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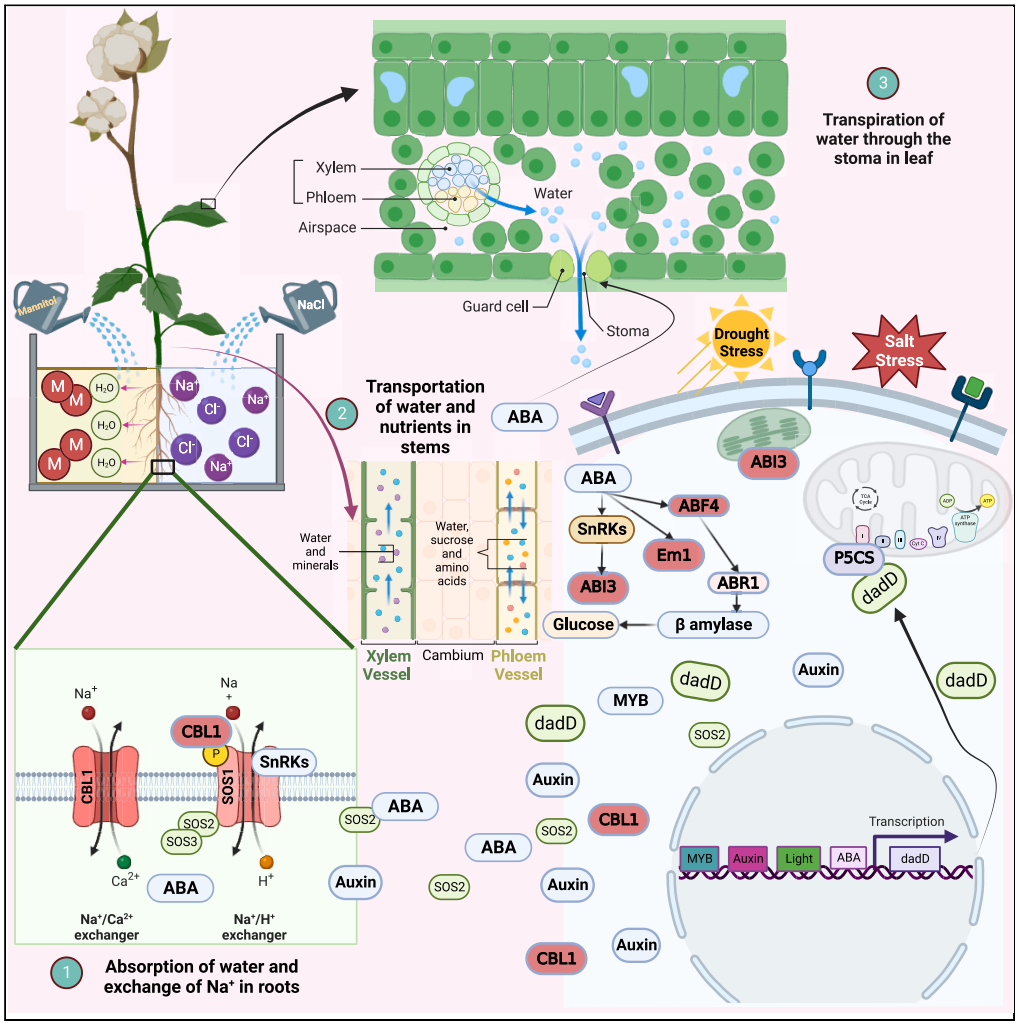

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Highlights

GhdadD responded to drought and salt stresses in cotton

GhdadD gene silencing decreased drought and salt stresses tolerance in cotton

GhdadD overexpressed Arabidopsis increased drought and salt stresses tolerance

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Overexpression and knockdown of cotton *GhdadD* gene reveals its drought and salt stress tolerance role

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SUMMARY

The 5′-deoxyadenosine deaminase (DADD), a member of the amidohydrolase family regulates biological purine metabolism. In this study, bioinformatic analysis, overexpression and knockdown of *GhdadD* gene were detected to identify its potential role in drought and salt stress tolerance. The results revealed that *GhdadD* was induced by ABA, Auxin, MBS and light responsive elements. In transgenic Arabidopsis, seed germination rate and root length were increased under drought or salt stress. *GhdadD* overexpressed seedlings resulted in higher plant height, less leaf damage and lower ion permeability. The expression of osmotic stress and ABA-responsive genes were up regulated. While in *GhdadD*-silenced cotton seedlings, CAT, SOD activity and soluble sugar content were reduced, MDA content was increased, and the stoma opening was depressed under drought or salt stress. Some osmics stress marker genes were also up regulated. These data indicating that GhdadD enhanced plant resistance to drought and salt stress through ABA pathways.

INTRODUCTION

Abiotic stresses such as cold, drought, salt, waterlogging, heavy metals, and other environmental factors have threatened plant growth and development. Among them, drought and salt stresses are causing more than 45% yield loss globally.[1] With the continuous increase of global greenhouse gas emissions, the arid and semi-arid areas will spread more than 50% by the end of this century. In China, 10% of the existing arable land has been affected by salinization, especially in Northwest China,[2] which threatened the cotton production.

Due to the long-term artificial domestication, cotton cultivars remained sensitive to drought and salt resistance due to lack of essential traits, although the yield and fiber quality has improved. Wild cotton germplasm resources such as *Gossypium tomentosum* are abundant in genetic variation, especially disease and insect resistance, drought and salt resistance and other characteristic.[3] In previous work, F2 genetic population[4] and BC2F2 population of interspecific crosses between *Gossypium hirsutum* and *Gossypium tomentosum* were constructed. Using drought stress treatment, a high-density genetic linkage map was constructed by genotyping-by-sequencing (GBS) on 188 lines of BC2F2 population. Thirty stable drought tolerance QTLs contained several drought tolerance related genes were screened. *GhdadD* encoded deoxy-adenosine deaminase (dadD) which is involved in biological purine metabolism, was located in a 0.75MB interval.[5]

DadD catalyzes the deamination of three SAM-derived enzyme products, which is an important part of amino acid biosynthesis.[6] The adenosine (AMP) deaminase has the same function as dadD; most of them are related to human diseases.[7–9] When the human body lacks AMP deaminase, it may cause skeletal muscle disease.[10,11] As enzyme inhibitors, AMP deaminase protein can be used as a binding target of chemical herbicides,[12,13] which can block the purine metabolism by inhibiting the activity of adenosine deaminase, thus achieving the goal of weed elimination. In tea, AMP deaminase inhibitor can reduce the synthesis of caffeine,[14] but it has not been reported in cotton yet.

Considering that there was no report about *GhdadD* and its stress resistance function, this study aimed to explore the potential role of *GhdadD* in drought and salt stress tolerance. Bioinformatics methods were used to observe the characteristics of *GhdadD*, including gene structure, protein characteristics, *cis*-acting elements, and evolution among different species. This study also explored its expression pattern in different tissues and the expression level of osmotic stress and ABA-responsive marker genes by qRT-PCR. In addition, we conducted transgenic and virus-induced gene silencing (VIGS) technology to get *GhdadD* transgenic Arabidopsis and *GhdadD*-silenced cotton

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plants to identify its potential regulatory roles in drought and salt stress tolerance. This research will help to understand how *GhdadD* improves the drought and salt tolerance in cotton seedlings. And this research is of great value for the mining and utilization of tolerance gene sources, so as to provide new drought and salt resistance cotton materials.

RESULTS

Gene structure and protein analysis of GhdadD

In this study, *GhdadD* was located on chromosome D01, within 1742062–1746905 bp range with 10 exons. Prediction of the conserved domains of GhdadD showed that it has two typical amide hydrogen domains (Figure 1A), indicating that GhdadD belongs to the amidohydrolase family, which is a superfamily of metal-dependent hydrolases.

The physicochemical analysis of protein showed that the theoretical relative molecular mass of GhdadD was 53.146kd, and the theoretical isoelectric point was 5.74. The total number of negatively charged residues in GhdadD was 58 and that of positively charged residues was 44, with the molecular formula $C_{2316}H_{3723}N_{655}O_{707}S_{34}$. The fatty acid coefficient of GhdadD was 94.82, which showed its thermal stability and the mean hydrophilicity of it was −0.050, suggesting that it was a hydrophilic protein.

The signal peptide prediction showed that GhdadD did not contain a signal peptide and it was a non-secreted protein. The transmembrane structure analysis speculated that GhdadD had no transmembrane structure. The secondary structure prediction of GhdadD showed that the proportion of α helix and random coil was the largest, accounting for 39.71% and 34.10%, respectively, followed by 17.67% of the extended chain and 8.52% of the β-turn (Figure 1B). The 3D structure of GhdadD was predicted by SWISS-MODEL homology modeling, and its main structures were also α-helix and random coil (Figure 1B), which was consistent with the predicted secondary structure.

Multiple sequence alignment analysis of *GhdadD* in *Gossypium arboreum*, *Gossypium barbadense*, *Gossypium raimondii*, *Hibiscus syriacus*, *Durio zibethinus*, *Herrania umbratica*, *Corchorus capsularis*, *Oryza sativa*, *Zea mays*, and *Theobroma cacao* showed that GhdadD had a highly conserved region among these species (Figure 1C). Homology analysis found that GhdadD has high homology with these species, indicating that dadD gene is highly conserved among different species. Based on phylogenetic tree, GhdadD had the strongest relationship with the homologous gene of *G. barbadense*, and had a high homology, while compared with other species it had the highest homology to *H. syriacus* (Figure 1D).

