Silencing GhJUB1L1 (JUB1-like 1) reduces cotton (Gossypium hirsutum) drought tolerance

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Abstract

Drought stress massively restricts plant growth and the yield of crops. Reducing the deleterious effects of drought is necessary for agricultural industry. The plant-specific NAC (NAM, ATAF1/2 and CUC2) transcription factors (TFs) are widely involved in the regulation of plant development and stress response. One of the NAC TF, JUNGBRUNNEN1 (JUB1), has been reported to involve in drought resistance in Arabidopsis. However, little is known of how the JUB1 gene respond to drought stress in cotton. In the present study, we cloned GhJUB1L1, a homologous gene of JUB1 in upland cotton. GhJUB1L1 is preferentially expressed in stem and leaf and could be induced by drought stress. GhJUB1L1 protein localizes to the cell nucleus, and the transcription activation region of which is located in the C-terminal region. Silencing GhJUB1L1 gene via VIGS reduced cotton drought tolerance, and retarded secondary cell wall (SCW) development. Additionally, the expression of some drought stress-related genes and SCW synthesis-related genes were altered in the GhJUB1L1 silencing plants. Collectively, our findings indicate that GhJUB1L1 may act as a positive regulator in response to drought stress and SCW development in cotton. Our results enriched the roles of NAC TFs in cotton drought tolerance and laid a foundation for the cultivation of transgenic cotton with higher drought tolerance.

Introduction

Plants often suffer from multifarious biotic and abiotic stresses with a sessile lifestyle. Drought is one of the most devastating environmental factors which largely restrict the growth and yield of crop plants all over the world. Cotton (Gossypium hirsutum) is an important fiber and oil crop worldwide. The yield of cotton is strongly limited by drought stress. Water is essential in every phase of cotton growth and development. Therefore, breeding excellent drought-resistant cotton is the dream pursued by many breeding researchers.
Plants have evolved a range of tolerance mechanisms at the molecular, biochemical, physiological, and developmental levels to reduce water loss [1–4]. Many stress-related transcription factors (TFs) and their target genes formed a complex regulatory network for plant stress response, which had been extensively studied and been well reviewed previously [5–13]. In the drought response experiment, the recovery rate of drought resistance of the *ataf1-1* and *ataf1-2* mutants was 7 times higher than that of the wild type. At the same time, real-time quantitative PCR analysis found that the expression of *COR47*, *ERD10*, *KIN1*, *RD22* and *RD29A* related to drought stress response was enhanced [8]. It was found that the overexpression of *DgNAC1* significantly improved the salt tolerance of tobacco [7].

NAC TF is one of the largest families of plant unique transcription factors which were named as the NAM (No Apical Meristem) in *Petunia hybrida* [14] and the ATAF1/2 and CUC2 in *Arabidopsis thaliana* [15]. A large number of NAC genes were found in *Arabidopsis thaliana* [14], soybean (*Glycine max*) [16], wheat (*Triticum aestivum*) [17], alfalfa (*Medicago truncatula*) [18], cotton (*Gossypium spp.*), [19] and many other species, which play essential roles in response to abiotic and biotic stresses. JUNGBRUNNEN1 (JUB1, ANAC042) is a multifunctional member of the NAC TF family in *Arabidopsis*, which participates in the regulation of plant longevity and stress tolerance. The JUB1-overexpressing plants (JUB1ox) were tolerant to multiple stresses, while the *jub1-1* mutants showed hypersensitivity to these stresses. AtJUB1 directly regulates the expression of stress-responsive transcription factors such as *DREB2A*, and reduces the level of reactive oxygen species, which contributes to the enhancement of stress tolerance [20, 21]. It was recently reported that overexpression of JUB1 also enhances drought tolerance in tomato [22] and banana [23]. AtJUB1 directly inhibits the expression of gibberellic acid (GA) and brassinosteroid (BR) biosynthetic genes, leading to the accumulation of DELLA protein, which inhibits growth and increases plant resistance to stresses [24, 25]. Reduced *SlJUB1* expression in tomato plants resulted in more sensitive to drought and exhibited higher levels of oxidative stress than that of control plants. In addition, ectopic expression of AtJUB1 led to enhanced stress tolerance and reduced oxidative damage. Furthermore, *SIDREB1*, *SIDREB2* and *SIDElla* are potential target genes of SlJUB1 under drought stress [22]. In conclusion, as a growth and stress response regulator, JUB1 provides great hope for genetic engineering to improve crop drought tolerance.

Here, we report that *GhJUB1L1* (*JUB1-like 1*) is a drought induced TF with a positive role in drought resistance in cotton. Compared with the control plants, the *GhJUB1L1* silenced cotton plants were more sensitive to drought stress. In addition, silencing *GhJUB1L1* reduces SCW synthesis in cotton. Our results provided new clues for further study of potential direct target genes for JUB1 TF.

