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A cysteine-rich transmembrane module peptide *GhCYSTM9* is involved in cold stress response

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Abstract

Background Cysteine-rich transmembrane module (CYSTM) peptides, which are widely distributed and highly conserved in eukaryotes, are largely involved in stress response and defence. However, the role of cotton *CYSTM* genes in the stress response has not been functionally characterized.

Results In this study, we identified *GhCYSTM9* as a cold stress-responsive *CYSTM* member from upland cotton. Compared with that in control cotton plants, *GhCYSTM9* silencing in cotton resulted in reduced tolerance under cold stress, accompanied by higher MDA contents and lower proline contents and SOD activities in leaves. Overexpressing *GhCYTMS9* in *Arabidopsis* significantly increased the seed germination rates and root elongation at the germination stage. Compared with wild-type seedlings, *GhCYSTM9*-overexpressing seedlings presented lower MDA contents and greater proline contents in leaves under cold stress. Transcriptome analysis of transgenic *Arabidopsis* revealed that *GhCYSTM9* may contribute to the cold response by regulating oxidative stress-related genes to mediate ROS levels. Yeast two-hybrid and bimolecular fluorescence complementation assays confirmed that GhCYSTM9 interacted with the light-harvesting chlorophyll *a/b*-binding protein GhLHBC2A1.

Conclusions Overall, our results revealed a positive role of *GhCYSTM9* in cold stress defence and suggested candidate genes for the genetic breeding of cold defence.

Keywords *GhCYSTM9*, Cysteine-rich peptide, Cold stress, *Gossypium hirsutum*

Background

As sessile organisms, plants often encounter adverse environmental conditions such as extremely low and high temperatures, drought, and high salinity. Cold stress is an important adverse factor for plants, which impacts not only agricultural production, such as seed germination, seedling emergence, and plant growth and development, but also geographical distribution [1]. After exposure to cold stress, cell membrane fluidity decreases; thus, membrane proteins that sense these changes trigger a series of signal transductions [2, 3]. As major signal messengers that mediate the cold stress response, ROS play dual roles, with a low level activated the defence responses at early stages, and with a high level began to injure the cell membrane by triggering oxidative stress and leading to the breakdown of defence [4]. The intricate transcriptional regulatory mechanism of the cold response is far from fully understood. Mining for novel cold tolerance-related candidate genes is highly important. Plant small

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peptides, which are composed of fewer than 100 amino acids, are brought into focus on account of their key roles in regulating biological processes related to plant development and stress defence [5]. For example, rapid alkalinization factor (RALF) [6, 7], phytosulfokine-α (PSK) [8, 9], plant peptide containing sulfated tyrosine (PSY) [10], clavata3/embryo-surrounding region-related (CLE) [11, 12] and plant elicitor peptide (PEP) [13] have been revealed to modulate various stress defence processes.

The cysteine-rich transmembrane module (CYSTM) family peptides are speculated to constitute a group of poorly characterized cysteine-rich non secreted small peptides [14, 15]. Studies have revealed that CYSTMs are widely distributed and conserved in eukaryotes and that they possess a cysteine-rich C-terminal domain and a unique non secreted N-terminal cytoplasmic element that differs from the N-terminal secretion signal peptide of a cysteine-rich secreted peptide [14, 15]. The C-terminal CYSTM module is composed of a transmembrane (TM) helix with 5–6 cysteines and a widely conserved acidic residue among this family, which is a hallmark of the CYSTM family [15]. The conserved TM module of the CYSTM peptide might be crucial for its conserved roles in the stress response [15, 16].

Xu et al. [13] identified 13 *CYSTM* genes in *Arabidopsis thaliana* and addressed their dramatic response to various abiotic stresses, which suggested a potential function in stress defence. Moreover, there are 10 *CrCYSTM* members in *Canavalia rosea*, whose expression obviously differs under extreme abiotic stress, indicating their diverse roles [17]. In addition, members of this family from many plants have been independently implicated in a broad range of biological processes related to plant development and stress defence. These include the *bs5* gene from pepper, which encodes a 2 amino acids deletion variant in the C-terminal TM domain that has been shown to be a good candidate for resistance against *Xanthomonas euvesicatoria (Xe)*, indicating that deletion of the TM domain affects gene function [18]. Furthermore, CRISPR/Cas9-mediated editing of *Bs5* and *Bs5L* in tomato led to *Xanthomonas* resistance [19]. A group of *CYSTM* members with SA-responsive expression, known as pathogen-induced cysteine-rich transmembrane proteins (PCMs), were shown to increase disease resistance and hypocotyl elongation [20]. *PCC1* encodes *Pathogen and Circadian Controlled 1* in *Arabidopsis* and is one of the most extensively studied *CYSTM* members. *PCC1* participates not only in response to pathogen-induced defence [21] but also in the modulation of the polar lipid content, stress-induced flowering, and ABA-mediated response at different developmental stages [22–24]. *AtCYSTM3* negatively regulates salt resistance by increasing ROS levels [25], whereas *AtCYSTM1* (*At1g05340*) and its paralogue *AtCYSTM6* (*At2g32210*) improve thermotolerance against oxidative stress by balancing ROS levels [26]. Although *CYSTM* members are important for protecting plants from various stresses, the possible regulatory modes of *CYSTM* genes remain unclear. The diverse functions and potential molecular regulatory mechanisms of *CYSTMs* in stress defence, particularly in cold stress, deserve to be elucidated.

As a plant originating from tropical and subtropical regions, cotton is sensitive to low temperatures during the seed germination and emergence stages in spring and the flowering and boll maturation stages in autumn. It has a series of adverse impacts on plant growth, which ultimately leads to low yield and quality of cotton production [27]. Recently, major cold-related genes and transcription factors identified in model plants were characterized by transgenic or gene editing methods in cotton as well [28–33], leading to a better understanding of the signaling transduction and regulatory networks of cotton at low temperatures. However, research on the gene identification and molecular regulation of low-temperature stress in cotton is insufficient. Mining more novel genes that function in the cold tolerance of cotton and elucidating the underlying molecular mechanism will help in the development of effective strategies to compensate for the negative impacts of cold on cotton production.

