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A histone deacetylase, GhHDT4D, is positively involved in cotton response to drought stress

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Abstract
Acetylation and deacetylation of histones are important for regulating a series of biological processes in plants. Histone deacetylases (HDACs) control the histone deacetylation that plays an important role in plant response to abiotic stress. In our study, we show the evidence that GhHDT4D (a member of the HD2 subfamily of HDACs) is involved in cotton (*Gossypium hirsutum*) response to drought stress. Overexpression of *GhHDT4D* in Arabidopsis increased plant tolerance to drought, whereas silencing *GhHDT4D* in cotton resulted in plant sensitivity to drought. Simultaneously, the H3K9 acetylation level was altered in the *GhHDT4D* silenced cotton, compared with the controls. Further study revealed that GhHDT4D suppressed the transcription of *GhWRKY33*, which plays a negative role in cotton defense to drought, by reducing its H3K9 acetylation level. The expressions of the stress-related genes, such as *GhDREB2A*, *GhDREB2C*, *GhSOS2*, *GhRD20-1*, *GhRD20-2* and *GhRD29A*, were significantly decreased in the *GhHDT4D* silenced cotton, but increased in the *GhWRKY33* silenced cotton. Given these data together, our findings suggested that GhHDT4D may enhance drought tolerance by suppressing the expression of *GhWRKY33*, thereby activating the downstream drought response genes in cotton.

Keywords Cotton (*Gossypium hirsutum*) · Histone deacetylase (HDAC) · Drought stress · H3K9 acetylation

Introduction

In plants, modifications of histones, including acetylation, methylation, phosphorylation, ubiquitylation, and adenosine diphosphate ribosylation, can cause dynamic changes of chromatin structure and gene expression (Peterson and Laniel 2004). Acetylation and deacetylation of histones play vital roles in regulating a series of important biological processes in plants (Pfluger and Wagner 2007). The positive charges of histone tails can be neutralized by acetylation, leading to the decreased ability of the histone binding to negatively charged DNA, and making the chromatin structure more loosely to facilitate the entry of transcriptional regulators (Annunziato and Hansen 2001). In contrast, eliminating the acetyl groups by histone deacetylation can lead to an increased affinity of histone binding to DNA, and thence block the availability of transcriptional regulators (Chrun et al. 2018).

The level of histone acetylation is closely related to gene expression and can be used as a marker to understand the transcriptional activity of the related genes. Histone acetylation is modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Legube and Trouche 2003). HATs catalyze hyperacetylation by adding acetyl groups to the lysine residues of histone, while HDACs bring about deacetylation by eliminating acetyl groups from the lysine residues of histone (Koprinarova et al. 2016).

HDACs are a class of important enzymes widely distributed in plants. They are involved in regulating many biological processes, such as determination of plant cell-type specificity, transition between developmental stages and response to environmental stress (Liu et al. 2014). HDACs can be divided into three subfamilies including RPD3/HDA1 (Reduced Potassium Dependence 3/Histone Deacetylase 1), SIR2 (Silent Information Regulator 2), and HD2 (Histone Deacetylase 2)-related proteins. The SIR2 subfamily has a catalytic domain whose function required nicotine adenine

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dinucleotide (NAD) as a cofactor, while members of the RPD3/HDA1 subfamily share sequence homology in the HDAC domain, and need Zn^{2+} cofactor for deacetylase activity (Liu et al. 2014). Besides, the HD2 subfamily is a class of plant-specific HDACs, and have four members in Arabidopsis, including HD2A, HD2B, HD2C and HD2D (Hollender and Liu 2008). It has been indicated that HDACs play vital roles in plant response to environmental stresses. SRT2 acts as a negative regulator that participates in plant basic defense response by suppressing salicylic acid (SA) biosynthesis (Wang et al. 2010). HDA19 forms a protein complex with AtERF7 and AtSin3 to regulate expressions of the abiotic stress-responsive genes, and the *hda19* mutant increases the sensitivity to ABA and salt stress (Luo et al. 2012a). Similarly, the T-DNA insertion mutant of HDA6 also shows a highly sensitive phenotype to ABA and salt stress, and the transcription levels of ABA-responsive genes are declined in *hda6* mutant (Chen and Wu 2010). The lysine residue 9 of histone H3 (H3K9) is an important acetylation modification site. Lysine 9 acetylation of histone H3 (H3K9ac) is mainly occurred around the transcript start site (TSS) regions, indicating H3K9ac is closely related to the transcriptional activity of genes (Zhou et al. 2010). Previous study showed that the higher expression levels of some water deprivation-related genes were detected in *hda19* mutant, and also the higher levels of H3K9 acetylation at promoters of these genes were found in the *hda19* mutant, relative to wild-type plants (Chen and Wu 2010). These results illustrate that the H3K9ac level is associated with the expression change of drought-related genes.

Upland cotton (*Gossypium hirsutum*) is an important crop planted worldwide, and provides natural fiber materials for the textile industry. Additionally, cotton seeds are also utilized as an important source of edible oil, and therefore have important value for the food industry (Gotmare et al. 2004). Drought is an important environmental limiting factor for agricultural production. The water source is very scarce in more than half of the cotton planting area (Pettigrew 2004). Thus, understanding the molecular mechanism and genetic basis in cotton response to drought stress is significant for basic cotton biology, and will contribute to cultivating drought-tolerant cotton cultivars. Although some plant HDACs have been reported to participate in plant response to drought stress (Luo et al. 2012a), the roles of HDACs in cotton response to drought stress remain unclear and need to be explored in details. In this study, we revealed that *GhHDT4D* participates in cotton response to drought stress. Overexpression of *GhHDT4D* in Arabidopsis increased drought tolerance of the transgenic plants, whereas silencing *GhHDT4D* in cotton resulted in the reduced tolerance to drought. Further study revealed that *GhHDT4D* functions in cotton response to drought stress possibly by modulating the H3K9 acetylation level at the promoter region of *GhWRKY33*, and thereby suppressing *GhWRKY33* expression.

Results

GhHDT4D expression is repressed in cotton by drought

To study the roles of the histone deacetylases in cotton, a total of 33 HDAC genes were identified in upland cotton (*Gossypium hirsutum*), and the HDAC homologous genes were also identified in diploid A genome species (G. *arboreum*) and D genome species (G. *raimondii*) (Table S1). Among them, we found a gene (designated as *GhHDT4D*, Ghir_D11G035640) may be involved in cotton response to drought stress. The evolutionary analysis revealed that GhHDT4D clusters to the branch with AtHD2A, AtHD2AB, AtHD2C and AtHDT2C (Fig. 1a), and motif analysis indicated that GhHDT4D shares similar motif composition to HD2 subgroup of AtHDACs (Fig. 1b), demonstrating that GhHDT4D belongs to the HD2 subgroup. We further analyzed *GhHDT4D* expression in cotton based on the cottonFGD database (https://cottonfgd.org/), and found the expression level of *GhHDT4D* is increased in cotton 1–3 h after PEG treatment, and then declined in cotton tissues under PEG treatment for 6–12 h (Fig. 1c). Furthermore, the 4-week-old cotton seedlings were subjected to water-withholding treatment for 7 days. The leaves of cotton seedlings with normal watering and water-withholding treatment were collected to extract RNA for expression analysis. The results showed that the transcription of *GhHDT4D* was inhibited in leaves of cotton after drought stress (Fig. 1d), suggesting that *GhHDT4D* may participate in cotton response to drought stress. We also investigated the expression pattern of HDAC genes under drought stress using the previously published transcriptome data from our laboratory (Chen et al. 2013). Based on transcriptome data, we can find that the expression of *GhHDT4D* is obviously altered under drought stress (Fig. S1), corresponding to the results above. Besides, the transcriptome data showed that expressions of some other HDAC genes are also altered under drought (Fig. S1), indicating that some other HDAC genes may also be involved in cotton drought response.

