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CabHLH55 Negatively Regulates Cold Tolerance in Pepper by Modulating Reactive Oxygen Species Homeostasis and Cold-Responsive Gene Expression

Jianwei Zhang [1,2,†], Rong Ning [2,†], Jiaxiang Luo [2,†], Liwei Bai [3], Fenglian Wu [4], Qin Yang [1,*], Jianwen He [4,*] and Huanxiu Li [5,*]

[1] Guizhou Key Laboratory of Molecular Breeding for Characteristic Horticultural Crops, Kaili 556011, China
[2] School of Life and Health Science, Kaili University, Kaili 556011, China
[3] Chili Pepper Research Institute, Guizhou Academy of Agricultural Sciences, Guiyang 550006, China
[4] Vegetable Research Institute, Qiandongnan Academy of Agricultural Sciences, Kaili 556000, China
[5] College of Horticulture, Sichuan Agricultural University, Chengdu 611130, China
* Correspondence: yangqin1028518@126.com (Q.Y.); hejianwen1022@126.com (J.H.); huanxiuli62@163.com (H.L.)
† These authors contributed equally to this work.

Abstract

Low temperature is a major abiotic stress that constrains the early-spring and late-season cultivation of pepper (*Capsicum annuum* L.). Basic helix–loop–helix (bHLH) transcription factors play important roles in plant stress responses; however, their functions in pepper cold tolerance remain poorly understood. In this study, we characterized *CabHLH55* and investigated its role in cold stress through bioinformatic analysis, subcellular localization, quantitative reverse transcription PCR (RT-qPCR), heterologous overexpression in Arabidopsis, and virus-induced gene silencing (VIGS) in pepper. CabHLH55 encodes a 257-amino-acid nuclear protein containing a conserved bHLH domain. Its promoter contains multiple cis-acting elements associated with phytohormone and stress responses. Expression analysis showed that *CabHLH55* responded to multiple abiotic stresses and was markedly downregulated under cold stress. Under cold conditions, *CabHLH55*-overexpressing Arabidopsis plants exhibited reduced chlorophyll content and increased relative electrolyte leakage and malondialdehyde (MDA) levels compared with WT plants, indicating enhanced cold-induced damage. Conversely, silencing *CabHLH55* in pepper alleviated cold injury, reduced reactive oxygen species (ROS) accumulation and MDA levels, and increased peroxidase (POD) activity. Moreover, the expression of the cold-responsive genes *CaCBF1a*, *CaKIN*, and *CaCOR47* was significantly increased in *CabHLH55*-silenced plants under cold stress. Collectively, these results indicate that *CabHLH55* functions as a negative regulator of cold tolerance in pepper, potentially by affecting ROS homeostasis and cold-responsive gene expression. This study provides a basis for further elucidating bHLH-mediated cold stress responses in pepper and may facilitate the molecular breeding of cold-tolerant pepper cultivars.

Keywords: pepper; CabHLH55; cold stress; VIGS; arabidopsis

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1. Introduction

Low temperature constitutes a significant abiotic stressor that adversely impacts plant growth, development, yield, and quality. It compromises cellular membrane integrity, inhibits photosynthetic activity and normal metabolic processes, and induces the excessive accumulation of reactive oxygen species (ROS). Under severe conditions, such stress can

impede plant growth and may ultimately result in plant mortality [1,2]. To mitigate the effects of cold stress, plants have developed intricate defense mechanisms encompassing signal perception and transduction, osmotic adjustment, ROS homeostasis, and the regulation of stress-responsive gene expression [3–5]. Transcription factors are integral to these processes, serving as critical mediators that connect stress signaling pathways to downstream gene expression, thereby facilitating cold acclimation through the modulation of stress-responsive gene transcription. Among these transcription factors, basic helix–loop–helix (bHLH) proteins play important roles in regulating plant adaptation to adverse environmental conditions.

