Cloning of *Gossypium hirsutum* Sucrose Non-Fermenting 1-Related Protein Kinase 2 Gene (*GhSnRK2*) and Its Overexpression in Transgenic *Arabidopsis* Escalates Drought and Low Temperature Tolerance

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**Abstract**

The molecular mechanisms of stress tolerance and the use of modern genetics approaches for the improvement of drought stress tolerance have been major focuses of plant molecular biologists. In the present study, we cloned the *Gossypium hirsutum* sucrose non-fermenting 1-related protein kinase 2 (*GhSnRK2*) gene and investigated its functions in transgenic *Arabidopsis*. We further elucidated the function of this gene in transgenic cotton using virus-induced gene silencing (VIGS) techniques. We hypothesized that *GhSnRK2* participates in the stress signaling pathway and elucidated its role in enhancing stress tolerance in plants via various stress-related pathways and stress-responsive genes. We determined that the subcellular localization of the *GhSnRK2*-green fluorescent protein (GFP) was localized in the nuclei and cytoplasm. In contrast to wild-type plants, transgenic plants overexpressing *GhSnRK2* exhibited increased tolerance to drought, cold, abscisic acid and salt stresses, suggesting that *GhSnRK2* acts as a positive regulator in response to cold and drought stresses. Plants overexpressing *GhSnRK2* displayed evidence of reduced water loss, turgor regulation, elevated relative water content, biomass, and proline accumulation. qRT-PCR analysis of *GhSnRK2* expression suggested that this gene may function in diverse tissues. Under normal and stress conditions, the expression levels of stress-inducible genes, such as *AtRD29A*, *AtP5CS1*, *AtABI3*, *AtCBF1*, and *AtAB15*, were increased in the *GhSnRK2*-overexpressing plants compared to the wild-type plants. *GhSnRK2* gene silencing alleviated drought tolerance in cotton plants, indicating that VIGS technique can certainly be used as an effective means to examine gene function by knocking down the expression of distinctly expressed genes. The results of this study suggested that the *GhSnRK2* gene, when incorporated into Arabidopsis, functions in positive responses to drought stress and in low temperature tolerance.

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**Introduction**

Plants have developed complex signaling pathways in response to various environmental stresses, such as salt, drought, and cold, and have acquired metabolic functions and developmental methods to survive changing environmental conditions [1]. Improving crop resistance to drought stress would be the most valuable means to improve agricultural productivity and to reduce crop loss caused by environmental stress. As a result, understanding the mechanisms of drought tolerance and developing drought-resistant crop plants have been major targets of plant molecular biologists and biotechnologists. Low-temperature constraints have been progressively overcome by the recognition of cold-tolerant genes for applications in transgenic plants. Transgenic approach has reveal many possibilities to improve cold stress in plants by incorporating or deleting genes that regulate a particular characteristic [2]. These approaches also provide unique opportunities to improve the genetic quality of plants via the development of particular crop varieties that exhibit enhanced resistance to biotic and cold stresses and improved nutritional quality. The plant response to salt stress typically results in osmotic alterations, which play a major role in ensuring osmotic balance in plant cells. During plant stress responses, the regulation of gene expression involves both universal and unique changes in the transcript levels of certain plant genes [3]. Plants directly or indirectly respond to stresses by initiating signal transduction pathways. Various abiotic stresses result in both general and specific effects on plant growth and development. For example, drought limits plant growth due to difficulties in maintaining turgor pressure, photosynthetic decline, osmotic stress-imposed constraints on plant processes and interference with nutrient availability as the soil dries [4].
Materials and Methods

Protein kinases and phosphatases are major elements of stress signals which are transmitted to different cellular regions via specialized signaling pathways. Some of the protein kinases involved in stress signal transduction in plants, such as mitogen-activated protein kinases (MAPks) [5,6,7,8], glycogen synthase kinase 3 (GSK3) [9,10], and S6 kinase (S6k) [11], are similar among all eukaryotic organisms, whereas others, including calcium-dependent protein kinases (CDPKs) [12,13,14] and SNF1-related kinases (SnRKS), are plant-specific.

SnRK2 is an important stress-related protein kinase in plants that has been implicated in stress and abscisic acid-mediated signaling pathways [15]. Previously, it was reported that SnRK2C/SnRK2.8, a subclass II member, was strongly activated by drought stress and that plants overexpressing SnRK2C exhibited improved drought tolerance as a result of the up-regulation of many stress-inducible genes [16]. SnRK2s have a molecular weight of approximately 40 kDa and are monomeric serine/threonine protein kinases [17,18]. Based on phylogenetic analysis, three groups of SnRK2 family members have been identified. Group I consists of kinases not activated by ABA, group 2 consists of kinases not activated or activated very weakly by ABA, and group 3 consists of kinases strongly activated by ABA. The amino acid sequences of all SnRK2s can be separated into two regions, the highly conserved N-terminal kinase domain and the regulatory C-terminal domain, which contains stretches of acidic amino acids. Furthermore, the C-terminal domain consists of two subdomains, Domain I and Domain II. Domain I is characteristic of all SnRK2 family members and is required for activation by osmotic stress. Based on our investigation, we found that the expression of GhSnRK2 was induced by PEG. PEG was quite commonly used in physiological experiments to induce controlled drought stress. In the present study, we generated a gene construct containing GhSnRK2 driven by the constitutive cauliflower mosaic virus (CaMV) 35S promoter and transformed this construct into Arabidopsis in order to investigate the functional analysis of GhSnRK2 gene. We monitored the activities of this gene with respect to drought and low temperature tolerance in transgenic plants. We further elucidated the function of this gene in transgenic cotton using virus-induced gene silencing (VIGS) techniques. We showed that GhSnRK2, the cotton SnRK2 gene, is involved in multi-stress responses. Our results promote the elucidation of functional analysis in G. hirsutum to facilitate the exploitation of desirable genes from this species. The elucidation of GhSnRK2 gene function contributes to our understanding of the mechanism by which this plant adapts to abiotic stress and provides a valuable gene resource for plant breeders.

Cloning of the GhSnRK2 gene

Total RNA was extracted from CCRI24 cotton tissues using Trizol (Sigma-Aldrich) according to the manufacturer's instructions. Reverse transcription (RT) was performed using total RNA extracted from seedlings oligo(dT)16 primer, and SuperScript II reverse transcriptase (Promega). The RT product was used in PCRs to amplify the predicted GhSnRK2 open reading frame using primer star polymerase (enzymes). The cDNA regions of GhSnRK2 were cloned into the T-simple vector. All of the clones were confirmed via sequencing. Primers specific to the sequence of GhSnRK2 were designed, synthesized, and used to clone the SnRK2 gene. For plant transformation, GhSnRK2 cDNAs were introduced into the modified pCAMBIA2301 plant transformation vector under the control of the CaMV 35S promoter.