Ectopic expression of *GhHDT4D* improves transgenic Arabidopsis drought tolerance

To explore the function of *GhHDT4D* in response to drought, the coding sequence of *GhHDT4D* under the control of CaMV35S promoter was ectopically expressed in Arabidopsis Col-0 plants. The expression levels of *GhHDT4D* in the transgenic Arabidopsis lines were detected

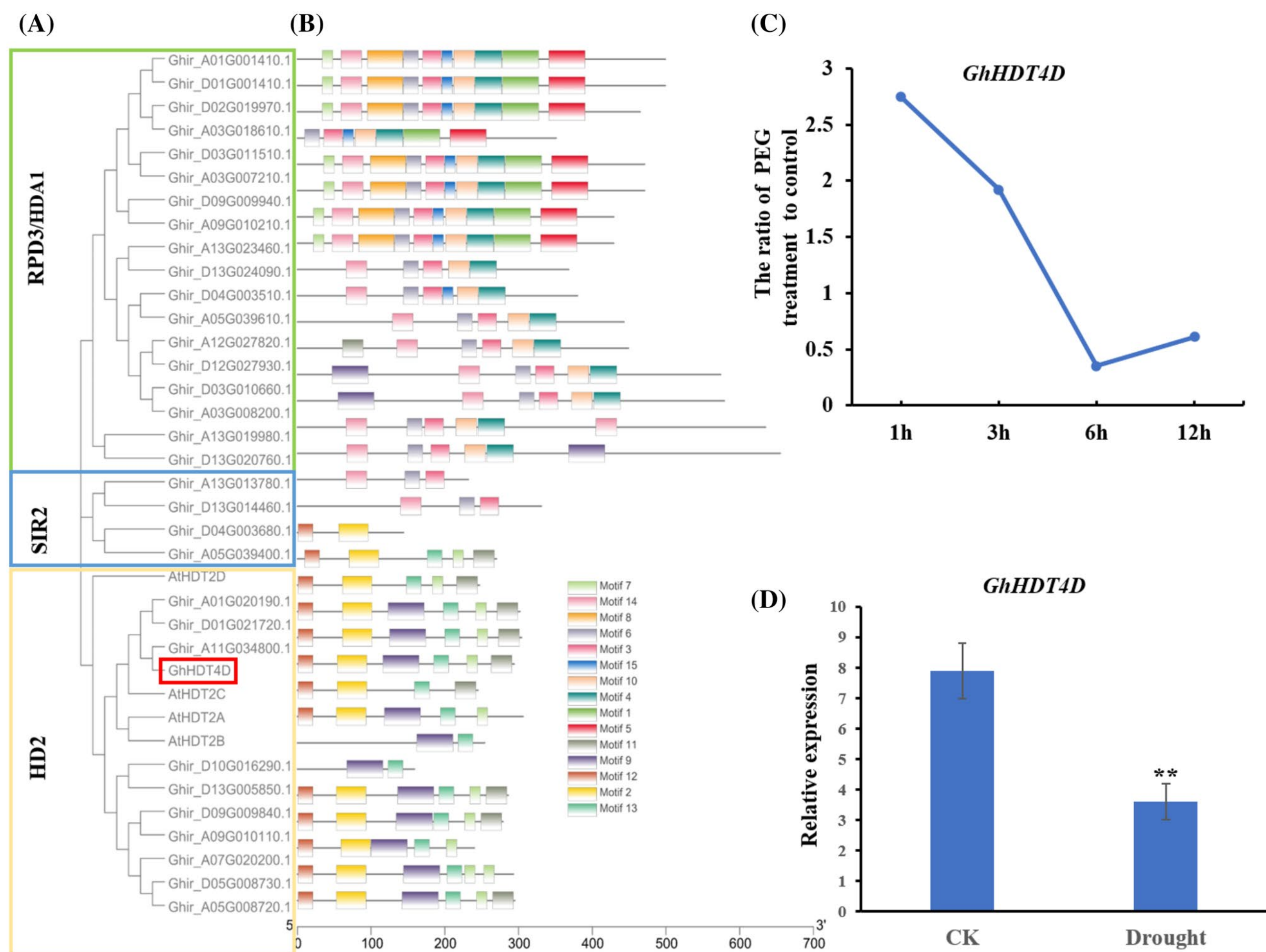

Fig. 1 Characterization and expression analysis of *GhHDT4D*. **a** Phylogenetic analysis of *GhHDACs* and HD2 subgroup of Arabidopsis. The unrooted phylogenetic tree was constructed using MEGA 6.0 by Neighbor-Joining method and the bootstrap test was performed with 1000 iterations. **b** Motif structure comparison using MEME Suite (https://meme-suite.org/tools/meme) with a set of parameters as follows: the optimum motif width was set to ≥ 6 and ≤ 50; the maximum number of motifs was set to 15. Different motifs are represented by various colored boxes. Motif 1/3/4/6/8/10/13/14/15 were annotated as the conserved histone deacetylase domain (pfam00850). Motif 2/11/12 were annotated as the nucleoplasmin-like domain (pfam17800). **c** Expression profile of *GhHDT4D* in cotton under PEG treatment. **d** Expression of *GhHDT4D* in cotton under drought stress. The experiments were repeated three times, and error bars denote the standard deviation calculated from three independent experiments. Asterisks represent Student's *t* test in statistical analysis for significant differences: $^{*}P < 0.05$; $^{**}P < 0.01$

by quantitative RT-PCR (Fig. 2a), and the transgenic line L1, L7 and L15 with higher expression levels of *GhHDT4D* were selected for further analysis. To observe the phenotype of the transgenic Arabidopsis, the four-week-old *GhHDT4D* transgenic plants and wild type were subjected to water-withholding treatment for 15 days. As shown in Fig. 2b, the growth status of the transgenic lines was better than that of wild type. Besides, the contents of MDA, proline, chlorophyll, and activities of SOD (Superoxide dismutase) and POD (Peroxidase) were measured in leaves of the transgenic plants and wild type under drought stress and normal growth condition. The experimental results showed that no significant difference was found between wild type and *GhHDT4D* transgenic lines under normal growth condition. However, the contents of proline and chlorophyll, and activities of SOD and POD in leaves of the *GhHDT4D* transgenic lines were higher, but MDA content in the transgenic plants were lower than those in wild type under drought treatment (Fig. 2c–g). Reducing the stomatal opening is an important way for plant to reduce water loss when facing drought stress. So we observed the stomatal aperture of wild type and the transgenic plants under normal condition and drought treatment. As shown in Fig. 2h, i, there was no obvious difference in stomatal aperture opening between wild type and the transgenic plants under normal growth condition. However, the stomata apertures of wild type plants opened wider than those of the transgenic plants under drought stress, suggesting that the transgenic lines had