Promoter analysis of *GhdadD*

Sequence analysis of the 1500bp upstream promoter of *GhdadD* showed that the promoter contained some core elements, such as TATA box and CAAT box. There were also many elements related to hormone and environmental stress responses (Table 1); hormone-responsive elements such as abscisic acid-responsive element (ABRE), auxin-responsive element (TGA-element), salicylic acid-responsive element (TCA-element) and jasmonic acid responsive elements (CGGTA-motif, TGACG-motif); environmental stress responsive elements, such as anaerobic sensing element (ARE), drought stress responsive element (MBS) and light responsive elements (G-Box, GT1-motif, Box4, I-Box). These showed that the expression of *GhdadD* may be related to abiotic stress and phytohormone induction.

Expression patterns of *GhdadD*

qRT-PCR results showed that *GhdadD* was expressed in the roots, stems, leaves, calycles, torus, petals, stamens, and pistils. The expression level of *GhdadD* was higher in roots, stems, leaves, and torus, but lower in the stamens and pistils (Figure 2A).

The expression level of *GhdadD* in the leaves gradually increased with the increase of stress treatment duration. The expression in the stress treatment was significantly upregulated over control group at 3h and 6h, while at 12 h after stress, the gene expression level of the control group was relatively higher (Figure 2B). These results suggested that the *GhdadD* may respond to drought and salt stress.

GhdadD is located in the nucleus and cytoplasm

Under laser confocal microscope, plasmid eGFP and GhdadD-eGFP can excite green fluorescence. Transient protein expression of GhdadD-eGFP vector was localized in the nucleus and cytoplasm of the Arabidopsis protoplasts (Figure 2C).

Overexpression of *GhdadD* in Arabidopsis enhanced drought and salt stress tolerance during germination

qPCR and qRT-PCR were conducted to identify transgenic seedlings. In total, 18 positive seedlings were obtained and the expression levels of *GhdadD* in OE9, OE10 and OE11 were higher (Figure 3), these three lines were selected for phenotyping.

The seed germination rate was decreased gradually with the increase of mannitol or NaCl concentrations in medium (Figure 4A), while the three OE lines showed a higher germination rate (Figure 4B), which suggests that OE plants were less inhibited than wild type (WT) plants. When 100 mM mannitol or NaCl was added, the root growth of all genotypes was slightly inhibited, but the root length of OE lines was longer over WT. When the concentrations of mannitol or NaCl were increased, the root growth were significantly inhibited, but the OE lines had longer root lengths compared to WT. Root growth of WT plants was completely inhibited under 300 mM mannitol or 200 mM NaCl treatment (Figures 4C and 4D).

Together, these results showed that of *GhdadD* overexpressed Arabidopsis are more tolerant to drought and salt stress during germination stage.

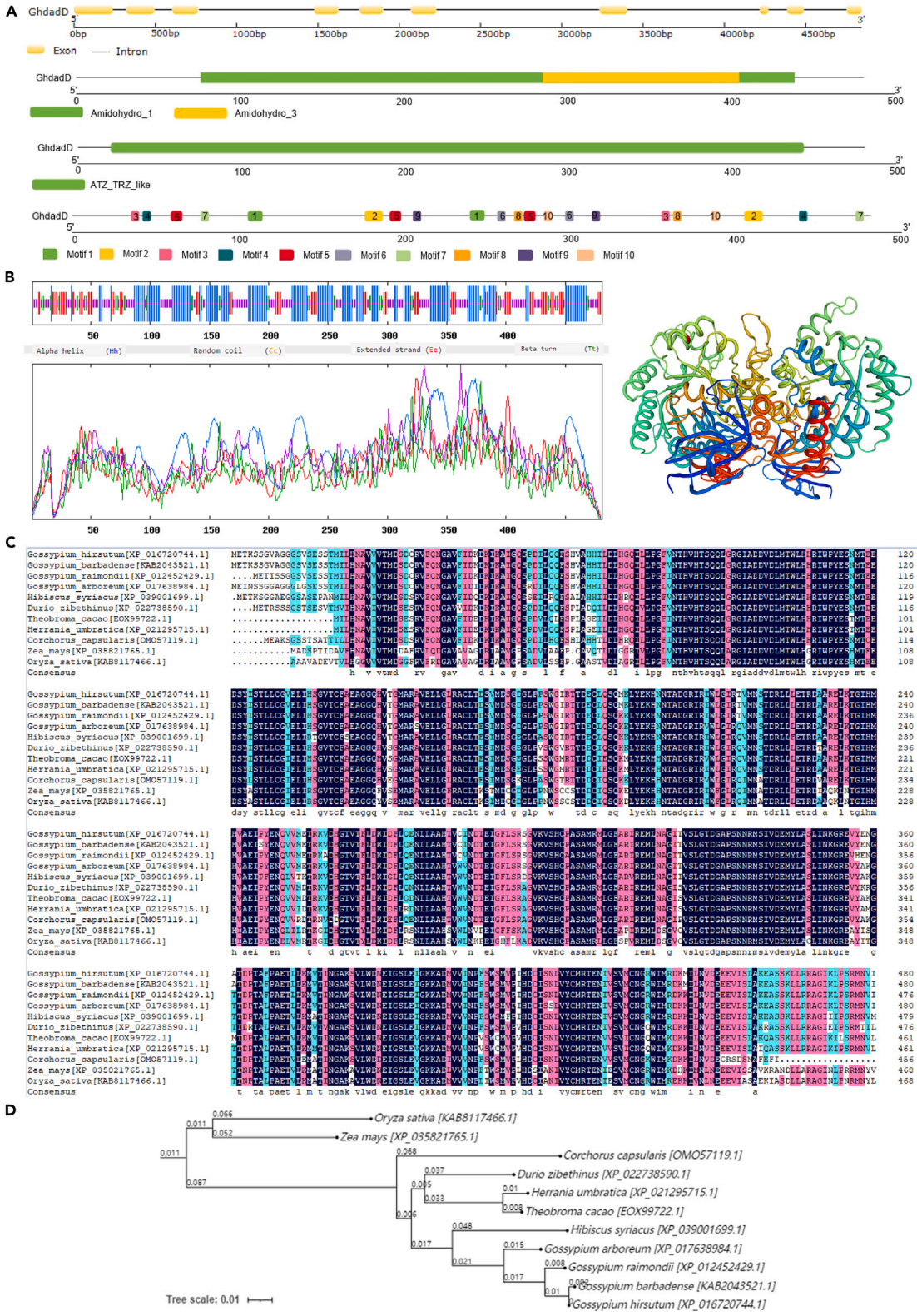

Figure 1. Bioinformatics analysis of *GhdadD* in *Gossypium hirsutum*

(A) Gene structure analysis and conserved domain analysis; (B) Secondary structure and tertiary structure prediction; (C) Amino acid sequence alignment; (D) Phylogenetic tree analysis.

Table 1. Prediction of *cis*-elements of *GhdadD* promoter

Elements	Number	Function
ABRE	7	*cis*-acting element involved in the abscisic acid responsiveness
G-Box	7	*cis*-acting regulatory element involved in light responsiveness
GT1-motif	6	light responsive element
MBS	2	MYB binding site involved in drought-inducibility
ARE	1	*cis*-acting regulatory element essential for the anaerobic induction
Box 4	1	part of a conserved DNA module involved in light responsiveness
I-box	1	part of a light responsive element
TCA-element	1	*cis*-acting element involved in salicylic acid responsiveness
TGA-element	1	auxin-responsive element
CGTCA-motif	1	*cis*-acting regulatory element involved in the MeJA-responsiveness
TGACG-motif	1	*cis*-acting regulatory element involved in the MeJA-responsiveness

Overexpression of *GhdadD* in Arabidopsis enhanced drought and salt stress tolerance at the seedling stage

At the seedling stage, all genotypes were severely damaged when exposed to stressors. After drought treatment, leaves of all seedlings were obviously wilted and chlorotic. Most leaves of WT plants dehydrated and yellowed, or even dried up and died. However, the leaves of OE lines only showed water loss and wilting, which showed lighter damage symptoms (Figure 5A). Drought stress also affected the plant height. After 10 days of drought treatment, the OE lines were bolted, however, their tidbits were reduced by drought stress. WT plants could be bolted, but the stems were weak and the plant height was significantly suppressed compared with OE lines (Figure 5A). The leaves of all lines were obviously chlorotic and yellowed under salt stress. Most leaves of WT plants withered and died, and the salt damage symptoms were obvious. However, only a few leaves of OE lines were chlorotic, and most leaves were still alive, especially OE12, with only mild symptoms of damage

