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MbbHLH93, a transcription factor associated with cold and drought tolerance in *Malus baccata*

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Abstract

The bHLH transcription factor is known to regulate cold signals and stress tolerance. In the present study, a new bHLH gene *MbbHLH93*, located in the nucleus, was isolated from *Malus baccata*, whose up-regulated expression were strongly induced by cold and drought treatment, and *MbbHLH93*-overexpressed heterologous lettuce plants displayed cold and drought stress-tolerant phenotypes. Determination of physiological and biochemical indexes associated with abiotic stress responses showed that overexpression of *MbbHLH93* increased the activities of antioxidant enzymes superoxide dismutase, peroxidase, and catalase in lettuce plants treated with cold and drought stress, and decreased the contents of H_2O_2, $O_2^{\cdot-}$, and malondialdehyde, which contributed to reducing cell membrane lipid peroxidation. Meanwhile, the accumulation of proline in transgenic plant cells increased, regulating cell osmotic pressure. Furthermore, quantitative expression analysis indicated that overexpression of *MbbHLH93* improved the expression levels of *LsCBFs,* which were positive functional genes in response to cold and drought stress, enhancing plant tolerance. This research demonstrates that the *MbbHLH93* is a key regulator in plant tolerance to cold and drought stresses, providing new knowledge for plant tolerance regulation.

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Introduction

Cold and drought stresses are key abiotic environmental factors that strongly affect plant growth, development, and yield[1,2]. When plants are subjected to cold and drought stresses, it will cause an imbalance of free radical metabolism in cells, leading to the excessive accumulation of reactive oxygen species (ROS)[3]. And then leads to oxidative stress, which can cause oxidative damage to proteins, lipids, and pigments[4], and even lead to plant death. Therefore, it is of significance to research the effects of cold and drought stresses on plant growth and development and improve plant tolerance to cold and drought stresses.

A series of plant responses to environmental stress were regulated by transcription factors, which receive stress signals and modulated the expression of stress-related functional genes. The pathway 'CBF-COR' among many stress regulation pathways was the focus of researchers. CBF (C-repeat binding transcription factor), also known as DREB1, is a class of plant-specific transcription factors activated by cold stress or ICE. Plant cold-responsive (COR) genes can produce cold regulatory proteins to improve plant cold tolerance. The CCGAC sequence of CBF can bind to the CRT/DRE cis-element in the COR gene promoter to activate the cold-resistance response. Tobacco NtbHLH123 confers tolerance to cold stress by regulating the *NtCBF* pathway and reactive oxygen species homeostasis[5]. The bHLH transcription factor ICE1 induced the expression of *CBF3/DREB1A* and COR genes under cold stress by combining with the cis-acting element MYC (CANNTG) of their promoter regions, to improve the tolerance of transgenic plants to stress[6]. *MaNAC1* from banana (*Musa acuminata*) is directly bound to the *MaICE1* promoter to target *MaCBF1* and enhance the cold tolerance of banana plants through a CBF-dependent pathway[7]. Overexpression of wild rice *OrbHLH2*, which is highly homologous to ICE1, induces the up-regulation of salt stress-related genes such as *DREB1A/CBF3*, *COR15A*, and enhances the tolerance to salt stress in transgenic plants[8]. Members of the bHLH gene family might regulate environmental stress response *via* the CBF-dependent pathway. However, it is not clear whether bHLH93, which belongs to the same bHLH IIIb subgroup is also involved in the CBF pathway playing a role in defense against abiotic plant stresses.

Apple is one of the most important economic fruit trees in the world. So far, there has been a certain basis for the research on the stress tolerance regulation mechanism of the bHLH gene in cultivated apple (*Malus domestica*). The MdCIbHLH1 protein binds to the promoter of *MdCBF2* to up-regulate the expression of *MdCBF2*, which contributes favorably to the cold tolerance of transgenic apple plants[9]. Under cold conditions, MdbHLH4 represses the expression of *MdCBF1* and *MdCBF3* by directly binding to their promoters. MdbHLH4 also interacts with MdCICE1L, a homolog of *AtICE1* in apple, and represses the

binding of MdCCE1L to the *MdCBF1/3* promoter, which inhibits its expression negatively regulating cold tolerance in plants[10]. MdABI4 positively regulated apple cold tolerance by interacting with MdICE1, which activated the downstream cold stress-crucial gene *MdCBF1*. While *MdJAZ1/2* interferes with the interaction between MdABI4 and MdICE1 to negatively regulate apple cold tolerance. Therefore, *MdABI4* accurately regulates cold tolerance of apple plants by integrating JA and ABA signals to form a JAZ-ABI4-ICE1-CBF cascade pathway[11]. Similarly, overexpression of bHLH transcription factor MdbHLH33 increased the expression levels of cold stress-related genes *MdCBF2*, *MdCOR15A-1*, and *MdCOR15A-2* in apple calli, and MdbHLH33 could bind to LTR cis-acting elements of *MdCBF* promoter to positively regulate low-temperature stress[12].

Wild apple (*Malus baccata*) as an important germplasm resource of apple tolerance is rich in valuable resistance genes, which is of great significance for apple resistance breeding. *MxNAS1* contributes to transgenic tobacco plants' tolerance to Fe stress by increasing the plant's antioxidant capacity and *MbCBF1* also contributes to plant tolerance against cold and salt stress[13,14]. In the present study, another cold- and drought-induced transcription factor *MbbHLH93* was identified and cloned. Phylogenetic and structural analysis revealed that MbbHLH93 is a typical bHLH family protein. To determine the function of *MbbHLH93* in plant tolerance to stress, the *MbbHLH93*-overexpressed heterologous lettuce (*Lactuca sativa*) was obtained to investigate its effect on plant tolerance. The results confirm that *MbbHLH93* functions to enhance the cold and drought tolerance of lettuce. Heterologous expression of *MbbHLH93* up-regulated the expression levels of its downstream stress response crucial genes *LsCBFs* and increased the contents of chlorophyll and proline as well as the activities of antioxidant enzymes but reduced the accumulation of MDA, H_2O_2, and $O_2^{\cdot-}$ in lettuce leaves, alleviating cold and drought damage to lettuce plants. The present study provides important candidate genes for further study of plant tolerance regulation and lays a foundation for the genetic improvement of tolerance plants.

Materials and methods

Plant materials

Apple plants (*Malus baccata*) from previous research[15] were precultured in Hoagland hydroponic solution at 25 °C and 80% relative humidity for a 16-h light/8-h dark cycle. The nutrient solutions were replaced every 3 d during the experiment. A total of 50 seedlings of *M. baccata* displaying similar growth (well-developed roots and 10-12 leaves) were separated into five groups for control, cold (4 °C), heat (37 °C), salt (200 mM NaCl), and drought (20% PEG6000) treatments for 12 h[16]. Roots and new leaves of various treatments were sampled at 0, 1, 3, 6, 9, and 12 h.

The Hong Kong iceberg lettuce (*Lactuca sativa* L.) was used in the present study. Lettuce seeds were disinfected with 75% ethanol and 10% sodium hypochlorite solution and seeded in 1/2 MS (Murashige & Skoog) solid medium. After 1 d of vernalization at 4 °C, then grown at 25 °C and 70% relative humidity for a 16-h light/8-h dark cycle.

Cloning and characterization of *MbbHLH93*

Total RNA was extracted from the leaves of *M. baccata* using an OminiPlant RNA Kit (Kangweishiji, Beijing, China) and the cDNA synthesis was conducted with HiFiScript gDNA Removal RT MasterMix (Kangweishiji, Beijing, China). *MbbHLH93* was cloned with the specific primer shown in Supplementary Table S1.

