be observably increased (Fig. 5D, E, and F). Thus, *GhTLUP30* silencing decreased the tolerance of cotton to drought stress.

GhTULP30 interacts with GhSKP1B and GhXERICO

GhTULP30 was used as bait to screen the TM-1 cDNA library of four-leaf-period for yeast two-hybrid (Y2H) to identify potential proteins that interact with GhTULP30. The *GhTULP30* CDS was cloned into the bait vector pGBKT7, and the plasmid was transformed into Y2H Gold yeast to detect GhTULP30 auto-activation. Although the transformant of GhTULP30 grew normally on the SD/-Trp/-Leu medium, it failed to grow on the SD/-Trp/-Leu/-Ade/-His medium, indicating the absence of GhTULP30 auto-activation (Fig. 6A). Two proteins, namely GhSKP1B and GhXERICO, interacting with GhTULP30 were selected by increasing the yeast

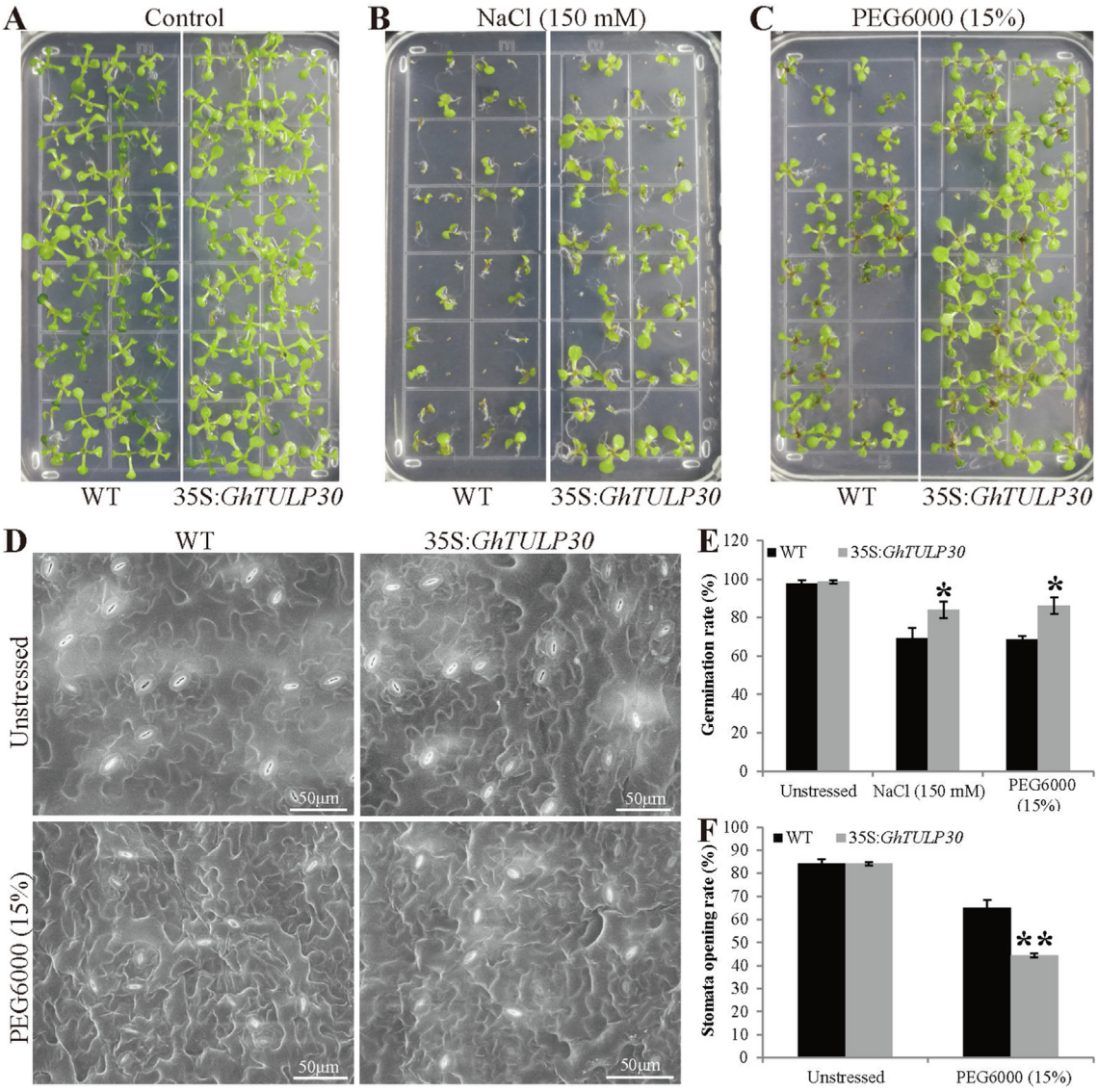

Fig. 3. *GhTULP30* affects seed germination and stomatal movement in *Arabidopsis*. (A, B and C) The germination status of 35S:*GhTULP30* and WT *Arabidopsis* seeds on empty MS medium and MS medium containing NaCl or PEG6000. (D) The stomata of transgenic and WT *Arabidopsis* were opened and closed after drought stress. The red and blue circles indicate closed and open stomata, respectively. (E) The germination rate of 35S:*GhTULP30* and WT *Arabidopsis* seeds on empty MS medium and MS medium containing NaCl or PEG6000. (F) The stomatal opening ratio of transgenic and WT *Arabidopsis* under drought stress condition. * and ** denote $p \leq 0.05$ and 0.01, respectively.

screening pressure. The CDS of the two genes was cloned, inserted into pGADT7, and transformed into Y2H Gold to verify the interaction results. Their ability to auto-activate was assessed, and they failed to survive on the selection medium SD/-Trp/-Leu/-Ade/-His (Fig. 6A). Then, BD-*GhTULP30* was co-transformed into Y2H Gold with AD-*GhSKP1B* and AD-*GhXERICO*. Yeasts carrying BD-*GhTULP30*/AD-*GhSKP1B* or BD-*GhTULP30*/AD-*GhXERICO* grew well on the screening medium (SD/-Leu/-Trp/-His/-Ade) (Fig. 6A). The split-luciferase experiments confirmed the interaction of GhTULP30 with GhSKP1B and GhXERICO (Fig. 6B).