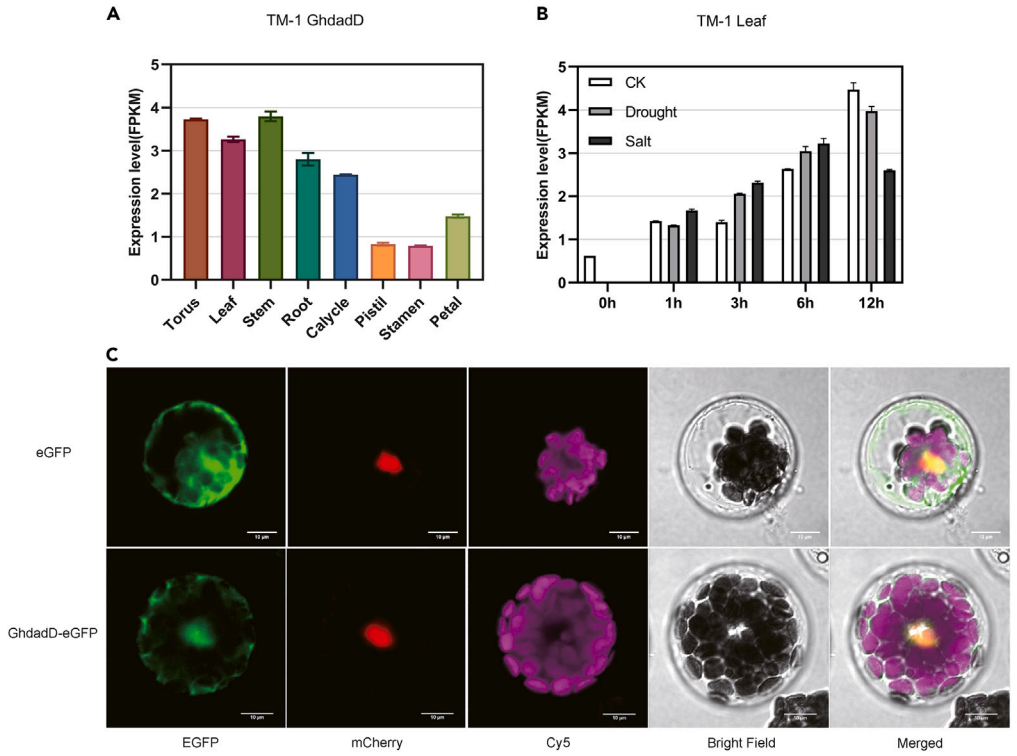

Figure 2. Expression analysis of *GhdadD* in *Gossypium hirsutum*

(A) Relative expression of *GhdadD* in different tissues of TM-1; (B) Relative expression of *GhdadD* in TM-1 leaves at different time of stress treatment; (C) Subcellular location of GhdadD. Bars, 10 μm.

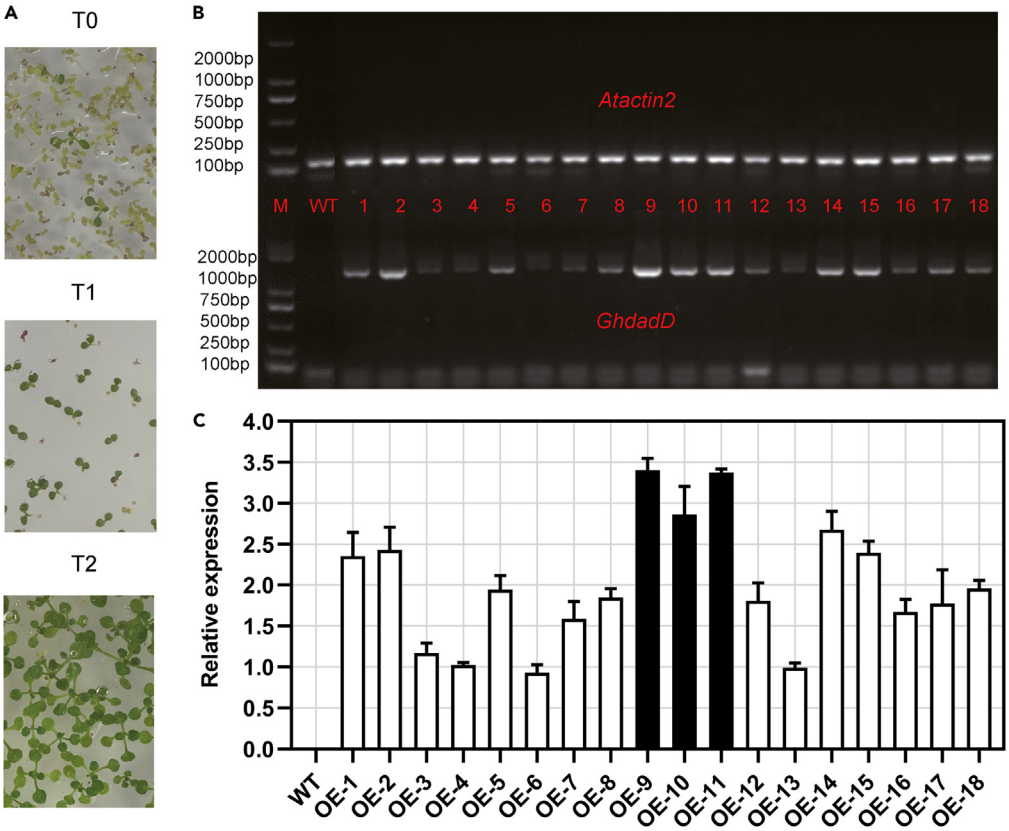

Figure 3. Screening of transgenic Lines in *Arabidopsis thaliana*

(A) Screening process of positive seedlings; (B) PCR detection; (C) Relative expression of *GhdadD* of different positive lines.

(Figure 5A). Salt stress inhibited plant height more obviously than drought stress. Most of the WT plants withered and died at the top of the new sprouts and could not continue to grow. The OE lines were also significantly affected, but more slightly compared with WT, and the inflorescence still grew (Figure 5B).

These results showed that overexpression of GhdadD in Arabidopsis enhanced the resistance to drought stress and salt stress at the seedling stage.

GhdadD overexpressed Arabidopsis were less damaged by drought and salt stress

Cotton leaves were stained with trypan blue to record the degree of damage. The results showed that leaves of all genotypes were obviously colored after exposure to stress. Leaves of WT plants had darker staining and more dead cells, while OE lines had lighter coloring (Figure 6A); this suggests that the *GhdadD* overexpressed plants suffered less damaged by drought and salt stress than WT.

DAB staining was performed to observe the accumulation of ROS in leaves and judge the degree of oxidative damage. The leaves of all genotypes dyed brown when subjected to drought and salt stress, while the brown region in the leaves of OE lines was substantially smaller than WT, and the dyeing depth was lighter (Figure 6B). This suggests that OE lines were less oxidatively damaged.

The determination result of ion permeability and MDA content showed that these two indexes were both increased significantly after exposure to drought and salt stress, and they increased more apparently under drought stress. The ionic permeability of OE increased less than WT, and the leaf MDA content after drought treatment was significantly lower than WT (Figures 6C and 6D). Under stress, the SOD activities of all lines were significantly increased, and the increase was greater under drought treatment. Besides, the SOD activities of OE lines were higher than WT (Figure 6E); this showed that *GhdadD* overexpressed plants were less oxidatively stressed, which was consistent with the results of DAB staining.

Overexpression of *GhdadD* altered the expression of ABA- and osmotic stress-responsive genes

To better understand the roles of *GhdadD* during plant stress response, the relative expression of osmotic stress marker genes including *AtABF4*, *AtCBL1*, *AtDREB1*, and the ABA-related genes *AtABI3*, *AtEm1* was measured by qRT-PCR in OE lines and WT seedlings. Results showed that these response genes were significantly upregulated after exposure to stress, and the upregulation of OE lines was more obvious

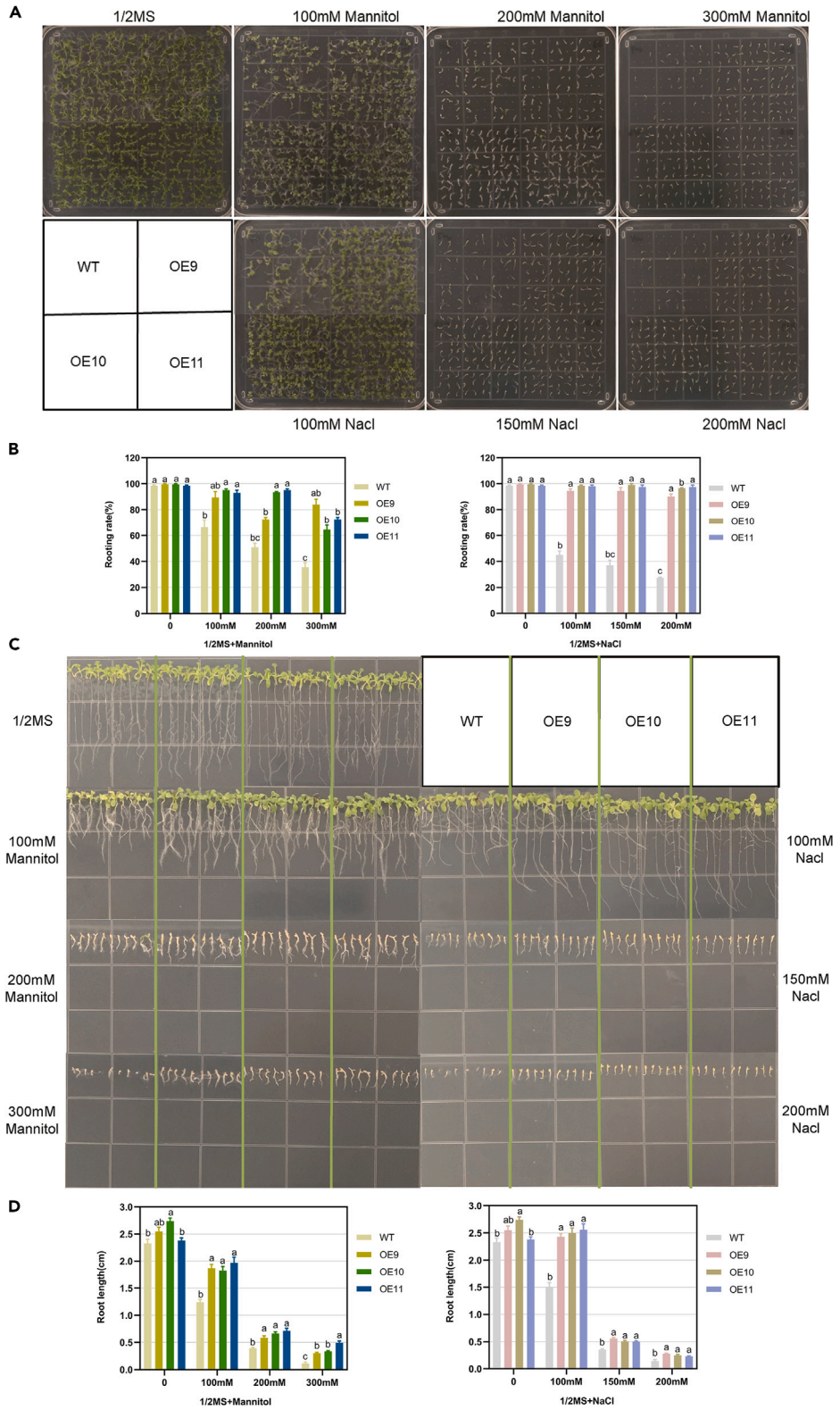

Figure 4. Overexpression of *GhdadD* in *Arabidopsis* enhances drought and salt tolerance during germination stage

(A) Seed germination; (B) Germination rate statistics; (C) The main root growth; (D) Root length statistics; (A and B) calculated by Student's *t* test with $p < 0.05$. Three biological replicates were used. Error bars indicate standard deviation.