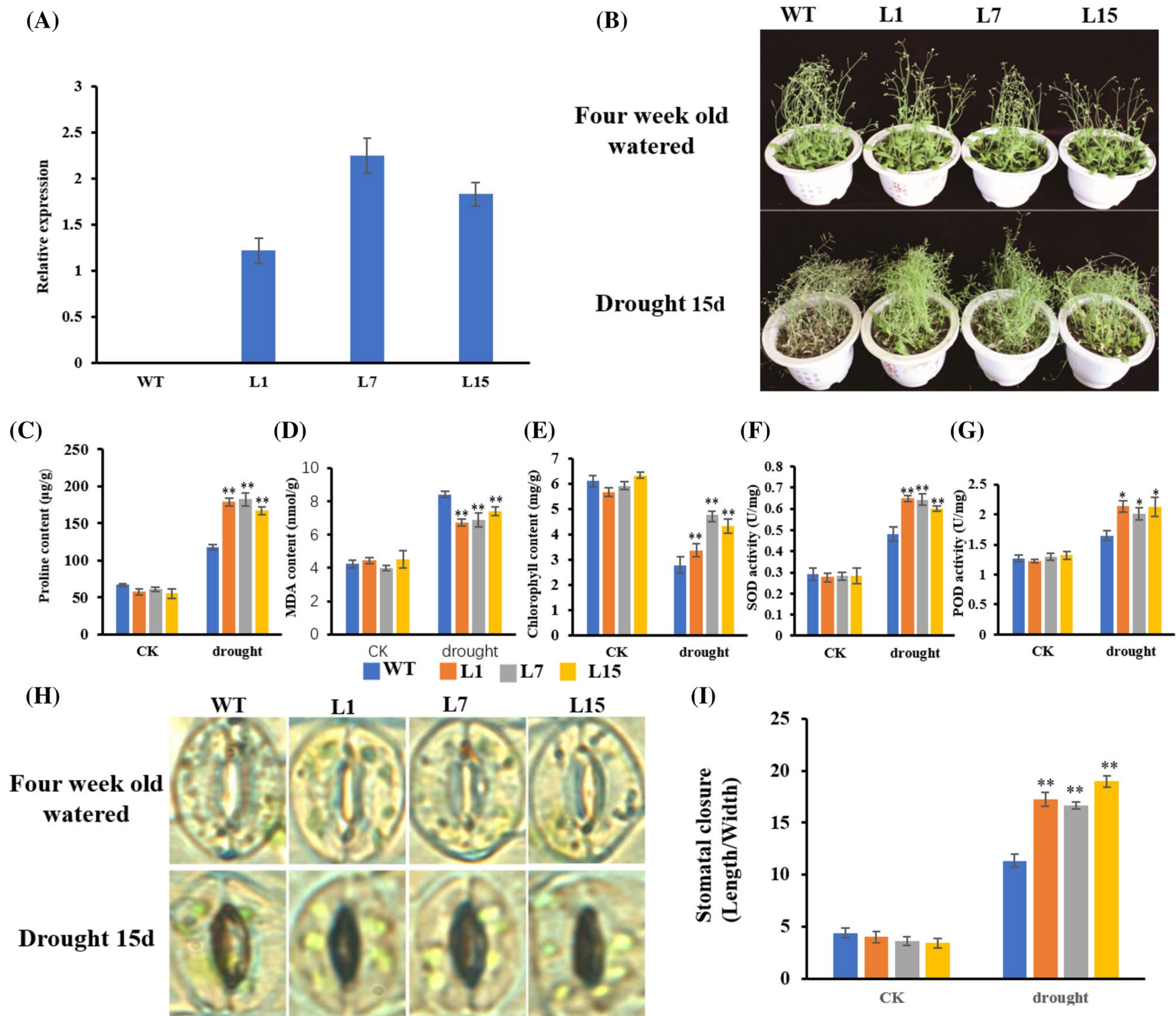

Fig. 2 Phenotypic analysis of *GhHDT4D* overexpression transgenic Arabidopsis under drought stress. **a** Quantitative RT-PCR analysis of *GhHDT4D* expression in the transgenic lines and wild type. **b** Phenotypic analysis of 4-week-old wild type and *GhHDT4D* transgenic Arabidopsis grown in soil under drought stress by withholding water for 15 days. **c**–**g** Several physiological indexes measured in the transgenic plants and wild type under normal growing condition (control) and drought treatments. **c** Proline content. **d** MDA content. **e** Chlorophyll content. **f** SOD activity. **g** POD activity. **h** Images of stoma in wild type and transgenic lines under normal growing conditions (control) and drought treatment. **i** Comparative stomatal aperture measurements (ratio of width to length). The experiments were repeated three times, and error bars denote the standard deviation calculated from three independent experiments. Asterisks represent Student's t-test in statistical analysis for significant differences: *$P<0.05$; **$P<0.01$. WT, wild type; L1, L7, L15, three *GhHDT4D* overexpression transgenic lines

lower rate of water loss. The above results indicated that the transgenic plants enhanced their drought tolerance owing to the ectopic expression of *GhHDT4D* in Arabidopsis.

GhHDT4D positively regulated drought tolerance in cotton

To further explore the role of *GhHDT4D* in cotton response to drought, we conducted a virus induced gene silencing (VIGS) experiment. The plants infected with TRV2:*GhCLA* appeared the albino phenotype, indicating the success of the VIGS experiment (Fig. 3a). Then the expression level of *GhHDT4D* was detected in TRV2:00 and TRV2:*GhHDT4D* plants to confirm the expression of *GhHDT4D* was effectively suppressed. Quantitative RT-PCR analysis revealed that the expression levels of *GhHDT4D* were significantly reduced in TRV2:*GhHDT4D* plants compared with the TRV2:00 plants (Fig. 3b). After