Localization of the GhSnRK2-GFP fusion protein

The method of [19] was adopted to perform the subcellular localization assay. The GhSnRK2 coding sequence was cloned and ligated into the XbaI and SpeI sites of the PCAMBIA2301-GFP vector to generate PCAMBIA2301-GhSnRK2-GFP, which expressed the GhSnRK2-GFP fusion protein under the control of the CaMV35S promoter. The construct was used for transient transformation of onion. Onion epidermal peels were bombarded with DNA-coated gold particles, and GFP expression was visualized 24 h later. Transformed onion cells were observed under a confocal microscope.

Effect of polyethylene glycol (PEG) treatment on GhSnRK2 gene expression

The effect of PEG on the expression level of the GhSnRK2 gene was evaluated via qRT-PCR. The root of a three-week-old upland cotton plant was submerged in 10% PEG solution, and samples were collected from the root at one hour intervals for 6 hours. RNA was extracted from the samples, and RT was performed as described above to generate cDNA for qRT-PCR analysis.

Arabidopsis transformation and screening of transgenic plants

pCAMBIA2301 carrying GhSnRK2 was introduced into Agrobacterium tumefaciens strain GV3101. The transgenic Arabidopsis plants were generated using the flower dipping method [20], and transgenic plants were selected based on their growth in 0.8% agar containing half-strength MS salts and kanamycin. The transformants were transferred to soil and allowed to set seed. The T3 generation of the transgenic plants was used for all experiments.

Plant materials and growth conditions

Arabidopsis thaliana seeds and ecotype Col-0 wild-type, mutant, and transgenic seeds were surface-sterilized using bleach (5% Sodium hypochlorite) and 0.1% Triton X-100. After cold exposure at 4°C for 2 d, the seeds were germinated and cultured in plates containing 0.5× MS medium, 0.8% agar, and 1% sucrose under continuous light at 22°C. For plants grown in soil, 7-day-old seedlings were transferred from the MS plates to soil and cultured under 80–100 μmol m−2 s−1 photoperiodic cycles of 16 h light and 8 h dark at 22°C in a growth chamber under fluorescent light using one cool-light and one warm-light tube, each of which was suspended several inches above the plants. Mature seeds obtained from transformed plants (T0 generation) were cultured in MS medium containing the antibiotic kanamycin in order to screen for transformed plants. The seeds were allowed to grow in the medium for two weeks before transfer to soil for growth continuation. Cotton seeds were planted in moist soil and grown in a growth chamber with a 14 h photoperiod at a 20/30°C night/day temperature cycle, with a light intensity of 400 μmol m−2 s−2 and at 60% relative humidity.

Drought tolerance assay

Drought stress tolerance was measured by transferring 2-week-old plants cultured in Petri dishes to pots (10-cm diameter) filled with a 1:1 (v/v) vermiculite-perlite mixture. The seedlings were cultured for 2 weeks with constant watering before the water was withheld. After 9 days without water, all of the pots were re-watered simultaneously, and plant re-growth was scored 4 days later. Thirty-five plants for each individual transgenic line were used in each repeated experiment, and one representative image is
shown. Plants were scored as survivors if there were healthy green young leaves after re-watering treatment. The survival rate was calculated as the ratio of number of surviving plants to the total number of treated plants in the flower pot. Representative transgenic and WT plants were photographed before and after drought treatment. Each stress assay was repeated at least three times. The water use efficiency (WUE) of the plants was calculated as the amount of water needed to maintain the weight of each experimental pot containing the plants. To determine the soil water content (SWC), the pots were immediately weighed after saturation with water prior to the initiation of drought stress and then periodically weighed during the drought stress period. The SWC was calculated as (final fresh weight-dry weight)/initial weight×100.

Freezing and cold stress treatment
Transgenic plants overexpressing the *GhSnRK2* gene and wild-type *Arabidopsis* plants were cultured in pots filled with a 1:1 mixture of perlite and vermiculite under a long-day photoperiod at 22°C. Freezing stress was performed by transferring 4-week-old plants to a chamber in which the temperature was adjusted to −4°C and −8°C for 10 h, respectively, and then returned to the typical standard growth conditions. For cold stress, 4-week-old plants were transferred to 4°C for three weeks. The plants were analyzed after recovery for 7 days under normal growth conditions. The survival rate (%) under freezing and cold conditions was calculated as the percentage of clearly green plants after returning to normal conditions, and plants exhibiting clear sign of wilting were denoted as dead. Thirty-five plants for each condition was calculated as the percentage of clearly green plants after returning to normal conditions, and plants exhibiting clear sign of wilting were denoted as dead. Thirty-five plants for each

Measurement of stomatal closure in response to ABA treatment
Stomatal closure assays were conducted as described by [24]. Leaves from *GhSnRK2* transgenic and WT plants were immersed in a solution containing 50 μM CaCl₂, 10 mM KCl, and 10 mM 2-(N-morpholino)ethanesulfonic acid (MES)-Tris, pH 6.15, and were exposed to light for 2 h. Subsequently, various concentrations of ABA were added to the solution. The stomatal apertures were measured after 2 h of ABA treatment.

Determination of proline content
The free proline content was measured using the method described by [25]. Leaf segments were homogenized in 3% sulfosalicylic acid, and the homogenates were centrifuged at 3000 g for 20 min. Mixtures containing 2 ml of sample supernatant, 2 ml of acetic acid, and 2 ml of 2.5% acid ninhydrin solution were boiled for 30 min, and the absorbance at 520 nm (A520) was measured.

Chlorophyll content assay
The determination of chlorophyll content was performed according to the method of [26]. Extracts were obtained from 0.1 g (fresh weight) leaf samples from 4-week-old plants and were homogenized in 1 ml of 80% acetone to quantify the chlorophyll content via spectrophotometric analysis.

Seedling growth in response to ABA and NaCl treatment
The sensitivity of seedling germination to ABA and NaCl was assessed on MS agar plates containing various concentrations of ABA and NaCl solution [27]. Seedlings from WT and transgenic plants were placed on MS agar plates supplemented with distilled water or different concentrations of ABA or NaCl and were placed in the growth chamber at 22°C.

Germination assay
To evaluate seed germination, the method of [27] was employed. Seeds from wild-type and transgenic plants were surface-sterilized using sodium hypochlorite and were placed on Murashige and Skoog (MS) solid medium containing various concentrations of ABA (0 μM, 0.3 μM, or 0.5 μM). For the evaluation of seed germination under salt stress, the MS medium was supplemented with various concentrations of NaCl (0 mM, 50 mM, or 100 mM). The percentage of germinating seeds was recorded after 7 days.