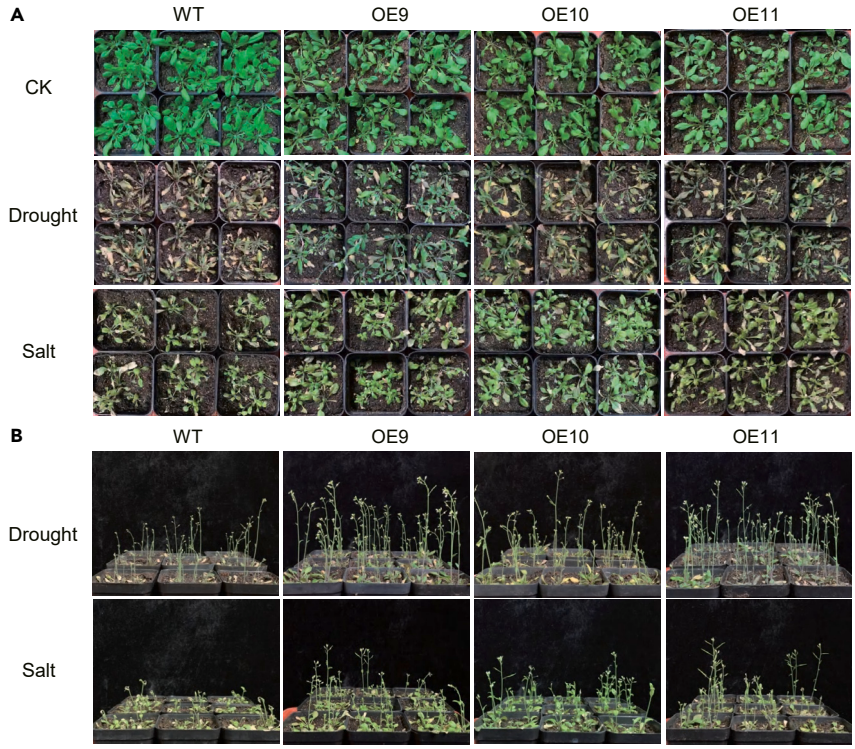

Figure 5. Overexpression of *GhdadD* in *Arabidopsis* enhances drought and salt tolerance during seedling stage
(A) Phenotype of WT and Transgenic *Arabidopsis*; (B) Plant height.

than WT (Figure 7). These results showed that overexpression of *GhdadD* in Arabidopsis could affect the expression of genes related to osmotic stress and ABA signaling pathway.

VIGS of *GhdadD* reduced drought and salt stress tolerance in cotton seedlings

The ClCrV-based VIGS system was used to generate *GhdadD*-silence plants. The etiolation phenotype appeared in ClCrV: *GhCLA1* seedlings one month after Agrobacterium tumefaciens transformed the plant (Figure 8A). The results of qRT-PCR showed that *GhdadD* expression level were reduced in *GhdadD*-silence plants (Figure 8B).

Under drought or salt stress conditions, the ClCrV: *GhdadD* seedlings displayed severe stress symptoms, such as leaf wilting and yellowing, comparing with ClCrV: 00 and CRI12 WT seedlings (Figure 8C). This showed that *GhdadD* was a positive regulator of drought and salt tolerance. Moreover, the physiological and chemical characteristics like excised leaf water loss (ELWL), relative leaf water content (RLWC), soluble sugar, SOD, MDA, and CAT exhibited significant difference between the *GhdadD*-silenced plants and control. The ClCrV: *GhdadD* seedlings showed a significant reduction in ELWL and RLWC compared with control, indicating that they suffered higher osmotic stress compared to ClCrV: 00 and WT. The soluble sugar content, SOD, and CAT level in ClCrV: *GhdadD* plants was significantly reduced compared with control whereas the MDA level was increased (Figure 8D). These results demonstrate that the silencing of *GhdadD* compromises the plant's effectiveness in tolerating drought and salt stress.

Effects of silencing of the *GhdadD* gene on the structure of cotton stems and leaves under salt and drought stress

Under drought or salt stress, the stomatal opening of the control and WT was decreased significantly. Although the stomatal opening of *GhdadD* silenced plants were also decreased to a certain extent, it was still larger than control plants (Figure 9A), explaining the leaf water retention ability to drop. The regulation and tolerance of *GhdadD* silenced plants to drought and salt stress was significantly decreased.

In addition, paraffin sectioning was used to observe the effect of osmotic stress on the structure of cotton leaves and stems. Under salt stress, compared with the control, the palisade cells of *GhdadD*-silenced plants had different degrees of longitudinal elongation, more and more closely arranged, and the thickness of palisade tissue became smaller and disordered. However, there were no significant changes in the upper and lower epidermal cells and spongy tissue of leaves (Figure 9B). From the perspective of stem transverse structure, the thickness of cortical parenchyma cells decreased and the thickness of pith was increased in the *GhdadD*-silenced plants compared with control (Figure 9C), suggesting that the structure of *GhdadD*-silenced plants changed greatly and the osmotic stress was more serious. These results showed that high salt stress could affect the structure of leaves and stems of cotton.

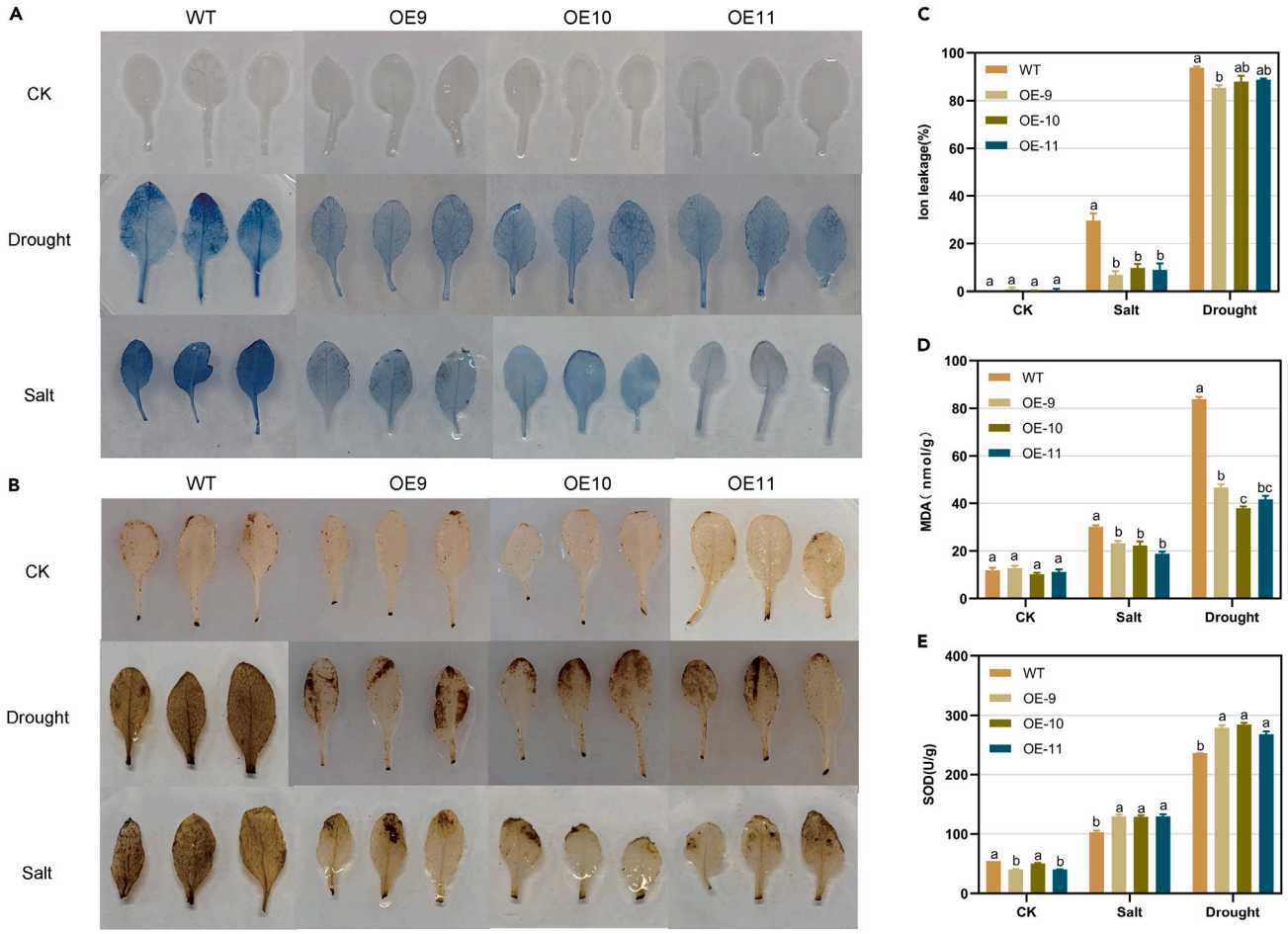

Figure 6. *GhdadD* overexpressed seedlings were less damaged by drought and salt stress

(A) Trypan blue staining; (B) DAB staining; (C) Ion permeability; (D) MDA content; (E) SOD activity. (A and B) calculated by Student's *t* test with $p < 0.05$. Three biological replicates were used. Error bars indicate standard deviation.