The protein sequences of MbbHLH93 (*M. baccata*) were obtained according to the MdbHLH93 (*Malus* × *domestica*), and were retrieved from the Genome Database for Rosaceae (GDR) (www.rosaceae.org). MbbHLH93 were used as query proteins to identify their homologous proteins in pear (*Pyrus communis*), peach (*Prunus persica*), and black raspberry (*Rubus occidentalis*) with a protein-protein BLAST (BLASTp) in GDR[17,18]. Structural motif annotation of MbbHLH93 was analyzed *via* the MEME program (http://meme-suite.org/). Phylogenetic analyses of MbbHLH93 orthologous proteins from these four *Rosaceae* species were conducted *via* MEGA6.0 software (www.megasoftware.net) by the maximum likelihood method based on 1,000 bootstrap replicates. Subsequently, the physiological and biochemical properties of tested proteins were conducted with the ProtParam online website (https://web.expasy.org/protparam/).

Subcellular localization of MbbHLH93

The coding sequences (CDS) of *MbbHLH93* without stop codon were inserted into the *BamH* I and *Sal* I sites downstream of GFP in the pSAT6-GFP-N1 vector, obtaining the fusion expression vector pro35S::MbbHLH93::GFP. Subsequently, the fusion expression vector and pSAT6-GFP-N1 empty vector were respectively transformed into tobacco leaves for 24 h according to a previous study[19]. 0.5 μg/mL 4,6-diamidino-2-phenylindole (DAPI) (Solarbio, China) served as cell nuclear dye. GFP and conjugate of DNA and DAPI fluorescence were observed respectively *via* a confocal laser scanning microscope (LSM 710; Carl Zeiss) at the wavelengths of 488 nm and 405 nm.

Expression analysis of *MbbHLH93*

The expression analysis of *MbbHLH93* was performed *via* qRT-PCR reactions based on a previous description[20]. Each reaction involved three biological replicates for error minimization, and the transcript level of *MbbHLH93* was determined *via* the $2^{-\Delta\Delta CT}$ method with *Actin* (EB127077) as an internal reference. Primer sequences crafted for this experiment are shown in Supplementary Table S1.

Gene transformation

To obtain *MbbHLH93* transgenic lettuce, the CDS of *MbbHLH93* was introduced into pCAMBIA2300 vectors under the control of the CaMV35S promoter, constructing the *MbbHLH93* overexpression vector. Genetic transformation of the lettuce plant was performed by the *Agrobacterium*-mediated leaf disk method[21]. The positive transgenic lettuce plants were screened in a selection medium containing kanamycin and identified by semi-quantitative RT-PCR analyses. The homozygous T_2 generation plants were used for subsequent experiments.

Stress treatments of lettuce plants and physiological measurements

A total of 30 seedlings of 25-day-old lettuce displaying similar growth were separated into three groups for control, cold (−7 °C for 6 h), and drought (no-watered for 7 d) treatments and lettuce plants were subjected respectively to 6 h and 7 d of low temperature and drought stress were collected for subsequent determination of genes and physiological indexes related to

low-temperature response. The plant phenotype and survival rates were analyzed after returning the stressed lettuces to normal growth for 15 d.

The content of chlorophyll, free proline, malondialdehyde (MDA), relative electrolyte leakage (EL), active oxygen (H_2O_2 and $O_2^{\cdot-}$), and the enzyme activity of superoxide dismutase (SOD), peroxidase POD, and catalase (CAT) were measured respectively based on previous research[22,23]. Each reaction involved three biological replicates for error minimization.

Detection of key stress-related genes

Total RNA extraction and cDNA synthesis from the leaves of cold- and drought-stressed lettuce refer to the methods above. The qRT-PCR was performed to analyze the expression levels of stress-related *LsCBF* genes in control and transgenic lettuce, with *LsIPP2* and *LsEIF2* as internal references[24,25]. Primer sequences crafted for this experiment are shown in Supplementary Table S1.

Statistical analysis

The experimental data were processed using IBM SPSS Statistics 21 software. One-way ANOVA followed by Tukey's multiple range test ($p < 0.05$) was used to compare significant differences between all the groups of each data set, and values are shown as the mean ± standard deviation (SD) of biological triplicates.

Results

Characterization and phylogenetic analysis of *MbbHLH93*

The coding region of *MbbHLH93* contains 1,428 bases encoding a predicted protein of 498 amino acids (aa). Physiological and biochemical properties of MbbHLH93 indicated that MbbHLH93 protein is probably an acidic hydrophilic protein (Supplementary Table S2). MbbHLH93 contained conserved bHLH and ACT-like domains at the C-terminal, which were consistent with other tested family members in *Rosaceae* species, indicating *MbbHLH93* belongs to the bHLH family gene (Fig. 1a). Further, phylogenetic analysis showed that bHLH93 of apple and pear are closely related (Fig. 1b).

Subcellular localization of MbbHLH93

To characterize the biological role of *MbbHLH93* in plant stress response, a MbbHLH93::GFP construct was transiently transformed into tobacco leaves to generate a fluorescently tagged MbbHLH93 protein for observation of protein subcellular localization. Green fluorescence was present in the whole cell of positive control, while only distinct fluorescence was observed in the nucleus (Fig. 2b, f), and the DAPI (4,6-diamidino-2-phenylindole) staining also confirmed that MbbHLH93 was targeted to the nucleus *in vivo* (Fig. 2a, e).

Expression patterns of *MbbHLH93* in various tissues of *Malus baccata*

To understand the expression patterns of *MbbHLH93* genes in *Malus baccata* plants, qRT-PCR analysis was conducted for evaluating *MbbHLH93* expression level in root, stem, new leaves, and mature leaves. The *MbbHLH93* showed significantly different expression levels in different tissues, and a high expression amount was observed in new leaves and roots (Supplementary Fig. S1). Subsequently, the variation trend of *MbbHLH93* gene expression was observed in roots and new leaves during the cold, drought, salt, and hot treatments. The results indicated that all four abiotic stresses could induce the expression of *MbbHLH93*, and with the extension of stress treatment time, the expression level of *MbbHLH93* in new leaves and roots showed a trend of first increasing and then decreasing (Fig. 3). It was worth noting that cold and drought treatments could rapidly induce large amounts of expression of *MbbHLH93*

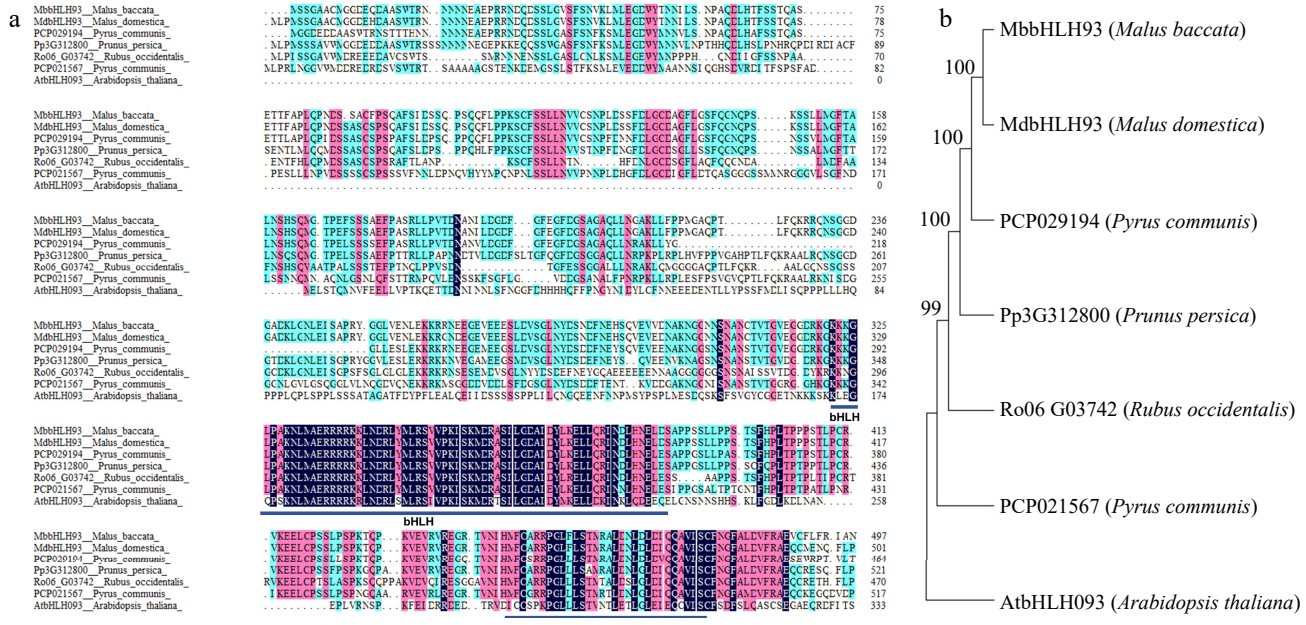

Fig. 1 Sequence alignment and phylogenetic analysis of bHLH93 in *Rosaceae* species. (a) Sequence alignment of MbbHLH93. The conserved amino acid residues are shown in purple. The bHLH and ACT-like domain are displayed respectively with rectangles in dark blue. (b) Phylogenetic analysis of MbbHLH93. The MbbHLH93 orthologous proteins from *Arabidopsis* and four *Rosaceae* species were obtained to build a phylogenetic tree *via* the maximum likelihood method based on the 1,000 bootstrap analyses of the MEGA6.0 software.