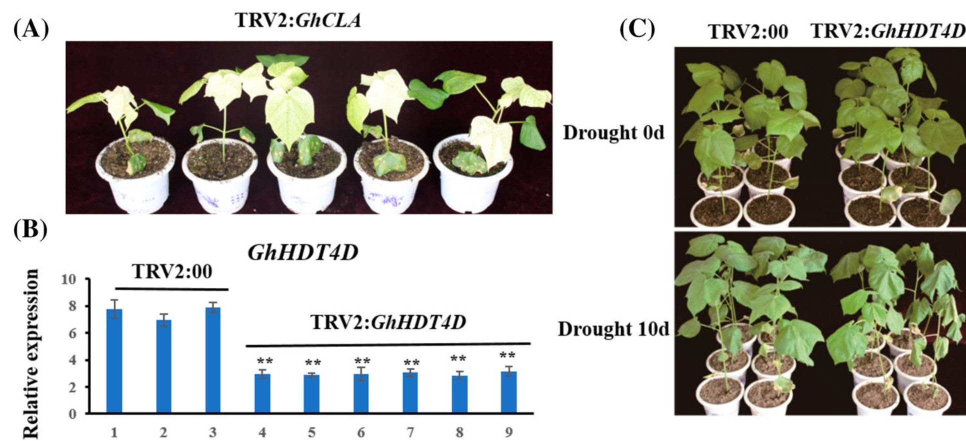

Fig. 3 Phenotypic analysis of *GhHDT4D* VIGS cotton plants under drought stress. **a** Albino phenotypes of positive control cotton plants. **b** Quantitative RT-PCR analysis of *GhHDT4D* expression in mock and *GhHDT4D* VIGS cotton plants. Experiments were repeated three times, and error bars denote the standard deviation calculated from three independent experiments. Asterisks represent Student's *t* test in statistical analysis for significant differences: *P < 0.05; **P < 0.01. **c** Phenotypic analysis of mock and *GhHDT4D* VIGS cotton plants under drought stress by withholding water for 10 days (bottom)

four weeks of growth, the TRV2:00 and TRV2*:GhHDT4D* plants were subjected to drought treatment. After water-withholding treatment for 10 days, we observed that the TRV2:*GhHDT4D* plants were more seriously wilted than those TRV2:00 plants (Fig. 3c), indicating that silencing *GhHDT4D* impared cotton tolerance to drought. These results suggested that *GhHDT4D* may have an important effect on cotton response to drought.

GhHDT4D modulates the H3K9 acetylation level of *GhWRKY33* promoter in cotton

H3K9 is an important epigenetic modification site that is positively correlated with transcriptional activity of stress-responsive genes in plants (Kim et al. 2015). To investigate whether silencing *GhHDT4D* can affect H3K9 acetylation level in cotton, we detected the H3K9ac level in the TRV2:00 and TRV2:*GhHDT4D* plants. As shown in Fig. 4a and b, H3K9ac level in TRV2:*GhHDT4D* plants was higher than that in TRV2:00 plants, suggesting that *GhHDT4D* could regulate H3K9 acetylation level in cotton. A previous report showed that overexpression of *GhWRKY33* resulted in the transgenic Arabidopsis sensitivity to drought stress (Wang et al. 2019). Here, we found that the transcription level of *GhWRKY33* is increased in TRV2:*GhHDT4D* plants compared with TRV2:00 plants (Fig. 4c). Therefore, the H3K9 acetylation level of *GhWRKY33* promoter was detected by the Chip-qPCR assay using the anti-H3K9ac antibody. As shown in Fig. 4d, the H3K9 acetylation level of *GhWRKY33* promoter was increased in TRV2:*GhHDT4D* plants relative to the controls. To determine whether *GhWRKY33* is the direct target gene of GhHDT4D, the GhHDT4D-GFP fusion construct was introduced into cott on hypocotyls by *Agrobacterium*-mediated DNA transfer. After three months, the transformed callus cells were used for the Chip-qPCR assay. As shown in Fig. 4e, GhHDT4D-GFP fusion proteins were expressed successfully in the callus cells. Chip-qPCR assay, using anti-GFP antibody, showed that GhHDT4D can directly bind to the promoter of *GhWRKY33* (Fig. 4f). The above results suggested that GhHDT4D negatively regulates *GhWRKY33* expression bydecreasing the H3K9 acetylation level of *GhWRKY33* promoter for cotton response to drought stress.

GhWRKY33 negatively regulates cotton drought tolerance

To further understand the role of *GhWRKY33* in cotton response to drought, similarly, we silenced *GhWKY33* in cotton via VIGS technique. As shown in Fig. 5a, the expressions of *GhWRKY33* and *GhHDT4D* were effectively repressed in the target gene-silenced plants. Then, the four-week-old TRV2:00, TRV2:*GhWRKY33* and TRV2:*GhHDT4D* plants were subjected to drought treatment. After 15 days of water-withholding treatment, the degree of leaf wilting in the TRV2:*GhHDT4D* plants was more serious compared with that in TRV2:00 and TRV2:*GhWRKY33* plants, whereas the TRV2:*GhWRKY33* plants displayed the best growth status (Fig. 5b). Additionally, we measured several physiological parameters (including contents of MDA, proline and chlorophyll and activities of SOD and POD) in leaves of plants under both drought stress and normal growth condition. As shown in Fig. 5c–g, no significant difference was observed in these parameters between the target gene-silenced plants and TRV2:00 controls under normal growth