Biomass accumulation
Plants were harvested for biomass measurements after 4 weeks of germination. The fresh weight of each individual plant shoot and root was measured immediately after harvesting. For fresh weight biomass, the fresh weight of the root and the shoot was measured immediately after harvesting, and the dry weight was recorded in [g] after drying in an oven to a constant weight at 70° for 48 h.

qRT-PCR
Total RNA was extracted from *Arabidopsis* seedlings using Trizol reagent (Takara) and was treated with RNase-free DNase (Promega). cDNA was synthesized using a Promega kit according to the manufacturer’s protocol. qPCR was performed using a SYBR green PCR master mix kit. After PCR, the data was quantified using the comparative CT method (2^(-ΔΔCT) method) based on the CT values [28]. *AtACT2* (gene accession At5G18780) and cotton histone3 (gene accession AF024716) were
used as internal controls, and the relative expression level of each target gene was quantified. Each RT-PCR measurement was performed in triplicate. All RT-PCR experiments were reproduced at least three times using independent cDNA samples.

Silencing construct development and cotton transformation

The method described by [29] was employed to construct the VIGS vector. Specific fragments (approximately 300 bp) were amplified via PCR and cloned into the T-simple vector according to the manufacturer’s specifications (Promega). The resulting clones were sequenced. The plasmids were digested using the restriction enzymes XbaI and BamHI and were subsequently ligated into the VIGS vector (PYL156:(pTRV-RNA2)-GhSnRK2). The resulting constructs were named according to the putative function of the stress-related gene. For transformation, Agrobacterium (GV3101) carrying GhSnRK2 derivatives were cultured at 28°C in LB medium containing appropriate antibiotics. Cells were harvested from cultures grown overnight, re-suspended in inoculation buffer (200 μM acetosyringone, 10 mM MES, pH 5.5), and incubated for 2 h at room temperature in a shaker. Agrobacterium strains containing the GhSnRK2 derivative PYL156:(pTRV-RNA2)-GhSnRk2, PYL192:(pTRV-RNA1), PYL156:(pTRV-RNA2)-GrCLA1/positive control, orPYL156:(pTRV-RNA2) vector (OD 1.5) were mixed at a 1:1 ratio in 5 mM MES buffer (pH 5.5) and inoculated into Gossypium hirsutum leaves using a needleless syringe. The inoculated plants were maintained in a greenhouse at 23±2°C for effective viral infection and spreading.

Experimental design and statistical analysis

The data obtained were subjected to statistical analysis and were expressed as the means and standard error (SE) of at least three replicates. All experimental data are presented as the means of at least three independent replicates, and the analysis for significance was performed using Student’s T-test. The sequences of primers used in this study are listed in Table S2.

Results

Phylogenetic analysis, subcellular localization, and expression pattern of GhSnRK2 in cotton plant

The phylogenetic tree constructed using the full length amino acid sequence of selected SnRK2 gene to analyze the evolutionary relationship between GhSnRK2 and other SnRK2 family genes is shown in the neighbor-joining tree developed based on an alignment of the complete protein sequences. The bootstrap values are shown on the branches. GhSnRK2 clustered with the known stress-related genes AtSnRK2.1, AED91326.1; AtSnRK2.4, AEE28666.1; AtSnRK2.5, AED97781.1; AtSnRK2.10, AEE33751.1; and Orzya sativa (RK1), ABB89146.1. SnRK2.10 and SnRK2.4 are closely related to GhSnRK2, suggesting that GhSnRK2 belongs to the SnRK2 family (Figure 1A). Alignment of the GhSnRK2 amino acid sequence with that of other SnRK2s revealed that the GhSnRK2 protein is highly similar to other SnRK2s; the relatively conserved motif is underlined. The deduced amino acid sequence displays relatively high homology with the monocot SnRk2 family members Orzya sativa (RK1), ABB89146 and with the dicot species AtSnRK2.10, AEE33751.1 (Figure S1A). The GhSnRK2-GFP fusion protein driven by the CaMV 35S promoter was transiently expressed in onion epidermal cells, and the green fluorescent GhSnRK2 protein signals were localized to the nuclei and the cytoplasm, whereas GFP alone was detected throughout the cell (Figure 1B). qRT-PCR was performed to quantify the expression level of the GhSnRK2 gene. Treatment of 3-week-old CCRI24 upland cotton cultivar plants with 10% PEG for different periods induced the expression of the GhSnRK2 gene for 3 h, after which the expression level declined (n = 3) (Figure 1C). To investigate the distribution of GhSnRK2 in different tissues, samples from root (RT), stem (ST), cauline leaves (CL), rosette leaves (RL) and flowers (FL) were analyzed. GhSnRK2 gene expression was detected in all of the examined tissues in distinct expression patterns and was highest in the root and lowest in the flower, suggesting that the GhSnRK2 gene may most actively function in the root (Figure 1D). Cotton histone3 (gene accession AF024716) was used as internal control to normalize expression data. The values are presented as the means of three replicates, and the error bars denote the SE. Different letters denote the means ± standard deviation displaying significant difference at P<0.05.

Plant transformation vector and the expression pattern of the GhSnRK2 gene in the transgenic lines

The schematic representation of the 35S-GhSnRK2 construct used for Arabidopsis transformation (Figure 2A). The full-length GhSnRK2 DNA was introduced into the Arabidopsis genome via the floral dip method using Agrobacterium GV3101; the XbaI-BamHI region of GhSnRK2 was inserted between the CaMV 35S promoter and the nopaline synthase gene terminator in the pCAMBIA2301 vector to generate the recombinant plasmid 35S-GhSnRK2-NOST. The neomycin phosphotransferase II (NPTII) gene, driven by the nopaline synthase gene promoter (NOSP), was carried by the pCAMBIA2301 vector for transgenic cell selection in kanamycin-containing LB medium. GhSnRK2 in transgenic Arabidopsis plants was confirmed by PCR analysis. Genomic DNA from the first generation of the plants (T1) was extracted and used as a template for gene-specific primers. Of the 43 plants evaluated, 35 were positive for GhSnRK2 amplification. No product was formed by the amplification reaction using untransformed plant DNA as the template (Figure S2B). At T1 generation, the ratio of dead to surviving plants was approximately 1:3 in kanamycin-containing LB medium. However, at T3 generation, all of the plants became homozygous, as their survival rate was 100% in kanamycin-containing LB medium (Figure S2A, Table S1). The values are expressed as the mean germination rate (%) of approximately 200 seeds. qRT-PCR analysis was performed to evaluate the differences in transgene expression between all of the independently generated transgenic lines. Various transgene expression patterns were detected in the transgenic lines overexpressing the GhSnRK2 gene, but no expression was detected in the WT line, indicating that the GhSnRK2 genes were successfully introduced into the transformed plants (Figure 2B). The values are presented as the means of three experimental replicates; the error bars indicate the standard deviations. AtACT2 (gene accession AtS01879) was used as internal control to normalize expression data. Three transgenic lines (L1, L2, and L4) were selected for further investigation based on their seed availability and relative expression pattern.