In this study, we investigated the function of *GhCYSTM9* in the cold stress response. Virus-induced gene silencing (VIGS) of *GhCYSTM9* in cotton resulted in decreased low-temperature tolerance in TRV2:*GhCYSTM9*-targeted plants. In addition, we observed its positive role under cold stress in transgenic *Arabidopsis*. Furthermore, transcriptome analysis of the *GhCYSTM9*-overexpressing transgenic lines suggested that *GhCYSTM9* enhanced cold tolerance primarily by modulating oxidative stress-related genes. To elucidate the possible molecular mechanism of GhCYSTM9, we characterized its interacting protein GhLHCB2A1 by yeast two-hybrid (Y2H) and biomolecular fluorescence complementation (BiFC) assays. Our study provides a theoretical foundation for understanding the regulatory mechanism of *GhCYSTM9* and suggests candidate genes for the genetic improvement of cotton.

Materials and methods

Plant materials

The coding sequence of *GhCYSTM9* was amplified from the leaf cDNA of *Gossypium hirsutum* acc. Texas Marker-1 (TM-1). The cotton cultivar *G. hirsutum* TM-1 was also used for inoculation and as the experimental control material in the VIGS assay. The TRV: 00-targeted cotton plants were used as the negative control. The VIGS-silenced TRV: *GhCYSTM9* cotton plants whose expression level of *GhCYSTM9* was less than 30% of that of the negative control plants were used for cold

treatment. The *Arabidopsis thaliana* ecotype Columbia (Col-0) was used for stable transformation. Seeds from the T_4 generation of three independent homozygous *GhCYSTM9*-overexpression transgenic *Arabidopsis* lines, OE5, OE10 and OE14, were used for cold tolerance analysis. The *Arabidopsis thaliana* ecotype Columbia (Col-0) in wild-type (WT) was used as an experimental control for functional analysis.

Silencing of *GhCYSTM9* in upland cotton

The SGN VIGS tool (https://vigs.solgenomics.net/) [34] was used to design the best targeted region. As a result, the full-length CDS of *GhCYSTM9* was returned and inserted into the TRV-based vector to obtain the TRV: *GhCYSTM9*. Primers used in this study are listed in Table S1. The VIGS assay was executed as described by Yang et al. [35]. The *Agrobacterium tumefaciens* strain GV3101 containing TRV: 00, TRV: *GhCLA1* and TRV: *GhCYSTM9* were respectively injected into the cotyledons of cotton seedlings with the help of pYL192-containing strain at a ratio of 1:1. The TRV: 00-targeted plants and TRV: *GhCLA1*-targeted plants were used as negative controls and positive controls, respectively. Samples were collected from the leaves of the negative control and VIGS-silenced TRV: *GhCYSTM9* seedlings at the two-leaf stage after inoculation for 14 days. The leaves of three cotton plants were mixed as a biological repeat. A total of three biological replicates were collected. The relative expression level of *GhCYSTM9* was detected via qRT−PCR to assess the VIGS efficiency.

Four weeks post-inoculation, the VIGS-silenced TRV: *GhCYSTM9* cotton plants and the TRV: 00-targeted at the four-leaf stage were introduced to a temperature of 4 °C for cold treatment. Phenotypic changes were observed after 10 days of treatment. Water loss assay was carried out by weighing the leaves of the 2nd leaves from the top every hour after 3 days of treatment. The water loss rate was calculated as the weight of water lost relative to the weight of fresh leaves. Physiological variation was evaluated by measuring the contents of the redox indicators MDA and proline in the 2nd leaf from the top after 5 days of treatment. Three independent plants were sampled and mixed as a biological replicate. Three biological replicates were detected for each indicator.

Overexpressing of *GhCYSTM9* in *Arabidopsis thaliana*

The 273-bp coding sequence of *GhCYSTM9* was amplified and inserted into the binary vector pART-CAM with the 35 S promoter for overexpression. The primers used in this study are listed in Table S1. The recombinant expression vector pART-CAM-GhCYSTM9 was transformed into *A. tumefaciens* strain GV3101. Plants of wild-type (WT) *Arabidopsis* Col-0 were transformed using the floral-dip method [36]. Seeds of the T_1 to T_3 generations were selected on half-strength Murashige and Skoog (1/2 MS) media supplemented with 50 $\mu g \cdot mL^{-1}$ kanamycin, and the plants were confirmed to be transgenic via reverse transcription polymerase chain reaction (RT−PCR). Three independent homozygous transgenic lines, OE5, OE10 and OE14, were used for cold tolerance analysis.

Functional analysis of *GhCYSTM9* Transgenic *Arabidopsis* under cold treatment

Seeds of the transgenic lines and WT were surface sterilized and inoculated on 1/2 MS media. The cultures were maintained at 4 °C with a 16 h photoperiod for the germination assay under cold stress, while cultures maintained at 22 °C with a 16 h photoperiod were used as controls. The plants grown under normal conditions for 7 days after inoculation were transferred to a new plate. The lengths of the roots of plantlets maintained at 4 °C for 2 weeks were examined, as well as those of the control plantlets that were maintained at 22 °C with a 16 h photoperiod. Germination rates and root length were examined on the 8th and 14th days of cold stress respectively. For the germination rate assay, 30 seeds of each line inoculated on the same plate were used as replicates. For the root length assay, the average root length of 10 seedlings was collected as a biological replicate. Three biological replicates were used to calculate the survival rate and root length.

Seedlings of the three *Arabidopsis* transgenic lines and the WT grown on the soil in the greenhouse at 22 °C for 4 weeks were subjected to cold treatment by holding at -10 °C for 3 h followed by 4 °C for 4 h. Samples for MDA and proline content measurements were collected immediately after cold stress treatment from each transgenic lines, and were assayed according to the instructions of kits (Suzhou Comin, Suzhou, China). The treated seedlings were then returned to 22 °C for 2 weeks to calculate the survival rates after recovery. Each replicate consisted of a minimum of 10 seedlings in three pots. Three biological replicates were used for survival rate calculations. Significant differences were analysed using DPS 15.0 software. The error bars represent the standard errors (SEs) of three biological replicates with three technical replicates.

Transcriptome analysis of transgenic *Arabidopsis*

Total RNA was extracted from 2-week-old seedlings of the *GhCYSTM9* transgenic line OE5 and WT plants treated at 4–22 °C for 6 h. Four RNA-seq library preparations and sequencing were performed by Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). Data analysis was carried out as previously described [37]. Differential expression analysis of genes was performed using the DESeq2 package. Differentially expressed genes

(DEGs) were defined as those with a value of| $\log_2$ (fold change)| ≥1 and p value < 0.05. The raw data have been deposited in NCBI under the BioProject accession number PRJNA1034306. The expression of ten selected DEGs was analysed by quantitative real-time PCR (qRT−PCR) to verify the RNA-seq results. All DEGs were subjected to enrichment analysis of Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways via KOBAS-i online tool (http://bioinfo.org/kobas) [38] with a threshold of p-value < 0.05.