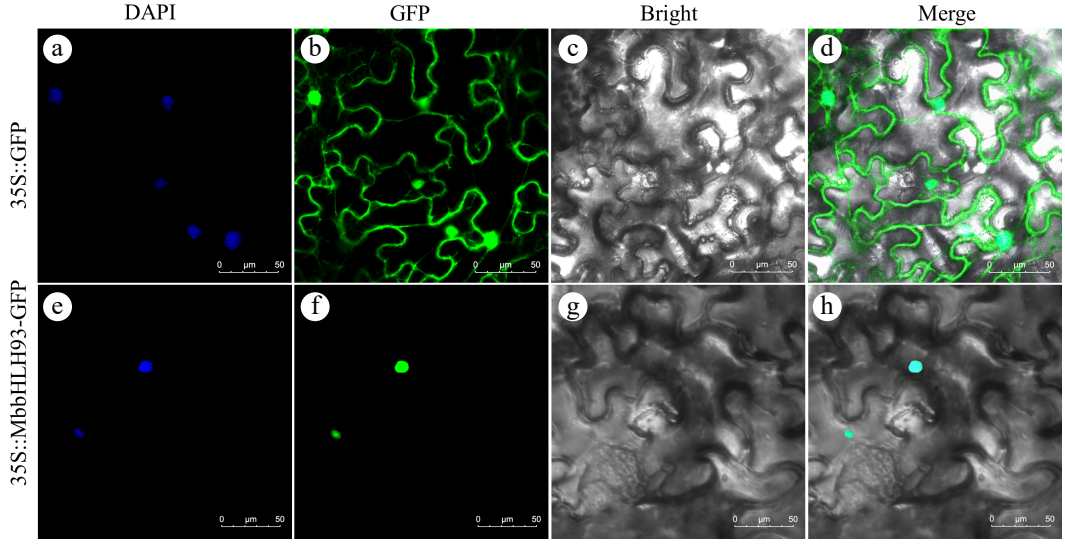

Fig. 2 Subcellular localization of MbbHLH93. 35Spro::MbbHLH93::GFP was expressed transiently into tobacco leaves with 35Spro::GFP as positive control. (a), (e) DAPI dyeing; (b), (f) GFP signals; (c), (g) bright field; (d), (h) merge. Scale bars: 50 μm.

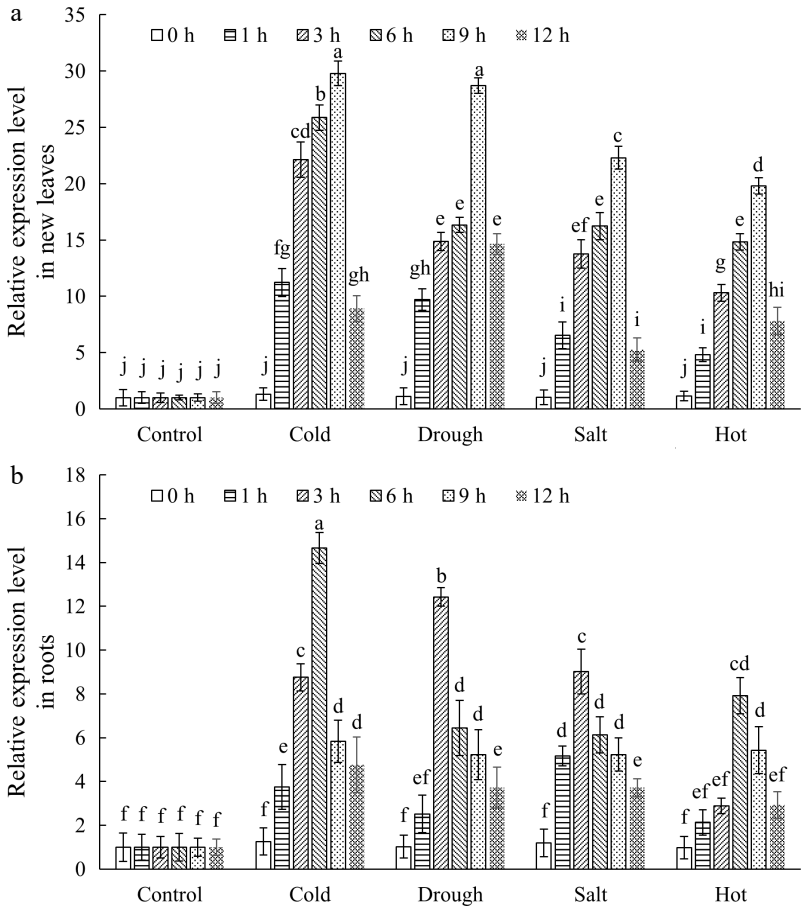

Fig. 3 The expression patterns of *MbbHLH93* in *Malus baccata* under various stress treatments. (a) The expression levels of *MbbHLH93* in new leaf under stress treatments. (b) The expression levels of *MbbHLH93* in root under stress treatments. The leaf and root samples were collected 0, 1, 3, 6, 9, and 12 h after treatments. Relative expression levels of *MbbHLH93* were calculated *via* the $2^{-\Delta\Delta CT}$ method and values were shown as the mean ± standard deviation (SD) based on three repetitions. Lowercase letters displayed the significant differences at $p < 0.05$ (one-way ANOVA followed by Tukey's multiple range test).

in the early stage of stress treatment. The highest expression abundance was detected in the roots induced by 6 h of low temperature and 3 h of drought, as well as in the new leaves induced by 9 h of low temperature and 9 h of drought (Fig. 3), indicating *MbbHLH93* might play an essential role in plant low temperature and drought tolerance.

Functional verification of *MbbHLH93* in enhancing cold tolerance of lettuce

To determine the function of *MbbHLH93* in affecting plant stress tolerance, the *MbbHLH93* overexpression vector pCAMBIA2300-MbbHLH93 was transformed into lettuce plants. The lettuce exhibits a short life cycle and the necessity of cold-resistant production in northeast China[25]. Six *MbbHLH93*-overexpressed lettuce lines (L1, L2, L3, L7, L8, and L11) were identified *via* kanamycin tolerance and semi-quantitative RT-PCR analyses (Supplementary Fig. S2). Among them, three lines (L1, L7, L8) were screened for following research with the wild type (WT) and empty vector line (VL) transformed lettuces as controls.

Subsequently, the phenotype of control and *MbbHLH93*-overexpressed lettuce lines after being treated with low temperature (−7 °C for 6 h, and recovered at room temperature for 15 d) were analyzed. The results showed control and transgenic lettuces suffered from freezing injury to different extents under longer duration of cold stress, while, obviously more severe damage was observed in control plants. Most of the transgenic lettuce lines rather than control lines after cold stress were able to resume normal growth when they were transferred to room temperature (Fig. 4a). These results confirmed that overexpression of *MbbHLH93* significantly improved plant adaptability to cold stress.