Overexpression of GhSnRK2 in transgenic plants improves their tolerance to drought and cold stresses

To investigate the roles of GhSnRK2 in stress response pathways, the responses of the three selected transgenic GhSnRK2-overexpressing Arabidopsis lines to drought and low temperature stresses were analyzed. Constitutive overexpression of
Figure 1. Phylogenetic analysis, subcellular localization, and expression pattern of GhSnRK2 in cotton plant. (A) A phylogenetic tree of GhSnRK2 and other SnRK2 proteins from different plants was constructed using the neighbor-joining method with MEGA 5. The sequences used for analysis are listed by accession number: Litchi chinensis (LcSnRK2), AFX72761.1; Arabidopsis thaliana (AtSNRK2.2), CP002686.1; Arabidopsis thaliana (AtSnRK2.3), AED98274.1; AtSnRK2.1, AED91326.1; AtSnRK2.9, AEO73961.1; AtSnRK2.10, AEE33751.1; AtSnRK2.4, AEE28666.1; AtSnRK2.5, AED97781.1; AtSnRK2.7, AEE87152.1; Populus tremula (PtreSnRK2.6a), AGW51610.1; Zea mays (ZmSnRK2.2), NM_001137717.1; Solanum tuberosum (StSnRK2.8), AFR68945.1; Oryza sativa (RK1), ABB89146.1; Oryza sativa (SAPK4), BAD18001.1; Sorghum bicolor (SAPK4), AGM39623.1; and Zea mays (SAPK5), ACG42286.1. The bootstrap values are shown on the tree branches. (B) Subcellular localization of the GhSnRK2-GFP protein. (1 and 2) GFP alone; (4 and 6) GhSnRK2-GFP in onion epidermal cells; (1 and 3) corresponding bright-field images. (C) The expression pattern of the GhSnRK2 gene in cotton plants subjected to 10% PEG stress. The gene expression data were normalized to that of the cotton histone 3 gene. The values are presented as the means of three experimental replicates. The vertical axis represents the relative expression level. The values from 0 to 6 indicate the time (h) of PEG treatment. Asterisk denotes a significant difference (P<0.05) compared with the control (0 h). (D) Relative expression levels of the GhSnRK2 gene in various cotton plant tissues. Samples from root (RT), stem (ST), cauline leaves (CL), rosettes leaves (RL) and flowers (FL) were analyzed. The vertical axis represents the relative expression level. The letters denote significant differences (P<0.05) based on Duncan’s multiple range tests. The cotton histone 3 gene was used as an internal control for normalization of the gene expression data. doi:10.1371/journal.pone.0112269.g001
the 35S-GhSnRK2 gene resulted in an increase in the drought tolerance of the transgenic plants. After withholding water for 9 days, some of the WT plants grew slowly, wilted severely and died, whereas few of the GhSnRK2 transgenic plants wilted. The recovery rate of the transgenic plants was higher than that of the WT plants after re-watering. Indeed, the survival rate was significantly different between the GhSnRK2 transgenic and wild-type plants: L2 (100%), L4 (94.4%), L1 (88.9%), and WT (4.4%); Student’s T-test (n = 3) (p < 0.05) (Figure 3A, B). These results suggested that the GhSnRK2 gene is involved in the response to drought tolerance.

Under low temperature stress conditions, the survival rate of the transgenic lines was significantly higher than that of the WT line. Most of the transgenic plants were intact and recovered from this stress, whereas most WT plants were found to have died and could not recover after transferring them to the normal growth conditions. The survival rates at 4°C were: WT (16.67%), L2 (80.55%), L1 (77.78%), and L4 (78.33%). The survival rates at −4°C were: WT (5.56%), L1 (54.44%), L2 (58.33%), and L4 (56.11%). At −8°C, only 3.3% of the WT plants survived, whereas the survival rates of transgenic lines ranged between 30% and 33.33% after 7 days under normal growth conditions (Figure 3C, D). However, the survival rate under different low temperatures clearly indicates the difference between the transgenic and WT plants, suggesting that the GhSnRK2 overexpression alleviates low temperature stress in transgenic Arabidopsis. Our findings revealed that the water use efficiency was higher in GhSnRK2-overexpressing plants compared to the corresponding WT plants, suggesting that GhSnRK2-overexpressing plants exhibit greater photosynthetic potential during drought stress treatment (Figure 3E). Normal turgor pressure which is regulated by the amount of water in the plant’s cells is required for healthy growth of a plant and is a powerful determinant of the plant’s drought tolerance. We determined the turgor of the GhSnRK2 transgenic plant to understand the role of turgor pressure in cellular signaling during water deficit condition. Turgor values of GhSnRK2 transgenic plant was notably higher compared to the corresponding WT plant and this may be attributable to osmotic adjustment, like stomatal closure, and the maintenance of water content which prevent plants from desiccation and turgor loss. Student’s T-test (n = 3) (p < 0.05) (Figure 3F).

**Biochemical and physiological assays of plants overexpressing the GhSnRK2 gene**

The rate of water loss and the RWC were investigated to further understand the tolerance of GhSnRK2-overexpressing plants to
Figure 3. Survival rates of *GhSnRK2* transgenic plants under drought and low temperature stresses. (A) WT and *GhSnRK2* transgenic plants before and after drought stress. (B) Seedlings were cultured for 2 weeks with constant watering before the withholding of water. After 9 days without water, all of the plants were irrigated, and plant re-growth was scored 4 days later. The plants were scored as survivors if there were healthy green young leaves after re-watering. The survival rate was calculated as the ratio of the number of surviving plants to the total number of treated plants in the flower pot. Asterisk denotes a significant difference (P<0.05). (C) WT and *GhSnRK2* transgenic plants before and after low temperature stress. (D) Survival rates under low temperature stress conditions. The survival rate after transferring 4-week-old *GhSnRK2* transgenic and WT plants to a low temperature chamber at 4°C, −4°C or −8°C for 10 h, followed by returning the plants to normal growth conditions. Clearly green plants after returning to the normal growth condition were scored as survivors, and plants exhibiting clear signs of wilting were denoted as dead. The mean survival rates of the WT line were compared with those of the transgenic lines using Student’s T-test. (E) The water use efficiency of the WT and *GhSnRK2* transgenic plants. Asterisk denotes a significant difference (P<0.05).