Silencing of *GhdadD* gene altered the expression of abiotic stress-responsive genes under drought and salt stress conditions

The expression levels of several stress-responsive genes were changed under stress conditions, such as *GhP5CS* (cotton Delta-1-pyrroline-5-carboxylate synthetase), *GhMYB(cotton myeloblastosis)*, *GhDTX27*, *GhCDKF4*, *GhTOM* and *GhGT-2* (Figure 10). Compared with the control, the expression level of *GhMYB*, *GhDTX27*, *GhCDKF4* and *GhTOM* in the *GhdadD* silenced plants were up-regulated under drought stress, *GhP5CS* and *GhGT-2* were down-regulated (Figure 10). As genes closely related to osmotic stress in cotton that have been reported, these significant changes of their relative expression over CK plant showed that the silence of *GhdadD* gene affected cotton to drought and salt stress tolerance.

DISCUSSION

In this study, *GhdadD* overexpressed Arabidopsis and *GhdadD* silenced cotton were obtained by biotechnology methods to change the transcription level of *GhdadD* gene. Based on phenotype, physiological and biochemical analysis, we explored the role of *GhdadD* gene in drought tolerance and salt tolerance of cotton.

Plants exhibit a variety of defense mechanisms in response to drought stress, involving various morphological and physiological changes.[15] The growth and elongation of plant roots can improve the ability of plants to absorb water and transport it in plants.[16] Under drought and salt stress, the seed germination rate and root length elongation of the overexpressed lines were significantly higher than WT (Figure 4). These results indicated that *GhdadD* could improve the inhibition of drought and salt stress on seed germination rate and reduce the inhibition of root elongation. In addition, under drought and salt stress, the *GhdadD* transgenic plants exhibited a better phenotype and the average plant height of these seedlings were significantly higher than WT (Figure 5). When overexpressed OsDHAR1 in rice, the

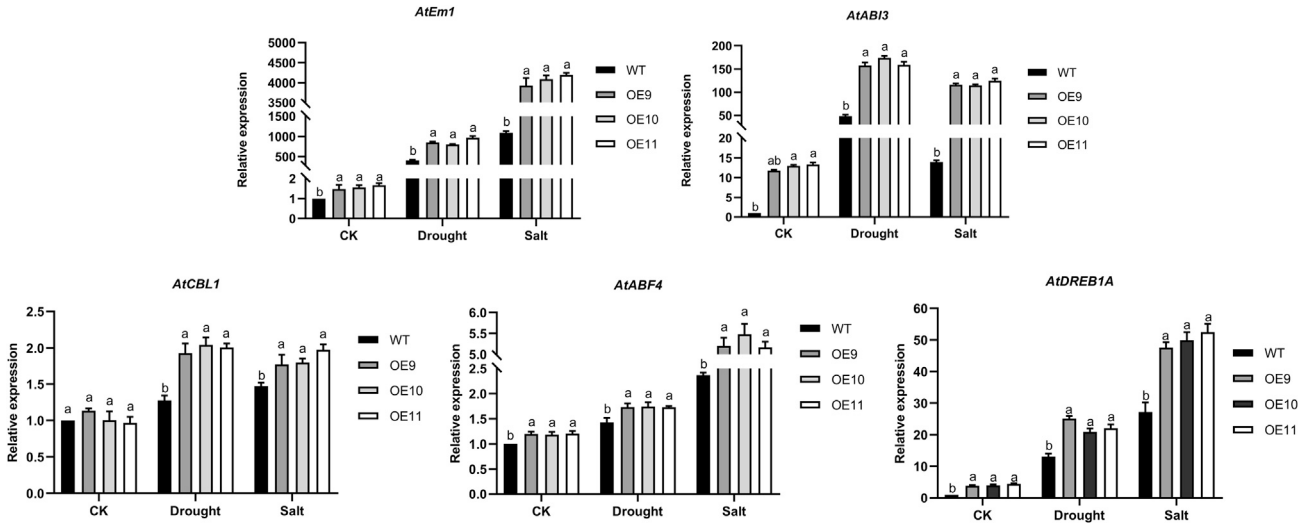

Figure 7. Expression of ABA and osmotic stress-related genes

All data calculated by Student's *t* test with $p < 0.05$. Three biological replicates were used. Error bars indicate standard deviation.

roots and shoots of the transgenic plants were longer and healthier than WT plants, which improved environmental adaptability of rice.[17] Their familiar phenotype indicated that the *GhdadD* transgenic Arabidopsis were more adaptable to the stress environment.

In plants, catalase (CAT), as an antioxidant enzyme, mainly scavenges H_2O_2 produced during mitochondrial electron transport, β-fatty acid oxidation and photorespiration to prevent damage caused by reactive oxygen free radicals.[18,19] CAT and superoxide dismutase (SOD) are components of biological antioxidant enzyme protection system.[20] In this study, SOD activity was significantly increased in *GhdadD* transgenic lines, while CAT and SOD activity decreased significantly in *GhdadD* silenced plants, leading to the increase of ROS accumulation and oxidative stress damage. This in turn damaged the membrane structure of plant cells. Malondialdehyde (MDA) and electrolyte permeability reflect the extent of cell membrane damage.[21] Under salt stress, the plant cell membrane was damaged, substances in cell membrane were extravasated, electrolyte permeability increased, and MDA content increased.[22] In this study, under drought and salt stress, the leaf MDA content and electrolyte permeability were decreased, indicating that transgenic plants can regulate the increase of reactive oxygen species elimination enzymes (CAT, SOD), thereby ensuring better cell membrane integrity and enhancing drought and salt tolerance. These results corresponded to the phenotype of plants under stress, indicating that the expression of *GhdadD* gene may affect the stress resistance of plants by affecting the degree of oxidative stress under osmotic stress.

An important response of plants to drought stress is stomatal closure, which can reduce water loss and improve water use efficiency.[23] Under drought stress, the stomata in *GhdadD*-silenced plants remained more open than WT (Figure 9A). This is consistent with the stomatal changes in Arabidopsis under osmotic stress reported by Yuan et al.,[24] indicating that gene silenced plants by impaired *GhdadD* may not induce the accumulation of abscisic acid (ABA), thereby triggering stomatal closure.

Leaf is the most important vegetative organ for photosynthesis, gas exchange, and transpiration in plants. Stress conditions can cause leaf changes in morphology and microanatomy. Leaf structure also reflects the adaptability of plants to the environment.[25] Wang Shuai[26] reported that with the increase of palisade tissue thickness and chloroplasts attachment area, the utilization of plants light energy can be improved. In this study, the leaf thickness of WT was increased under drought or salt stress, which reflects the adaptation of WT plants to osmotic stress. However, the leaf thickness of *GhdadD*-silenced plants decreased due to water loss of parenchyma cells in the palisade tissue, resulted in thinning and tight arrangement of palisade tissue, reflecting the damage were more serious in *GhdadD*-silenced plants.

Plant stems transport water and minerals absorbed by roots upward through the xylem to various vegetative organs, while transporting the photosynthesis products downward through the phloem.[27] The pith area in alfalfa stems significantly decreased with the increase of salt concentration by observing the cellular structure of alfalfa stems.[28] Our study found that under salt stress, the pith area within the stems of *GhdadD*-silenced plants decreased than WT (Figure 9), which is similar to the above results. This may be an indicative feature of plant stems responding to salt stress. This change increases the resistance of water flow from roots to leaves,[29] and reduces the transport capacity of vascular tissue, The transportation of dissolved salt ions absorbed by the root system to the aboveground portion is greatly limited.[30] In this study, severe water loss in the stems of *GhdadD*-silenced plants led to thinning of cortical parenchyma cells and reduction in stem diameter, reflecting the damage caused by stress, indicating that *GhdadD*-silenced plants were more sensitive to salt tolerance.