**Materials and methods**

**Plant materials**

The cotton variety used in this experiment is Upland cotton J14 (*Gossypium hirsutum L. acc. Jimian14*) provided by Professor Ma Zhiying of Hebei Agricultural University. For gene expression analysis, the cotton plants were grown under natural field conditions in Chongqing. The cotton roots, stems, leaves and flowers (0 days post-anthesis, DPA) were cut and stored at -80°C after quick-freezing with liquid nitrogen; For drought treatment, cotton seeds were planted in humus soil and cultured in plant light incubator at 25–28 °C. After the plants grew to 3 true leaves, they were kept away from water for 1 week. Normally watered plants were used as controls. The cotton aboveground parts of treatment group and control group were cut and stored at -80°C after quick-freezing with liquid nitrogen. For VIGS experiment, cotton seeds were planted in humus soil and cultured in plant light incubator at 25–28 °C. The
cotyledons were infiltrated after they were fully expanded. The tobacco variety used for instantaneous expression is *Nicotiana benthamiana* and is preserved in our laboratory.

**Cloning of GhJUB1L1**

The gene ID and the sequence of *GhJUB1L1* was downloaded from the Upland cotton genome database, CottonFGD (https://cottonfgd.org). Primers were designed according to the downloaded sequence and the full length of the target gene was amplified using the cotton leaf cDNA as the template. The amplified primers were listed in S1 Table. Sequence alignment and phylogenetic analysis was performed using DNAMAN and MEGA7.0 software [26], respectively. The accession numbers are: GhJUB1-like (Gh_D06G2096), GhJUB1L1 (Gh_D06G2096.1), DzJUB1 (XP_022724113), HsJUB1 (KAE8725843), AtJUB1 (NP_181828), OsJUB1 (XP_015628846.1), and ZmJUB1 (XP_008644461.1). The evolutionary history was inferred using the Neighbor-joining method [27]. The optimal tree with the sum of branch length = 286.31250000 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [28]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method [29] and are in the units of the number of amino acid differences per sequence.

**Real-time qRT-PCR analysis**

Total RNA was extracted using the RNAprep pure Plant Kit (TIANGEN, Beijing, China). First-strand cDNA was synthesized using the PrimeScript RT reagent Kit with gDNA Eraser (TAKARA, Kyoto, Japan). qRT-PCR analysis was performed using Novostar-SYBR Supermix (Novoprotein, Shanghai, China): 94°C for 2 min followed by 40 cycles of 94 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 1 min. Three biological repetitions were performed. Relative expression levels were calculated using the \( 2^{-\Delta\Delta Ct} \) method [30]. The specific primers of the selected genes and the internal control, HISTONE3 (GenBank accession No. AF024716), are listed in S2 Table.

**Subcellular localization**

The full-length CDS (Coding sequence) of *GhJUB1L1* (without terminating codon) was ligated into the binary vector 35S::GFP (modified from pCAMBIA2300) (S1 Table). The resultant 35S::GhJUB1L1::eGFP plasmid was introduced into agrobacterium tumefaciens strain GV3101 and infiltrated into tobacco (*Nicotiana benthamiana*) leaves for transient assays. The GFP fluorescence was observed via a confocal laser scanning microscopy (Leica SP8).

**Analysis of transcriptional activity in yeast**

The transcription activation assay in yeast was performed using Yeastmaker™ Yeast Transformation System 2 (Clontech). Different regions of GhJUB1L1 were cloned into pGBK7T7 vector (S1 Table). These plasmids and the control pGBK7T7 were introduced into yeast strain Y2HGold, and the transformed strains were cultured on either SD (Synthetic Dropout Media)/-Trp, TDO (Triple Dropout Supplements, SD/-Trp-His-Ade) and TDO/x-α-gal (SD/-Trp-His-Ade/x-α-gal) incubated at 30°C for 3 days to detect the response of GAL4 reporter.

**Virus-induced gene silencing**

To silence *GhJUB1L1* through the VIGS (Virus-induced gene silencing) system mediated by tobacco rattle virus (TRV), a 228 bp sequences (S1 Table) were selected from the CDS of
GhJUB1L1 and ligated into the pTRV2 vector. The agrobacterium (GV3101) containing pTRV1+TRV:GhJUB1L1 were co-infiltrated into the cotton cotyledon with the pTRV1 +TRV:00 group as the negative control. Silencing the chlorophyll biosynthesis gene GhCLA1 affected the synthesis of chlorophyll in cotton leaves, which resulted in the production of albino seedlings. Therefore, pTRV1+TRV:GhCLA1 group was used as the positive control in this study to test the effectiveness of the VIGS system. After the positive control leaves showed albino phenotypes (about 1 week after receiving injection), the expression level of GhJUB1L1 in the silenced lines was detected by real-time qRT-PCR (2 weeks after receiving injection), and the down-regulated plants were selected for subsequent observation and research. For drought treatment, the GhJUB1L1 silenced plants and controls were kept away from water for 10 days, normally watered plants were used as controls, then phenotypes were recorded. Then the materials were rewatered, phenotypes were recorded after 2 days.

**Determination of ion leakage**

For ion leakage measurements, the second leaves in the top were immersed in 10 ml deionized water with a hole punch and soaked at room temperature for 12 h. Electrical conductivity (R1) was measured at 25 °C, using a conduct meter (Shanghai INESA & Scientific Instrument CO. LTD). The samples were boiled for 15 min, cooled down to 25°C, and conductivity (R2) was measured again. Ion leakage was calculated through the expression R1/R2×100. Three independent experiments were performed.

**Determination of Relative Water Content (RWC)**

Plant material leaves of the same position) was weighed (fresh weight, FW), then put in a Petri dish containing water and kept at room temperature for 24 h. Then, leaves were weighed again (turgid weight, TW). Then put the leaves into the drying box to dry and weigh the dry weight (DW). Relative water content (RWC) was calculated using the following formula: RWC [%] = (FW–DW)/(TW–DW)×100. Three independent experiments were performed.