Y2H and BiFC assays

The bait vector pGBKT7-GhCYSTM9 was constructed to screen the cotton abiotic stress-induced cDNA library in yeast. GhLHCB2A1, a highly reliable candidate screened from the yeast library, was subsequently cloned and inserted into pGADT7 as prey. The bait and prey vectors were co-transformed into yeast strain Y2H cells. The interaction relationship was determined by the growth of yeast cells on SD/-Leu/-Trp (DDO) media and SD/-Leu/-Trp/-His/-Ade/X—gal/AbA (QDO/X/A) media at 30 °C for 3 days.

For the Bimolecular Fluorescence Complementation (BiFC) assay, the CDSs of *GhCYSTM9* and *GhLHCB2A1* were cloned and inserted into the pSm35s-ccdb-cYFP and pSM35s-nYFP-ccdb vectors and transformed into *A. tumefaciens* strain GV3101, respectively. The BiFC assay was performed as previously described by Song et al. [39]. YFP fluorescence in the epidermal cells of *Nicotiana benthamiana* was observed after incubation for 48 h using a confocal microscope (Leica, TCS SP8, Wetzlar, Germany).

RNA extraction and qRT–PCR

Total RNA extracted by the RNAprep Pure Kit (Tiangen, Beijing, China) was transcribed to first-strand cDNA using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). The resulting cDNA was diluted to 100 ng. The qRT−PCR reactions were performed using a TB Green® Premix Ex Taq™ II kit (TaKaRa, Dalian, China) on a CFX96 Real-Time PCR System (Bio-Rad, CA, USA) to assess the relative expression levels via the $2^{-\Delta\Delta Ct}$ method [40]. *GhHistone3* (AF024716) and *AtActin2* were used as housekeeping genes. Primers used are listed in Table S1. Three biological replicates were employed for gene expression analysis before data normalization. Significant differences were analysed at the $p < 0.05$ level using DPS 15.0 software with one-way ANOVA.

Results

Silencing of *CYSTM9* by VIGS in cotton reduced resistance to cold stress

GhCYSTM9 was found to be induced under cold, drought and salt stress and was cloned in a previous study [41]. This study aimed to identify the potential role of the *GhCYSTM9* gene in the cold stress response. Owing to the long period of gene transformation in cotton, a VIGS assay was performed to explore the function of *GhCYSTM9* in cold defence in cotton. At 14 d after inoculation, the leaves of the TRV: *GhCLA1*-targeted positive control plants had lost their green colour Fig. 1A). Meanwhile, qRT−PCR analysis of the *GhCYSTM9* expression level in the negative control plants TRV:00 and TRV: *GhCYSTM9*-silenced plants was conducted to evaluate the silencing efficiency of the VIGS array. The results revealed highly effective silencing of *GhCYSTM9* in cotton plants Fig. 1B). The *GhCYSTM9*-silenced plants and control plants were then exposed to cold stress after inoculation for 4 weeks Fig. 1C). Water loss and eventual drying of the leaves of both the silenced plants and the control plants were observed after cold treatment for 7 d Fig. 1D). However, there were obvious differences in the symptoms of younger leaves between the silenced plants and the controls. The outer margin of the leaf blade turned atrophic and coiled heavily in the silenced plants compared with the controls. Compared with that in TRV:00 leaves, cold stress resulted in significantly greater water loss rates in silenced plant leaves ($p < 0.05$) Fig. 1E). Cold stress causes damage to plant cells and eventually leads to increases in the MDA and proline contents. Differential analysis between the silenced and control plants revealed that cold stress resulted in significantly greater MDA contents and lower proline contents in the leaves of the silenced plants than in those of the control plants ($p < 0.05$) Fig. 1F, 1G). The results of the VIGS assay indicated that *GhCYSTM9*-silenced plants presented a loss of cold tolerance.

Overexpression of *CYSTM9* in *Arabidopsis* resulted in increased cold tolerance

An overexpression vector was generated and transformed into *A. thaliana*. Then, 3 transgenic lines of *GhCYSTM9*-overexpressing *Arabidopsis* (OE5, OE10 and OE14) that were identified by reverse transcription PCR (RT−PCR) (Fig. 2A) and qRT−PCR (Fig. 2B) were planted until the T_3 homozygous generation was reached. The cold tolerance of the OE lines and WT plants at the germination stage was evaluated by examining the germination ratios and root length under cold stress at 4 °C. Seeds of the WT and OE lines were 100% germinated and grew normally under control conditions (22 °C) (Fig. 2C). However, obvious inhibition of germination and growth was observed in seeds treated at 4 °C. After 8 d under 4 °C stress, the seeds of the OE lines began to germinate and turn white, whereas most of the WT seeds maintained the same status as those at the time of inoculation (Fig. 2C). The germination rates of the OE lines on the 8th day after inoculation were significantly greater than