The bHLH family constitutes one of the most extensive groups of transcription factors in plants. Members of this family possess a basic domain responsible for DNA binding and a Helix 1–Loop–Helix 2 motif that facilitates protein dimerization [6,7]. Typically, bHLH proteins recognize E-box (CANNTG) or G-box (CACGTG) sequences within the promoters of target genes. Through the formation of homo- or heterodimers, they modulate downstream gene expression and engage in intricate transcriptional regulatory networks [6–8]. Prior research has established that bHLHs play critical roles in regulating flowering, fruit development, and secondary metabolism. For example, in tomato, *SlbHLH22* exhibits high expression levels in flowers and fruits; its overexpression induces early flowering, accelerates fruit ripening, promotes carotenoid accumulation, and influences the expression of flowering-related genes such as *SlSFT* and *SlLFY* [9]. In rice, *OsbHLH107* governs grain size by enhancing longitudinal cell proliferation in the hull, thereby increasing grain length and weight [10]. Furthermore, bHLH are implicated in plant responses to abiotic stresses including cold, drought, and salinity. In rice, *OsbHLH148* is involved in jasmonic acid signaling pathways, with its overexpression conferring improved drought tolerance [11]. Similarly, in sugar beet, overexpression of *BvbHLH93* mitigates Na^+ and malondialdehyde (MDA) accumulation under salt stress, while augmenting K^+ content and antioxidant enzyme activities, collectively enhancing salt tolerance [12]. In sweet cherry, *PavbHLH28* overexpression reduces electrolyte leakage, MDA accumulation, and ROS levels during cold stress; it also directly activates peroxidase 2 (POD2) by binding to its promoter, thereby bolstering cold tolerance [13]. Collectively, these studies highlight the important roles of bHLH transcription factors in plant responses to various abiotic stresses, suggesting their potential involvement in the regulation of plant cold stress responses.

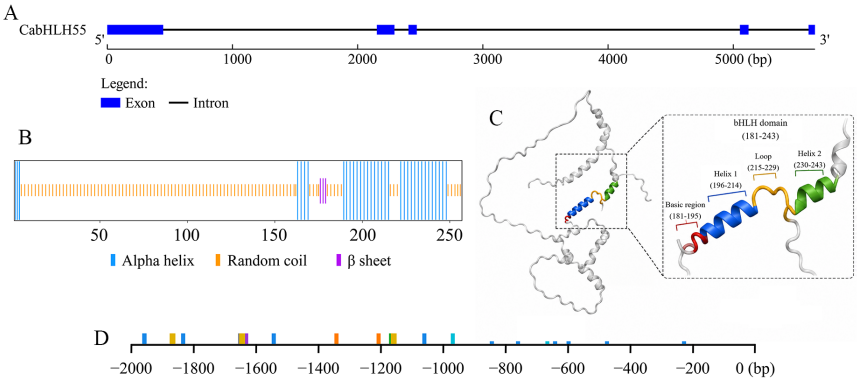
Pepper (*Capsicum annuum* L.), a member of the *Solanaceae* family, is an economically important vegetable and spice crop cultivated worldwide. In northern China, pepper cultivation during the winter and early spring seasons is frequently compromised by cold stress, which adversely affects plant growth and development, ultimately leading to yield reductions [14,15]. Consequently, the identification of key genes involved in pepper's response to cold stress, along with elucidation of their regulatory mechanisms, is critical for the genetic enhancement of cold tolerance. Several bHLHs have been implicated in mediating pepper's response to cold stress. Previous studies have demonstrated that *CabHLH79* expression is induced by cold conditions; silencing of *CabHLH79* diminishes cold tolerance in pepper, whereas its overexpression confers enhanced tolerance. Further investigations revealed that *CabHLH79* directly binds to the promoter region of CaNAC035, thereby modulating its expression during cold stress [16]. Similarly, suppression of *CabHLH035* increases pepper susceptibility to cold stress, while its heterologous overexpression in Arabidopsis improves cold tolerance. *CabHLH035* also plays a role in ROS homeostasis and the regulation of cold-responsive (COR) genes [15]. Despite these advances, the roles and regulatory mechanisms of bHLH transcription factors in pepper cold stress responses remain insufficiently understood, particularly those of bHLHs that function as negative regulators of cold tolerance. In our previous transcriptomic analysis, *CabHLH55* was identified as a cold-responsive

candidate gene that was markedly downregulated under cold stress [17]. Based on its strong transcriptional response to cold and the limited understanding of negative regulatory bHLHs in pepper, *CabHLH55* was selected for further functional characterization. The present study therefore investigated the role of *CabHLH55* in cold tolerance using heterologous overexpression and gene silencing, with particular attention to ROS homeostasis and COR gene expression. The outcomes of this research contribute a candidate gene for advancing the understanding of cold response mechanisms in pepper and for facilitating the improvement of cold tolerance through molecular breeding strategies.