**Staining of lignin and cellulose**

Cut the the second internode stem (3 weeks after receiving injection) of the plant material. Place the material on a clean slide and slice it continuously to form many thin section, placing them in clear water for later use. For Phlorotriphenol/HCl staining method, put a drop of 5% phloroglucinol solution (0.05 g phloroglucinol dissolved in 1 mL 95% alcohol, keep in dark place) in the middle of a clean slide, pick a section into the dye solution, then put a drop of concentrated hydrochloric acid. Once the material turns red, cover the glass quickly and observe under a normal microscope (BX41TF). The degree of lignification of the material was accorded to the intensity of pigmentation. The volatilization of concentrated hydrochloric acid and ethanol leads to the precipitation of resorcinol, it is browning of the material. Therefore, the material can be temporarily placed in 75% (V/V) ethanol for photographic observation. For Toluidine blue staining method, the material was dyed with 0.05% toluidine blue (0.05 g toluidine blue dissolved in 100 mL water) for 1 min. For the rest, please refer to the resorcinol/HCl staining method [31, 32].

**Dual-luciferase (Dual-LUC) assays**

Dual-luciferase (Dual-LUC) assays were performed according to the manual instruction of the Dual-Luciferase Reporter Assay System kit (Promega, USA). The promoter deletion fragments were fused with firefly luciferases (LUC) gene in the pGreen0800-LUC vector [33] (S1 Table).
Effector construct was generated by introducing the GhJUB1L1 gene into the overexpression vector pCambia2300-35S-eGFP. Mixes of the agrobacteria strains harboring effector and reporter (effector: reporter, 2: 1, v/v) were introduced into 4-wk-old tobacco leaves by infiltration [34]. After 40–48 h, leaves were collected for the dual-luciferase assay.

### Statistical data analysis

Data are presented as means ± SD. Statistical analysis were performed by the one-tailed Student’s t-test. *, **, and *** indicates significant differences at p < 0.05, p < 0.01, and p < 0.001, respectively.

### Results

#### Characters of GhJUB1L1

Searching the public database of upland cotton genome, we found that there are 16 JUB1-like genes in cotton. In order to clarify the roles of 16 JUB1-like genes in cotton growth, their tissue expression was analyzed using the Cotton FGD website (https://cottonfgd.org/) Gene Profile transcriptome database. The results (Fig 1) showed that the expression levels of the 16 JUB1-like genes were generally low in ovules and fibers at different stages, and were only predominantly expressed in leaves and stems. The expression levels of Gh_D06G2096 and Gh_A06G1947 were the highest in stem, the important tissue for water transport, suggesting that Gh_D06G2096 and Gh_A06G1947 may be related to drought resistance in cotton. Based on the above analysis, Gh_D06G2096, whose expression level in stem was higher than Gh_A06G1947, was selected for further study, and was cloned and renamed as GhJUB1L1 (JUB1-like 1).

In order to clone the GhJUB1L1 (Gh_D06G2096) gene, the specific primers were designed to amplify the full ORF of this gene. The resulting sequence is 948 bp (S1 Fig) in length and encodes a protein with 315 amino acid residues, with a predicted molecular weight of 36.8 kD and an isoelectric point of 6.219 (S2 Fig).

GhJUB1L1 contains the typical sequence of NAC transcription factor (TF) (S2 Fig). Compared with other JUB1 proteins from Arabidopsis thaliana, durian (Durio zibethinus), and hibiscus (Hibiscus syriacus), the N-terminal of GhJUB1L1 is highly conservative while the C-terminal is variable (S2 Fig). In order to clarify the phylogenetic relationship of GhJUB1L1, we selected homologous proteins from dicotyledonous plants, cotton (Gossypium hirsutum), durian (Durio zibethinus), hibiscus (Hibiscus syriacus), and Arabidopsis (Arabidopsis thaliana) and monocotyledonous plants, rice (Oryza sativa) and maize (Zea mays) for phylogenetic analysis. The results (S3 Fig) showed that GhJUB1L1 was closely related to JUB1 protein of cotton, durian, hibiscus and Arabidopsis, but was far related to rice and maize. The JUB1 proteins from monocotyledons and dicotyledons were divided into two groups, indicated that the gene evolved independently after the differentiation of monocotyledons and dicotyledons.

#### Expression patterns of GhJUB1L1

To explore the functions of GhJUB1L1 in cotton, we analyzed the expression levels of GhJUB1L1 gene in different tissues and organs. The results indicated that the expression level of GhJUB1L1 was higher in stem and leaf, while it was lower in flowers and roots under non-stress conditions. The expression of this gene in these tissues increased when plants were subjected to drought (Fig 2). Given that stem and leaf are the key tissues for water transport and release (transpiration) in cotton, our expression results implying that GhJUB1L1 might be important for drought stress for cotton.
Subcellular localization of GhJUB1L1

To examine the subcellular localization of GhJUB1L1, we constructed the 35S::GhJUB1L1::eGFP vector (S4 Fig), and introduced this vector in a transient expression system in *N. benthamiana* leaves by an agrobacterium-mediated method. The eGFP fluorescence signal was detected by a confocal laser scanning microscopy, and the results showed that the signal was found to be exclusively present in the nucleus, and was overlapped with the fluorescent signal of nuclear dye DAPI (Fig 3). These results indicate that GhJUB1L1 localizes in the nucleus.