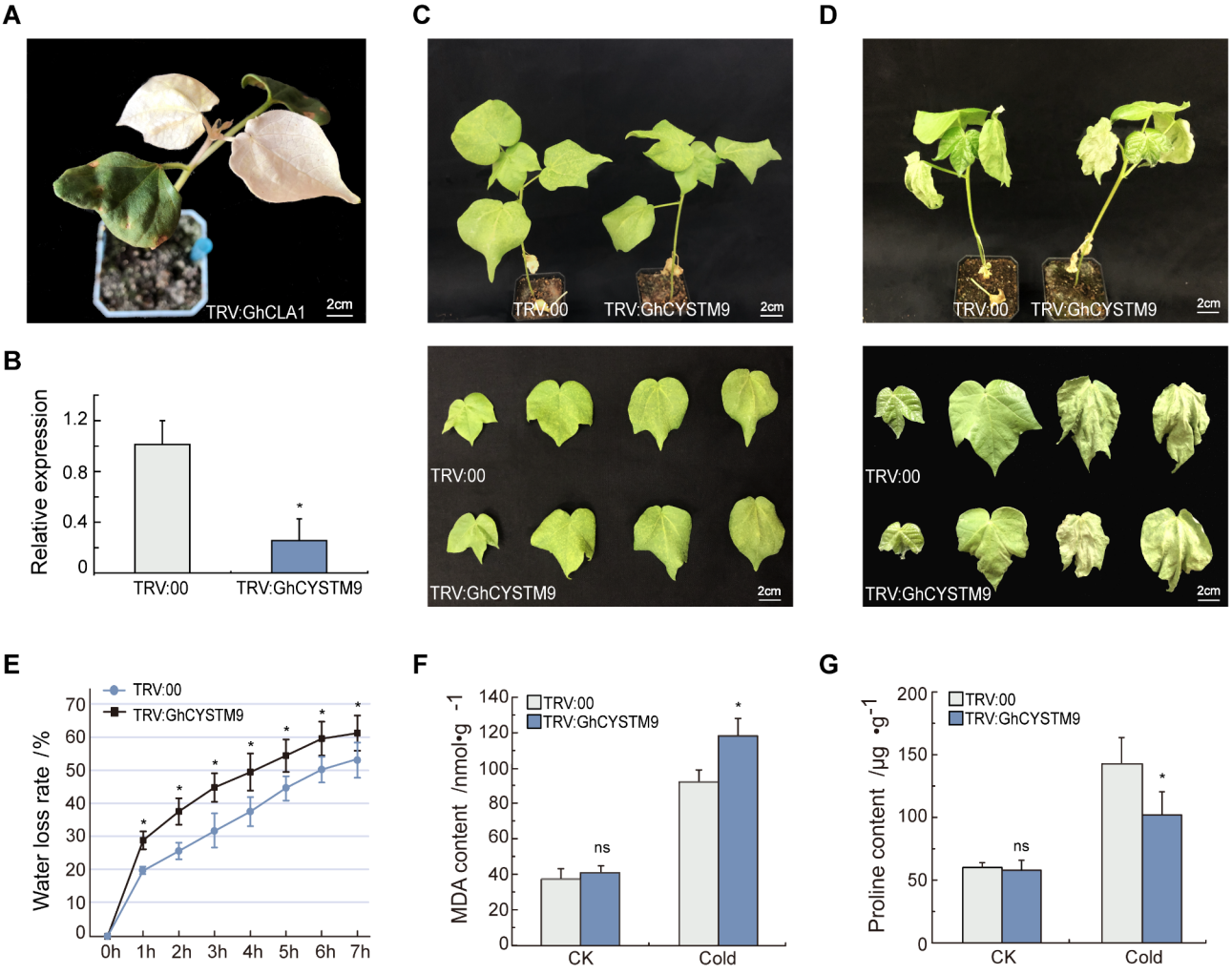

Fig. 1 *GhCYSTM9* silencing in cotton by VIGS resulted in decreased resistance to cold stress. (**A**) TRV: *CLA1*-injected plants showed a bleached phenotype in their leaves. (**B**) *GhCYSTM9* expression in silenced plants was validated via qRT–PCR. (**C**) Phenotype of plants before cold stress treatment. (**D**) Phenotypes of the plants 10 days after cold treatment. (**E**) Water loss, (**F**) the MDA contents and (**G**) the proline contents were measured after 5 days of cold treatment. The * symbol indicates a significant difference between *GhCYSTM9*-silenced plants and control plants injected with TRV:00 ($p < 0.05$)

those of the WT plants ($p < 0.05$) (Fig. 2D). Similarly, the root length of the OE lines was significantly longer than that of the WT after they were grown on media for 14 d at 4 °C ($p < 0.05$) (Fig. 2E and F).

The WT and OE lines at the seedling stage were also observed to further confirm the potential role of *GhCYSTM9* in cold resistance. The phenotypes of the WT and OE seedlings under normal conditions were not obviously different. However, just at the end of cold treatment, the leaves of the transgenic and WT plants that experienced stress were frostbitten and looked duller in colour. After away from cold treatment for 10 d, some plants recovered from cold injury to maintain growth (Fig. 3A). The survival rates of the OE lines varied from 21 to 35%, which was significantly greater than that of the WT (5%) (Fig. 3B). The MDA and proline contents were measured to evaluate physiological resistance to cold stress. Following cold treatment at -10 °C for 4 h and then at 4 °C for 4 h, the MDA content in OE plants was significantly lower than that in WT plants, and the proline content in OE plants was significantly higher than that in WT plants (Fig. 3C and D). The results of a cold tolerance assay of transgenic and WT *Arabidopsis* revealed that the overexpression of *GhCYSTM9* confers enhanced cold tolerance in *Arabidopsis*.

Transcriptome analysis revealed that *GhCYSTM9* regulates oxidation-reduction-related genes

RNA-seq analysis of WT and OE5 transgenic seedlings exposed to 4 °C for 6 h was conducted to determine the expression profile and potential regulatory network of *GhCYSTM9*. qRT–PCR analysis of ten randomly selected genes confirmed the reliability of the RNA-seq data (Fig. S2). Principal component analysis (PCA) results