Cold tolerance is closely related to a series of physiological parameters in plants[26,27]. To analyze the basis for the altered tolerance to stress in *MbbHLH93*-overexpressed transgenic materials, cold-related physiological indicators were analyzed.

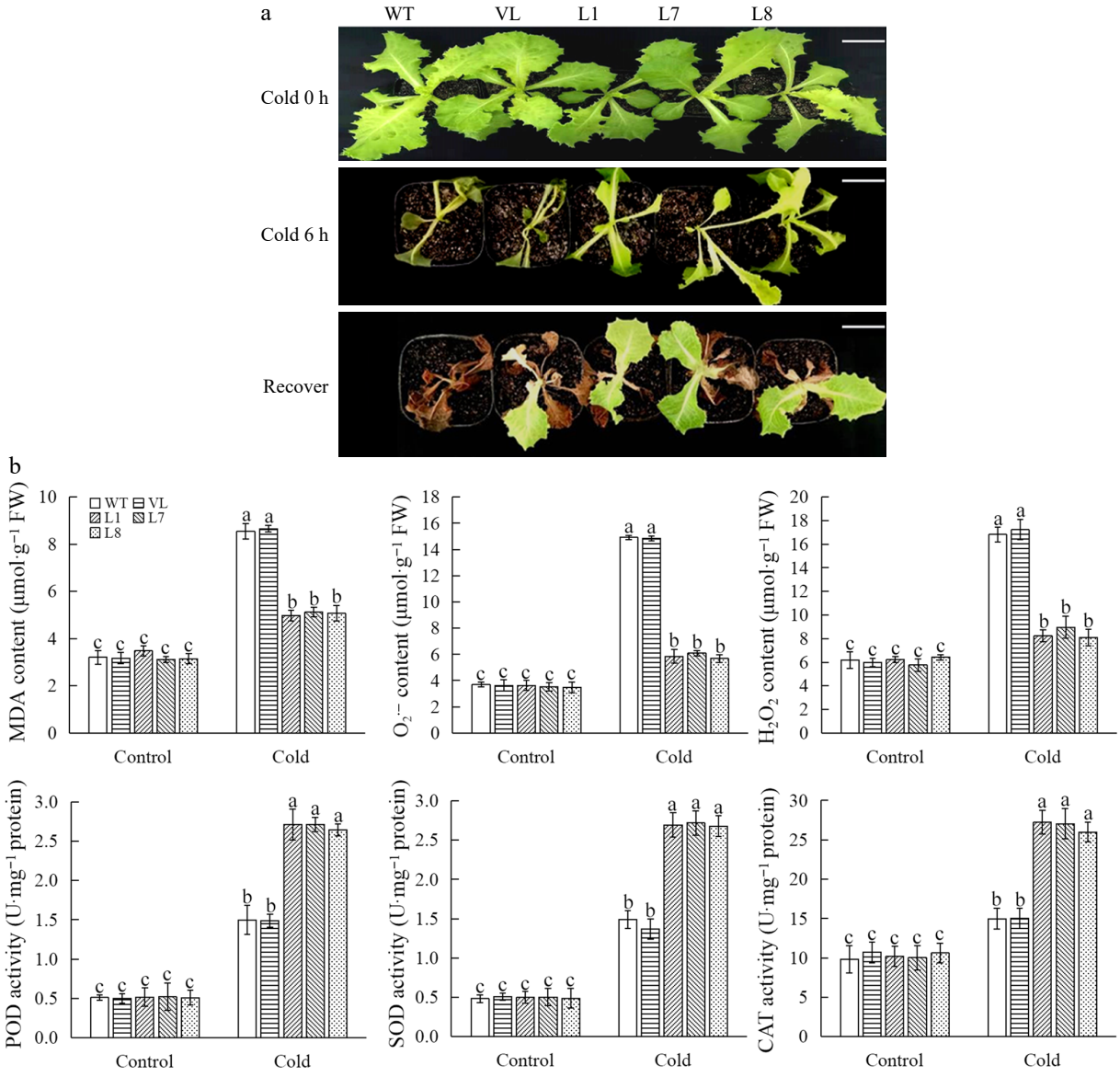

Fig. 4 *MbbHLH93* enhances cold resistance of lettuce plants. (a) Phenotype of wild line (WT), vector line (VL), and *MbbHLH93*-overexpressed lettuce (L1, L7, L8) under cold for 0 h (control), cold for 6 h, and recovery for 15 d. Scale bar: 3 cm. (b) Determinations of physiological indices associated with cold stress in wild line (WT), vector line (VL), and *MbbHLH93*-overexpressed lettuces (L1, L7, L8) under cold for 0 h (control) and cold for 6 h. Values are shown as the mean ± standard deviation (SD) based on three repetitions. Lowercase letters show the significant differences at $p < 0.05$ (one-way ANOVA followed by Tukey's multiple range test).

Under normal growth conditions, the relative electrolyte leakage of control and transgenic lettuce plants was similar (Supplementary Fig. S3). After low-temperature stress, electrolyte leakage of control was significantly higher than that of *MbbHLH93*-overexpressed lettuce lines, indicating that control plants were more subjected to cell membrane damage during low-temperature stress than transgenic plants. Proline content increased and chlorophyll content declined significantly in lettuce plants after experiencing low-temperature stress, while the contents of proline and chlorophyll in *MbbHLH93*-overexpressed strains were both significantly higher than that of control after cold stress (Supplementary Fig. S3). Similar to the change in chlorophyll, the overexpression of *MbbHLH93* also significantly increase the antioxidant enzyme (SOD, POD, CAT) activities under cold stress (Fig. 4b). Additionally, low temperature caused an increased accumulation of H_2O_2, $O_2^{\cdot-}$ as well as MDA in lettuce cells, but the heterologous expression of *MbbHLH93* in lettuces relieved their accumulation to some extent (Fig. 4b).

Functional verification of *MbbHLH93* in enhancing drought tolerance of lettuce

To further explore the function of *MbbHLH93* in affecting plant drought tolerance, a waterless stress assay was conducted for 7 d to assess the phenotype and survival rate of lettuces. In keeping with the results of cold stress, after drought treatment, control lines were subjected to more serious damage than transgenic lettuces. Most of the control seedlings nearly died when they were returned to room temperature for 7 d, while *MbbHLH93*-overexpressed transgenic lettuces could resume growth (Fig. 5a). These results displayed the drought tolerance function of *MbbHLH93*.

Moreover, drought-related physiological indicators were analyzed in control and transgenic lines. The results showed that drought stress led to an increase in the relative electrolyte leakage and proline, MDA, H_2O_2, and $O_2^{\cdot-}$ content as well as antioxidant enzyme activity, while a decline of chlorophyll content was seen. After drought treatment, *MbbHLH93*-overexpressed lettuces showed a lower relative electrolyte leakage and MDA content but higher proline and chlorophyll contents compared to the control (Supplementary Fig. S3). In addition, drought stress contributed to the activity of antioxidant oxidase increased more and the accumulation of reactive oxygen species (ROS) decreased in transgenic lettuce compared to the control (Fig. 5b).

Over-expression of *MbbHLH93* enhanced the expression of the stress response gene

To genetically clarify if the effect of *MbbHLH93* overexpression on plant tolerance is dependent on the classical CBF pathway, the expression levels of *LsCBF* genes were determined in transgenic lines. Low temperature induced the up-regulated expression of most *Ls*CBF genes, except for *LsCBF2* and *LsCBF13*. After low-temperature stress, the expression level of most *LsCBFs* in transgenic lettuce was significantly higher than that in control. Similarly, the expression levels of most *CBF* genes were positively induced after drought treatment, and drought treatment also significantly increased the expression level of *LsCBFs* in transgenic lettuce compared to the control (Fig. 6). These results indicated that *MbbHLH93* could positively regulate the expression of *LsCBF* genes, thereby improving the cold and drought tolerance of plants.