2. Results

2.1. Cloning and Bioinformatic Analysis of CabHLH55

We identified a bHLH gene, designated *CabHLH55*, which was significantly downregulated under cold stress and subsequently amplified via PCR using cDNA synthesized from pepper leaves (Figure S1). Sequence analysis showed that the coding sequence of *CabHLH55* was 774 bp in length and encoded a protein of 257 amino acids. The predicted molecular weight of the protein was 28.11 kDa, with a theoretical isoelectric point of 7.02. The instability index was calculated to be 54.27, indicating that CabHLH55 is likely an unstable protein (Table S1). The *CabHLH55* gene consists of five exons and four introns (Figure 1A). Secondary structure prediction using SOPMA revealed that the protein predominantly consisted of random coils and α-helices, with α-helices constituting approximately 26.07%, extended strands 1.56%, and random coils 72.37% (Figure 1B). The predicted tertiary structure featured a characteristic helical domain at the C-terminus, consistent with the conserved structural motifs typical of the bHLH protein family (Figure 1C).



Cis-acting elements (motifs)	Function	Number
ATC-motif, chs-CMA1a, G-box, MRE, GT1-motif, LS7, Box 4	Light responsiveness	12
circadian	Circadian control	1
ARE	Anaerobic induction	2
LTR	Low-temperature responsiveness	2
O2-site	Zein metabolism regulation	1
ABRE	Abscisic acid responsiveness	1
TGA-box	Auxin-responsive element	1
CGTCA-motif, TGACG-motif	MeJA-responsiveness	6

Figure 1. Bioinformatics analysis of CabHLH55 in pepper. (**A**) gene structure; (**B**) predicted protein secondary structure; (**C**) predicted protein tertiary structure; (**D**) analysis of cis-acting elements in the *CabHLH55* promoter.

An analysis of a 2000 bp region upstream of the *CabHLH55* coding sequence was conducted to identify putative cis-acting elements. This analysis revealed multiple elements associated with hormone signaling pathways and stress responses. Light-responsive elements were the most prevalent, with twelve distinct motifs identified, including ATC-motif, chs-CMA1a, G-box, MRE, GT1-motif, LS7, and Box 4. Additionally, six methyl jasmonate

(MeJA)-responsive elements, such as the CGTCA-motif and TGACG-motif, were detected. Both anaerobic response elements (ARE) and low-temperature-responsive elements (LTR) were present twice. Furthermore, single occurrences of elements related to circadian regulation, zein metabolism, abscisic acid (ABA) responsiveness, and auxin responsiveness were also identified (Figure 1D). The presence of LTR elements suggests that *CabHLH55* may be involved in plant responses to cold stress and other environmental stresses.

2.2. Conserved Domain Analysis and Phylogenetic Relationships of CabHLH55

To elucidate the structural characteristics of CabHLH55, an analysis of its conserved bHLH domain was conducted. The CabHLH55 protein was found to possess a canonical bHLH domain comprising the Basic region, Helix 1, Loop, and Helix 2 (Figure 2A). Subsequently, multiple sequence alignment was performed using homologous proteins from 18 different species. This analysis revealed a high degree of amino acid sequence conservation within the bHLH domain among CabHLH55 and its homologs, suggesting that this domain is evolutionarily conserved across diverse taxa (Figure 2B). Phylogenetic analysis further demonstrated that CabHLH55 clusters closely with homologs from Solanaceae species, whereas it exhibits a more distant relationship with homologs from monocotyledonous plants. These findings indicate that CabHLH55 shares a closer phylogenetic affinity with *Solanaceae* homologs (Figure 2C).