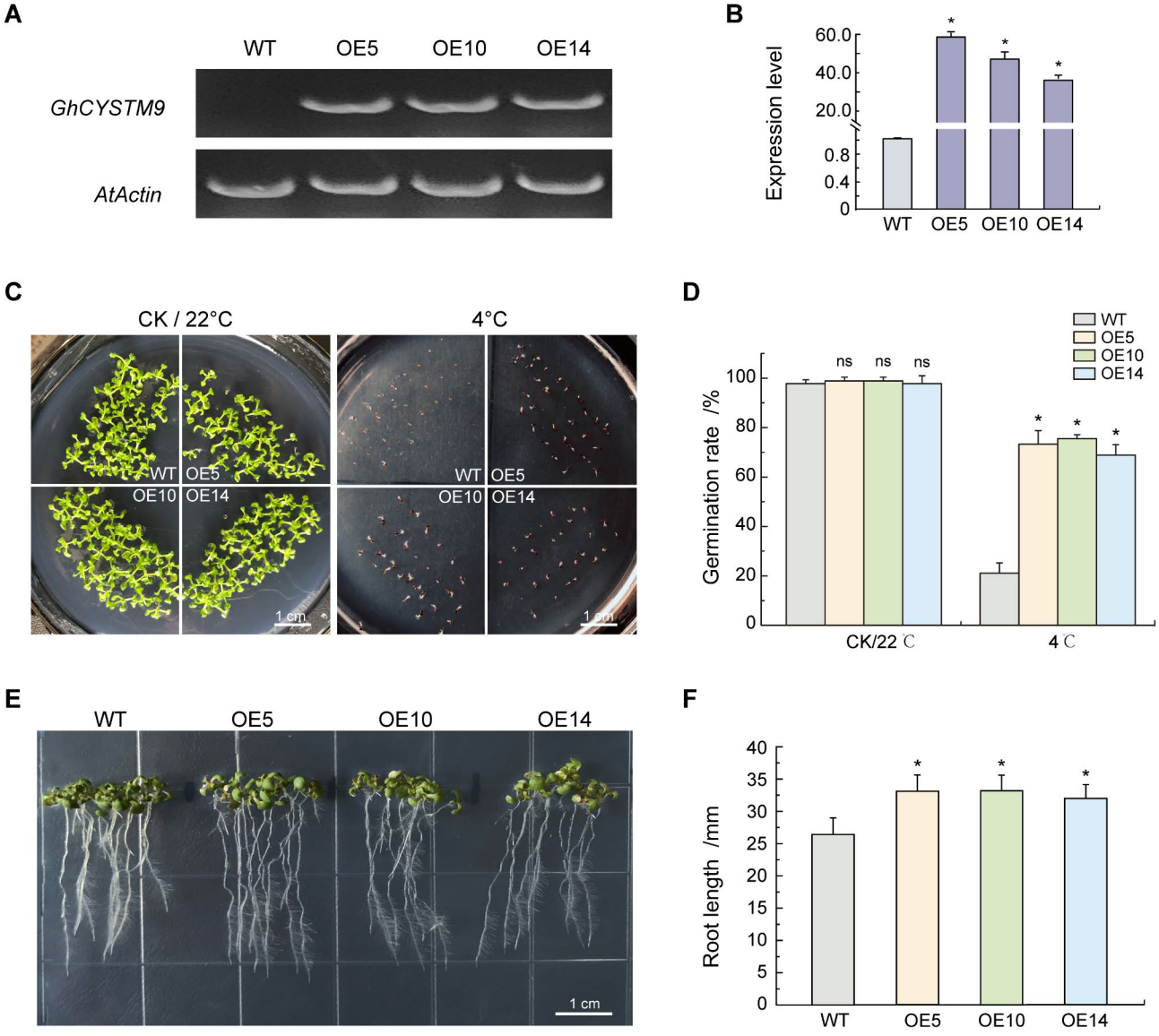

Fig. 2 *GhCYSTM9* transgenic lines of *Arabidopsis* exhibited enhanced cold tolerance at the germination stage. (**A**) RT–PCR identification and (**B**) qRT–PCR analysis of the *GhCYSTM9* gene expression in the wild-type *Arabidopsis* and different *GhCYSTM9*-overexpressing transgenic lines. (**C**) Phenotypes of transgenic and wild-type *Arabidopsis* at the germination stage. (**D**) Germination rates evaluated 8 days after sowing. (**E**) Root elongation under cold stress. (**F**) Root length after cold stress. The * symbol indicates a significant difference between different transgenic lines and the wild-type ($p < 0.05$)

indicated that the correlation between replicates of the same group was normal (Fig. S1). There was a total of 155 DEGs between WT and OE under normal conditions (WTctrl vs. OEctrl), including 126 up-regulated genes and 29 down-regulated genes (Table S2, Fig. S1). After cold treatment, a total of 665 DEGs in the OEcold vs. WTcold comparison were identified, of which 146 genes were up-regulated and 519 genes were down-regulated (Table S3, Fig. S1). A Venn diagram revealed that 57 DEGs overlapped between the two comparison groups (Fig. 4A). The number of DEGs in the OEcold vs. WTcold comparison group was greater than that in the WTctrl vs. OEctrl comparison group, which implies that *GhCYSTM9* is highly involved in the cold response.

GO enrichment analysis revealed that there were significant enrichments in GO terms related to response to stimulus, response to chemicals, response to stress, glutathione transferase activity, response to oxidative stress and so on (Fig. 4B) in the comparison group WTctrl vs. OEctrl. GO terms such as response to hypoxia, response to oxygen levels, response to stimulus and response to stress were significantly enriched in the OEcold vs. WTcold comparison groups (Fig. 4B). These results demonstrate that the overexpression of *GhCYSTM9* in *Arabidopsis* resulted in greater antioxidant capacity by the