We also analyzed the co-expression network of *GhTULP30* using the ccNET database and found a total of 35 genes co-express with *GhTULP30* (Fig. 6C, Table S2). Among these genes, 6 *XERICO* genes were involved in plant response to salt and drought stress, as well as ABA metabolism. The co-expression network also comprised 3 *SKP1B*, 1 *SKP1A*, and 2 *ASK4* genes, which specifically interact with the F-box domain and are core components of the SCF-type E3 ubiquitin ligase involved in the ABA-dependent stress signaling transduction pathway. Additionally, the NB-ARC domain-containing resistance proteins (Gh_A11G2772 and Gh_Sca004942G02) and 3 *NTF3* genes encoding mitogen-activated kinases that are involved in innate immunity were identified. Mitogen-activated kinases are also key proteins affecting plant leaf development and stomatal movement [43]. More importantly, this was consistent with the results of the screened interaction proteins. These results indicated that *GhTULP30* is involved in plant response to both abiotic stress, such as drought and salt, and biotic stress.

Discussion

Cotton is a vital economic crop. Upland cotton is the main cultivated species that exhibits low resistance to various stresses. Frequently occurring natural disasters such as drought, salinity, and extreme temperature severely restrict the growth and development of cotton. Therefore, the genes involved in response to adverse conditions in cotton should be identified, and specific gene functions in overcoming stress and growth and development in cotton should be studied to provide breeders with valuable genetic resources.