Discussion

Abiotic stress seriously damages plant growth and crop yield[28]. Transgenic technology has become a mature and rapid method to cultivate resistant crops with the development of biotechnology, which highly relies on the extraction and functional identification of key genes associated with stress regulation[29,30]. To date, researchers have demonstrated that bHLH family proteins play an important role in abiotic stress responses, but there are few studies on the anti-stress application of bHLH family proteins in wild apple (*Malus baccata*). In the present study, the wild apple bHLH family gene *MbbHLH93* was cloned and transferred into lettuce to identify its biological function of regulating plant cold and salt tolerance. The analyses of gene structure and phylogenetics showed that *MbbHLH93* was a bHLH family gene (Fig. 1a). Under low temperature and drought stress, the *MbbHLH93* was significantly induced and the survival rate of *MbbHLH93* transgenic lettuces was significantly higher than that of controls (Figs 4a & 5a), indicating that *MbbHLH93* functioned in significantly enhancing plant low temperature and drought tolerance.

The bHLH transcription factors play crucial roles in various biological processes, such as plant flowering, pollen fertility, plant stomata, embryo and seed development, and abiotic stress response[31,32]. *AtbHLH093*, belongs to the ICE1 family bHLH-LZs, has a role in controlling flowering time and is required for apical meristem function[33,34]. Furthermore, overexpression of *AtbHLH093* leads to a weak decrease in the number of mature stomatal phenotype[35]. Some studies have also pointed out that bHLH93 plays an important role in tolerance to plant stress. Ding et al. predicts that *Prunus mume* PmbHLH06 (bHLH93) can directly interact with PmbHLH38 (FBH4) thereby forming a dimer to function under low temperature stress[36]. Knockdown of *bHLH93* in tobacco impaired disease tolerance by reducing the expression of the defense gene *PDF1.2*[37]. MdbHLH093 increases H_2O_2 accumulation and activates the SA signaling pathway in interaction with MdMYB116 to improve apple tolerance to powdery mildew[38]. Another recent study showed that MdbHLH093 in apple positively regulates dopamine accumulation through transcriptional regulation of *MdTyDC* conferring drought tolerance[39]. To further understand the function of *MbbHLH93* in plant stress response, the expression levels of *LsCBF* genes, and a series of stress-related physiological indicators in *MbbHLH93*-overexpressed lettuce were analyzed, indicating that *MbbHLH93* changed the stress-related physiological indicators in lettuce cells by up-regulating the expression of *LsCBFs*, thereby positively regulating the cold and drought tolerance of plants (Fig. 7).

CBF cold response pathway plays a crucial role in the cold regulatory network[14,40]. Heterologous expression of *AtCBF3* or *AtCBF1* could increase the low-temperature tolerance of transgenic eggplant[41], potato[42], and petunia[43]. At low temperature, ICEs can bind to the MYC recognition site of the *CBF* gene promoter to enhance the expression of the *CBF* gene and its downstream stress-related target gene[44]. Heterologous overexpression of grapevine *VabHLH1* and *VvbHLH1* in *Arabidopsis* could induce the expression of *AtCBF1, AtCBF2*, *AtCBF3,* and other cold response genes[45]. In this study, *MbbHLH93* could not induce the expression of *CBF* genes in lettuce plants grown at room temperature, which was consistent with previous

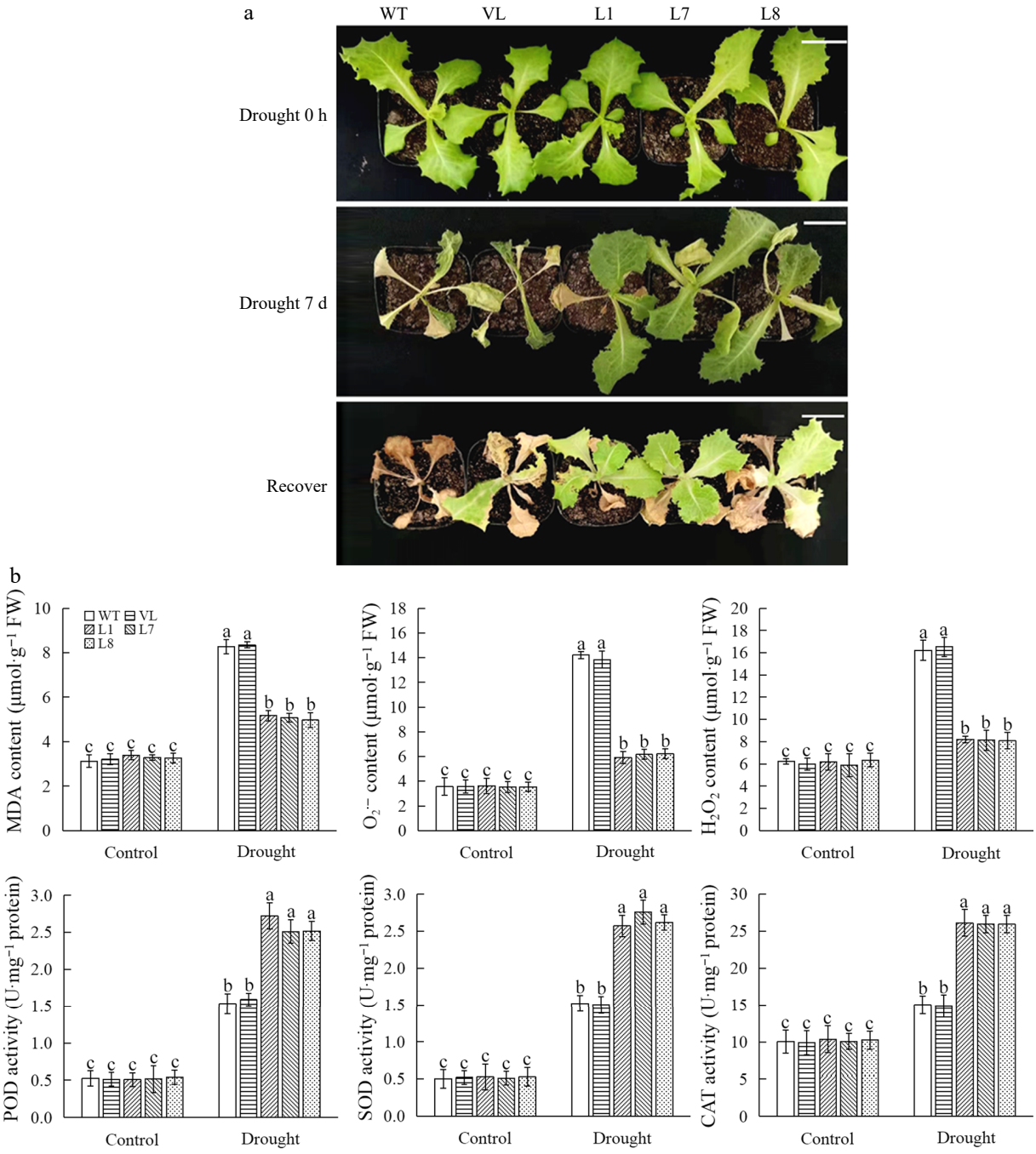

Fig. 5 *MbbHLH93* enhances drought resistance of lettuce plants. (a) Phenotype of wild line (WT), vector line (VL), and *MbbHLH93*-overexpressed lettuces (L1, L7, L8) under drought for 0 h (control), drought for 7 d and recovery for 15 d. Scale bar: 3 cm. (b) Determinations of physiological indices associated with drought stress in wild line (WT), vector line (VL), and *MbbHLH93*-overexpressed lettuce lines (L1, L7, L8) under drought for 0 h (control) and drought for 7 d. Values are shown as the mean ± standard deviation (SD) based on three repetitions. Lowercase letters indicate the significant differences at $p < 0.05$ (one-way ANOVA followed by Tukey's multiple range test).

findings[6]. After low temperature and drought treatment, the overexpression of *MbbHLH93* induced significantly the up-regulated expression of a large number of *LsCBFs* gene in lettuce (Fig. 6), suggesting that *MbbHLH93* could enhance cold tolerance by activating *LsCBFs* expression. Research conducted on different plants has shown variations in the response of CBF proteins to different environmental stresses. Cold-treated *Arabidopsis* plants showed higher expression of *CBF1*, *CBF2*, and *CBF3* genes, in contrast to salinity and drought treatments[46]. While *CBF1*, *CBF2*, and *CBF3* are more tolerance to drought, the *CBF4* gene in grapes is typically activated by cold treatment[47]. In *MbbHLH93* transgenic lines, *LsCBF1/3/5/6* were significantly induced by both cold and drought, whereas *LsCBF2/7* and *LsCBF8/9/10/12* responded better to a single drought or cold (Fig. 6). This result suggested functional differences among different *LsCBF* genes in stress response and provided potential CBFs for the *MbbHLH93* transgenic lines in response to drought and cold stress.