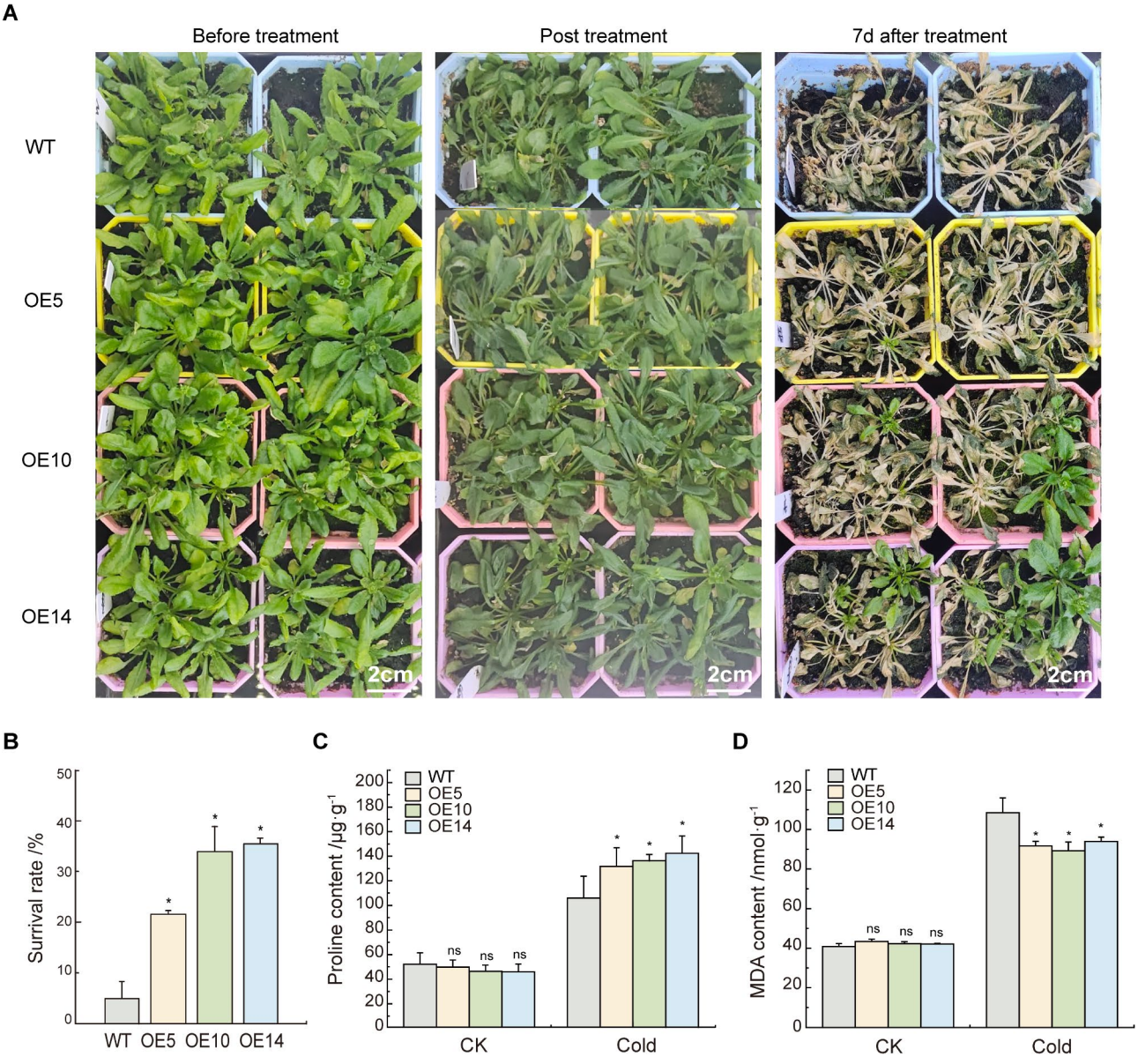

Fig. 3 Seedlings of the *GhCYSTM9* transgenic lines in *Arabidopsis* presented increased cold stress tolerance. (**A**) Phenotypes of transgenic and wild-type seedlings grown in potted soil under cold stress. (**B**) Survival rates were calculated after the plants were protected from cold stress for 10 days. (**C**) Proline content of seedling leaves. (**D**) MDA content of seedling leaves. The * symbol indicates a significant difference between different transgenic lines and the wild-type ($p < 0.05$)

regulation of genes related to processes such as glutathione metabolism and the oxidative stress response.

The top 5 significantly enriched KEGG pathways in the WTctrl vs. OEctrl Glutathione metabolism, Linoleic acid metabolism, ABC transporters, Biosynthesis of unsaturated fatty acids and the MAPK signalling pathway (Fig. 4C). In the OEcold vs. WTcold group, pathways related to the MAPK signalling pathway, Plant-pathogen interaction, Glutathione metabolism, Plant hormone signal transduction and ABC transporters were the most significant (Fig. 4C). Glutathione metabolism, ABC transporters and the MAPK signalling pathway were common in the two comparison groups, suggesting that *GhCYSTM9* is implicated in cold resistance by participating in pathways associated with MAPK signalling cascade transduction, membrane transport, ROS removal and oxidative reactions under cold stress.

Moreover, we found that several key DEGs related to detoxification and ROS elimination processes, such as *DETOXIFICATION/DTX*, *Glutathione S- transferase/GST*, *UDP-glycosyltransferase/UGT* and *Cytochrome P450/CYP*, were significantly induced in OE plants (Fig. 4D). Meanwhile, some key marker genes involved in the abiotic stress response (*RD29A*, *COR15A*, *COR15B*,