Osmotic stress responsive genes *CBL1*,[31] *AREB2/ABF4*[32] and *DREB1A*[33] are involved in plant response to water and salt stress, and regulates the expression of many functional genes responding to abiotic stress. The expression level of *CBL1* was significantly up-regulated under salt stress.[34] CBL protein can interact with CIPK to form CBL-CIPK protein complex and participate in response to osmotic stress.[35] Osmotic stress induced the expression of *ABF4*.[36] Under drought and high salt conditions, bZIP transcription factors combined with ABFs and AREBs regulates downstream target genes.[37] Overexpression of *DREB1A* in plants can improve the frost and drought resistance of plants.[38]

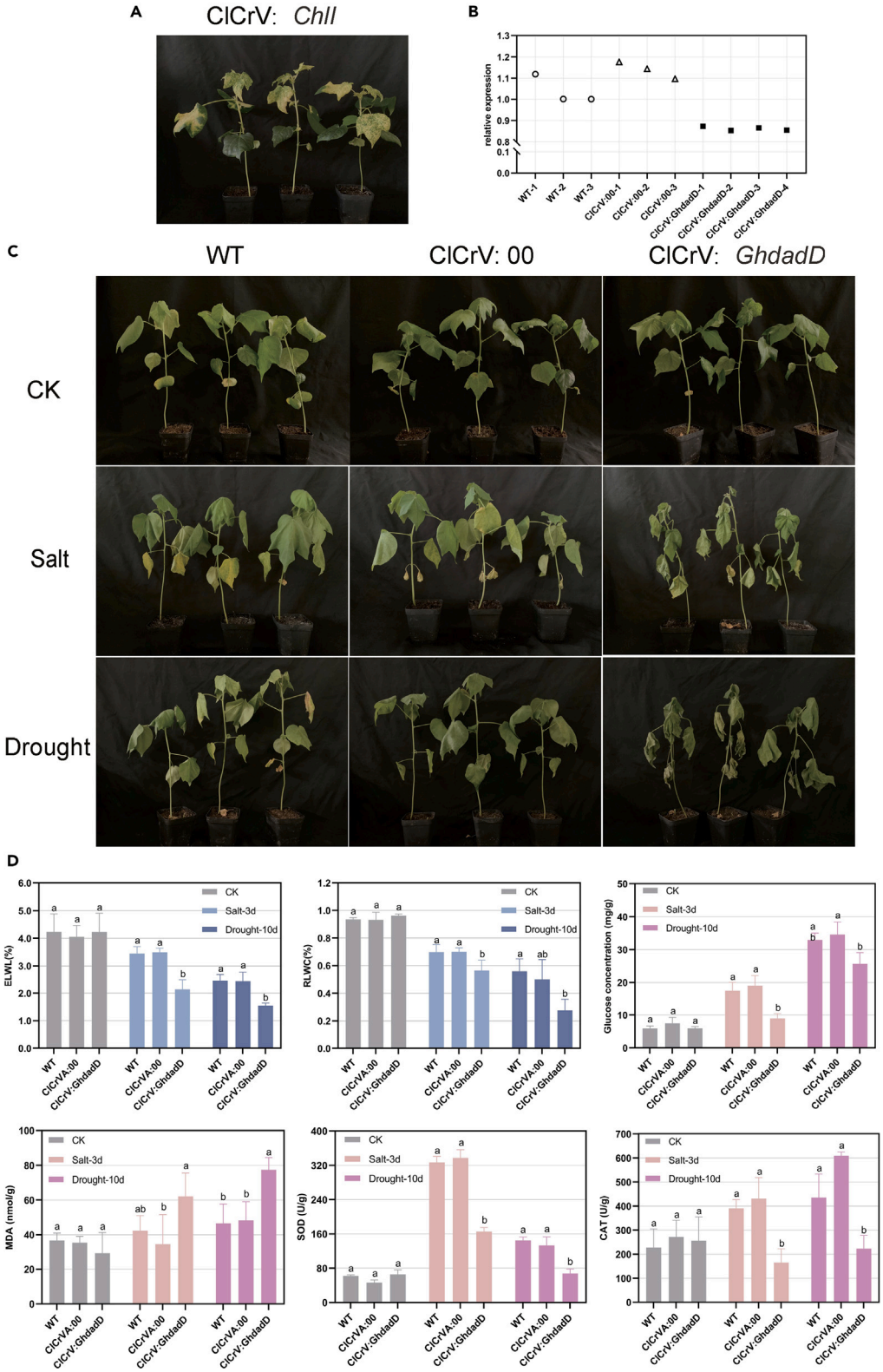

Figure 8. *GhdadD*-VIGS cotton plants show reduced drought and salt sensitivity

(A) Etiolation phenotype; (B) Detection of cotton silence efficiency; (C) Phenotype of WT and *GhdadD*-silence plants; (D) Determination of physiological and biochemical indexes. (A and B) calculated by Student's *t* test with $p < 0.05$. Three biological replicates were used. Error bars indicate standard deviation.

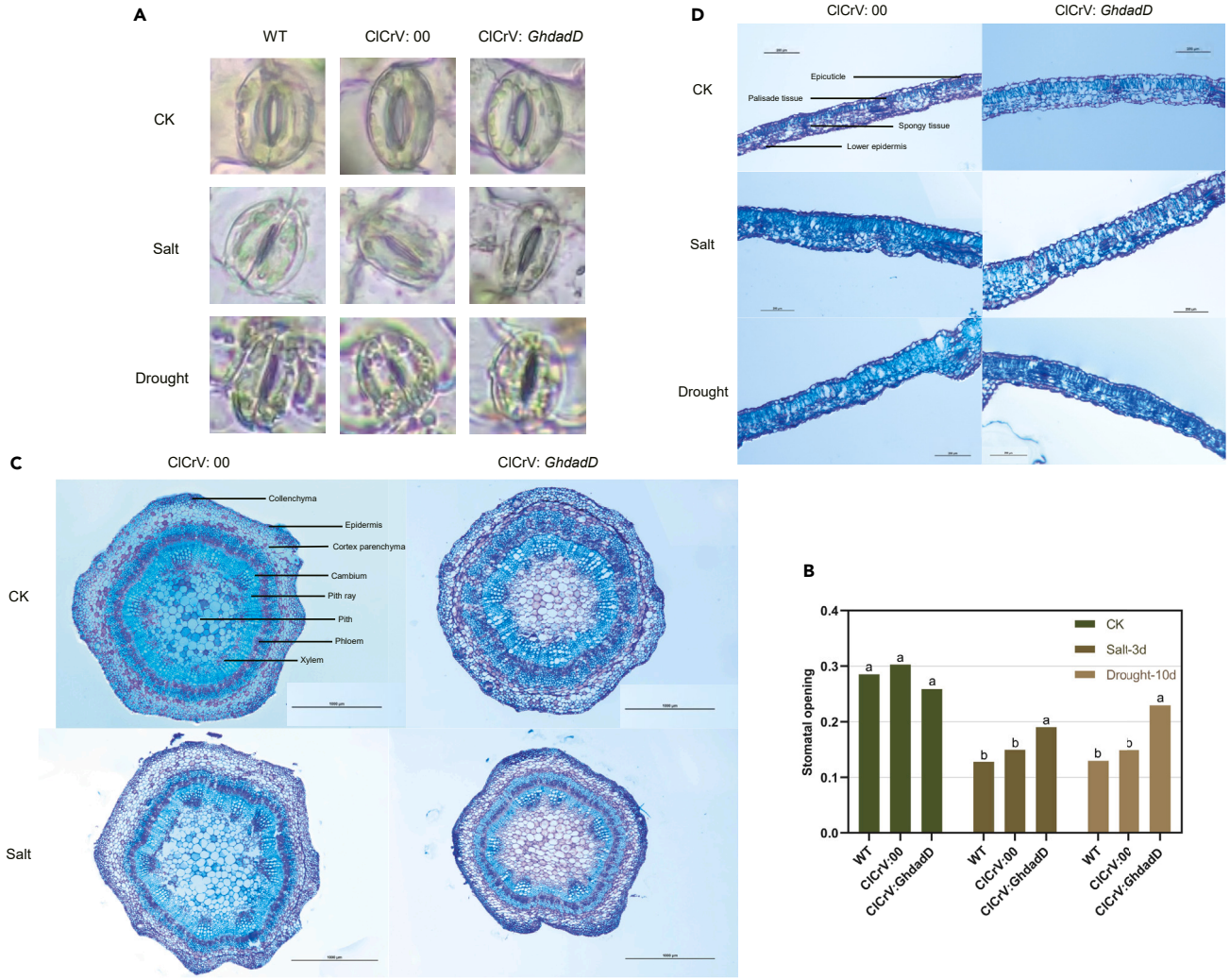

Figure 9. *GhdadD*-VIGS cotton plants show reduced drought and salt sensitivity

(A) Stomatal opening of cotton leaves; (B) Measurement of stomatal opening; (C) Cross-section of cotton stem; (D) Longitudinal section of cotton leaf. (A and B) calculated by Student's *t* test with $p < 0.05$. Three biological replicates were used. Error bars indicate standard deviation.

DREB-like transcription factors specifically bind with DRE *cis*-acting elements in the promoter region of stress-resistant genes, regulates the expression of downstream stress-response genes under salt, drought, cold conditions, and participate in the regulation of plant growth and development and response to abiotic stress of plants.[39] In this study, overexpression of *GhdadD* in Arabidopsis changed the expression levels of genes related to osmotic stress and ABA pathway under stress conditions, indicating that *GhdadD* may play a regulatory role in enhancing osmotic stress tolerance in plants. In *GhdadD* silenced plants, the expressions of *GhMYB*, *GhDTX27*, *GhCDKF4* and *GhTOM* genes were significantly up-regulated when subjected to drought stress. The MYB genes have a profound effect on enhancing stress tolerance in plants. For instance, ZmMYB30 gene a member of the R2R3-MYB gene family has been found to be highly inducted under abiotic stress conditions in maize and its ectopic expression in transgenic Arabidopsis plants promoted salt-stress tolerance.[40] The P5CS gene has been reported to for inducing stress tolerance in wheat.[41] As a key role in the pathway of proline biosynthesis, P5CS enzymes catalyze the activation of glutamate by phosphorylation and also reduction of labile intermediate, γ glutamyl phosphate, into glutamate semialdehyde.[42] Its downregulation in the VIGS plants showed that the knockdown of the *GhdadD* genes affected the proline biosynthesis pathways, further influenced cotton drought and salt stress tolerance. Among the existing research results in our laboratory, *GhDTX27*,[43] *GhCDKF4*,[44] and *GhTOM*[45] play an important role in drought stress tolerance in cotton. As the genes in the QTL mapping interval of drought in cotton, they may compensate each other in response to osmotic stress, which further indicates the important role of *GhdadD* in participating in drought and salt stress.