Transactivation activity assay of GhJUB1L1 in yeast

In order to identify whether GhJUB1L1 has transcriptional activity, the transcription activation assay in yeast was performed by using the Yeastmaker™ Yeast Transformation System 2 (Clontech). Different regions of GhJUB1L1 CDS were cloned into pGBKT7 vector (Fig 4a).
The results showed that yeast cells carrying BD-TAR (Transcriptional activation region) grew well in SD/-Trp, TDO and TDO/x-α-gal medium, and the x-α-gal chromogenic reaction presented blue color (Fig 4b), indicating that the TAR region of GhJUB1L1 had self-activation activity. Conversely, yeast cells carrying BD-full or BD-NAC domain could only grow on SD/-Trp medium. In summary, the GhJUB1L1 active region is located in the TAR region of its C-terminal. Its full-length transcriptional activity is inhibited, indicating that the N-terminal might play a regulatory role in its transcriptional activation activity.

**Silencing GhJUB1L1 gene via VIGS reduces cotton drought tolerance**

To elucidate the functions of GhJUB1L1 in drought stress, we silenced the GhJUB1L1 gene by the VIGS system. A 228 bp fragment was selected from the full length of GhJUB1L1 gene and
subjected to construct the TRV-GhJUB1L1 vector (S5 Fig). The cotton cotyledon was then impregnated with agrobacteria (GV3101) containing pTRV1 and TRV-GhJUB1L1, and pTRV:00 (pTRV1+pTRV2) was injected as a negative control. The GhCLA1 gene affects the synthesis of chlorophyll in cotton leaves, silencing this gene leads to the production of albino seedlings. Therefore, TRV:GhCLA1 (pTRV1+TRV:GhCLA1) was used as a positive control. The results showed that after 1 week of injection, the leaves of TRV-GhCLA1 plants showed albino phenotypes, indicating that the VIGS system was operational (Fig 5a). We further detected the expression level of GhJUB1L1 gene in GhJUB1L1 silenced plants by qRT-PCR. Compared with the negative control, the transcript abundance of GhJUB1L1 in GhJUB1L1 silenced plants was significantly reduced, indicating that we obtained GhJUB1L1 down-regulated cottons by this VIGS system (Fig 5b).

To further clarify the role of GhJUB1L1 in drought tolerance of cotton, we silenced GhJUB1L1 gene in cotton seedlings by VIGS. GhJUB1L1 silenced plants and controls were kept away from water for 10 days (S6 Fig). As shown in Fig 7c, GhJUB1L1 silenced plants appeared to be yellowing and more withered than controls which were still green. After re-watered, almost all control plants survived, as few silenced plants survived (Fig 5d and S7 Fig). Furthermore, RWC in leaves was determined after 10 days of withholding water. A significantly higher RWC was observed in control plants than in GhJUB1L1 silenced plants (Fig 5e). Membrane stability under water deficit conditions was assessed by measuring ion leakage in both, control and treated plants. When water was withheld for 10 days, GhJUB1L1 silenced plants showed a higher ion leakage than control plants (Fig 5f). Collectively, our results reveal that Silencing GhJUB1L1 gene significantly reduced the drought tolerance of cotton plants.

Silencing GhJUB1L1 gene negatively regulates SCW development

The vascular bundle exists in stem, leaf (the vascular bundle in leaf is also called vein) and other organs, play crucial roles in the transport of water and minerals in plant tissues. To observe the xylem development of the GhJUB1L1 silenced plants, we detected the change of lignin and cellulose by phloroglucinol-HCl and toluidine blue stain, respectively. Compared with the control group (Fig 6), the tracheary element was significantly smaller, and the pink of GhJUB1L1 silenced plant xylem cells was weaker than that of control. Meanwhile, the intensity of toluidine blue stain staining of GhJUB1L1 gene silencing plants tracheary element was lighter. The results were similar when we stained the re-watered cotton plants (S8 Fig). Based on above analysis, silencing GhJUB1L1 gene can lead to decreased SCW deposition in plants tracheary element.

Silencing GhJUB1L1 gene suppressed the expression of drought-related and SCW synthesis-related genes

To further confirm the response of GhJUB1L1 silenced plants to drought stress, we analyzed expression of several drought-related genes (such as CBP1, P5CS, ABI1, SOS2) in GhJUB1L1 silenced plants and controls. As shown in Fig 7a–7d, the expression level of ABI1 was increased, whereas the expression of CBP1, P5CS, and SOS2 were reduced in the GhJUB1L1 silenced plants. These results indicated that silencing GhJUB1L1 gene can inhibit the expression of drought-related genes, suggesting that GhJUB1L1 may be a positive regulator of cotton drought tolerance.

Silencing GhJUB1L1 gene retarded tracheary element SCW development, we further detected the expression levels of genes involved in SCW formation. The results (Fig 7e–7k) showed that the expression levels of lignin biosynthesis genes 4CL1, CCoAOMT1, and hemicellulose biosynthesis genes IRX9, IRX14 were significantly down-regulated in the GhJUB1L1
silenced plants. Meanwhile, the expression levels of cellulose biosynthesis genes CesA4, CesA7 and CesA8 genes were also significantly down-regulated. These results indicated that silencing GhJUB1L1 can inhibit the expression of genes involved in cellulose and lignin synthesis, suggesting that GhJUB1L1 may be a positive regulator in SCW biosynthesis of cotton.