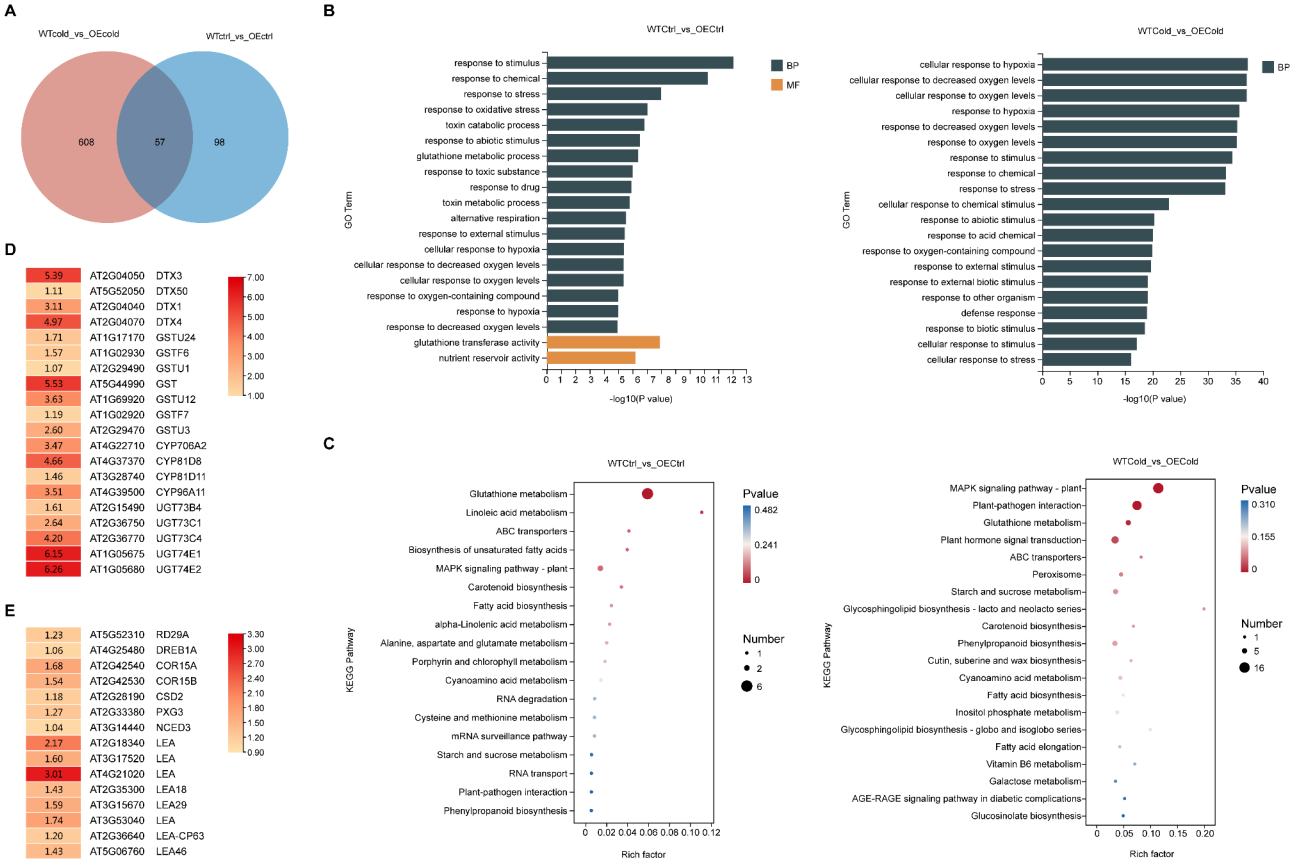

Fig. 4 Transcriptome analysis of *GhCYSTM9* transgenic *Arabidopsis* and wild-type plants. (**A**) Venn diagram displaying the overlap of DEGs from the two comparison groups. (**B**) The top 20 enriched GO terms from the two groups. (**C**) DEG enrichment of KEGG pathways from the two groups. (**D**) DEGs related to detoxification and the ROS elimination process according to RNA-seq. (**E**) DEGs of key marker genes involved in the abiotic stress response and LEA-encoded genes according to RNA-seq

DREB1A, *CSD2*, *PXG3* and *NCED3*) were significantly up-regulated in the samples from the OEcold group (Fig. 4E). These results demonstrated that the ability of *GhCYSTM9* overexpression to increase cold tolerance may be related to oxidation–reduction processes via the activation of antioxidant defence genes. Interestingly, multiple genes encoding late embryogenesis-abundant proteins (LEAs) were significantly induced in OE plants under cold stress (Fig. 4E), suggesting that the overexpression of *GhCYSTM9* may result in the accumulation of many LEA proteins, which help cells resist cold stress by protecting membrane lipids, nucleic acids and proteins.

GhCYSTM9 interacted with GhLHCB2A1

Roots and leaves from seedlings at the 3rd leaf stage treated for 6 h at 4 °C for cold stress, 17% PEG for drought stress and 200 mM NaCl for salt stress were sampled and used for total RNA extraction. Equal amounts of RNA from the samples were mixed and used for abiotic stress-induced cDNA library construction. We screened the cotton cDNA library in yeast using pGBKT7-GhCYSTM9 as the bait vector. Y2H cells containing pGBKT7-GhCYSTM9 and pGADT7 grew and turned blue on QDO/X/A media, which indicated that the GhCYSTM9 protein in yeast clones can be self-activated (Fig. S3A). Further assays on the inhibition of self-activation revealed that self-activation of pGBKT7-GhCYSTM9 could be efficiently inhibited by adding 15 mmol·L^{-1} 3-amino-1,2,4-triazole (3-AT) to the QDO/X/A medium (Fig. S3B). Therefore, QDO/X/A media supplemented with 15 mmol·L^{-1} 3-AT were used to determine the growth of yeast cells for library screening. Finally, the light-harvesting chlorophyll *a/b*-binding protein GhLHCB2A1 (XP_016746917.1) was screened out and considered a promising candidate for interaction with GhCYSTM9, which is involved in the cold stress response. Here, Y2H and BiFC assays were conducted to confirm their interaction relationships. Y2H assays revealed that yeast clones with GhCYSTM9 and GhLHCB2A1 grew and turned blue on QDO/X/A media supplemented with 15 mmol·L^{-1} 3-AT, indicating their veritable interaction relationship in vitro (Fig. 5A). For the BiFC assays, yellow YFP fluorescence

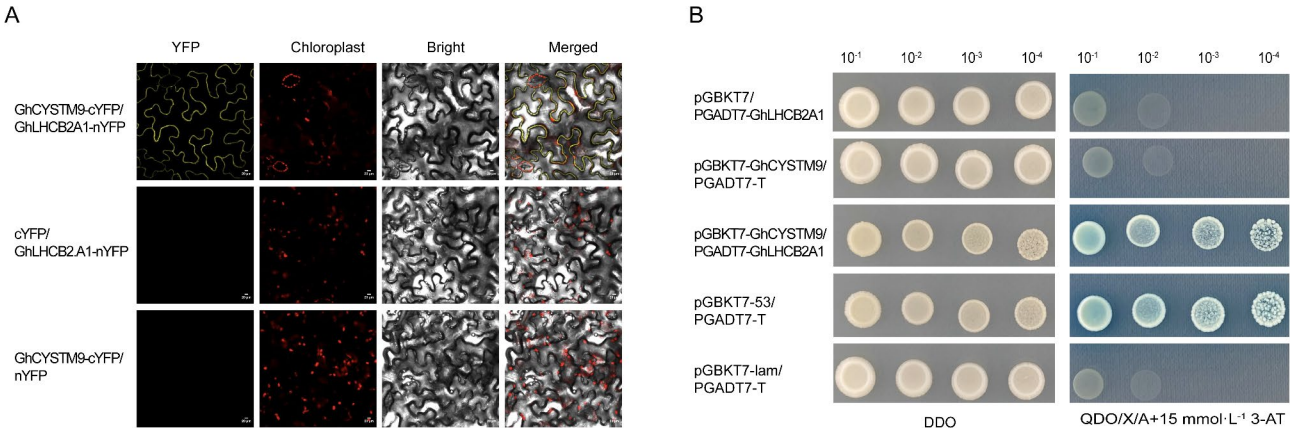

Fig. 5 Protein interactions between GhCYSTM9 and GhLHCB2A1. (**A**) BiFC assays showing yellow fluorescence in the epidermal cells of tobacco leaves transiently expressing GhCYSTM9-nYFP and GhLHCB2A1-cYFP. (**B**) Y2H assay showing yeast growth on QDO/X/A + 15 mmol·L^{-1} 3-AT by co-transforming pGBKT7-GhCYSTM9 and pGADT7-GhLHCB2A1 into yeast cells

was visualized in epidermal cells by transient expression of GhCYSTM9-nYFP and GhLHCB2A1-cYFP in tobacco mesophyll (Fig. 5B), suggesting that GhCYSTM9 and GhLHCB2A1 interact in vivo.

Discussion

GhCYSTM9 served a positive role in cold stress through its involvement in the regulation of oxidative stress

Plant small peptides, which have been proposed as a new class of plant peptide hormones, have recently emerged as major players in multiple stress responses and development [42]. *CYSTM* a novel non-secreted cysteine-rich peptide family, reportedly functions in disease resistance [20, 22], responds to multiple abiotic stresses, such as salt, drought, cold, heat and UV [14, 25, 26], and is related to photomorphogenesis and development [20]. *GhCYSTM9*, a homologue of *AtCYSTM9*, was previously cloned and found to be preferentially expressed under abiotic stresses such as cold, drought and salt [41], which warrants further research.