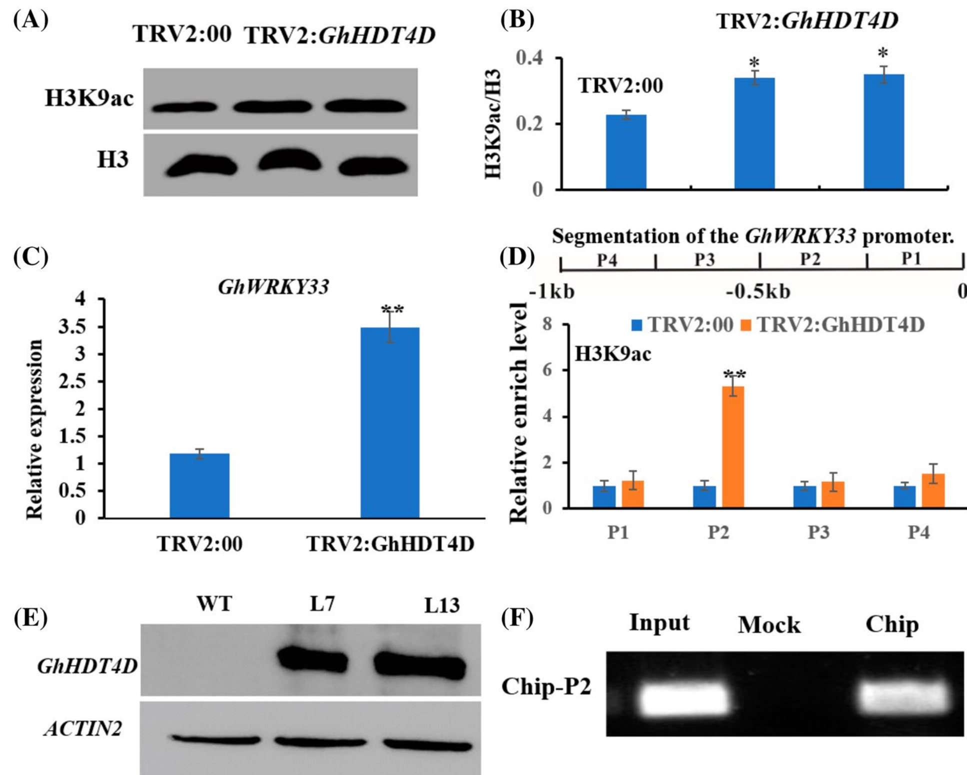

Fig. 4 Silencing of *GhHDT4D* changes the expression and H3K9 acetylation level of *GhWRKY33* in cotton. **a** and **b** Comparison of H3K9 acetylation levels between mock and silenced *GhHDT4D* cotton plants. **a** The expression analysis of *GhWRKY33* in mock and silenced *GhHDT4D* cotton plants. **b** Immunoblot assay for histone acetylation levels in mock and silenced *GhHDT4D* cotton plants, probed with anti-H3K9ac and antiH3 antibodies. The intensities of blotting signals were quantified using Image J software. **c** Quantitative RT-PCR analysis of *GhHDT4D* expression in mock and silenced *GhHDT4D* cotton plants. Experiments were repeated three times, and error bars denote the standard deviation calculated from three independent experiments. Asterisks represent Student's *t* test in statistical analysis for significant differences: $^*P<0.05$; $^{**}P<0.01$. **d** Chip-qPCR analysis of H3K9 acetylation level of *GhWRKY33*. The 1000 bp promoter sequence of *GhWRKY33* was divided into four segments (P1–P4) for Chip-qPCR. Experiments were repeated three times, and error bars denote the standard deviation calculated from three independent experiments. Asterisks represent Student's *t* test in statistical analysis for significant differences: $^*P<0.05$; $^{**}P<0.01$. **e** Western blot analysis of expression of GhHDT4D-GFP fusion protein in transgenic cotton callus cells. **f** ChIP-PCR assay of GhHDT4D binding to *GhWRKY33* promoter. The transgenic callus cells overexpressing *GhHDT4D*-GFP were used for the ChIP assay with anti-GFP antibody, and the precipitated DNA was quantified by semiquantitative RT-PCR analysis

condition. After drought treatment, however, contents of chlorophyll and proline, and activities of SOD and POD were higher, but the MDA content was lower in leaves of the TRV2:*GhWRKY33* plants, compared with the TRV2:00 controls. Conversely, the TRV2:*GhHDT4D* plants showed the opposite phenotype. Besides, the stomatal aperture was observed in TRV2:00, TRV2:*GhWRKY33* and TRV2:*GhHDT4D* plants. The experimental results revealed that there was no significant difference in stomatal aperture opening among the target gene-silenced plants and TRV2:00 controls under normal growth condition. However, the opening of stomatal apertures in the TRV2:*GhWRKY33* plants was smaller than that in the TRV2:00 plants, whereas the opening of stomatal apertures in TRV2*:GhHDT4D* plants was bigger than that in the TRV2:00 plants under drought stress (Fig. 5h, i). As the opening size of stomatal apertures was closely related to the rate of water loss in plants, our results indicated that silencing *GhWRKY33* resulted in the enhanced drought tolerance of cotton owing to the decreased water loss in the plants.

GhHDT4D affects expressions of the drought stress-related genes in cotton

It was reported that *DREB2C*, *DREB2A*, *RD20*, *SOS2* and *RD29A* play critical roles in plant response to drought stress (Kasuga et al. 1999). To elucidate the regulatory mechanism of GhHDT4D-GhWRKY33-mediated cotton response to drought stress, we analyzed the expression levels of these drought-related genes in cotton. The experimental results showed that the transcription levels of *DREB2C*, *DREB2A*, *RD20*, *SOS2*

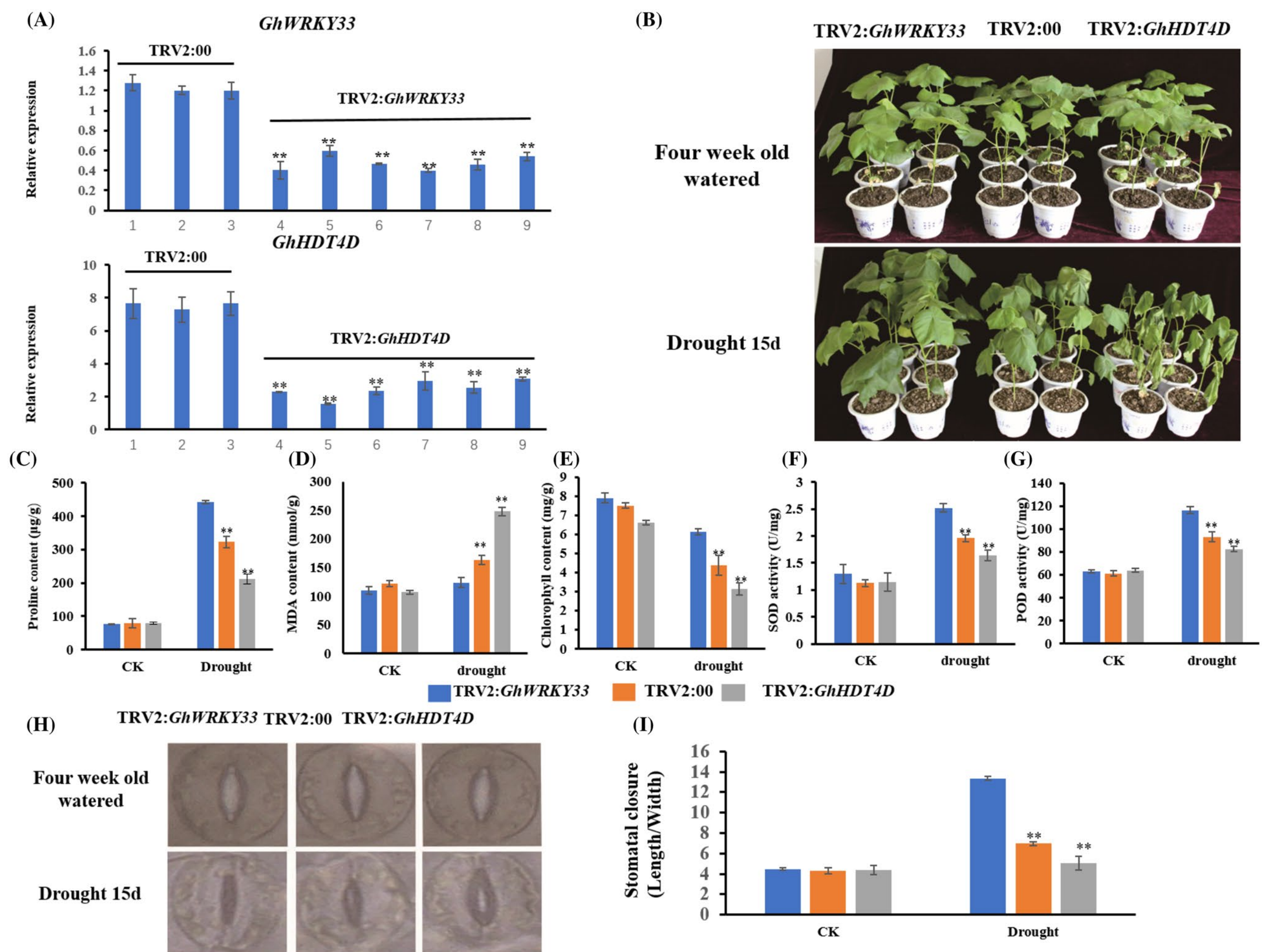

Fig. 5 Phenotypic analysis of the silenced *GhWRKY33* and *GhHDT4D* cotton plants under drought stress. **a** Quantitative RT-PCR analysis of *GhWRKY33* and *GhHDT4D* expressions in mock and silenced *GhWRKY33* and *GhHDT4D* cotton plants. **b** Phenotypic analysis of mock and silenced *GhWRKY33* and *GhHDT4D* cotton plants under drought stress by withholding water for 15 days. **c–e** Several physiological indexes measured in the target gene-silenced cotton plants and mock controls under normal growing condition and drought treatments. **c** Proline content. **d** MDA content. **e** Chlorophyll content. **f** SOD activity. **g** POD activity. **h** Images of stoma in silenced *GhWRKY33* and *GhHDT4D* cotton plants and mock controls under normal growing condition and drought treatment. **i** Comparative stomatal aperture measurements (ratio of width to length). The experiments were repeated three times, and error bars denote the standard deviation calculated from three independent experiments. Asterisks represent Student's *t* test in statistical analysis for significant differences: $*P<0.05$; $**P<0.01$