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water stress via the maintenance of a higher RWC and a reduced rate of water loss. Our results revealed that due to their smaller stomata aperture, the transgenic Arabidopsis plants overexpressing the GhSnRK2 gene lost water more slowly than the WT plant in the same period under normal conditions, which may underlie the capacity of the transgenic plants to maintain a higher leaf RWC and to tolerate water stress conditions: “tstat”>1 Critical two-tail (Figure 4A). The GhSnRK2-overexpressing plants exhibited a higher RWC than the WT plants, which is most likely due to the reduction in transpiration-mediated water loss. The RWCs in each line were L2(74.56%), L4(72.09%), L1 (70.04%), and WT (53.98%); Student’s t-test (n = 3) (p<0.05) (Figure 4B). It is known that during the plant response to abiotic stress, stomata typically close to reduce the rate of water loss due to transpiration. Moreover, abscisic acid (ABA) regulates the stomatal aperture under water-deficient conditions. We found that the stomata apertures of the GhSnRK2 transgenic plants in response to exogenous ABA treatment were smaller than those of the WT plants, indicating the protective effect of the GhSnRK2 protein in response to ABA. At 0 µM ABA, the stomata aperture of the WT plants was observed slightly wider than that of the GhSnRK2 transgenic plants. However, when the concentration of ABA was increased to 1 µM, the aperture size of the transgenic plants was significantly reduced. A similar pattern was detected at 5 µM ABA (n = 5) (Figure 4C, D). Free proline is an osmoprotective molecule that accumulates under stress conditions. Our results revealed that the proline contents of the GhSnRK2 transgenic lines were significantly higher than those of the WT line, indicating that the transgenic plants accumulate a higher amount of proline than the WT plants. Moreover, the upregulation of Arabidopsis pyrroline-5-carboxylate synthetase 1, a proline biosynthesis gene, in GhSnRK2-overexpressing plants suggested that proline production may be improved by GhSnRK2 via the regulation of proline biosynthesis genes; Student’s T-test (n = 3) (p<0.05) (Figure 4E). Greater leaf chlorophyll content at all stages of plant development has been associated with improved transpiration efficiency under drought stress, and this trait may indicate the presence of drought avoidance mechanisms. Based on our results, the chlorophyll content of the GhSnRK2 transgenic lines was higher than that of the WT line, suggesting that GhSnRK2-overexpressing plants exhibit greater photosynthetic potential (P<0.05) (Figure 4F).

Overexpression of GhSnRK2 in transgenic plants enhances seedling growth in response to NaCl and exogenous ABA treatment

To investigate the effect of GhSnRK2 overexpression in response to NaCl and exogenous ABA treatment, a root growth experiment was conducted to elucidate the physiological differences between the transgenic and WT plants. The transgenic plants overexpressing GhSnRK2 exhibited enhanced seedling growth under NaCl and ABA stress treatments, suggesting that GhSnRK2 may be involved in the oxidative stress response pathway. Seedlings of WT and GhSnRK2 transgenic lines grew normally in 0 mM NaCl. In 100 mM and 150 mM NaCl, the transgenic seedlings formed longer roots and displayed significantly larger growth than the WT seedlings. When the NaCl concentration was increased to 200 mM, the growth of the WT seedlings was completely inhibited, and the color of these seedlings was found to turn brownish, whereas the transgenic seedlings remained green and continued to grow, although at a slower rate (n = 3) (Figure 5A, B).

The seedlings from the WT and GhSnRK2-overexpressing lines cultured under normal conditions displayed no significant difference. However, when various concentrations of ABA (0.5 µM, 1 µM, or 2 µM) were introduced to the MS medium and the plants were allowed to grow vertically, the root growth of the GhSnRK2-overexpressing lines, although slightly inhibited, displayed greater elongation than the WT line, the root growth of which was severely inhibited (n = 3). These results indicated that GhSnRK2-overexpressing plants were more tolerant to ABA than the WT plants (Figure 5C, D).

GhSnRK2 overexpression in transgenic plants enhances seed germination in response to NaCl and exogenous ABA treatment

We investigated the germination of GhSnRK2-overexpressing plants under exogenous ABA and salt stresses to determine whether GhSnRK2 is involved in stress response pathways. The germination rates of WT and GhSnRK2-overexpressing seeds were similar at 0 mM NaCl and ABA. However, when different concentrations of ABA or NaCl were introduced, the germination of the WT and GhSnRK2-overexpressing seeds was inhibited. When 50 mM NaCl was introduced to the “MS” medium, the germination rate was 47% for the WT line and 85%, 89%, and 86% for the L1, L2, and L4 lines, respectively. At 100 mM NaCl, the germination rates were: WT (20.5%), L1 (52%), L2 (56%), and L4 (54%) (Figure 6A, B). These results suggested that the GhSnRK2-overexpressing plants were more tolerant to salt stress than the corresponding WT plants. ABA plays a prominent role in the regulation of germinating and post-germinating growth arrest and mediates the adaptation of the plant to stress. To investigate this hypothesis, we measured the response of GhSnRK2-overexpressing plants to different concentrations of ABA during the germination stage. At 0.3 µM ABA the seed germination rates were WT (45%), L1 (69%), L2 (73%), and L4 (70%), respectively. When the concentration of ABA was increased to 0.5 µM, the germination rate of the WT line was clearly reduced (27%), whereas the germination rate of the transgenic lines ranged from 43% to 48% (Figure 6A, C). In summary, our findings revealed that the GhSnRK2-overexpressing mutants were more tolerant to NaCl and exogenous ABA stresses than the corresponding WT plants. Student’s t-test was used to compare the mean germination rates. Asterisks denote a significant difference compared to the control (P≤0.05).

We further investigated the biomass accumulation of GhSnRK2 transgenic and WT plants. The transgenic plants exhibited highly significant increases in both the fresh and dry weight biomasses compared with the corresponding WT plants (Figure 6D, E). These increases were all statistically significant; suggesting that overexpression of the GhSnRK2 gene increases biomass accumulation in plants. The increased biomass accumulation may be attributable to the increased chlorophyll content in GhSnRK2 overexpressing plant, as chlorophyll in plant cells carries out the photosynthesis process and is probably the most-frequently used estimator of plant biomass.