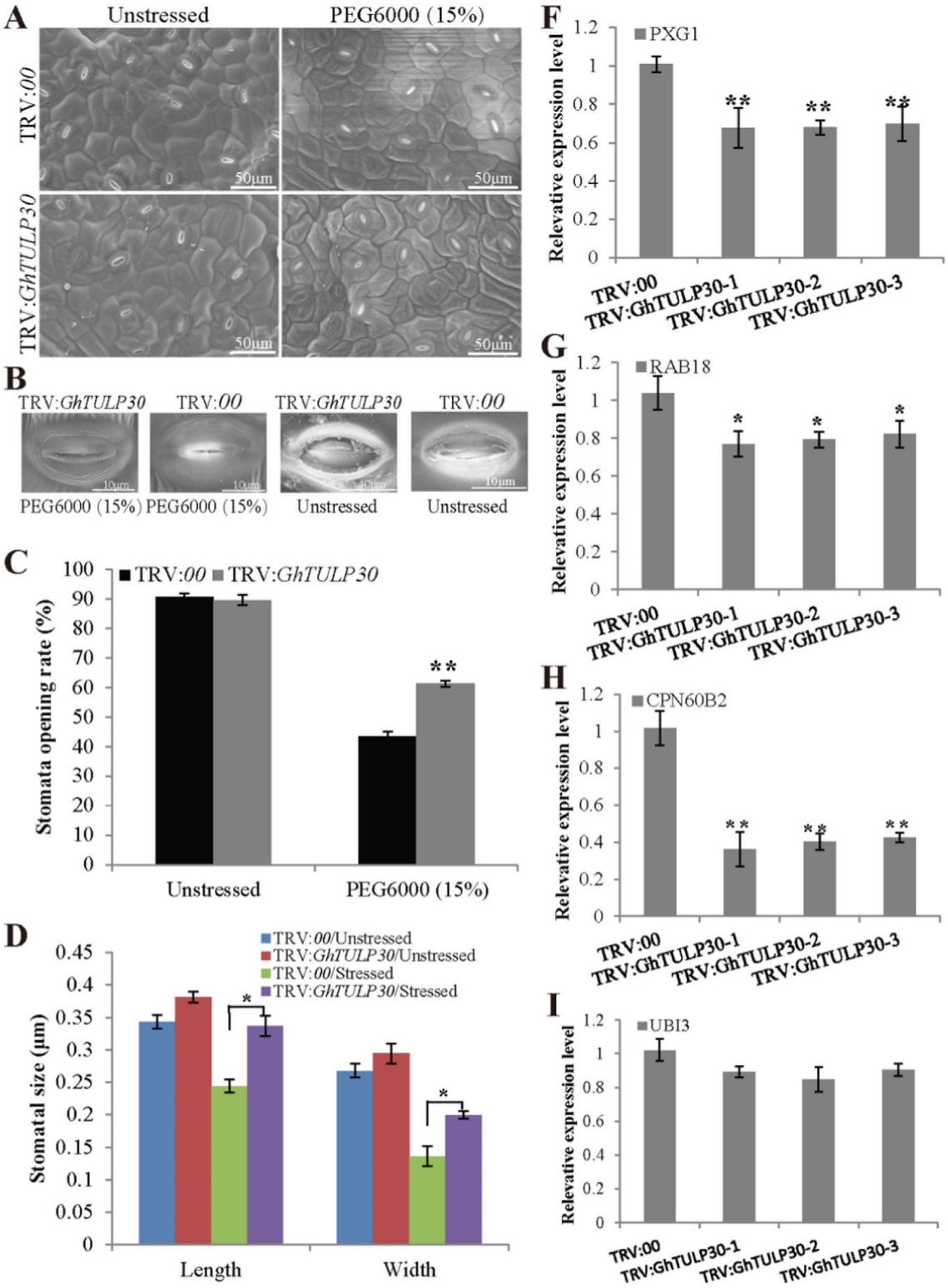

Fig. 4. Silencing *GhTULP30* affects the movement and size of cotton stomata. (A) Stomatal closure of leaves of control and VIGS plants under drought conditions after silencing *GhTULP30*. The red and blue circles indicate closed and open stomata, respectively. (B) Single stomatal closure in leaves of control and VIGS plants under drought conditions after silencing *GhTULP30*. (C) Stomatal opening rate of control and VIGS cotton under drought stress. (D) Statistics of stomatal size of control and VIGS cotton plants under drought stress. The expression levels of stress response genes *PXG1* (F), *RAB18* (G), *CPN60B2* (H), and *UBI3* (I) in VIGS and WT plants under drought stress. * and ** denote $p \leq 0.05$ and 0.01, respectively.