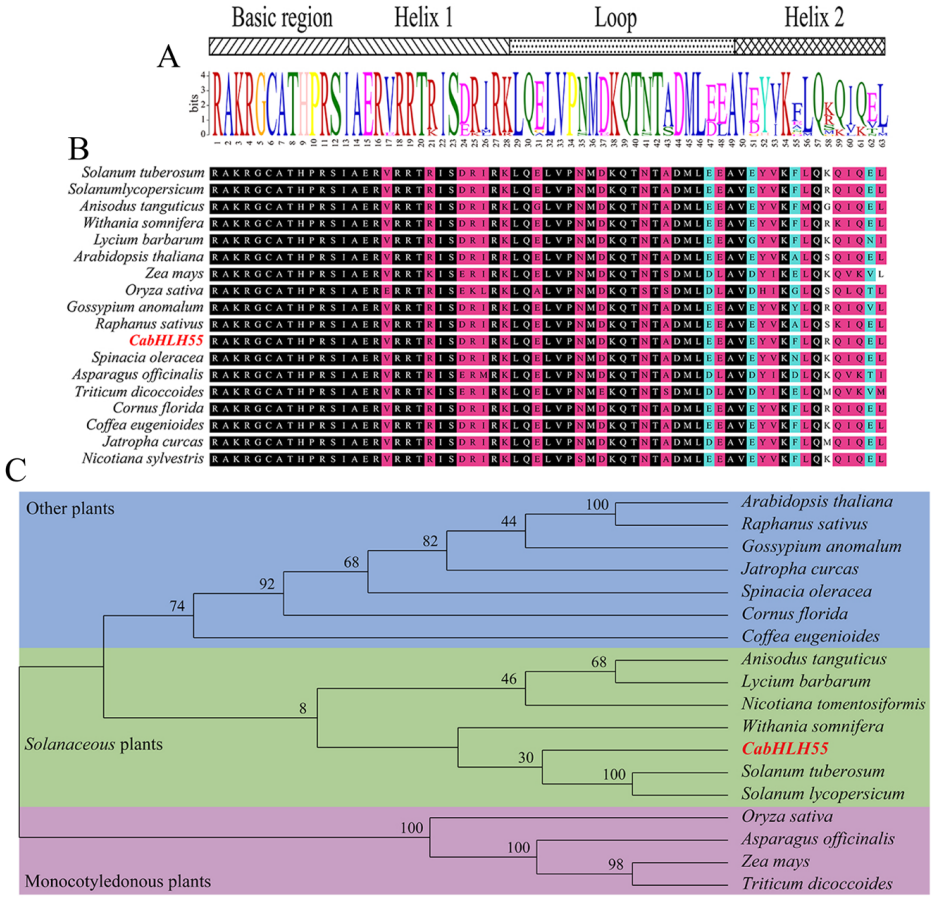


Figure 2. Multiple sequence alignment and phylogenetic analysis of the CabHLH55 protein. (**A**) Sequence conservation of the bHLH domain. (**B**) Multiple sequence alignment of CabHLH55 and its homologous proteins: *Solanum tuberosum* (XP_006356502.1), *Solanum lycopersicum* (NP_001315191.2), *Anisodus tanguticus* (KAK4365714.1), *Withania somnifera* (CAN4097992.1), *Lycium barbarum* (XP_060216897.1), *Arabidopsis thaliana* (NP_174776.1), *Zea mays* (XP_008670486.1), *Oryza sativa* (XP_015636683.1), *Gossypium anomalum* (KAG8472723.1), *Raphanus sativus* (XP_018447615.1),

Spinacia oleracea (XP_021847922.1), *Asparagus officinalis* (XP_020265966.1), *Triticum dicoccoides* (XP_037434938.1), *Cornus florida* (XP_059630951.1), *Coffea eugenioides* (XP_027150216.1), *Jatropha curcas* (XP_012075470.1), and *Nicotiana tomentosiformis* (XP_009605833.1). CabHLH55 is highlighted in red. (**C**) Phylogenetic tree of CabHLH55 and its homologous proteins. Blue, green, and purple backgrounds indicate other plants, *Solanaceae* species, and monocotyledonous plants, respectively.

2.3. Analysis of CabHLH55 Subcellular Localization

To investigate the subcellular localization of CabHLH55, a p1300-*CabHLH55*-eGFP fusion construct was generated and transiently expressed in tobacco epidermal cells (Figure 3A). The fluorescence signal of free eGFP was observed to be diffusely distributed throughout the cellular compartments. In contrast, the fluorescence associated with the *CabHLH55*-eGFP fusion protein was predominantly confined to the nucleus, co-localizing with the red fluorescent protein (RFP) nuclear localization marker (Figure 3B). These findings demonstrate that CabHLH55 is localized within the nucleus.