ROS play an important role in the plant acclimation process. Moderate amounts of ROS are beneficial to plants, enabling them to make positive adjustment of metabolism during stress [43]. Excessive ROS accumulation can trigger a series of physiological damage to cells, including DNA, RNA, and proteins injury and membranes oxidation [44]. SOD is an important enzyme in the ROS scavenging mechanism [45, 46]. The MDA content and proline content are two key indicators used to assess the level of oxidative stress [47, 48]. In this study, we overexpressed *GhCYSTM9* in *Arabidopsis*, which helps increase the accumulation of proline and inhibit MDA production in *Arabidopsis* seedlings under cold stress (Fig. 3C and D). The results of these indicators in VIGS-silenced cotton plants were reversed (Fig. 1F and G). These results demonstrate that the enhanced cold tolerance of the *GhCYSTM9*-overexpressing plants was typically associated with decreased ROS production and efficient ROS scavenging, which affected ROS-mediated redox signalling.

Transcriptome analysis revealed significant enrichment of GO terms related to the response to stimulus, oxidative stress and glutathione transferase activity in the two comparison groups (Fig. 4B). These findings suggest that *GhCYSTM9* is involved in the cold stress response by reducing oxidative stress through the regulation of ROS levels, which is in accordance with previous findings concerning some *CYSTM* members [14, 25, 26]. KEGG pathway enrichment analysis showed that Glutathione metabolism, ABC transporters and MAPK signalling pathway were common in both comparison groups, implying that *GhCYSTM9* is closely associated with MAPK signalling cascade transduction, membrane transport, ROS removal and oxidative reactions. However, the potential elaborate mechanism provided by *GhCYSTM9* is worthy of attention.

AtCYSTM3, as a negative regulator, was found to be involved in salt tolerance by suppressing the activities of ROS-scavenging enzymes [25]. *AtCYSTM1* and *AtCYSTM6* enhance heat stress tolerance by reducing ROS levels [26]. Pereira Mendes et al. [20] reported that the *Arabidopsis* overexpression lines of *PCM1–PCM8* (except for *PCM6*) resulted in decreased resistance to the bacterium *H. arabidiopsis* (*Hpa* Noco2), whereas overexpressing *PCM1–PCM3* but not other genes reduced the spore formation of *P. syringae* pv. *tomato* DC3000 (Pto DC3000), suggesting that only *PCM1–PCM3* is responsible for protection against *Pto* DC3000. In this study, we demonstrated that *GhCYSTM9* enhanced cold tolerance by participating in the activation of antioxidant defence genes in the oxidative stress response. On the basis of these results, it could be speculated that specific *CYSTM* genes from specific species engage in different stress

defences through diverse regulatory pathways, which supports the findings of previous studies [49].

PCC1, the homologue of *GhCYSTM9* in *Arabidopsis*, has been demonstrated to be involved in many biological processes. It not only displays a protective effect against pathogens, as originally identified [21] but also regulates the response to abiotic stress and plant development, such as seed germination, root elongation and flowering [22, 23]. In this study, we experimentally demonstrated that the overexpression of *GhCYSTM9* in *Arabidopsis* resulted in increased cold tolerance and that *GhCYSTM9*-silenced cotton plants presented decreased cold tolerance. These results indicate a positive function of *GhCYSTM9* in cold defence. Considering the homology of *GhCYSTM9* to *PPC1*, it can be hypothesized that the diverse effects and mechanisms of *GhCYSTM9* in the fight against pathogens and other stresses may be prospective and intricate, which merits further research. Transgenic and CRISPR/Cas gene-editing cotton lines of *GhCYSTM9* may help evaluate performance under various stresses in future.

Protein interaction of GhCYSTM9 in the cold stress response

GhLHCB2A1 was experimentally confirmed to interact with GhCYSTM9 by Y2H and BiFC in vivo. GhLHCB2A1 encodes a light-harvesting chlorophyll *a/b*-binding protein with 265 amino acids. Moreover, *GhLHCB2A1*, which is highly expressed in leaves and up-regulated in leaves and roots under cold and drought stress, was found to positively control cold and drought tolerance in a previous study (Cai et al. unpublished observations). LHCB proteins are reportedly involved in photosynthesis [50], photoprotection [51, 52], circadian rhythms [50] and environmental stimuli such as cold [53–55], drought [16, 56, 57] and salt [58, 59].

Previous studies have highlighted the critical role of *CsLHCB2* as an indicator of temperature sensitivity in the low-temperature response [60]. Moreover, *LHCB* genes in *Arabidopsis* are vital for low-temperature adaptation [61, 62]. The overexpression of *LeLhcb2* in tobacco contributed to increased tolerance to chilling stress by alleviating the photooxidation of PSII [53]. Since *LHCB* genes are critically involved in the cold response, it was strongly speculated that GhLHCB2A1 interacted with GhCYSTM9 to synergistically regulate resistance during cold stress defence. However, limited information is available regarding their interaction modes and regulatory pathways in response to cold stress, which should be the focus of further research.

Conclusion

In the present study, we revealed that the cysteine-rich transmembrane module peptide GhCYSTM9 positively modulated cold stress tolerance. *GhCYSTM9*-silenced cotton plants are sensitive to cold stress, whereas the overexpression of *GhCYSTM9* in *Arabidopsis* results in enhanced cold tolerance. Transcriptome analysis of transgenic *Arabidopsis* revealed that *GhCYSTM9* may contribute to cold defence by regulating oxidation–reduction-related genes. On the basis of the results of the Y2H and BiFC assays, the light-harvesting chlorophyll *a/b*-binding protein GhLHBC2A1 was further confirmed to interact with GhCYSTM9. These findings provide a theoretical foundation for the genetic improvement of cold resistance in cotton.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06271-w.

Supplementary Material 1

Supplementary Material 2

Author contributions

Z. J. and C. X. conceived and designed the research. L. C., T. L., Z. S. L. X. and W. H. performed the experiments and analyzed the data. C. X. wrote the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets supporting the conclusions of this study are available in the following repository. The RNA-seq data used in this study are available from the NCBI under accession number PRJNA1034306. Other data and materials generated in this study are included in this article and its supplementary materials.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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