*TULP*s constitute a multi-gene family, which ars ubiquitous in plants and respond to environmental stress [44]. *GhTULP*s are involved in cotton response to environmental stress and tissue development. For example, under salt stress, the germination rate of *Arabidopsis* seeds overexpressing *GhTULP34* is significantly reduced, and under mannitol stress, root development and stomatal closure are inhibited. Additionally, GhTULP34 interacts with the GhSKP1A subunit of the SCF-type complex [21]. The *GhTULP30*

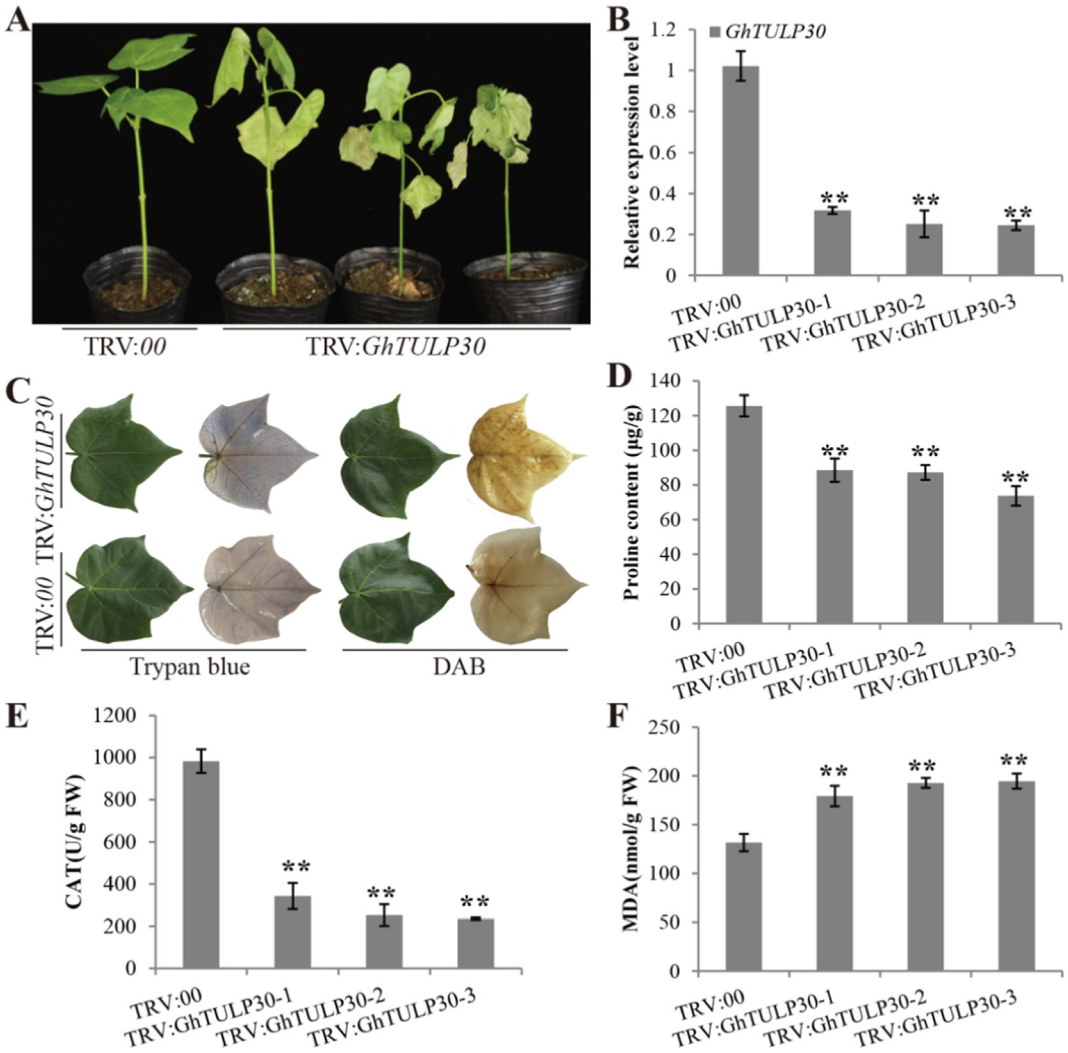

Fig. 5. Drought tolerance of cotton decreases after *GhTULP30* silencing. (A) Phenotypes of drought-stressed VIGS and control cotton plants. (B) *GhTULP30* expression level in VIGS and control cotton plants under drought stress. (C) Trypan blue and DAB staining of VIGS and control leaves after drought stress. (D, E and F) Proline, CAT, and MDA content of VIGS and control cotton plants under drought stress. ** denotes $p \leq 0.01$.

transcription level was notably increased in response to drought and salt stress in cotton [21]. Thus, the function of *GhTULP30* was further studied.