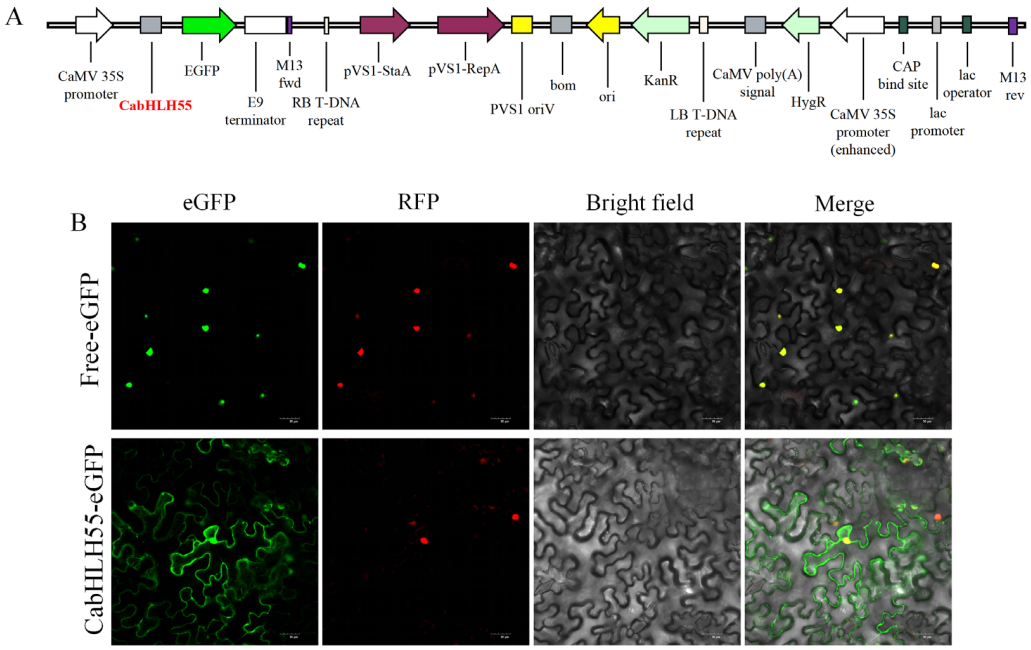


Figure 3. Subcellular localization of CabHLH55. (**A**) Construction of the p1300-*CabHLH55*-eGFP fusion vector. (**B**) Subcellular localization of CabHLH55 in tobacco leaves. eGFP and the fusion protein are shown in green, and the nuclear marker is shown in red. Scale bar = 30 μm.

2.4. Tissue-Specific Expression of CabHLH55

To elucidate the potential role of *CabHLH55*, its transcript abundance was quantified across various tissues using quantitative reverse transcription PCR (RT-qPCR). Tissue samples were obtained at three developmental stages: seedling, flowering, and fruiting. Specifically, seedling samples comprised cotyledons, roots, stems, and leaves; flowers were harvested during the flowering stage; and green fruits, red fruits, roots, stems, and leaves were collected at the fruiting stage. As shown in Figure 4, *CabHLH55* transcripts were detected in all examined tissues, although expression levels exhibited considerable variation. The highest expression was observed in cotyledons, whereas green and red fruits displayed comparatively lower transcript levels. Among roots, stems, and leaves, stems demonstrated the most elevated expression at both the seedling and fruiting stages, with expression in stems significantly surpassing that in roots and leaves.

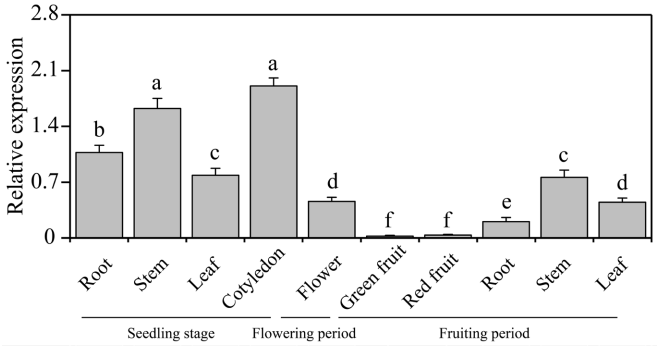


Figure 4. Expression patterns of *CabHLH55* in different tissues. The expression level in roots at seedling stage was used as the calibrator and set to 1. Data are presented as the mean ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test. Different lowercase letters indicate significant differences among tissues ($p < 0.05$).

2.5. CabHLH55 Responds to Multiple Phytohormone Treatments

To elucidate the response of *CabHLH55* to various phytohormones, publicly available transcriptomic datasets were employed to assess its expression profiles. The analysis encompassed treatments with ABA, MeJA, salicylic acid (SA), and ethylene (ET). *CabHLH55* exhibited differential transcriptional responses across these phytohormone treatments, with notable expression changes observed at multiple time points (Figure 5A). Specifically, expression levels remained generally low under ABA treatment, albeit with increases at certain intervals. MeJA treatment induced pronounced fluctuations in *CabHLH55* expression, characterized by significant upregulation at several time points. In contrast, expression alterations under SA treatment were relatively modest, whereas a marked increase in *CabHLH55* expression was detected during the later stages of ET treatment. These response of *CabHLH55* to phytohormone treatments was further examined by RT-qPCR (Figure 5B). Compared with CK, *CabHLH55* expression showed slight fluctuations under ABA treatment, with a decrease at 6 h followed by a gradual increase, but no significant differences were observed. Both MeJA and SA treatments resulted in a significant downregulation of *CabHLH55* at 6 h post-treatment, followed by a gradual restoration of expression levels. Under gibberellic acid (GA) treatment, *CabHLH55* expression initially declined, reaching its nadir at 6 h, before subsequently increasing.