To verify whether GhJUB1L1 directly activates these genes expression in vivo, transient dual-luciferase assay was performed in tobacco epidermis cells. It was confirmed (Fig 8) that GhJUB1L1 can directly bind to the GhABI11, GhSOS2, GhCCoAOMT1, GhCesA7, and GhIRX14 promoter, and then induce the expression of these five genes.

**Discussion**

Plants should develop a complex regulatory circuit to respond to biotic and abiotic stresses, because the living environment is not always suitable. The regulatory circuit consists of transcriptional activators and repressors that regulate the expression of defense genes. In recent years, various NAC TFs in different plant species have been reported to be suitable targets for
improving plant responses to dehydration/drought stress. For example, anac016 mutants exhibit high drought tolerance, while those with ANAC016 overexpression are sensitive to drought and exhibit accelerated aging [35]. In Arabidopsis, transgenic plants overexpressing ANAC019, ANAC055, or ANAC072/RD26 show enhanced expression of stress response genes and improved tolerance to drought and salt stress [36]. The transgenic plants overexpressing RD26 cDNA were hypersensitive to ABA, and inversely, the transgenic plants with RD26 repressed were insensitive to ABA. The expressions of many ABA- and stress-induced genes including RD20 and GLY genes were upregulated in plants overexpressing RD26 and repressed in plants with RD26 repressed. In Arabidopsis protoplasts, RD26 activated a promoter of the GLY gene that was upregulated in plants overexpressing RD26 [37]. Microarray analysis of transgenic plants overexpressing either ANAC019, ANAC055, or ANAC072 revealed that several stress-inducible genes were upregulated in the transgenic plants, and the plants showed significantly increased drought tolerance [36]. Microarray analysis of transgenic plants overexpressing ZFHD1 revealed that several stress inducible genes were upregulated in the transgenic plants. Transgenic plants exhibited a smaller morphological phenotype and had a significant improvement of drought stress tolerance. Moreover, co-overexpression of the ZFHD1 and NAC genes restored the morphological phenotype of the transgenic plants to a near wild-type state and enhanced expression of ERD1 in transgenic Arabidopsis plants [38]. We isolated and identified GhJUB1L1, a homologue gene of AtJUB1. GhJUB1L1 preferentially expressed in stem and leaf and could be induced by drought stress. These data give us a possible clue that GhJUB1L1 may be involved in drought response. In this study, we found that cotton plants with silenced GhJUB1L1 were more sensitive to drought stress than the control plants. Given that TFs regulate the expression of stress-related target genes and participate in various plant stresses [39–41]. We analyzed the expression of several drought-related genes in GhJUB1L1 silenced cottons. It has been reported that the expression of CBF genes in many plants increases significantly when subjected to abiotic stresses such as drought, high salinity, and exogenous hormones [42, 43]. Yamchi et al. [44] transferred Arabidopsis P5CS gene into tobacco. Under drought and salt stress, transgenic plants accumulated more proline and...
showed more stress tolerance than wide type control. Previous studies have shown that SOS2 has the effect of improving drought resistance [45]. In this study, compared with the control, the expression levels of CBF1, P5CS and SOS2 in GhJUB1L1 silenced plants were significantly reduced. Type 2C protein phosphatase ABI1 (ABA insensitive 1) is a key negative regulator of ABA signal transduction and plays a negative role in stomatal closure induced by ABA [46–48]. The results of our analysis showed that the transcription level of ABI1 in GhJUB1L1 silenced plants was relatively higher than that in the control plants, suggesting that GhJUB1L1 may be a positive regulator of ABA signaling. These data suggest that GhJUB1L1 may be involved in drought response by regulating drought stress-related genes.

Fig 7. Genes expression level in GhJUB1L1 silenced plants. The expression level of drought-related (a-d) and SCW-synthesis related genes (e-k). Error bars represent SD of three independent replicates. *, ** and *** indicates significant differences at p < 0.05, p < 0.01, and p < 0.001, respectively. https://doi.org/10.1371/journal.pone.0259382.g007
SCW provide support for plants and forms a mechanical barrier against pathogen infection and abiotic stress. High expression of SCW biosynthesis related genes is necessary for plant tolerance to abiotic stress [49, 50]. Interestingly, the SCW development of \textit{GhJUB1L1} silenced cottons was significantly reduced compared with the control, which resulted in decreased drought resistance of the plants. Therefore, we analyzed the expression of \textit{CESA4}, \textit{CESA7}, \textit{CESA8}, \textit{4CL}, \textit{CCoAOMT1}, \textit{IRX9}, and \textit{IRX14}, and found that all these genes were significantly down-regulated in the \textit{GhJUB1L1} silenced plants. In summary, our data suggest that \textit{GhJUB1L1} may be involved in drought stress response and SCW development by regulating drought stress-related and SCW biosynthesis genes, which positively regulating plant response to drought stress.