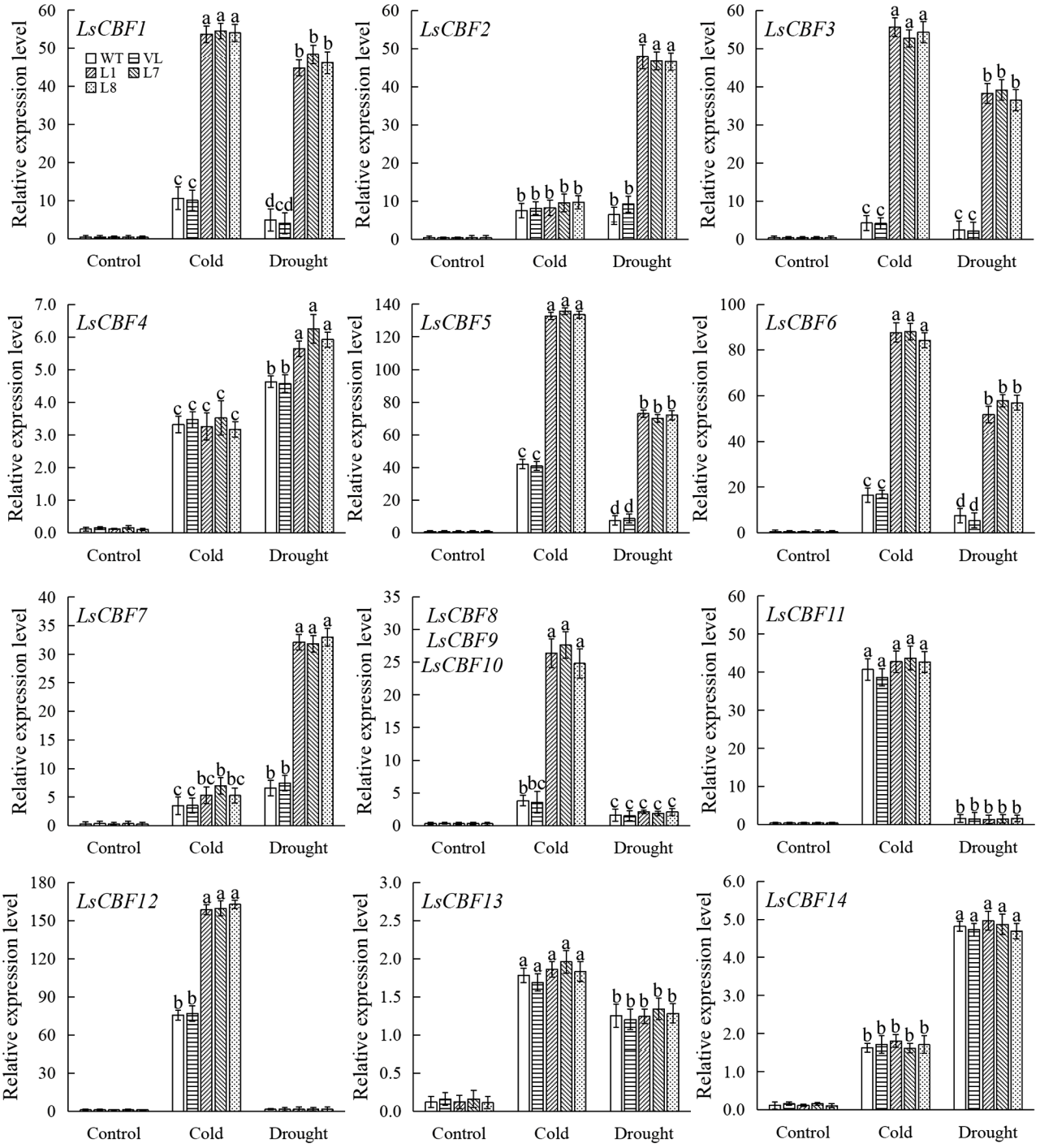

Fig. 6 Expression of *LsCBF* genes in wild line (WT), vector line (VL), and *MbbHLH93*-overexpressed lettuce lines (L1, L7, L8) after control, cold and drought stresses. *LsCBF8*, *LsCBF9*, and *LsCBF10* were quantitatively amplified with the same set of primers. Relative expression levels of *LsCBFs* were calculated *via* the $2^{-\Delta\Delta CT}$ method and values are shown as the mean ± standard deviation (SD) based on three repetitions. Lowercase letters indicate the significant differences at $p < 0.05$ (one-way ANOVA followed by Tukey's multiple range test).

Abiotic stress will cause many physiological and biochemical changes in plant cells, such as electrolyte leakage and MDA content are indexes to assess membrane damage[15,48], and proline content affects osmotic regulation to protect proteins and cell membranes in response to environmental stresses[49,50]. *SlbHLH96* and a bHLHm1 transcription factor gene *MdSAT1* caused declined electrolyte leakage and MDA contents, and increased cold tolerance[51]. The overexpression of *MdbHLH130* in tobacco also enhanced the plant tolerance *via* regulating the level of physiological indexes[52]. In accordance with the above results, overexpression of *MbbHLH93* also decreased the electrolyte leakage and contents of MDA, but increased proline contents in lettuce plants after cold and drought treatment (Supplementary Fig. S3). It suggests that the higher tolerance of *MbbHLH93* overexpression lines to low-temperature and drought may be attributed to the regulation

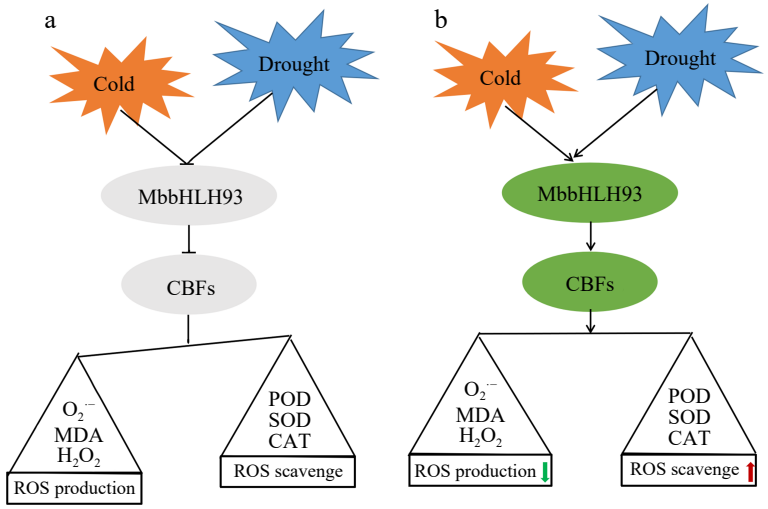

Fig. 7 Working model of MbbHLH93 function on the cold and drought adaptation in lettuce. (a) There is no *MbbHLH93* in wild lettuce lines, and a large number of ROS accumulate in the cells under low temperature and drought treatment, threatening the normal growth of plants. (b) Cold and drought stress treatments rapidly induced large expression of transcription factor *MbbHLH93* in *MbbHLH93*-overexpressed lettuce lines, and regulated lettuce plants' adaptation to stress through the classic CBF pathway. Primarily, membrane lipid peroxidation caused by large accumulation of reactive oxygen species in cells was reduced *via* improving the activities of antioxidant enzymes in transgenic lines, increasing plant stress resistance.

of osmotic homeostasis, which may be one of the mechanisms of tolerance to abiotic stresses.