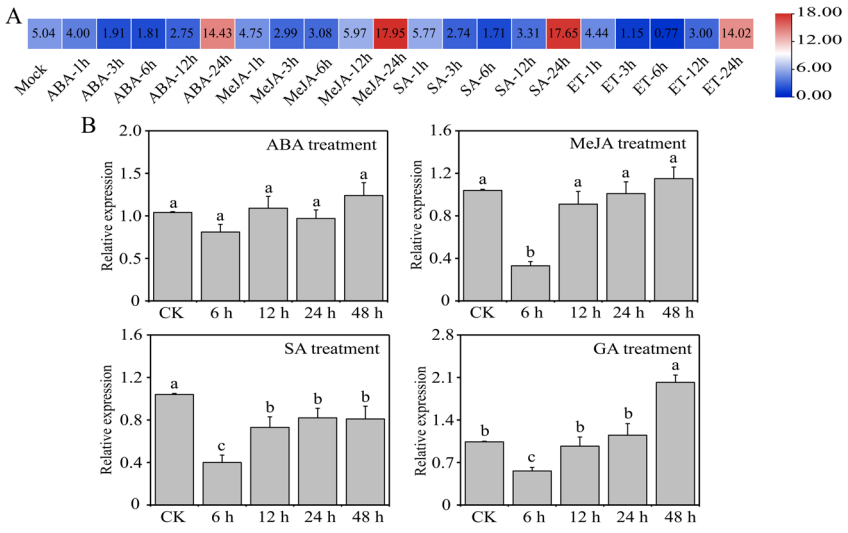


Figure 5. Expression patterns of *CabHLH55* under different phytohormone treatments. (**A**) Transcriptome analysis of *CabHLH55* expression levels. The color gradient from blue to red corresponds to the

normalized expression values ranging from 0.00 to 24.00 as indicated by the color bar on the right, and the number inside each block represents the exact transcript abundance of *CabHLH55* under the corresponding treatment. (**B**) RT-qPCR analysis of *CabHLH55* expression levels. The expression data under ET treatment were obtained from the public transcriptome dataset and were not further validated by RT-qPCR. Data are presented as the mean ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test. Different lowercase letters indicate significant differences among treatment times within each stress treatment ($p < 0.05$).

2.6. CabHLH55 Responds to Multiple Abiotic Stresses

CabHLH55 demonstrated distinct expression profiles in response to various abiotic stress conditions. Transcriptome analysis revealed a gradual downregulation of *CabHLH55* expression under cold stress, indicating a pronounced inhibitory effect. Conversely, its expression was significantly upregulated during the later phases of high-temperature exposure. Additionally, salt and drought treatments elicited differential modulation of *CabHLH55* expression (Figure 6A).

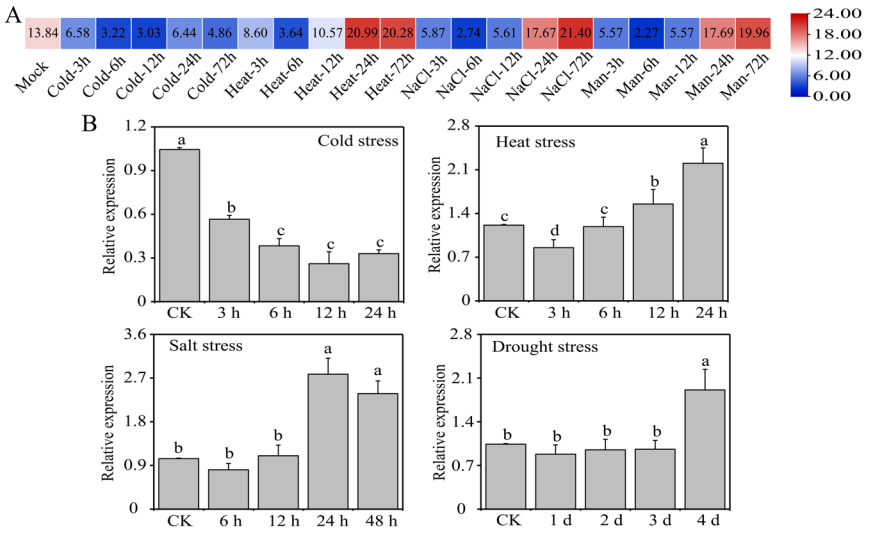


Figure 6. Expression patterns of *CabHLH55* under different abiotic stresses. (**A**) Transcriptome analysis of *CabHLH55* expression levels. The color gradient from blue to red corresponds to the normalized expression values ranging from 0.00 to 24.00 as indicated by the color bar on the right, and the number inside each block represents the exact transcript abundance of *CabHLH55* under the corresponding treatment. (**B**) RT-qPCR analysis of *CabHLH55* expression levels. Data are presented as the mean ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test. Different lowercase letters indicate significant differences among treatment times within each stress treatment ($p < 0.05$).