\textbf{Supporting information}

\textit{S1} Fig. Cloning of \textit{GhJUB1L1}. M, Marker DL2000. (TIF)

\textit{S2} Fig. Multiple alignment comparison of \textit{GhJUB1L1} with other JUB1 proteins. Horizontal lines, mazarine shading, and pink shading represent conserved NAC binding domains, conserved amino acid residues, and similar amino acid residues, respectively. (TIF)

\textit{S3} Fig. Evolutionary relationships analysis of \textit{GhJUB1L1}. ▲ represents \textit{GhJUB1L1}. (TIF)

\textit{S4} Fig. 35S::\textit{GhJUB1L1}::eGFP vector construction. CaMV35S, promoter. (TIF)

\textit{S5} Fig. TRV::\textit{GhJUB1L1} vector construction. CaMV35S, promoter. (TIF)

\textit{S6} Fig. Field capacity (%). Error bars represent SD (standard deviation) of three independent replicates. (TIF)

\textit{S7} Fig. Survival rates of the plants after re-watering. Error bars represent SD of three independent replicates. *** represent p<0.001. (TIF)
S8 Fig. Silencing GhJUB1L1 gene inhibits SCW lignin in cotton. Phloroglucinol/HCl (a-b) and toluidine bluestaining (c-d) analysis for re-watered plants. Scale bar = 200 μm.

(TIF)

S1 Table. Gene-specific primers used in isolation of GhJUB1L1 genes and vector construction.

(PDF)

S2 Table. Gene-specific primers used in RT-PCR analysis.

(PDF)

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Conceptualization: Qian Chen, Qigao Guo, Ming Luo.
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References
1. Basu S, Ramegowda V, Kumar A, Pereira A. Plant adaptation to drought stress. F1000Res. 2016; 5. Epub 2016/07/22. https://doi.org/10.12688/f1000research.7678.1 PMID: 27441087
2. Lata C, Prasad M. Role of DREBs in regulation of abiotic stress responses in plants. J Exp Bot. 2011; 62(14):4731–48. Epub 2011/07/09. https://doi.org/10.1093/jxb/err210 PMID: 21737415.
3. Li C, Yue J, Wu X, Xu C, Yu J. An ABA-responsive DRE-binding protein gene from Setaria italica, SiARDP, the target gene of SiAREB, plays a critical role under drought stress. J Exp Bot. 2014; 65 (18):5415–27. Epub 2014/07/30. https://doi.org/10.1093/jxb/eru302 PMID: 25072121
4. Tamura T, Hara K, Yamaguchi Y, Koizumi N, Sano H. Osmotic stress tolerance of transgenic tobacco expressing a gene encoding a membrane-located receptor-like protein from tobacco plants. Plant Physiol. 2003; 131(2):454–62. Epub 2003/02/15. https://doi.org/10.1104/pp.102.011007 PMID: 12586870
5. Ingram J, Bartels D. The molecular basis of dehydration tolerance in plants. Annu Rev Plant Phys. 1996; 47:377–403. https://doi.org/10.1146/annurev.arplant.47.1.377 PMID: 15012294
6. Zhu JK. Salt and drought stress signal transduction in plants. Annual Review of Plant Biology. 2002; 53:247–73. https://doi.org/10.1146/annurev.arplant.53.091401.143329 PMID: 12221979
7. Liu QL, Xu KD, Zhao LJ, Pan YZ, Jiang BB, Zhang HQ, et al. Overexpression of a novel chrysanthemum NAC transcription factor gene enhances salt tolerance in tobacco. Biotechnol Lett. 2011; 33 (10):2073–82. https://doi.org/10.1007/s10529-011-0659-8 PMID: 21660574
8. Lu PL, Chen NZ, An R, Su Z, Qi BS, Ren F, et al. A novel drought-inducible gene, ATAF1, encodes a NAC family protein that negatively regulates the expression of stress-responsive genes in Arabidopsis. Plant Mol Biol. 2007; 63(2):289–305. Epub 2006/10/13. https://doi.org/10.1007/s11103-006-9089-8 PMID: 17031511.
9. Chen HY, Hsieh EJ, Cheng MC, Chen CY, Hwang SY, Lin TP. ORA47 (octadecanoid-responsive AP2/ERF-domain transcription factor 47) regulates jasmonic acid and abscisic acid biosynthesis and
signaling through binding to a novel cis-element. New Phytol. 2016; 211(2):599–613. Epub 2016/03/15. https://doi.org/10.1111/nph.13914 PMID: 26974851.

10. Joshi R, Wani SH, Singh B, Bohra A, Dar ZA, Lone AA, et al. Transcription Factors and Plants Response to Drought Stress: Current Understanding and Future Directions. Front Plant Sci. 2016; 7:1029. Epub 2016/07/30. https://doi.org/10.3389/fpls.2016.01029 PMID: 27471513

11. Rabara RC, Tripathi P, Rushton PJ. The potential of transcription factor-based genetic engineering in improving crop tolerance to drought. OMICS. 2014; 18(10):601–14. Epub 2014/08/15. https://doi.org/10.1089/omi.2013.0177 PMID: 25118806

12. Todaka D, Shinozaki K, Yamaguchi-Shinozaki K. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. Front Plant Sci. 2015; 6:84. Epub 2015/03/06. https://doi.org/10.3389/fpls.2015.00084 PMID: 25741357

13. Vermeirssen V, De Clercq I, Van Parys T, Van Breusegem F, Van De Peer Y. Arabidopsis JUNGBRUNNEN1, a reactive oxygen signaling mediator, enhances drought tolerance in tomato. Plant Physiol. 2014; 164(6):354–66. Epub 2017/06/24. https://doi.org/10.1104/pp.114.241745 PMID: 28109443

14. Souer E, van Houwelingen A, Kloos D, Mol J, Koes R. The no apical meristem gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell. 1996; 85(2):159–70. Epub 1996/04/19. https://doi.org/10.1016/0092-8674(96)81093-4 PMID: 8612269.

15. Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. Plant Cell. 1997; 9(6):841–57. Epub 1997/06/01. https://doi.org/10.1105/tpc.9.6.841 PMID: 9212461

16. Le DT, Nishiyama R, Watanabe Y, Mochida K, Yamaguchi-Shinozaki K, Shinozaki K, et al. Genome-wide survey and expression analysis of the plant-specific NAC transcription factor family in soybean during development and dehydration stress. DNA Res. 2011; 18(4):263–76. Epub 2011/06/21. https://doi.org/10.1093/dnares/dsr015 PMID: 21685489

17. Borrill P, Harrington SA, Uauy C. Genome-Wide Sequence and Expression Analysis of the NAC Transcription Factor Family in Polyploid Wheat. G3 (Bethesda). 2017; 7(9):3019–29. Epub 2017/07/13. https://doi.org/10.1534/g3.117.043679 PMID: 28698232

18. Ling L, Song L, Wang Y, Guo C. Genome-wide analysis and expression patterns of the NAC transcription factor family in Medicago truncatula. Physiol Mol Biol Plants. 2017; 23(2):343–56. Epub 2017/05/04. https://doi.org/10.1007/s00709-017-0421-3 PMID: 28461723

19. Sun H, Hu M, Li J, Chen L, Li M, Zhang S, et al. Comprehensive analysis of NAC transcription factors uncovers their roles during fiber development and stress response in cotton. BMC Plant Biol. 2018; 18(1):150. Epub 2018/07/26. https://doi.org/10.1186/s12870-018-1367-5 PMID: 30041622

20. Shahnejat-Bushehri S, Mueller-Roeber B, Balazadeh S. Arabidopsis JUNGBRUNNEN1 affects thermomemory-associated genes and enhances heat stress tolerance in primed and unprimed conditions. Plant Signal Behav. 2012; 7(12):1518–21. Epub 2012/10/18. https://doi.org/10.4161/psb.22092 PMID: 23073024

21. Wu A, Allu AD, Garapati P, Siddiqui H, Dortay H, Zanor MI, et al. JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. Plant Cell. 2012; 24(2):482–506. Epub 2012/02/22. https://doi.org/10.1105/tpc.111.090894 PMID: 22354491

22. Thirumalaikumar VP, Devkar V, Mehterov N, Ali S, Ozgur R, Turkan I, et al. NAC transcription factor JUNGBRUNNEN1 enhances drought tolerance in tomato. Plant Biotechnol J. 2018; 16(2):354–66. Epub 2017/06/24. https://doi.org/10.1111/pbi.12776 PMID: 28640975

23. Tak H, Negi S, Ganapathi TR. Banana NAC transcription factor MusaNAC042 is positively associated with drought and salinity tolerance. Protoplasma. 2017; 254(2):403–16. Epub 2016/06/29. https://doi.org/10.1007/s00709-016-0991-x PMID: 27352311.

24. Daviere JM, Achard P. A Pivotal Role of DELLAS in Regulating Multiple Hormone Signals. Mol Plant. 2016; 9(1):10–20. Epub 2015/09/30. https://doi.org/10.1093/mp/mpi092 PMID: 26415696.

25. Shahnejat-Bushehri S, Tarkowska D, Sakuraba Y, Balazadeh S. Arabidopsis JUNGBRUNNEN1 enhances drought tolerance in tomato. Nat Plants. 2016; 2:16013. Epub 2016/06/02. https://doi.org/10.1038/nplants.2016.13 PMID: 27248348.

26. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016; 33(7):1870–4. Epub 2016/03/24. https://doi.org/10.1093/molbev/msw054 PMID: 27044904.

27. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987; 4(4):406–25. Epub 1987/07/01. https://doi.org/10.1093/oxfordjournals.molbev.a040454 PMID: 3447015.
28. Felsenstein J. Confidence-Limits on Phylogenies—an Approach Using the Bootstrap. Evolution. 1985; 39(4):783–91. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x PMID: 28561359

29. Nei M, Rogozin IB, Piontkivska H. Purifying selection and birth-and-death evolution in the ubiquitin gene family. Proc Natl Acad Sci U S A. 2000; 97(20):10866–71. Epub 2000/09/27. https://doi.org/10.1073/pnas.97.20.10866 PMID: 11005860

30. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods. 2001; 25(4):402–8. https://doi.org/10.1006/meth.2001.1262 PMID: 11846609

31. Huang J, Chen F, Guo Y, Gan X, Yang M, Zeng W, et al. GhMYB7 promotes secondary wall cellulose deposition in cotton fibres by regulating GhCesA gene expression through three distinct cis-elements. New Phytol. 2021. Epub 2021/07/11. https://doi.org/10.1111/nph.17612 PMID: 34245570.

32. Huang J, Chen F, Wu S, Li J, Xu W. Cotton GhMYB7 is predominantly expressed in developing fibers and regulates secondary cell wall biosynthesis in transgenic Arabidopsis. Sci China Life Sci. 2016; 59(2):194–205. Epub 2016/01/25. https://doi.org/10.1007/s11187-015-4991-4 PMID: 26803299.