and *RD29A* were all increased in the *TRV2:GhWRKY33* plants, but decreased in the *TRV2:GhHDT4D* plants compared with those in the TRV2:00 plants (Fig. 6). Therefore, we supposed that *GhWRKY33* may negatively regulate the expressions of these drought-related genes in cotton. Possibly, *GhHDT4D* plays a positive role in cotton response to drought by negatively regulating *GhWRKY33* to affect expressions of the drought-related genes.

GhHDT4D interacts with GhHDA19

Previous study reported that HD2 proteins could interact with RPD3-type histone deacetylase for forming a corresponding dimer (Luo et al. 2012a, b, c). Thus, the yeast two-hybrid assay was conducted to search potential proteins interacting with GhHDT4D. Results showed that GhHDT4D can interact with GhHDA19 in yeast (Fig. 7a). To further determine the interaction between GhHDT4D and GhHDA19, the coding sequences of *GhHDT4D* and *GhHDA19* were inserted into JW771 and JW772 vectors, respectively, to perform the bimolecular fluorescence complementation (BiFC) assay. As shown in Fig. 7b, GhHDT4D could interact with GhHDA19 in tobacco leaves. The above results showed that GhHDT4D can interact with GhHDA19, an RPD3-type histone

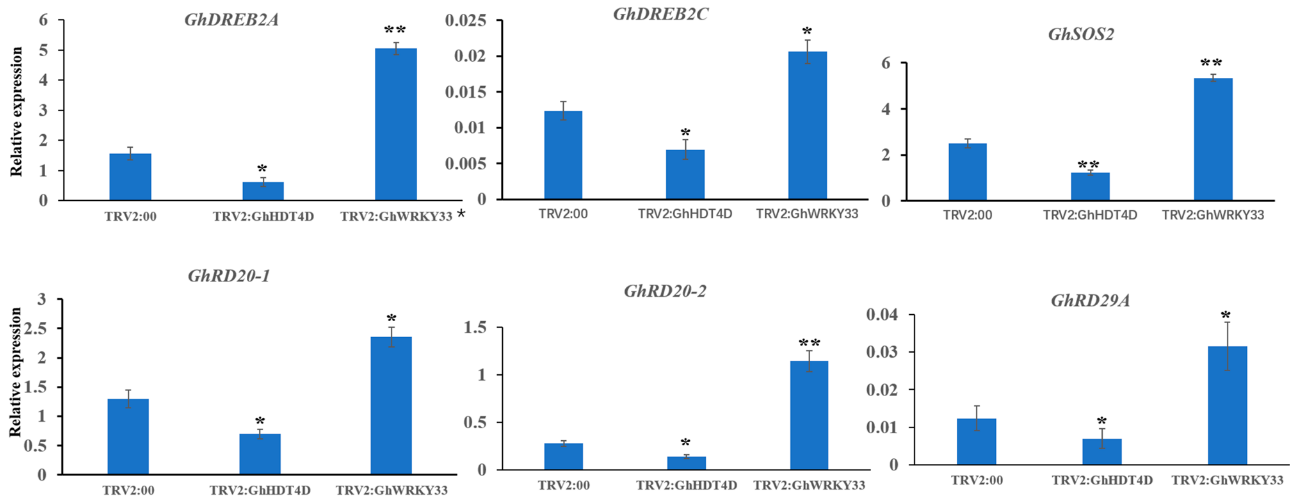

Fig. 6 Quantitative RT-PCR analysis of expression of the drought stress-related genes in the silenced *GhWRKY33* and *GhHDT4D* cotton plants. Transcript levels of *GhDREB2A*, *GhDREB2C*, *GhSOS2*, *GhRD20-1*, *GhRD20-1* and *GhRD29A* in mock and silenced *GhWRKY33* and *GhHDT4D* plants were determined by quantitative RT-PCR using *GhUBI1* as a quantification control. Mean values and standard errors (bars) are shown from three independent experiments. Asterisks represent Student's *t* test in statistical analysis for significant differences: *P < 0.05; **P < 0.01

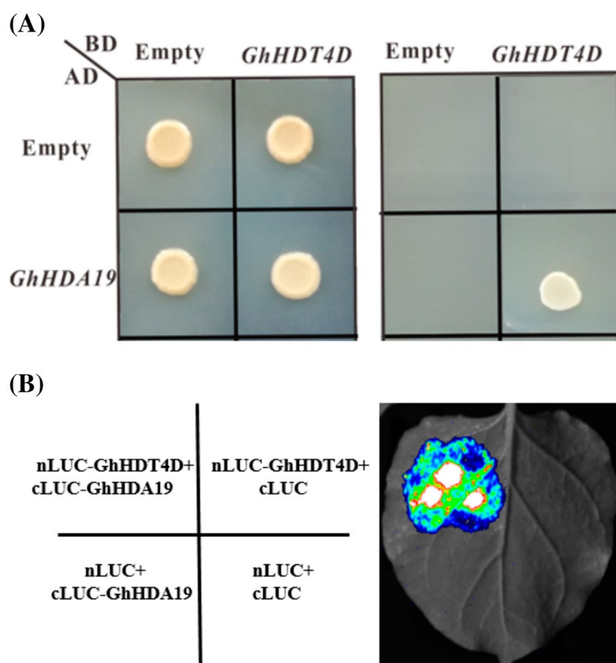

Fig. 7 GhHDT4D interacts with GhHDA19 in vitro and in vivo. **a** Yeast two-hybrid assay of GhHDT4D interacted with GhHDA19. BD, GAL4 DNA binding domain; AD, GAL4 activation domain. Left panel: The yeast cells grown on 2-dropout medium. Right panel: The yeast cells grown on selective 4-dropout medium. **b** Bimolecular fluorescence complementation (BiFC) assay of GhHDT4D interacted with GhHDA19. cLUC, the carboxy terminus of luciferase; nLUC, the amino terminus of luciferase

deacetylase, suggesting that GhHDT4D may form a dimer with GhHDA19 for its function in cotton.