In addition, ROS accumulation was used to evaluate stress damage[53]. Excessive accumulation of ROS led to cell oxidative damage, affecting plant growth and development. The present study found that cold and drought stress induced excessive ROS accumulation in lettuce plants (Figs 4b & 5b). The lower H_2O_2 and $O_2^{\cdot-}$ accumulation in *MbbHLH93* overexpressed lines indicated that these transgenic materials suffered less stress damage. These results were consistent with lower electrolyte leakage and MDA accumulation in transgenic plants[54]. Plants have evolved essential defense mechanisms, such as enzyme scavenging systems, aimed at preventing the excessive accumulation of ROS[55]. The enzyme activities of CAT, POD, and SOD in *MbbHLH93* transgenic lettuces were significantly higher than those of the control under cold and drought stress (Figs 4b & 5b), indicating that *MbbHLH93* can improve the activity of antioxidant enzymes in lettuce cells under stress, which is the main reason for less ROS accumulation in transgenic materials. SOD, as an important protective enzyme for ROS scavenging in plants, scavenged excess $O_2^{\cdot-}$ and directly mitigated $O_2^{\cdot-}$ induced damage to cell membranes in the *MbbHLH93* transgenic line. CAT and POD were able to act synergistically to break down H_2O_2, and the increase in their activity in *MbbHLH93* lines maximized the scavenging of potentially harmful ROS and protected the structure of the cell membrane. These results also indicated that *MbbHLH93* could enhance the scavenging ability of ROS by regulating cell osmotic potential and enhancing plant tolerance to low temperature and drought.

Conclusions

In summary, the present study demonstrates the function of transcription factor gene *MbbHLH93* in enhancing plant tolerance to cold and drought. *MbbHLH93* was rapidly induced under low temperature and drought stress, and its overexpression up-regulated the expression of a series of *LsCBFs*, the key genes that respond to abiotic stress, in lettuce and probably regulated the physiological and biochemical changes of cells *via* the 'CBF' pathway to cope with stress response. Specifically, the overexpression of *MbbHLH93* increased the content of intracellular osmotic regulatory substances and maintained the balance of cellular and external osmosis. Moreover, membrane lipid peroxidation caused by a large accumulation of ROS in cells was reduced *via* improving the activities of antioxidant enzymes in transgenic lines, increasing plant stress tolerance. These findings enrich our understanding of tolerance regulation of *Malus baccata* and lay a foundation for plant tolerance regulation and genetic improvement.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Han D, Huo J; data collection: Zhang L, Xu Y, Lv L, Wang T; analysis and interpretation of results: Zhang L, Liu W, Li X, Li W; draft manuscript preparation: Zhang L, Xu Y. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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References

1. Zhao C, Wang P, Si T, Hsu CC, Wang L, et al. 2017. MAP Kinase cascades regulate the cold response by modulating ICE1 protein stability. *Developmental Cell* 43:618–629.e5
2. Li H, Ding Y, Shi Y, Zhang X, Zhang S, et al. 2017. MPK3- and MPK6-mediated *ICE1* phosphorylation negatively regulates *ICE1* stability and freezing tolerance in *Arabidopsis*. *Developmental Cell* 43:630–642.e4
3. Han D, Zhang Z, Ding H, Wang Y, Liu W, et al. 2018. Molecular cloning and functional analysis of *MbWRKY3* involved in improved drought tolerance in transformed tobacco. *Journal of Plant Interactions* 13:329–37
4. Bhatt D, Negi M, Sharma P, Saxena SC, Dobriyal AK, et al. 2011. Responses to drought induced oxidative stress in five finger millet varieties differing in their geographical distribution. *Physiology and Molecular Biology of Plants* 17:347–53
5. Zhao Q, Xiang X, Liu D, Yang A, Wang Y. 2018. Tobacco transcription factor *NtbHLH123* confers tolerance to cold stress by regulating the *NtCBF* pathway and reactive oxygen species homeostasis. *Frontiers in Plant Science* 9:381
6. Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, et al. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Genes and Development* 17:1043–54
7. Shan W, Kuang JF, Lu WJ, Chen JY. 2014. Banana fruit NAC transcription factor MaNAC1 is a direct target of *MaICE1* and involved in cold stress through interacting with *MaCBF1*. *Plant, Cell & Environment* 37:2116–27
8. Zhou J, Li F, Wang JL, Ma Y, Chong K, et al. 2009. Basic helix-loop-helix transcription factor from wild rice (*OrbHLH2*) improves tolerance to salt- and osmotic stress in *Arabidopsis*. *Journal of Plant Physiology* 166:1296–306
9. Feng X, Zhao Q, Zhao L, Qiao Y, Xie X, et al. 2012. The cold-induced basic helix-loop-helix transcription factor gene *MdCIbHLH1* encodes an ICE-like protein in apple. *BMC Plant Biology* 12:22
10. Yang J, Guo X, Mei Q, Qiu L, Chen P, et al. 2023. MdbHLH4 negatively regulates apple cold tolerance by inhibiting *MdCBF1/3* expression and promoting *MdCAX3L-2* expression. *Plant Physiology* 191:789–806
11. An JP, Xu RR, Liu X, Su L, Yang K, et al. 2022. Abscisic acid insensitive 4 interacts with ICE1 and JAZ proteins to regulate ABA signaling-mediated cold tolerance in apple. *Journal of Experimental Botany* 73:980–97
12. Wang S, Zhang Z, Li LX, Wang HB, Zhou H, et al. 2022. Apple MdMYB306-like inhibits anthocyanin synthesis by directly interacting with *MdMYB17* and *MdbHLH33*. *The Plant Journal* 110:1021–34
13. Han D, Yang G, Xu K, Shao Q, Yu Z, et al. 2013. Overexpression of a *Malus xiaojinensis Nas1* gene influences flower development and tolerance to iron stress in transgenic tobacco. *Plant Molecular Biology Reporter* 31:802–09
14. Liang X, Luo G, Li W, Yao A, Liu W, et al. 2022. Overexpression of a *Malus baccata* CBF transcription factor gene, *MbCBF1*, increases cold and salinity tolerance in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* 192:230–42
15. Han D, Wang Y, Zhang L, Ma L, Zhang X, et al. 2012. Isolation and functional characterization of *MxCS1*: a gene encoding a citrate synthase in *Malus xiaojinensis*. *Biologia Plantarum* 56:50–56
16. Han D, Wang L, Wang Y, Yang G, Gao C, et al. 2013. Overexpression of *Malus xiaojinensis CS1* gene in tobacco affects plant development and increases iron stress tolerance. *Scientia Horticulturae* 150:65–72
17. Verde I, Jenkins J, Dondini L, Micali S, Pagliarani G, et al. 2017. The Peach v2.0 release: high-resolution linkage mapping and deep resequencing improve chromosome-scale assembly and contiguity. *BMC Genomics* 18:225
18. Zhu L, Su J, Jin Y, Zhao H, Tian X, et al. 2021. Genome-wide identification, molecular evolution, and expression divergence of the hexokinase gene family in apple. *Journal of Integrative Agriculture* 20:2112–25
19. Ren C, Luo G, Li X, Yao A, Liu W, et al. 2023. *MxFRO4* confers iron and salt tolerance through up-regulating antioxidant capacity associated with the ROS scavenging. *Journal of Plant Physiology* 285:154001
20. Zhang L, Ma B, Wang C, Chen X, Ruan YL, et al. 2022. MdWRKY126 modulates malate accumulation in apple fruit by regulating cytosolic malate dehydrogenase (*MdMDH5*). *Plant Physiology* 188:2059–72
21. Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* 16:735–43
22. Han D, Shi Y, Yu Z, Liu W, Lv B, et al. 2015. Isolation and functional analysis of *MdCS1*: a gene encoding a citrate synthase in *Malus domestica* (L.) Borkh. *Plant Growth Regulation* 75:209–18
23. Li Y, Zhong J, Huang P, Shao B, Li W, et al. 2022. Overexpression of *MxFRO6*, a FRO gene from *Malus xiaojinensis*, increases iron and salt tolerance in *Arabidopsis thaliana*. *In Vitro Cellular & Developmental Biology - Plant* 58:189–99
24. Xu W, Jiao Y, Li R, Zhang N, Xiao D, et al. 2014. Chinese wild-growing *Vitis amurensis ICE1* and *ICE2* encode *MYC*-type *bHLH* transcription activators that regulate cold tolerance in *Arabidopsis*. *PLoS One* 9:e102303
25. Park S, Shi A, Mou B. 2020. Genome-wide identification and expression analysis of the *CBF/DREB1* gene family in lettuce. *Scientific Reports* 10:5733
26. Liu L, Duan L, Zhang J, Zhang Z, Mi G, et al. 2010. Cucumber (*Cucumis sativus* L.) over-expressing cold-induced transcriptome regulator *ICE1* exhibits changed morphological characters and enhances chilling tolerance. *Scientia Horticulturae* 124:29–33
27. Li J, Wang L, Zhu W, Wang N, Xin H, et al. 2014. Characterization of two *VvICE1* genes isolated from 'Muscat Hamburg' grapevine and their effect on the tolerance to abiotic stresses. *Scientia Horticulturae* 165:266–73
28. Manzoor MA, Xu Y, Iv Z, Xu J, Wang Y, et al. 2023. Fruit crop abiotic stress management: a comprehensive review of plant hormones mediated responses. *Fruit Research* 3:30
29. Lobato-Gómez M, Hewitt S, Capell T, Christou P, Dhingra A. et al. 2021. Transgenic and genome-edited fruits: background, constraints, benefits, and commercial opportunities. *Horticulture Research* 8:166
30. Anjanappa RB, Gruissem W. 2021. Current progress and challenges in crop genetic transformation. *Journal of Plant Physiology* 261:153411
31. Lee JH, Jung JH, Park CM. 2017. Light inhibits COP1-mediated degradation of ICE transcription factors to induce stomatal development in *Arabidopsis*. *The Plant Cell* 29:2817–30