Analysis of the GhTULP30 structural characteristics revealed that GhTULP30 contains typical F-Box and Tub domains (Fig. 1) and that it is a typical plant TULP [44]. Alignment with amino acid sequences in other species exhibited that GhTULP30 contains a conserved PIP_2 binding site (Fig. 1A), suggesting that GhTULP30 may be located on the plasma membrane [22,42]. The interaction between TULP and PIP_2 is reversible. When TULP interacts with the subunit $G\alpha_q$ of the G-protein, TULP is released by the plasma membrane and transported to the nucleus, where it acts as a transcription factor for the regulation of gene expression [15]. Nuclear Factor Y subunit C3 interacts with AtTLP2 and transports it to the nucleus [25]. Under mannitol, salt, and H_2O_2 stress, plasma membrane releases AtTLP3 into the nucleus [16], and CaTLP1 accumulates in the plant nucleus under osmotic stress [45]. Phylogenetic tree and motif analysis also indicated that GhTULP30 is highly similar to the TULPs present in other species, indicating that they may have similar functions. Additionally, the *cis*-acting element analysis exhibited that the *GhTULP30* promoter region comprises several elements that respond to abiotic stress and hormones (Fig. 1C).

Relieving the reactive oxygen species toxicity is a vital mechanism of plant tolerance to stress. Reactive oxygen species regulates seed germination. Excessive release of reactive oxygen species destroys the macromolecular structures such as protein and DNA in seeds and inhibit seed germination [46]. Studies have exhibited that *CaTLP1* improved the tolerance of yeast and *Arabidopsis* to stress by regulating antioxidant defense genes [17]. The qRT-PCR results in the present study exhibited that the *GhTULP30* transcription level was markedly induced by NaCl and PEG6000 (Fig. 2). Ectopic expression of *GhTULP30* in yeast notably improved yeast cell tolerance to NaCl and mannitol (Fig. 2). Overexpression of *GhTULP30* in *Arabidopsis* observably increased the germination rate and stomatal closure rate of seeds under drought and salt stress (Fig. 3). Therefore, *GhTULP30* improved the tolerance of yeast and *Arabidopsis* to drought and salt stress and was also related to the accumulation of reactive oxygen species.

Leaf morpho-anatomical traits and stomatal opening affect carbon input and net photosynthesis, ultimately affecting crop productivity [3,6–9]. Stomatal size and movement are important factors affecting plant drought tolerance and water use efficiency [47]. Studies have shown that many genes regulate ABA concentration, and ABA affects stomatal opening in guard cells and stomatal