Discussion

GhHDT4D is involved in cotton response to drought stress

The HD2 genes are a class of plant-specific genes that have been reported to be involved in plant response to abiotic stress (Zhou et al. 2004; Luo et al.2017). In Arabidopsis, the transcription of HDT1 (HDT2A), HDT2 (HDT2B), HDT3 (HD2C) and HDT4 (HD2D) is suppressed under ABA treatment and high salt stress (Luo et al. 2012a; Zhou et al. 2004). In rice, the transcription level of *HDT701* is reduced dramatically 1 h after ABA, NaCl and PEG treatments, but recovered after ABA and PEG exposure for 3 h. Similarly, expression of *HDT702* in rice is reduced 1 h after ABA treatment, but dramatically enhanced after ABA, NaCl, and PEG exposure for 3 h, indicating that the expressions of *HDT701* and *HDT702* could be regulated by abiotic stresses (Zhao et al. 2015). In this study, we found that the expression of *GhHDT4D* is repressed in cotton under drought treatment, suggesting that *GhHDT4D* may be involved in cotton drought response. Moreover, previous study revealed that overexpression of *AtHD2D* in Arabidopsis enhanced plant tolerance to drought and salt stresses (Han et al. 2016; Farhi 2015). Overexpression of *AtHD2C* also increased plant resistance to drought and salt stresses by regulating the expressions of some ABA-responsive genes (Sridha and Wu 2006). Similarly, overexpression of *HDT701*

in rice enhanced plant salt and osmotic stress resistance. On the contrary, *hdt701* mutant seedlings displayed the increased sensitivity to both salt and osmotic stresses with the decreased expression levels of stress-inducible genes (Zhao et al. 2015; Wai and An 2018). Likewise, we found that overexpression of *GhHDT4D* strengthened the drought tolerance of the transgenic Arabidopsis, while virus-induced gene silencing of *GhHDT4D* remarkably reduced cotton drought resistance, suggesting that *GhHDT4D* may play an important role in cotton response to drought stress.

GhHDT4D regulates the H3K9ac level in cotton

Histone deacetylases regulate the histone acetylation levels on DNA for influencing the transcription activities of genes (Ma et al. 2013; Liu et al. 2014). It has been demonstrated that lysine residue 9 of histone H3 (H3K9) is a critical site for the acetylation/deacetylation (Zhou et al. 2010). This epigenetic modification site is positively correlated with transcriptional activity of stress-responsive genes in plants (Kim et al. 2015; Zhou et al. 2010). In Arabidopsis, the *HDA9* mutation increases the expressions of 47 stress-related genes that are accompanied by higher H3K9ac in their promoters (Zheng et al. 2016). The *hda19* mutant has higher levels of H3K9ac in the *PR1* and *PR2* promoter regions compared with wild type (Choi et al. 2012). Overexpression of *HDC1* increased the transcript levels and H3K9K14ac levels of the salt stress-regulated genes (Perrella et al. 2013). In *hd2c* mutant, the expression and H3K9K14ac levels of *ABI1* and *ABI2* are higher than those in wild type plants (Luo et al. 2012a, b, c). In *srt1-1/srt2-1* double mutant, the H3K9ac level was significantly increased compared with that in wild type. *SRT1* and *SRT2* suppress expressions of ethylene-repressed genes by regulating H3K9ac levels of them (Zhang et al. 2018). In *Brachypodium*, overexpression of *BdHD1* leads to a decrease of H3K9ac level. *BdHD1* acts as a global regulator to link the H3K9ac level and gene expression to respond to drought stress (Song et al. 2019). In this study, we found that the H3K9ac level in the TRV2:*GhHDT4D* plants was higher than that in TRV2:00 plants, indicating that *GhHDT4D* may regulate the H3K9ac level for responding to drought stress in cotton.

GhHDT4D plays a positive role in cotton response to drought by suppressing *GhWRKY33* expression

It is known that the histone deacetylases can repress the expressions of the target genes by decreasing the histone acetylation levels of these genes (Chen et al. 2015; Liu et al. 2014). Therefore, we speculated that the histone acetylation levels and expression levels of putative target genes of *GhHDT4D* should be increased in the *GhHDT4D* silenced cotton. Furthermore, a study indicated that histone deacetylase 701 enhances rice abiotic stress resistance at the seedling developmental stage by suppressing expression of *OsWRKY45* (Wai and An 2018). Additionally, WRKY transcription factors play essential roles in plant responsed to drought stress (Chen et al. 2012). In Arabidopsis, three WRKY genes, *WRKY46*, *WRKY54* and *WRKY70*, participate in drought stress responses as negative regulators, and the triple mutant *wrky46/wrky54/wrky70* exhibits the enhanced drought stress tolerance (Chen et al. 2017). Overexpression of *GhWRKY33* increased plant sensitivity to drought (Wang et al. 2019). In this study, silencing *GhWRKY33* increased cotton tolerance to drought, suggesting that *GhWRKY33* plays a negative role in cotton response to drought stress. So we detected the H3K9 acetylation level and expression of *GhWRKY33* in TRV2:00 and TRV2:*GhHDT4D* plants. As expected, both acetylation level and expression level of *GhWRKY33* were increased in the *GhHDT4D* silenced plants compared with TRV2:00 controls. Furthermore, Chip-PCR analysis showed that *GhHDT4D* could directly bind to the promoter region of *GhWRKY33*. These results suggested that GhHDT4D suppresses the expression of *GhWRKY33* by modulating its H3K9ac level.

As WRKY transcription factors can bind to W-box *cis*-elements existed in promoter regions of the target genes to regulate their transcription activities (Chen et al. 2012), to elucidate the regulatory pathway governed by GhHDT4D depended on GhWRKY33, we analyzed the expressions of some drought-related genes, including *DREB2A*, *DREB2C*, *RD20*, *SOS2* and *RD29A*, with W-box motifs on their promoters, in TRV2:00, TRV2:*GhHDT4D* and TRV2:*GhWRKY33* plants. These genes have been reported to contribute to drought tolerance in different plants (Nakashima et al. 2000; Sakuma et al. 2006; Xiu et al. 2016; Je et al. 2014; Zhao et al. 2013; Aubert et al. 2010; Liu et al. 2000; Xiao et al. 2009). In this study, the transcriptional levels of *DREB2A*, *DREB2C*, *RD20*, *SOS2* and *RD29A* were decreased in the TRV2:*GhHDT4D* plants, but increased in the TRV2:*GhWRKY33* plants compared with those in the TRV2:00 plants. The above results indicate that the increased sensitivity of *GhHDT4D* silenced cotton to drought stress is due to the reduced expression of these drought-related genes. It also means that increased expression of drought stress-related genes contributes to increase the drought tolerance of *GhWRKY33* silenced cotton. *GhHDT4D* can enhance expressions of the drought stress-related genes, but *GhWRKY33* can suppress their expressions. Also, because *GhHDT4D* can repress the expression of *GhWRKY33,* we infer that *GhHDT4D* may enhance the expression of these drought-related genes by suppressing the transcription activity of *GhWRKY33*. In brief, our data suggest that GhHDT4D plays a positive role in cotton response to drought stress. GhHDT4D might enhance the osmotic stress tolerance of cotton via

suppressing the expression of *GhWRKY33*, thereby releasing the inhibition of the downstream drought-related genes by GhWRKY33.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana (Columbia ecotype) seeds were surface-sterilized with 10% NaClO for 5 min, followed by washing three times with sterile water. Then the seeds were plated on Murashige and Skoog (MS) medium. After placed at 4 °C for 72 h in darkness, the seeds were transferred to the growth incubator for germination and development (22 °C, 16 h light/8 h dark). Seven days later, the seedlings were transplanted into soil and grew in the growth chamber (22 °C, 16 h light/8 h dark).