Expression analysis of stress-responsive marker genes

To further elucidate the biological function and molecular mechanisms of the GhSnRK2 gene, we determined the transcript levels of several stress-associated genes in GhSnRK2-overexpressing lines. Seedlings from 10-day-old GhSnRK2 transgenic and WT Arabidopsis lines cultured in MS medium were in the presence or absence of NaCl (250 mM) for 2 h. qRT-PCR was performed as described in the Materials and Methods section. Our findings revealed that GhSnRK2 regulates the expression levels of ABA and stress-responsive marker genes, suggesting that GhSnRK2 may positively affect ABA signaling and plant stress
Figure 4. Biochemical and physiological assays of plants overexpressing the GhSnRK2 gene. (A) Rate of water loss from GhSnRK2 transgenic plants. Six plants of each transgenic and WT line were analyzed. Three biological replicates produced similar results. Asterisk denotes a significant difference (P < 0.05). (B) The RWC of GhSnRK2 transgenic plants. (C) Stomata aperture size of WT and GhSnRK2 transgenic plants treated with different concentrations of ABA (D) The stomata apertures were measured after 2 h of treatment with different concentrations of ABA, and the mean values of the WT and transgenic lines at each ABA concentration were compared using Student’s T-test (P < 0.05). Asterisk denotes a significant difference (P < 0.05). (E) Proline accumulation in WT and GhSnRK2 transgenic plants. The proline content of GhSnRK2 transgenic lines was consistently higher than that of the WT line. Student’s T-test revealed a significant difference (P < 0.05) between the transgenic and WT lines (n = 3). (F) The chlorophyll content of the WT and GhSnRK2 transgenic plants. The mean values were compared using Student’s “t-test”. Asterisk denotes a significant difference (P < 0.05).

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responses. The expression levels of C-repeat binding factor-1 (AtCBF1; accession: ABV27087), delta-1-pyrroline-5-carboxylate-synthase-1 (AtP5CS1; accession:NP001189715), protein abscisic acid-insensitive-5 (AtABI5; accession:NP565840), protein abscisic acid-insensitive-3 (AtABI3; accession:CAA48241), desiccation-responsive genes (Atrd29B; accession: BAA02375 and Atrd29A; accession:BAA02376) in the GhSnRK2-overexpressing lines were significantly higher than those of the WT lines (p < 0.05), revealing that GhSnRK2 is actively involved in stress signaling pathways (Figure 7). Three experimental replicates produced similar results (n = 3); Student’s T-test.

VIGS efficiency and the transcript level of GhSnRK2 in gene silenced plants

We employed the VIGS technique to further dissect the function of the GhSnRK2 gene in transgenic cotton. The TRV VIGS vectors were modified based on a pTRV1 containing RNA-dependent RNA polymerase (RdRp), movement protein (MP), a 16 kDa cysteine-rich protein (16K), CaMV 35S promoters (2X35S) and a NOS terminator (NOSI) T-DNA vector. pTRV2 contains the coat protein (CP), multiple cloning sites (MCSs), CaMV 35S promoters (2X35S) and a NOS terminator (NOSI) T-DNA vector. Both vectors contain Rz, which is designated as a
Figure 6. Seed germination of the WT and GhSnRK2 plants subjected to NaCl and exogenous ABA treatment and biomass accumulation of these plants. (A) Seed germination frequency of the WT and GhSnRK2 transgenic plants cultured on MS medium supplemented with different concentrations of NaCl (50 mM or 100 mM) or ABA (0 μM, 0.3 μM, or 0.5 μM). (B) The survival rate of the WT and GhSnRK2 transgenic plants cultured in MS medium containing 50 mM or 100 mM NaCl. The mean values were compared using Student’s T-test (p<0.05). (C) The
self-cleaving ribozyme, LB (left border) and RB (right border) of the T-DNA (Figure S1B). After inoculating Agrobacterium strains containing GhSnRK2 derivatives (PYL156: pTRV-RNA2-GrCLA1, PYL192: pTRV-RNA1, PYL156: pTRV-RNA2-GhSnRK2 (positive control), or PYL156: pTRV-RNA2 vector) into Gossypium hirsutum leaves, the phenotype of both silenced and non-silenced plants was monitored daily for gene silencing efficiency. Loss of normal green coloration in plant leaves and albino phenotype was detected in PYL156: pTRV-RNA2-GrCLA1 silenced plants, which served as the positive control indicating gene silencing efficiency. The GrCLA1 gene represents the ideal visual marker for gene silencing efficiency due to its involvement in chloroplast development, as its loss of function results in an albino phenotype in true leaves (Figure 8A). The transcript levels of GhSnRK2 in gene silenced and non-silenced plants were analyzed via qRT-PCR. The transcript level of GhSnRK2 in the gene silenced two plant cultivars were downregulated compared to the non-silence plants inoculated with empty vector and WT plants, which exhibited a higher expression level. The downregulation of GhSnRK2 indicated that the gene was successfully knocked down in the silenced plants (Figure 8B). qRT-PCR analysis of the distribution of the TRV-construct in the silenced plants revealed that its infection results in gene-specific transcript degradation, that pTRV-GhSnRK2 functions in diverse tissues, and that pTRV-GhSnRK2 is abundantly expressed in the root. The roots of gene silenced plants exhibited further down regulated expression levels compared to the stems and the leaves. The TRV-construct displays increased infectivity and meristem invasion, both of which are key requirements for efficient VIGS-based functional characterization of genes in root tissues (Figure 8C).

Physiological assay and responses of GhSnRK2 gene silenced plants to various stresses

To further elucidate the function of the GhSnRK2 gene in stress tolerance, we investigated the response of GhSnRK2 gene silenced plants to various stresses. Our findings validated the importance of this technique for stress tolerance studies. Under water-deficient conditions, the silenced plants wilted and drooped, regardless of the cultivar (Figure 9A). The phenotype of the non-silenced plants inoculated with empty vector (TRV) was indistinguishable from that of the WT plants. The two cotton cultivars (CRI99668 and CRI409) inoculated with the target gene displayed similar phenotypes and symptoms. The WT and control vector-treated plant survival rates were 80% and 77.3%, respectively, whereas the survival rate of the two gene silenced cotton cultivars (CRI99668 and CRI409) were 34.66% and 38.67%, respectively (Figure 9B). Student’s T-test revealed a significant difference (p< 0.05) between the survival rate of the gene silenced and non-silenced plants. The rate of water loss from the detached leaves was determined in both the gene silenced and non-silenced plants (Figure 9C). After monitoring the water loss for 6-h, we found that the GhSnRK2 gene silenced plants inoculated lost more water than the non-silenced plants inoculated with empty vector and the WT plants. The rate of water loss was slightly higher in the CRI99668 cultivars than in the CRI409 cultivars. Under similar conditions, the gene silenced plants, regardless of the cultivar, exhibited a substantially reduced water content compared with the non-silenced plants inoculated with empty vector and the WT plants (Figure 9D). Oxidative stress, including that mediated by salinity, alters the physiological and morphological responses of plants. To investigate the effect of salt stress on GhSnRK2 gene silenced plants, the gene silenced and non-silenced plants were treated with 150 mM NaCl for 7 days, and the effect of NaCl was measured following this stress. Our findings revealed that the chlorophyll content of the non-silenced plants was higher than that of the gene silenced plants, indicating that the GhSnRK2 gene may be involved in the oxidative stress response (Figure 9E). Growth retardation was detected in the GhSnRK2 silenced plants, and the effect of salt accumulation in the plant cells was detected as elevated blisters, which were visibly detectable on the leaf surface of GhSnRK2 gene silenced plants (Figure 9F).