RT-qPCR analysis further confirmed that *CabHLH55* expression significantly declined after 3 h of cold treatment and continued to decrease with extended exposure. Although a slight increase was observed at 24 h, this change was not statistically significant compared to the 12 h time point. Under high-temperature conditions, *CabHLH55* expression initially decreased but subsequently increased progressively, peaking at 24 h. Salt stress notably induced *CabHLH55* expression between 24 and 48 h. In the case of drought treatment, expression levels remained relatively stable during the early stages but exhibited a significant increase at 4 d (Figure 6B). Collectively, these results indicate that *CabHLH55* may play a vital role in plant abiotic stress responses, and we therefore further explored its specific biological functions under cold stress.

2.7. CabHLH55-Overexpression (CabHLH55-OE) Reduces Cold Tolerance in Arabidopsis

Arabidopsis plants were genetically transformed utilizing the floral dip technique. Homozygous T3 transgenic lines were validated through RT-qPCR analysis of *CabHLH55* transcript levels (Figure S2). Among these, three lines (OE-2, OE-5, and OE-7) with the highest expression levels were selected for further experimental investigation.

Under normal conditions, WT plants and the three OE lines displayed comparable growth patterns without discernible phenotypic differences. Following exposure to cold stress, growth inhibition was evident across all genotypes. Notably, the *CabHLH55*-OE lines manifested more pronounced physiological damage, including wilting of certain leaves (Figure 7A). Measurements of total chlorophyll content revealed no significant differences between WT and the OE lines under normal conditions. However, post-cold treatment, chlorophyll levels declined in all plants, with OE-2, OE-5, and OE-7 exhibiting significantly greater reductions compared to WT. These findings suggest that overexpression of *CabHLH55* exacerbates cold-induced chlorophyll degradation (Figure 7B). Furthermore, under normal conditions, relative conductivity and MDA content did not differ significantly among the genotypes tested. Both parameters increased substantially following cold exposure. Importantly, relative electrolyte leakage was significantly elevated in all three OE lines relative to WT, and MDA content was also markedly higher in the OE lines compared to WT (Figure 7C,D).

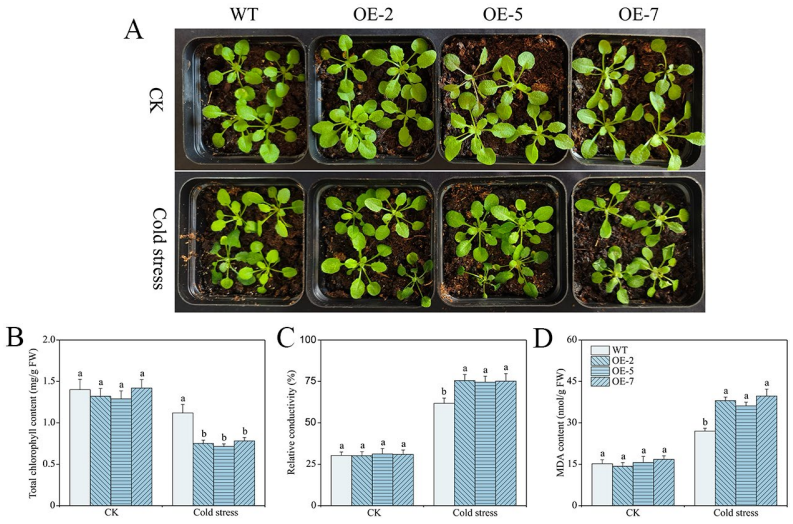


Figure 7. Phenotypic and physiological responses of *CabHLH55*-OE Arabidopsis plants under cold stress. (**A**) phenotype; (**B**) total chlorophyll content; (**C**) relative conductivity; (**D**) MDA content. Data are presented as the mean ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Duncan's multiple range test. Different lowercase letters indicate significant differences among WT and transgenic lines under the same temperature condition ($p < 0.05$).