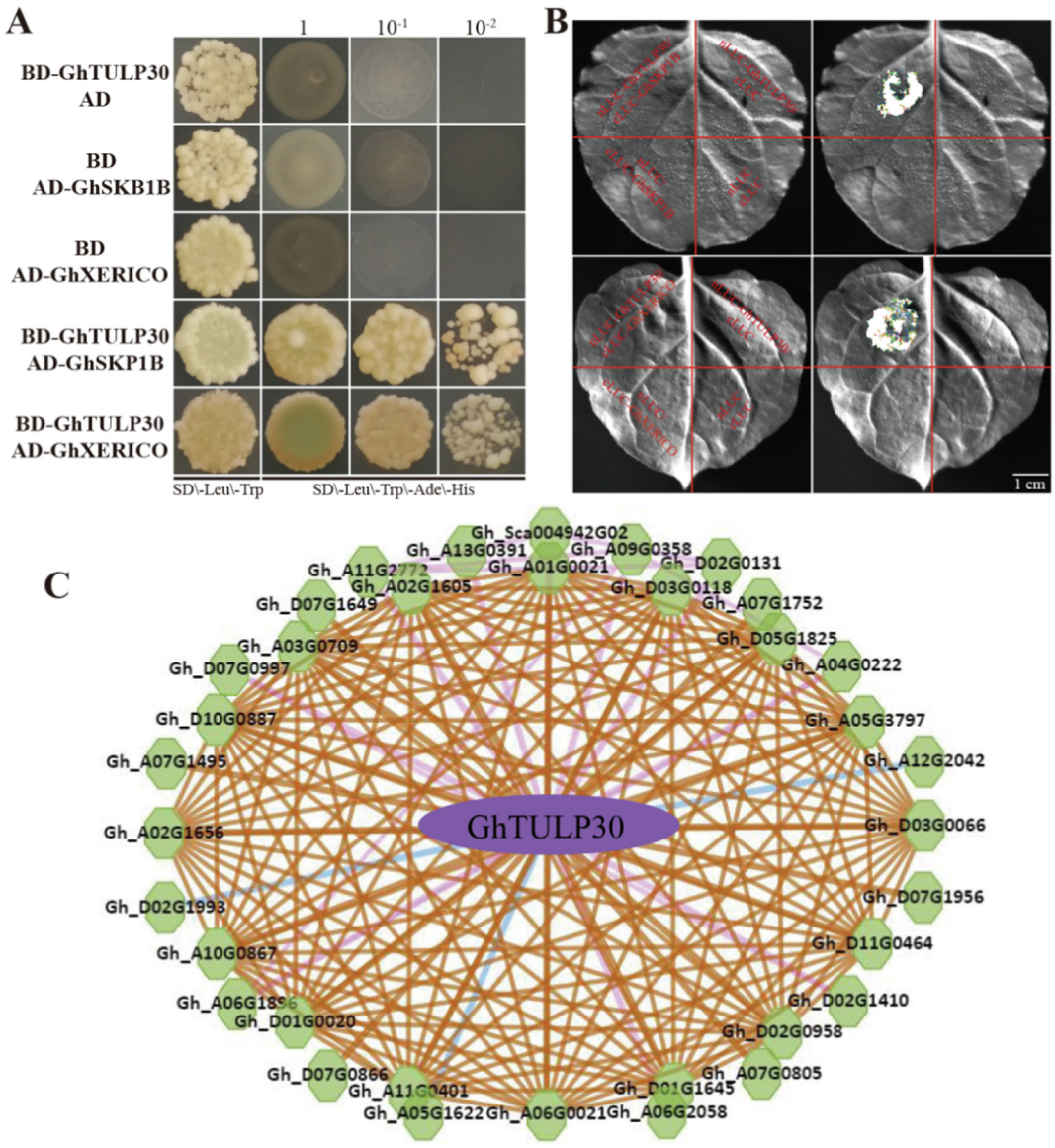

Fig. 6. Protein interaction and co-expression network analysis. (A) Yeast two-hybrid assay exhibiting auto-activation and interaction of GhTULP30 with GhSKB1B and GhXERICO. (B) Luciferase experiment confirmed the interaction of GhTULP30 with GhSKB1B and GhXERICO. (C) Co-expression network of *GhTULP30*. Nodes denote individual genes, and edges denote significant co-expression between genes. Pink lines denote positive co-expression relationship with the target protein; blue lines represent negative co-expression relationship with the target protein; and the orange line represents protein interaction with the target protein.

density [48,49]. Meanwhile, ABA also affects stomatal movement in response to drought stress through ion channel activity [50], microtubule pattern and cell wall structure and composition [43]. The movement and size of stomata regulate the response to drought and salt stress [51,52]. CaTLP1 regulates gene transcription in the stress resistance pathway and induces stomatal closure by interacting with the protein kinase CK1 [17]. *SlTLFP8* affects the stomatal size and density of leaf epidermis through endoreduplication, thereby reducing water loss [23]. Therefore, the size of the stomata and the opening and closing state of the cotton leaf epidermis were measured after silencing *GhTULP30* (Fig. 4). The silenced plants exhibited less stomatal closure, large stomata size, rapid water loss, and weak drought tolerance. Determination of the expression of stress response-related marker genes exhibited that the gene transcription level was markedly inhibited (Fig. 4), indicating that VIGS plants had reduced tolerance to drought and salt stress.

Proline participates in osmotic adjustment, which reflects stress resistance in plants [28]. The proline content in the VIGS lines decreased notably, indicating that their drought tolerance was reduced. The accumulation of reactive oxygen species increased after the plants were subjected to drought and salt stress, and CAT is the key enzyme for removing reactive oxygen species [53,54]. When the *GhTULP30* expression level was suppressed, the CAT content was observably reduced under drought conditions. Lipids are decomposed by undergoing peroxidation to produce MDA. Thus, MDA reflects the membrane damage caused to plants under stress [55]. The present study exhibited that the MDA content was significantly increased in VIGS plants. Simultaneously, staining with trypan blue and DAB exhibited the severity of leaf cell necrosis in VIGS plants (Fig. 5), indicating the crucial role of *GhTULP30* in promoting cotton response to drought stress.