33. Hellens RP, Allan AC, Friel EN, Bolitho K, Grafton K, Templeton MD, et al. Transient expression vectors for functional genomics, quantification of promoter activity and RNA silencing in plants. Methods. 2005; 1:13. Epub 2005/12/20. https://doi.org/10.1186/1746-4811-1-13 PMID: 16359558.

34. Sparks IA, Runions J, Kearns A, Hawes C. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. Nat Protoc. 2006; 1(4):2019–25. Epub 2007/05/10. https://doi.org/10.1038/nprot.2006.286 PMID: 17487191.

35. Sakuraba Y, Kim YS, Han SH, Lee BD, Paek NC. The Arabidopsis Transcription Factor NAC016 Promotes Turgor Stress Responses by Repressing ABE1 Transcription through a Trifurcated Feed-Forward Regulatory Loop Involving NAP. Plant Cell. 2015; 27(6):1771–87. https://doi.org/10.1105/tpc.15.00222 PMID: 26059204.

36. Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, et al. Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell. 2004; 16(9):2481–98. Epub 2004/08/21. https://doi.org/10.1105/tpc.022699 PMID: 15319476.

37. Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, et al. A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J. 2004; 39(6):863–76. Epub 2004/09/03. https://doi.org/10.1111/j.1365-313X.2004.02171.x PMID: 15341629.

38. Tran LSP, Nakashima K, Sakuma Y, Osakabe Y, Qin F, Simpson SD, et al. Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. Plant Journal. 2007; 49(1):46–63. https://doi.org/10.1111/j.1365-313X.2006.02932.x PMID: 17233795.

39. Eulgem T, Somssich IE. Networks of WRKY transcription factors in defense signaling. Current Opinion in Plant Biology. 2007; 10(4):366–71. https://doi.org/10.1016/j.pbi.2007.04.020 PMID: 17644023.

40. Butt HI, Yang ZE, Gong Q, Chen EY, Wang XQ, Zhao G, et al. GaMYB85, an R2R3 MYB gene, in transgenic Arabidopsis plays an important role in drought tolerance. BMC Plant Biology. 2017; 17. ARTN 142 https://doi.org/10.1186/s12870-017-1078-3 PMID: 28830364.

41. Luo X, Bai X, Sun XL, Zhu D, Liu BH, Ji W, et al. Expression of wild soybean WRKY20 in Arabidopsis enhances drought tolerance and regulates ABA signalling. Journal of Experimental Botany. 2013; 64(8):2155–69. https://doi.org/10.1038/jxb.erf073 PMID: 23606412.

42. Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, et al. A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J Biol Chem. 2006; 281(49):37636–45. Epub 2006/10/04. https://doi.org/10.1074/jbc.M605895200 PMID: 17015446.

43. Akhtar M, Jaiswal A, Taj G, Jaiswal JP, Qureshi MI, Singh NK. DREB1/CBF transcription factors: their structure, function and role in abiotic stress tolerance in plants. Journal of Genetics. 2012; 91(3):385–95. https://doi.org/10.1007/s12041-012-0201-3 PMID: 23271026.

44. Yamchi A, Jazi FR, Moussavi A, Karkhan A. Proline accumulation in transgenic tobacco as a result of expression of Arabidopsis Delta(1)-pyrroline-5-carboxylate synthetase (P5CS) during osmotic stress. J Plant Biochem Biot. 2007; 16(1):9–15. https://doi.org/10.1007/Bf03321922.

45. Xiao BZ, Chen X, Xiang CB, Tang N, Zhang QF, Xiong LZ. Evaluation of Seven Function-Known Candidate Genes for their Effects on Improving Drought Resistance of Transgenic Rice under Field Conditions. Molecular Plant. 2015; 2(1):73–83. https://doi.org/10.1093/mp/ssn068 PMID: 19529831.

46. Babula-Skowronska D, Ludwikow A, Ciesla A, Olejnik A, Cegielska-Taras T, Bartkowiak-Broda I, et al. Involvement of genes encoding ABI1 protein phosphatases in the response of Brassica napus L. to drought stress. Plant Molecular Biology. 2015; 88(4–5):445–57. https://doi.org/10.1007/s11103-015-0334-x PMID: 26059040.
47. Meyer K, Leube MP, Grill E. A Protein Phosphatase 2c Involved in Aba Signal-Transduction in Arabidopsis-Thaliana. Science. 1994; 264(5164):1452–5. https://doi.org/10.1126/science.8197457 PMID: 8197457

48. Sheen J. Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. P Natl Acad Sci USA. 1998; 95(3):975–80. https://doi.org/10.1073/pnas.95.3.975 PMID: 9448270

49. Hu P, Zhang KM, Yang CP. BpNAC012 Positively Regulates Abiotic Stress Responses and Secondary Wall Biosynthesis. Plant Physiology. 2019; 179(2):700–17. https://doi.org/10.1104/pp.18.01167 PMID: 30530740

50. Park SC, Kim YH, Jeong JC, Kim CY, Lee HS, Bang JW, et al. Sweetpotato late embryogenesis abundant 14 (IbLEA14) gene influences lignification and increases osmotic- and salt stress-tolerance of transgenic calli. Planta. 2011; 233(3):621–34. Epub 2010/12/08. https://doi.org/10.1007/s00425-010-1326-3 PMID: 21136074.