Based on these findings, the mechanism related to osmotic stress tolerance has been shown (Figure 11). Under salt and drought stress, *dadD* was activated by ABA, Auxin and light. It was located on nucleus and cytoplasm and a key enzyme for proline synthesis P5CS was

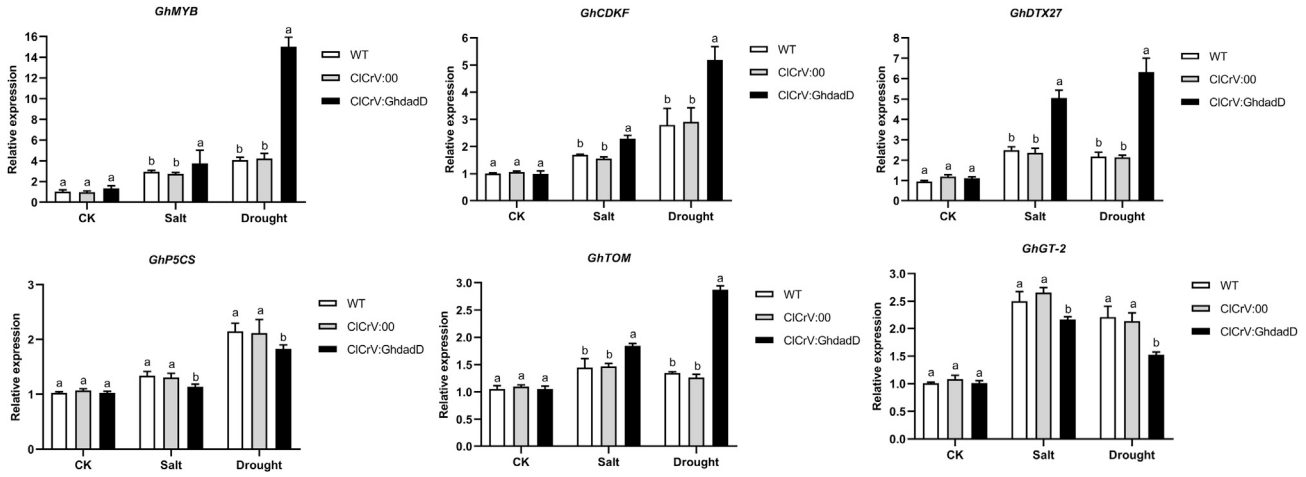

Figure 10. Expression of abiotic stress-responsive genes

All data calculated by Student's *t* test with $p < 0.05$. Three biological replicates were used. Error bars indicate standard deviation.

upregulated, which response to drought stress.[46] Proline can protect Complex II of the mitochondrial electron transport chain during salt stress and therefore stabilized mitochondrial respiration.[47] Osmotic stress promoted the biosynthesis of proline in ABA-dependent pathway through calcium binding protein (CaM/CML) and glutathione-S-transferase in wheat roots,[48] while in *GhdadD* transgenic Arabidopsis, CBL1 regulated the Na^+/Ca^{2+} exchange[49] and SOS2 were downregulated to suppress the Na^+/H^+ exchange,[50] which can keep the osmotic balance with longer roots (Figure 4) under drought and salt stress (Figure 7). ABF4 and ABR1 synergistically regulate amylase-mediated starch catabolism in drought tolerance, which results in increased glucose content (Figure 8D) to enhance water and nutrients transportation, as well as cortical parenchyma cells and pith in stem (Figure 9C). ABA regulates stoma movement in response to stress,[51] while some ABA response gene, such as ABI3 and Em1, are upregulated (Figure 7). In leaf, transpiration of water through the stoma open was higher in VIGS silence seedlings with significant reduction in ELWL and RLWC (Figures 8D and 9A). In brief, ion balance in longer root, more water and nutrients transportation in stem, and less transpiration in leaf can enhance the resistance to drought and salt stress through *GhdadD* pathway.

Conclusions

In this study, the gene structure, protein characteristics, and *cis*-acting elements of *GhdadD* gene were analyzed using bioinformatics methods. In this study, promoter region of *GhdadD* comprised of several elements related to hormone and environmental stress response. Homology analysis indicated that dadD protein was highly conserved among different species. The expression pattern analysis of *GhdadD* gene showed that it was specifically expressed in cotton tissues and was upregulated after drought and salt stress. Subcellular localization analysis revealed that the gene was located in the nucleus and cytoplasm. In addition, the role of *GhdadD* gene in improving drought and salt tolerance was verified through transgenic and VIGS technology. We found that *GhdadD* transgenic Arabidopsis was more tolerant to drought and salt stress, with longer root length, higher seed germination rate, higher plant height, and less ROS accumulation. However, *GhdadD*-silenced plants showed a high resistance reduction, ELWL, RLWC, soluble sugar, and decreased SOD and CAT content with increased MDA content. Moreover, stem and leaf microstructure also showed corresponding changes. The above results indicate that *GhdadD* gene may play a potential regulatory role in drought and salt resistance. This study will further help to explore the role of *GhdadD* gene in cotton.

Limitations of the study

In this study, subcellular localization analysis revealed that GhdadD was located in the nucleus and cytoplasm and it was specifically expressed in cotton tissues. But how they transmit signals within cells to reduce the accumulation of ROS in cells through osmotic stress and ABA pathways, thereby enhancing drought and salt stress tolerance of cotton, further discussion and research are still needed.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability

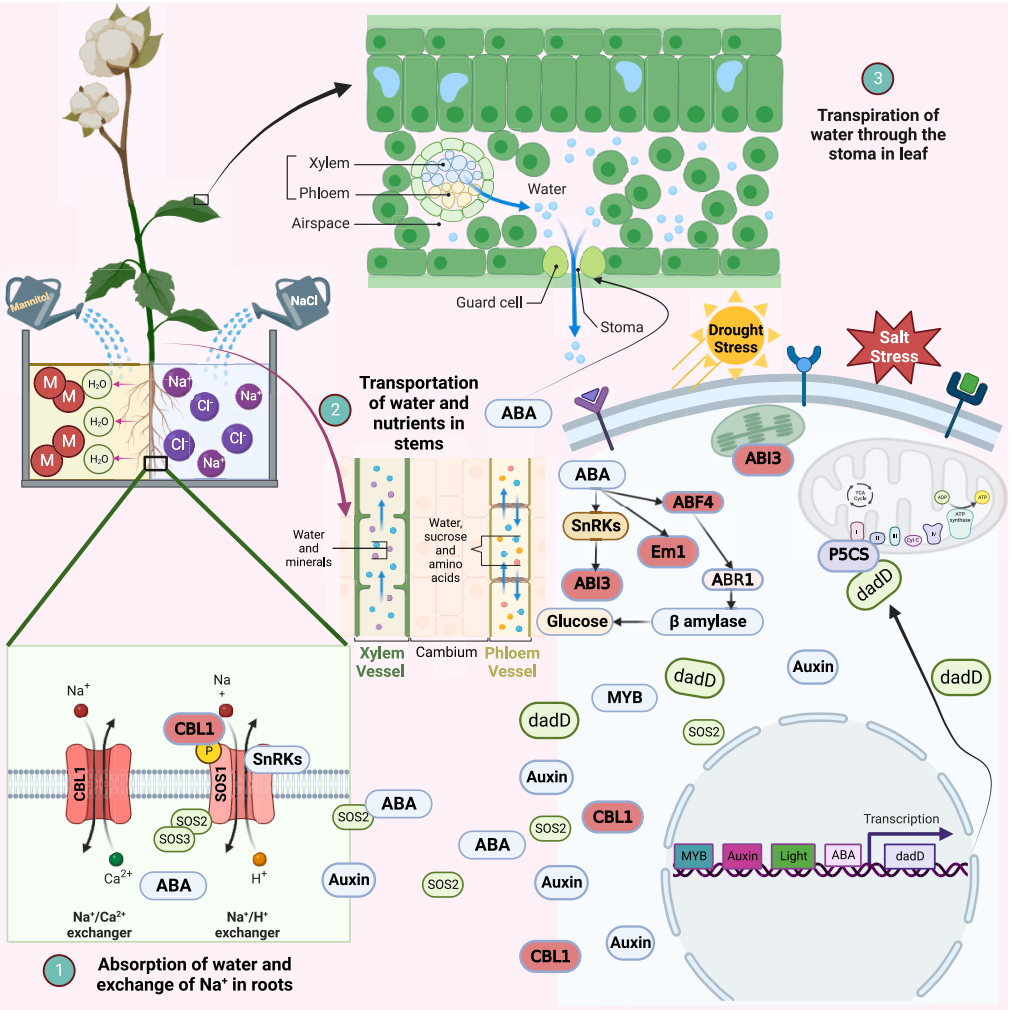

Figure 11. Schematic diagram of *GhdadD* osmotic stress resistance mechanism

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108664.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.L. and X.C.; methodology, Y.Z.; validation, Y.Z., S.M.L., and R.O.M.; resources, Z.Z.; writing—original draft preparation, Y.Z.; writing—review and editing, J.Z., and Y.H.; supervision, F.L.; project administration, F.L. and X.C.; funding acquisition, F.L. and X.C. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
DH5α Chemically Competent Cell	WEIDI	DL1001
GV3101(pSoup-p19) Chemically Competent Cell	WEIDI	AC1003
LBA4404 Chemically Competent Cell	WEIDI	AC1030
Chemicals, peptides, and recombinant proteins		
RNAprep Pure Plant Plus Kit (Polysaccharides & Polyphenolics-rich)	TIANGEN	DP441
TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix	TransGen	AT311
ChamQ Universal SYBR qPCR Master Mix	Vazyme	Q711
Phanta Max Super-Fidelity DNA Polymerase	Vazyme	P505
Peasy-Uni Seamless Cloning and Assembly Kit	TransGen	CU101
Plant Soluble Sugar Content Assay Kit	Solarbio	BC0030
Catalase (CAT) Activity Assay Kit	Solarbio	BC0200
Malondialdehyde (MDA) Content Assay Kit	Solarbio	BC0020
Superoxide Dismutase (SOD) Activity Assay Kit	Solarbio	BC5160
Trypan Blue	Solarbio	T8070
DAB chromogenic kit	Nanjing Jiancheng	W026-1
Surfactant L-77	Solarbio	S9430
PEG6000	Solarbio	P8250
Chloral hydrate	Aladdin	C104202
L-Lactic Acid	Aladdin	L107596
Phenol	Aladdin	P100762
Glycerol	Aladdin	G116203
Experimental models: Organisms/strains		
G. hirsutum cultivar CRI-12	Institute of Cotton Research of CAAS	N/A
A. thaliana (Col-0)	Institute of Cotton Research of CAAS	N/A
N. benthamiana	Institute of Cotton Research of CAAS	N/A
Oligonucleotides		
Primers used are shown in Table S1	This paper	N/A
Recombinant DNA		
Plasmid: p2300-eGFP-*GhdadD*	This paper	N/A
Plasmid: pClCrV-*GhdadD*	This paper	N/A
Plasmid: pBI121-*GhdadD*	This paper	N/A
Software and algorithms		
TBtools	Chen et al.[52]	https://github.com/CJ-Chen/TBtools/releases; RRID: SCR_023018
GraphPad Prism9	Open source	https://www.graphpad.com; RRID: SCR_002798
ImageJ	Open source	https://ImageJ.nih.gov/ij/; RRID: SCR_003070

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Xiaoyan Cai (caixy@cricaas.com.cn).

Materials availability

Materials generated in this study are available upon request. For further details contact the lead contact.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

The *G. hirsutum* cultivar CRI-12, *A. thaliana* (Col-0) and *N. benthamiana* were utilized in this study and obtained from the Institute of Cotton Research of Chinese Academy of Agricultural Science. Seeds of CRI-12 and tobacco were germinated at 28°C in dark conditions, and then transplanted into nutrient media in the 16h light/8h dark at 28°C. For Arabidopsis, the seeds were first sterilized and grown on a 1/2 Morishige and Skoog (MS) medium, vernalized at 4°Cin dark for 2 days and cultured in 22°C (16 h light/8 h dark) growth chamber for 1 week. Subsequently, transplanted into a mixture of nutrient soil and vermiculite with a 1:1 ratio.

For transgenic Arabidopsis, series concentration of NaCl and mannitol was added to 1/2MS medium to simulate salt and drought stress before germination. At the seedling stage, 200 mM NaCl was irrigated to soil for salt treatment, renewed every 5 days and 20% PEG6000 for drought treatment. After 10 days, phenotype photographs were taken. For cotton, 400 mM NaCl treatment was obtained at the 5 true leaf stages, and water thirsty for drought stress. After 3 days of salt stress and 10 days of drought stress, samples were collected for further detection and phenotypes observation.

METHOD DETAILS

Bioinformatics analysis of GhdadD

The cDNA sequence and genome sequence of the *GhdadD* were submitted to the GSDS2.0 (http://gsds.gao-lab.org/) website for gene structure analyze. The conserved domains of proteins were predicted by NCBI-CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and Pfam (http://pfam.xfam.org/), and online tool MEME (http://meme-suite.org/meme/) was used to identify the conserved motifs. The results were visualized by using TBtools software.[52] Physio-biochemical properties and secondary structure of GhdadD were analyzed with ProtParam (http://web.expasy.org/protparam/) and SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_sopma.pl) respectively. SignalP5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predicate the signal peptides. Transmembrane structures of GhdadD were analyzed with TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). SWISSMODEL (http://swissmodel.expasy.org/) was used to predict protein tertiary structure. Blastp (website) was used for searching homologous sequences of other species, and multiple sequence alignment by DNAMAN. The phylogenetic tree was plotted using MEGA7 software.

Subcellular localization of GhdadD in tobacco

P2300-eGFP-Flag was used to construct a fluorescent protein report plasmid i.e., GhdadD-GFP, and transfected into Arabidopsis protoplasts with the nuclear fluorescently-labeled organelle marker to investigate its subcellular localization. The procedure of the protoplasts transformation are shown in ref.[53] . Laser scanning confocal microscope (ZEISS LSM880) was used to observe the fluorescence signals.

Ectopic expression of *GhdadD* in Arabidopsis

The *GhdadD* coding sequence with *Xba* I and *Bam*H I (BioLabs, Beijing) were first amplified by Phanta Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing). Then, it was used to inserted into pBI121 plasmid to construct 35S: *GhdadD* vector by using Peasy-Uni Seamless Cloning and Assembly Kit (TransGen, Beijing). The resultant construct was sequenced (Sangon, Shanghai) to ensure that no mutations had been introduced and then introduced into Agrobacterium tumefaciens strain GV3101.Transgenic Arabidopsis were obtained using the floral-dip method.[54] The produced seeds were selected on 1/2 MS medium containing 50 μg/mL kanamycin until T2 generation. T3 generation homozygous lines were further confirmed by PCR reactions on T2 generation using specific primers (Table S1). qRT-PCR experiment was used to detect the expression level of *GhdadD* in T2 generation, and the lines with high expression levels were selected for phenotyping.

Seed germination and root elongation assays

The seeds of three transgenic lines-OE9, OE10 and OE11 were plated in the 1/2 MS media described in 2.2, 100 seeds per line. Seed germination rate was recorded after 10 days of growth. For root elongation assays, seeds were plated in a straight line on different 1/2 MS medias respectively, 10 seeds per line. The medium was placed vertically, root length was recorded after 10 days.

Measurement of cell damage and physio-biochemical attributes

Ion permeability was determined using 10 rosette leaves with identical size.[55] Soluble sugar content and antica oxidants include Catalase (CAT), Malondialdehyde (MDA) and Superoxide Dismutase (SOD) detection (Solarbio, Beijing) were performed before and after treatment following the procedures of manufacturer''s instructions.

DAB (3,3'-diaminobenzidine) and trypan blue staining experiment were performed to observe leaf cells injury caused by drought and salt stress. DAB chromogenic kit (Nanjing Jiancheng, Nanjing) was used to stain plant leaves to determine the accumulation of peroxidase in leaves, following the procedure described in ref.[56]. For trypan blue staining, the staining solution was first prepared according to the proportions of 10 ml lactic acid, 10 ml glycerol, 10 g phenol, 10 mg trypan blue (Solarbio, Beijing) and 10 ml distilled water; then soaked plant leaves in this staining solution, bath in a boiling water for 2 minutes, cooled at room temperature, and decolorized in chloral hydrate (1.25g/ml). Daily replacement of decolorization solution was performed until the background color is eliminated.

Virus-induced gene silencing (VIGS) of *GhdadD*

For VIGS experiment, the ClCrCA system was used. A 340-bp fragment of *GhdadD* with *Spe* I and *Asc* I was cloned into the plasmid to generate ClCrV: *GhdadD* vector. The resultant construct was then introduced into Agrobacterium tumefaciens strain LBA4404. We followed the procedures described by Corbin et al.[57] to prepare the bacteria inoculum and inoculate it on cotyledons of cotton. The ClCrV: *ChlI* vector was used as positive control to get reliable results and ClCrV: 00 was negative control.

Observation of cotton stem and leaf traits

For cotton stomata observation, tweezers were used to tear the cells of the upper epidermis from the cotton leaves directly and gently, then spread the torn cells on the slide with water and covered with coverslip,[58] observed and photos were taken under the fluorescent microscope, and use ImageJ software to measure the stomatal opening of the leaves.

According to the method described by Kong et al.,[59] paraffin sections of cotton stems and leaves were prepared, and after the paraffin wax in the tissues were eluted, the paraffin sections were observed and photographed under the stereomicroscope.

Quantitative RT-PCR analysis

Total RNA was extracted using RNAprep pure Plant Kit (Tiangen, Beijing). The obtained high-quality RNA was reverse-transcribed to synthesize first-strand cDNA using TransScript All-in-One First-Strand cDNA Synthesis Super Mix for qPCR (TransGen, Beijing). qRT-PCR experiments were performed using the 2×SYBR qPCR Master Mix (Vazyme, Nanjing) on ABI 7500 fast platform. Three biological replicates and three technical repetitions were used for each sample. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method.[60] The primer sequences are listed in Table S1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Bioinformatic analysis was described in the method details section. The statistical analysis was performed via LSD (Fisher's Least Significant Difference) method at the 5% probability level, to verify the significance of differences among samples. All the experiments were carried out in three replications.