Cotton (*Gossypium hirsutum* cv. Coker312) seeds germinated and grew under controlled condition (25 °C, 16 h light/8 h dark) in culture room. Tissues were harvested from these seedlings for further study.

Analyses of phylogenetic relationship and motifs of GhHDACs

The protein sequences of GhHDACs were searched in cotton (*Gossypium hirsutum* L., cultivar TM-1 (AD1)) genome database (https://cottonfgd.org/). To identify GhHDACs, the Arabidopsis HDAC proteins were employed as queries to search cotton database. The MEGA 6.0 was used to construct the unrooted phylogenetic tree. The protein sequences were subjected to the Motif Elicitation (MEME) online program (https://meme.sdsc.edu/meme/intro.html) to predict conserved motif (Bailey et al. 2015). The InterProScan (https://www.ebi.ac.uk/Tools/InterProScan/) was used to annotate the identified motifs (De Castro et al. 2006).

Transcriptome data for analyzing gene expressions in cotton

The transcriptomic data of cotton under drought stress used in this study was obtained from our laboratory (Chen et al. 2013). The fragments per kilobase per million reads (FPKM) value of genes is discarded if all three samples are less than one (Zhu et al. 2018). Then, the remaining expression data were used for generation of heatmap using TBtools (Chen et al. 2018).

Arabidopsis transformation and phenotypic analysis

The coding sequence of *GhHDT4D* was cloned into PK2GW7.0 vector to generate 35S:*GhHDT4D* construct. The construct was introduced into Arabidopsis through Agrobacterium-mediate transformation. Seeds of *GhHDT4D* homozygous lines (T3 generation) were selected by kanamycin resistance and were used for further study. For drought treatment, the four-week-old seedlings were subjected to water-deficit treatment for 15 days. The phenotypes of the plants were observed and photographed after drought treatments. Each of the experiments was performed at three independent biological replicates.

Test of virus induced gene silencing (VIGS) in cotton

The VIGS experiment was carried out by the method as described previously (Gao et al 2016). The fragments (400 bp) of *GhHDT4D* and *GhWRKY33* genes were inserted into TRV2 vector to generate TRV2:*GhHDT4D* and TRV2:*GhWRKY33* constructs, respectively. The constructs were transferred into *Agrobacterium tumefaciens* strain GV3101. The transformed agrobacteria solution containing the TRV1 and TRV2:00 or TRV2:*GhHDT4D* or TRV2:*GhWRKY33* was mixed and injected into cotyledons of cotton seedlings. After detecting the expression of *GhHDT4D* and *GhWRKY33* by quantitative RT-PCR analysis, the four-week-old cotton seedlings were subjected to water-deficit treatment. Each of the experiments was performed at three independent biological replicates. Nine individuals were used in each treatment and control, respectively.

Determination of drought stress-related physiological parameters

Malondialdehyde (MDA) content was determined from 0.1 g cotton leaf tissues by using MDA Quantification Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). For quantification of proline content, 0.1 g samples of cotton leaves were prepared and followed the procedure as described by the manufacturer of Proline Quantification Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The measurement of peroxidase (POD) and superoxide dismutase (SOD) enzyme activities in the stressed plants and controls was performed as described by the manufacturer of Peroxidase (POD) assay kit and Superoxide Dismutase (SOD) assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Stomatal aperture was observed in leaves of cotton under normal condition and

drought treatment by microscopy, and the ratio of stomatal length to width was measured (n > 50 stoma per sample).

Yeast two-hybrid assay

The coding sequence of *GhHDA19* was inserted into pGADT7 vector and transferred into yeast strain AH109, while the coding sequence of *GhHDT4D* was inserted into pGBKT7 vector and transferred into yeast strain Y187, using the high-efficiency lithium acetate transformation procedure following the manufacturer's instructions (Clontech). Then AH109 and Y187 yeast haploid strains were mated. The mated yeast diploid strains were identified on yeast double drop-out (DDO) medium lacking Leu and Trp, and were incubated on DDO medium at 30 °C for 3–4 days. The positive interactions were identified on yeast quadruple dropouts (QDO) medium lacking Leu, Trp, His and Ade.

Bimolecular fluorescence complementation (BiFC) assay

The bimolecular fluorescence complementation experiment was carried out as described by Chen et al (2008). The coding sequences of *GhHDT4D* and *GhHDA19* were cloned into JW771 and JW772 vectors, respectively. In the constructs, GhHDT4D was fused to the amino terminus of luciferase (nLUC) to generate nLUC-GhHDT4D, and GhHDA19 was fused to the carboxy terminus of LUC (cLUC) to generate GhHDA19-cLUC, respectively, using cLUC and nLUC as negative controls. The vectors were transferred into *Agrobacterium tumefaciens* stain GV3101. Resuspending *Agrobacterium* cells to OD1.0 with infiltration buffer (10 mM MgCl2, 10 mM MES (2-(N-morpholino) ethanesulfonic acid) pH 5.7, 150 mM acetosyringone). Then, the *Agrobacterium* cell solution was injected into tobacco (*Nicotiana benthamiana*) leaves. After 72 h of co-cultivation, the tobacco leaves were used for detecting luciferase activity using chemiluminescence image analysis system (Tanon 4600SF).

Western blot analysis

Total proteins were extracted from leaves of cotton seedlings or callus cells by the method described previously (Yao et al. 2006). The protein samples were loading onto SDS-PAGE gels. After separation by electrophoresis, proteins were transferred to a nitrocellulose membrane (Amersham) using a Trans-Blot Semi-DryElectrophoretic Transfer Cell (Bio-Rad). The primary antibodies anti-H3K9ac (Millipore), anti-H3 (Millipore) and anti-GFP (ABclonal) were incubated overnight at 4 degrees Celsius. The membrane was washed three times for 15 min each time by using TBST solution. Secondary rabbit antibody (CWBIO) was incubated with the membrane for 1.5 h. Then The membrane was washed three times for 15 min each time by using TBST solution. Proteins were detected using the eECL Western Blot Kit (CWBIO) and developed using chemiluminescence imaging system(LI-COR).

Chromatin immunoprecipitation (ChIP) assay

ChIP experiment was conducted as described previously (Liu et al. 2019). In brief, 3 g of leaves of cotton seedlings or callus cells were harvested and then fixed in 1% formaldehyde solution for 20 min in a vacuum. The cross-linking reactions were stopped by adding 0.125 M glycine. The samples were ground in liquid nitrogen and chromatin was extracted with extraction buffers as described in the protocol. Sonication was applied to shear chromatin into pieces with an average length of 500 bp. The chromatin fragments were immunoprecipitated with the specific antibody anti-H3K9ac and anti-GFP (Millipore and ABclonal). The DNA fragments were purified and used as templates for ChIP-PCR analysis. All the primers used in the experiments are listed in Table S2.

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Author contributions XBL and JBZ conceived and designed the research; JBZ, SPH, JWL, XPW and DDL performed the experiments; JBZ and XBL analyzed data and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no any competing interests.

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