Protein interaction experiments exhibited the interaction of GhTULP30 with GhSKP1B and GhXERICO (Fig. 6). Studies have exhibited that ABA is a prominent regulator of seed germination and directly affects ion transport in guard cells to rapidly alter stomatal aperture in response to changing water availability and abiotic stresses such as drought [56,57]. GhTULP30 interacts with

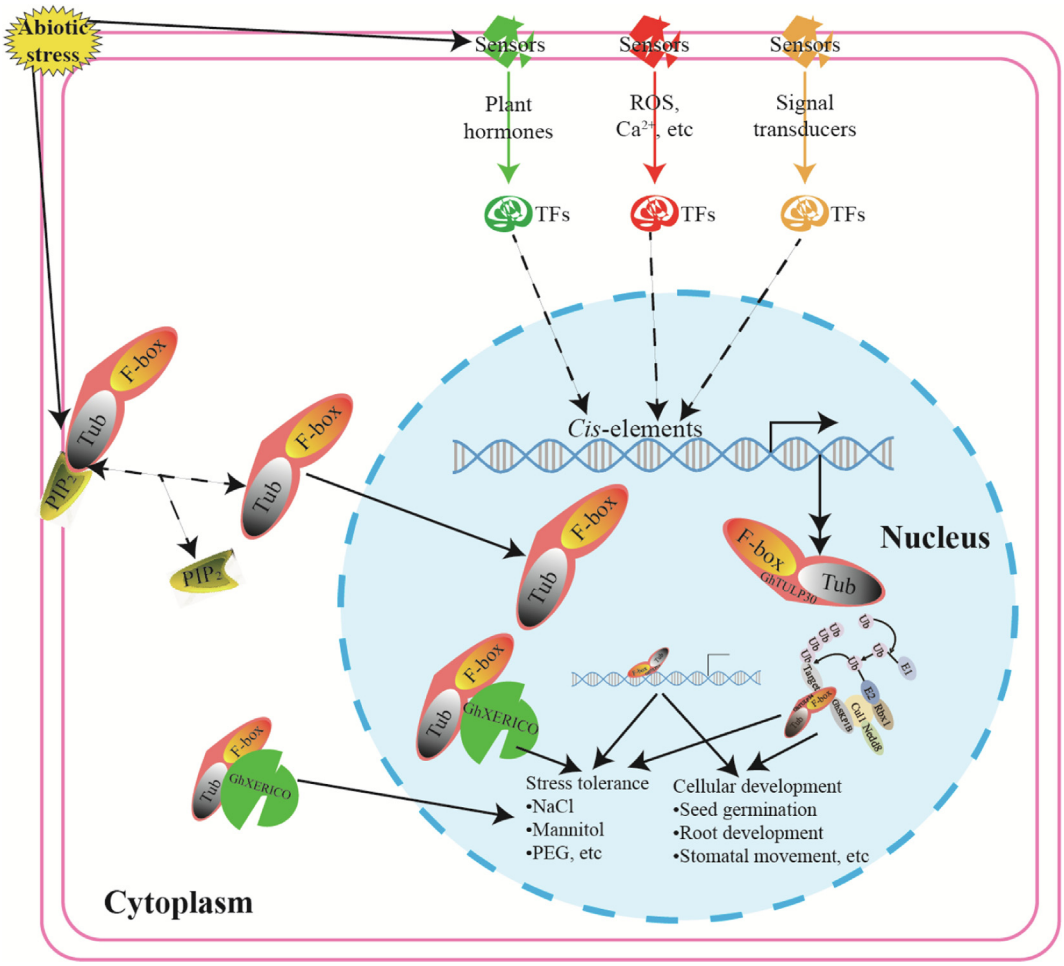

Fig. 7. Hypothetical *GhTULP30* functional mechanism model. Plant cells receive external stress signals through various sensors. Several secondary messengers such as reactive oxygen species and Ca^{2+}, plant hormones, and signal converters transmit these stress signals and act on related transcription factors. Transcription factors bind to the *GhTULP30* promoter region to induce their expression. The GhTULP30-PIP_2 complex is reversible in the G-protein signaling pathway. Under drought and salt stress, GhTULP30-PIP_2 disintegrates, and GhTULP30 is transferred from the plasma membrane to the nucleus to transmit signals in response to the corresponding stress and affect plant development. The interaction between GhTULP30 and GhXERICO also affects plant response to stress and development. The SCF-type complex and ubiquitination of target proteins regulate drought and salt stress. The SCF-type complex functions within the ubiquitination reaction through combined action with the E1 and E2 enzymes. TULPs bind to SKP and targets through their F-box domain and C-terminal domain, respectively, thereby presenting the ubiquitination target. PIP_2, phosphatidylinositol 4, 5-bisphosphate; Tub, Tub domain; F-box, F-box domain; TFs, transcription factors. Ub, ubiquitin. Rbx1, ring-box protein.

GhSKP1B, the subunit of the SCF-type complex, and participates in the regulation of ABA-dependent pathways by SCF-type E3 ligase in response to plant drought stress [16,58]. XERICO is a RING-H2 type zinc finger protein, and the RING domain of c-CBL has E3 ubiquitin protein ligase activity, which interacts with components in the ubiquitin-mediated protein degradation pathway (such as SCF complex), and participates in the ubiquitin degradation of ABA signaling pathway to regulate plant response to drought stress [59,60]. GhTULP30 interacts with the XERICO protein, and the XERICO RING E3 ligase may contribute to regulating ABA level, affecting plant responses to osmosis and salt stress. Simultaneously, the co-expression network analysis exhibited the co-expression of *GhTULP30* with *SKP1B* and *XERICO*, indicating its involvement in plant response to abiotic stress. Moreover, *Gh_A11G2772*, *Gh_Sca004942G02*, and *NTF3*, which are involved in the process of biotic stress, were also identified in the co-expression network, indicating that *GhTULP30* may also participate in plant response to biotic stress.