Discussion

The ability of plants to withstand water shortage while sustaining proper physiological activities can be associated with drought tolerance. In this study, we generated a gene construct containing GhSnRK2 driven by the CaMV 35S promoter and functionally characterized it via gene overexpression in Arabidopsis. We further elucidated the function of this gene in transgenic cotton plants using VIGS techniques. We found that the amino acid sequence of GhSnRK2 is highly similar to that of other SnRK2 proteins, and a neighbor-joining tree developed based on an alignment of the complete protein sequences revealed that GhSnRK2 clustered with known stress-related genes from other plants. The conserved motif detected demonstrated that GhSnRK2 is a functional protein. The conserved domain (CD) region in these proteins plays a crucial role in protein interactions, DNA binding, enzyme activity, and other important cellular processes. Furthermore, we found that GhSnRK2 was localized to the cytoplasm and the nucleus, suggesting that GhSnRK2 might perform diverse functions in cotton plants. Our findings revealed that GhSnRK2 is abundantly expressed in the root and is widely distributed throughout plant tissues, suggesting that it may function in diverse tissues. Transgenic Arabidopsis plants overexpressing GhSnRK2 exhibited enhanced tolerance to various abiotic stresses. Consistent with previous studies, SnRK2 genes have been implicated in the response to multi-environmental stresses [16,30,31]. The improved drought tolerance of plants overexpressing the GhSnRK2 gene may be due to the abundance of gene expression in roots. In addition, protein kinases that are specifically found in roots, such as SnRK2C, may perform certain important functions in root tissues as a sensor of water and nutrients in soil. The root tip plays a vital role in the response to water and nutrient detection via appropriate signal transduction [32]. Turgor regulation of the GhSnRK2 transgenic plant under water deficit condition may be attributable to the dynamic process of cell wall adjustment of the transgenic plant due to their enhanced drought-tolerant. Plants can maintain turgor by solute accumulation, i.e. by osmotic adjustment, and possibly by elastic adjustment of their cell walls [33].
Based on our results, the transgenic plants overexpressing GhSnRK2 exhibited enhanced low temperature tolerance compared to WT plants. This result may be attributable to the resistance of the membrane system of the transgenic plant cells to cold stress. These findings corroborated the results of [30], who reported enhanced multi-stress tolerance in Arabidopsis plants overexpressing TaSnRK2.8. Numerous studies have demonstrated that the membrane systems of the cell are the primary site of low temperature contusion in plants [34,35]. Indeed, it is well confirmed that low temperature-mediated membrane injury...
occurs primarily as a result of acute dehydration associated with low temperature [35,36].

Our findings revealed the accumulation of compatible osmo-lytes, such as free proline, in the GhSnRK2-overexpressing plants; therefore, we speculate that the GhSnRK2 transgenic plants exhibit increased stress tolerance by regulating downstream gene expression and accumulating a larger amount of compatible osmo-lytes, which may account for the increase in stress tolerance.

Figure 8. Silencing efficiency and transcript level of the GhSnRK2 gene silenced plants. (A) Phenotype of gene silenced and non-silenced plants; 14 dpi. Wild-type (WT), negative control Empty vector (pTRV), positive control (pTRV-GrCLA1), gene silenced CRI409 cotton cultivars (pTRV-GhSnRk2 (09)), and gene silenced CRI99668 cotton cultivars (pTRV-GhSnRk2 (68)). (B) The silencing efficiency as determined by the expression pattern of WT, vector control (pTRV) and GhSnRK2 gene silenced plants based on qRT-PCR. (C) The distribution of the TRV- construct in the gene silenced plants. Samples from the root, the stem and the leaves of gene silenced and non-silenced plants were analyzed via qRT-PCR. The values are presented as the means of three biological replicates. Asterisk denotes a significant difference (P<0.05).

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Figure 9. Physiological assay of GhSnRK2 gene silenced plants. (A) Phenotype of drought stressed plants. Water was withheld from GhSnRK2 gene silenced and non-silenced plants for 5 days approximately two weeks post-inoculation. Each of the ten treated groups consisted of five plants. A photograph of each group representative was captured. (B) The survival rate was determined by withholding water from GhSnRK2 gene silenced and non-silenced plants for 5 days approximately two weeks post-inoculation, and the survival rate was recorded as the percentage of plants that survived after re-watering for 3 days. (C) The relative water loss was determined two weeks after inoculation. The reduction in the fresh weight from the initial weight was determined at the indicated time and represented as the percentage of water loss. The experiment was replicated three times. (D) The mean RWC was analyzed after immersing the fresh detached leaves in distilled water for 4 h and oven drying at 80°C for 48 h. The values are presented as the means of three biological replicates. Asterisk denotes a significant difference (P < 0.05). (E) The change in the chlorophyll content of GhSnRK2 gene silenced and non-silenced plants under salt stress. (F) The phenotype of GhSnRK2 gene silenced and non-silenced plants under salt stress.

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of GhSnRK2 plants. Proline is an osmoprotective molecule that accumulates in response to water stress and salinity [37,38]. Proline is considered as a typical physiological parameter for evaluating abiotic stress tolerance and resistance in plants. Many plants reduce their cellular osmotic potential via the accumulation of intracellular organic osmolytes, such as proline, to maintain a stable intracellular environment when subjected to external environmental stresses [39,40,41].

We detected decreased stomatal apertures in the GhSnRK2 transgenic plants compared with the corresponding WT plants. When exposed to adverse environmental conditions, the stomata must close for the plant to survive [42]. Stomatal closure reduces water loss, particularly in plants that have been exposed to water stress conditions caused by high solute concentrations in the nutrient medium, such that minimizing the rate of water loss due to transpiration reduces the accumulation of toxic ions [43]. The reduced rate of water loss from GhSnRK2 transgenic plants may be due to the decrease in the size of the stomata aperture. ABA is involved in stomata movement, enhanced drought tolerance and plant osmoregulation. Under water stress conditions, ABA is released into leaves, inducing the release of potassium salts from the guard cells, resulting in stomatal closure. Plant guard cells modulate the opening and closure of the stomata in response to phytohormones and various environmental signals, such as light and temperature, thereby regulating gas exchange for photosynthesis and water status via transpiration [44]. The SnRK2 gene SrRK2E, or SnRK2.6, plays a significant role in stomatal closure in Arabidopsis leaves [45,46].