32. Huang X, Li K, Jin C, Zhang S. 2015. *ICE1* of *Pyrus ussuriensis* functions in cold tolerance by enhancing *PuDREBa* transcriptional levels through interacting with PuHHP1. *Scientific Reports* 5:17620
33. Sharma N, Xin R, Kim DH, Sung S, Lange T, et al. 2016. NO FLOWERING IN SHORT DAY (NFL) is a bHLH transcription factor that promotes flowering specifically under short-day conditions in *Arabidopsis*. *Development* 143:682–90
34. Poirier BC, Feldman MJ, Lange BM. 2018. *bHLH093/NFL* and *bHLH061* are required for apical meristem function in *Arabidopsis thaliana*. *Plant Signaling & Behavior* 13:e1486146
35. Ohashi-Ito K, Bergmann DC. 2006. *Arabidopsis* FAMA controls the final proliferation /differentiation switch during stomatal development. *The Plant Cell* 18:2493–505
36. Ding Y, Li H, Zhang X, Xie Q, Gong Z, et al. 2015. OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in *Arabidopsis*. *Developmental Cell* 32:278–89
37. Zhuo T, Wang X, Chen Z, Cui H, Zeng Y, et al. 2020. The *Ralstonia solanacearum* effector RipI induces a defence reaction by interacting with the bHLH93 transcription factor in *Nicotiana benthamiana*. *Molecular Plant Pathology* 21:999–1004
38. Ma H, Zou F, Li D, Wan Y, Zhang Y, et al. 2023. Transcription factor *MdbHLH093* enhances powdery mildew resistance by promoting salicylic acid signaling and hydrogen peroxide accumulation. *International Journal of Molecular Sciences* 24:9390
39. Liu X, Wang Y, Ma X, Zhang H, Zhou Y, et al. 2024. MdbHLH93 confers drought tolerance by activating *MdTyDC* expression and promoting dopamine biosynthesis. *International Journal of Biological Macromolecules* 258:129003
40. Zhou MQ, Shen C, Wu LH, Tang KX, Lin J. 2011. CBF-dependent signaling pathway: a key responder to low temperature stress in plants. *Critical Reviews in Biotechnology* 31:186–92
41. Wan F, Pan Y, Li J, Chen X, Pan Y, et al. 2014. Heterologous expression of *Arabidopsis C-repeat binding factor 3* (*AtCBF3*) and *cold-regulated 15A* (*AtCOR15A*) enhanced chilling tolerance in transgenic eggplant (*Solanum melongena* L.). *Plant Cell Reports* 33:1951–61
42. Pino MT, Skinner JS, Jeknić Z, Hayes PM, Soeldner AH, et al. 2008. Ectopic *AtCBF1* over-expression enhances freezing tolerance and induces cold acclimation-associated physiological modifications in potato. *Plant, Cell & Environment* 31:393–406
43. Walworth AE, Song G, Warner RM. 2014. Ectopic *AtCBF3* expression improves freezing tolerance and promotes compact growth habit in petunia. *Molecular Breeding* 33:731–41
44. Kidokoro S, Shinozaki K, Yamaguchi-Shinozaki K. 2022. Transcriptional regulatory network of plant cold-stress responses. *Trends in Plant Science* 27:922–35
45. Xu W, Zhang N, Jiao Y, Li R, Xiao D, et al. 2014. The grapevine basic helix-loop-helix (*bHLH*) transcription factor positively modulates *CBF*-pathway and confers tolerance to cold-stress in *Arabidopsis*. *Molecular Biology Reports* 41:329–42
46. Gilmour SJ, Zarka DG, Stockinger EJ, Salazar MP, Houghton JM, et al. 1998. Low temperature regulation of *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. *The Plant Journal* 16:433–42
47. Xiao H, Tattersall EAR, Siddiqua MK, Cramer G, Nassuth A. 2008. *CBF4* is a unique member of the CBF transcription factor family of *Vitis vinifera* and *Vitis riparia*. *Plant, Cell & Environment* 31:1–10
48. Campos PS, Quartin VN, Ramalho JC, Nunes MA. 2003. Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. *Journal of Plant Physiology* 160:283–92
49. Trovato M, Mattioli R, Costantino P. 2008. Multiple roles of proline in plant stress tolerance and development. *Rendiconti Lincei* 19:325–46
50. Yu D, Zhang L, Zhao K, Niu R, Zhai H, et al. 2017. *VaERD15*, a transcription factor gene associated with cold-tolerance in Chinese wild *Vitis amurensis*. *Frontiers in Plant Science* 8:297
51. Yang YY, Zheng PF, Ren YR, Yao YX, You CX, et al. 2021. Apple *MdSAT1* encodes a bHLHm1 transcription factor involved in salinity and drought responses. *Planta* 253:46
52. Zhao Q, Fan Z, Qiu L, Che Q, Wang T, et al. 2020. *MdbHLH130*, an apple bHLH transcription factor, confers water stress resistance by regulating stomatal closure and ROS homeostasis in transgenic tobacco. *Frontiers in Plant Science* 11:543696
53. Han D, Wang Y, Zhang Z, Pu Q, Ding H, et al. 2017. Isolation and functional analysis of *MxCS3*: a gene encoding a citrate synthase in *Malus xiaojinensis*, with functions in tolerance to iron stress and abnormal flower in transgenic *Arabidopsis thaliana*. *Plant Growth Regulation* 82:479–89
54. Chen M, Luo Z, Zhao X, Li S, Wu F, et al. 2022. Exogenously applied methyl jasmonate increased the resistance of postharvest pear fruit to blue mold. *Fruit Research* 2:11
55. Han D, Shi Y, Wang B, Liu W, Yu Z, et al. 2015. Isolation and preliminary functional analysis of *MxCS2*: a gene encoding a citrate synthase in *Malus xiaojinensis*. *Plant Molecular Biology Reporter* 33:133–42