Our previous study has exhibited that another member of the *TULP* family, *GhTULP34*, plays a negative regulatory role in the response of cotton to abiotic stresses such as drought and salt [21], which is opposite to the function of *GhTULP30*. Sequence alignment analysis exhibited that the amino acid sequence similarity between GhTULP30 and GhTULP34 is 67.73%. Further motif analysis indicated that GhTULP30 has one less motif than GhTULP34. Additionally, the phylogenetic tree analysis indicated that GhTULP30 and GhTULP34 share a close evolutionary relationship (Fig. S1). Further secondary and 3D model prediction analysis exhibited that the lack of motif8 in GhTULP30 may lead to the lack of a beta strand. Additionally, GhTULP30 has two more small alpha helix than GhTULP34 (Fig. S2). The *GhTULP30* promoter region exhibits more *cis*-acting elements (such as ABRE, LTR, MBS, and Myc) in response to stress than the *GhTULP34*, and light-responsive elements (such as G-box and GA-motif) than *GhTULP34* (Table S3). These differences may be the reasons for the differentiation of *GhTULP30* and *GhTULP34* in the process of executive function. This phenomenon also occurs in other plants such as *Arabidopsis*, cucumber, and tomato [12,16,21–23], and further research is required to explore the specific molecular mechanism.

Conclusion

In this study, we found that *GhTULP30* expression was induced by drought and salt stress; Ectopic expression significantly

improved yeast tolerance, and increased the seed germination rate and stomatal closure rate of *Arabidopsis* under drought and salt stress conditions; When *GhTULP30* was silenced in cotton, the closure of the leaf epidermis stomata was blocked, and the leaf wilting and necrosis were obvious; GhTULP30 interact with GhSKP1B and GhXERICO. In brief, *GhTULP30* acts as a molecular sensor, transcription factor, or protein subunit in response to drought stress in cotton (Fig. 7). The present study will provide references and ideas for further exploration of *TULP* functions.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Zhanshuai Li: Conceptualization, Methodology, Investigation, Writing – original draft. **Ji Liu:** Validation, Formal analysis, Visualization. **Meng Kuang:** Formal analysis. **Chaojun Zhang and Qifeng Ma:** Resources, Data Curation. **Longyu Huang and Huiying Wang:** Resources, Writing – review & editing. **Shuli Fan and Jun Peng:** Conceptualization, Data Curation, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.06.007.

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