The GhSnRK2 gene was upregulated after treatment with 10% PEG, which may suggest that the GhSnRK2 gene sufficiently confers drought tolerance, as one strategy that can be employed to mimic the effect of drought on plants is treatment with PEG. PEG was quite commonly used in physiological experiments to induce controlled drought stress.

We speculate that the GhSnRK2-overexpressing plants were more tolerant to NaCl than the WT plants, which may be due to the improved accumulation of Na+ in the vacuoles, increased sequestration of Na+ into the vacuoles, improved cellular Na+ exclusion, or their greater capacity for vacuolar osmoregulation. High salt can result in oxidative damage to cell membranes. The ability to avert such damage is consistent with the degree of tolerance exhibited by the plant [47]. Under salt stress, this damage is less extensive in transgenic plants overexpressing the GhSnRK2 gene than in WT plants because GhSnRK2 overexpression can tolerate the accumulation of Na+ in the vacuoles, thus preventing the toxicity of excess Na+. Hypersensitivity to ABA treatment during seed germination and early seedling development is typically followed by improved drought tolerance [48,49]. Our finding reveals that the transgenic plants overexpressing GhSnRK2 exhibited enhanced seedling growth under ABA stress treatment. This may be attributable to ABA-independent signaling pathway involving SnRK2 in root growth. This finding reveals the complexity in ABA-signaling, as different signaling components may function in different cells or tissues in ABA signal transduction. SnRK2.2 and SnRK2.3 double mutant showed strong ABA-insensitive phenotypes in seed germination and root growth inhibition [50].

The decreased stomatal apertures detected in the GhSnRK2 transgenic plants may be attributable to the role of SnRK2 in the regulation of ABA-induced activation of plasma membrane anion channels in guard cells and consequent response to their closure. These results suggest that SnRK2 functions in ABA signaling of stomatal closure. SnRK2E knockout mutant lost the ABA-dependent stomatal closure. SnRK2E affects ABA-signaling in stomatal closure, but not in germination stage [51]. ABA and PYR/PYLs inhibit protein phosphatase2C (PP2C), which in turn relieves the repression of positive factors, such as SnRK2s [52].

We analyzed the expression of the stress-inducible marker genes RD29A, RD29B, P5CS1, ABI3, CBF1, and ABI5 in GhSnRK2-overexpressing plants, and our results revealed increases in the transcript levels of these marker genes. Consistent with a previous study, the upregulation of stress response genes, such as P5CS1, can contribute to enhanced salt stress tolerance in plants [53]. ABI3 and ABI5 activation is necessary to sustain the germination of seedlings during intense drought stress [54]. ABI5 and ABI3 regulate numerous ABA responses, such as osmotic water permeability of the plasma membrane, stomatal closure, drought-induced resistance, germination, and inhibition of vegetative growth. We speculated that GhSnRK2 functions in the transcriptional regulation of ABA-inducible genes in seedlings, suggesting that SnRK2 protein kinases are involved in several processes of ABA signal transduction, including transcriptional as well as post-transcriptional regulation pathways. Therefore, we suggest that stress signaling pathways may be involved in GhSnRK2-mediated stress tolerance.

Our findings revealed the downregulation of GhSnRK2 in gene silenced plants, indicating that this gene was knocked down in the gene silenced plants. TRV spread vigorously throughout the entire plant, including meristem tissue, and the symptoms induced by TRV are not severe compared with those induced by other viruses [55].

The albino phenotype observed in the G. hirsutum gene silenced plants in this study is an indicator of effective VIGS function in inoculated plants, which is consistent with previous findings [56]. The results of this study revealed that GhSnRK2 gene silencing in G. hirsutum greatly reduced its tolerance to drought stress, corroborating the findings of [57], who reported that silencing of the function of the SpMPK1, SpMPK2, and SpMPK3 genes in tomato plants alleviates their tolerance to drought stress. The decrease in the chlorophyll content of silenced plants in response to salt stress indicates that GhSnRK2 may be involved in oxidative stress. High-concentration salt stress inhibits plant biochemical processes. Decreased chlorophyll content in salt-stressed pumpkin plants was detected by [58]. The stunted growth observed in salt-stressed GhSnRK2 gene silenced plants corroborate the findings of [59], who reported that the typical effect of salt stress in plants is growth retardation as a result of cell elongation inhibition.
Conclusions

Despite the significant innovations that have been made in elucidating the genetic mechanisms underlying drought tolerance, considerable challenges remain. The present study revealed that the genetic manipulation of the GhSnRK2 gene from cotton using a transgenic technique results in enhanced drought and cold stress tolerance in plants. Thus, GhSnRK2 is hypothesized to participate in the stress signaling pathway, and therefore, overexpression of GhSnRK2 may alleviate abiotic stress by regulating stress-responsive genes. Moreover, the findings in this study have helped to elucidate that GhSnRK2 enhances stress tolerance in plants by affecting various stress-related pathways. Thus, the GhSnRK2 gene represents a candidate gene for future research of abiotic stress signaling pathways and the genetic modification of novel *Gossypium hirsutum* varieties.

Supporting Information

Figure S1  Multiple sequence alignment of GhSnRK2 and closely related SnRK2s from other plants species and VIGS construct. (A) Alignment of GhSnRK2 and closely related SnRK2s. The relatively conserved motif is underlined. The deduced amino acid sequence displays relatively high homology with the monocot SnRK2 family member *Oryza sativa* (RK1), ABR9146 and with the dicot species *AtSnRK2.10*, AEE38731.1. (B) Virus-induced gene silencing construct. The TRV VIGS vectors were modified based on a pTRV1 containing RNA-dependent RNA polymerase (RdRp), movement protein (MP), a 16 kDa cysteine-rich protein (16K), CaMV 35S promoters (2X35S) and a NOS terminator (NOS) T-DNA vector. pTRV2 contains the coat protein (CP), multiple cloning sites (MCSs), CaMV 35S promoters (2X35S) and a NOS terminator (NOS) T-DNA vector. Both vectors contain Rz, which is designated as a self-cleaving ribozyme, LB (left border) and RB (right border) of the T-DNA.

**Figure S2** Survival rate in kanamycin-containing medium and PCR confirmation of GhSnRK2 gene expression

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