2.8. Silencing of CabHLH55 Enhances Cold Tolerance in Pepper

To elucidate the role of *CabHLH55* in pepper, plants with silenced *CabHLH55* expression were generated utilizing the virus-induced gene silencing (VIGS) system. Four weeks post-infiltration, plants inoculated with TRV2:*PDS* exhibited a pronounced photobleaching phenotype, whereas no such phenotype was observed in the TRV2:00 plants (Figure S3). RT-qPCR analysis showed that, compared to WT plants, the transcript level of *CabHLH55* in TRV2:*CabHLH55* plants was significantly decreased by 65.67%, confirming that the target gene was effectively silenced.

There were no discernible phenotypic differences between TRV2:00 and TRV2:*CabHLH55* plants under control conditions. Following exposure to cold stress, both groups exhibited varying degrees of wilting; however, TRV2:*CabHLH55* plants demonstrated superior leaf integrity and reduced visible damage relative to controls (Figure 8A). Histochemical staining with 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) revealed no significant differences under normal conditions. Post-cold treatment, leaves from TRV2:00 plants displayed more intense brown and blue staining, indicative of higher accumulation of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet-}$), compared to TRV2:*CabHLH55* leaves. These findings suggest that silencing *CabHLH55* mitigates ROS accumulation under cold stress (Figure 8B). Anatomical analysis of leaf cross-sections further demonstrated that, under cold conditions, TRV2:*CabHLH55* plants exhibited more orderly cellular arrangements than control plants. Additionally, the palisade and spongy mesophyll tissues retained relatively intact structural integrity (Figure 8C).

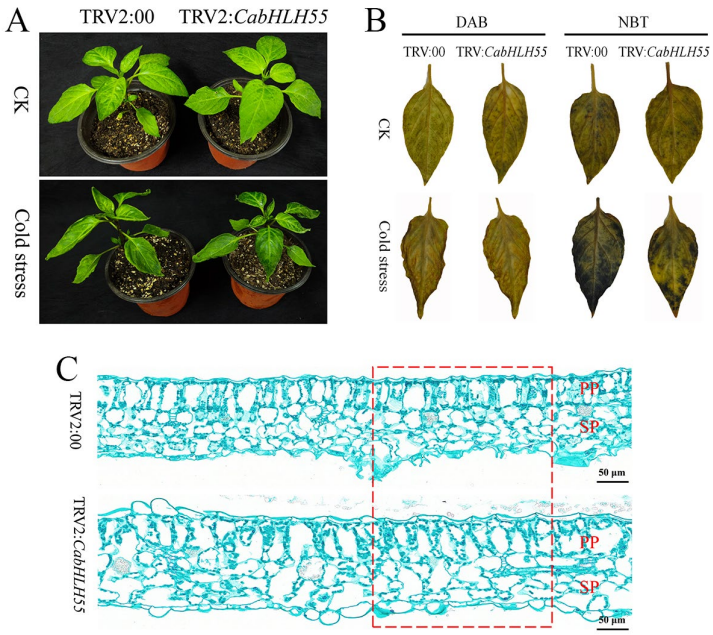


Figure 8. (**A**) Phenotypes, (**B**) ROS staining, and (**C**) leaf anatomical structure of *CabHLH55*-silenced pepper plants under cold stress. PP: palisade tissue; SP: spongy tissue. Scale bars = 50 μm.

2.9. Silencing of CabHLH55 Alleviates Oxidative Damage and Modulates POD Activity Under Cold Stress

ROS levels and the activities of antioxidant enzymes were quantitatively assessed. Under normal conditions, no significant differences were observed in the concentrations of H_2O_2, $O_2^{\bullet-}$, and MDA between TRV2:00 and TRV2:*CabHLH55* plants. Following exposure to cold stress, the levels of H_2O_2, $O_2^{\bullet-}$, and MDA increased in both TRV2:00 and TRV2:*CabHLH55* plants. Notably, these increases were significantly attenuated in TRV2:*CabHLH55* plants compared to TRV2:00 controls. These findings corroborate the results obtained from DAB and NBT staining assays (Figure 9A–C), suggesting that silencing of *CabHLH55* mitigates excessive ROS accumulation and reduces membrane lipid peroxidation under cold stress. Furthermore, POD activity was significantly elevated in TRV2:*CabHLH55* plants relative to TRV2:00 plants during cold treatment, whereas superoxide dismutase (SOD) and catalase (CAT) activities exhibited upward trends that did not reach statistical significance (Figure 9D–F).

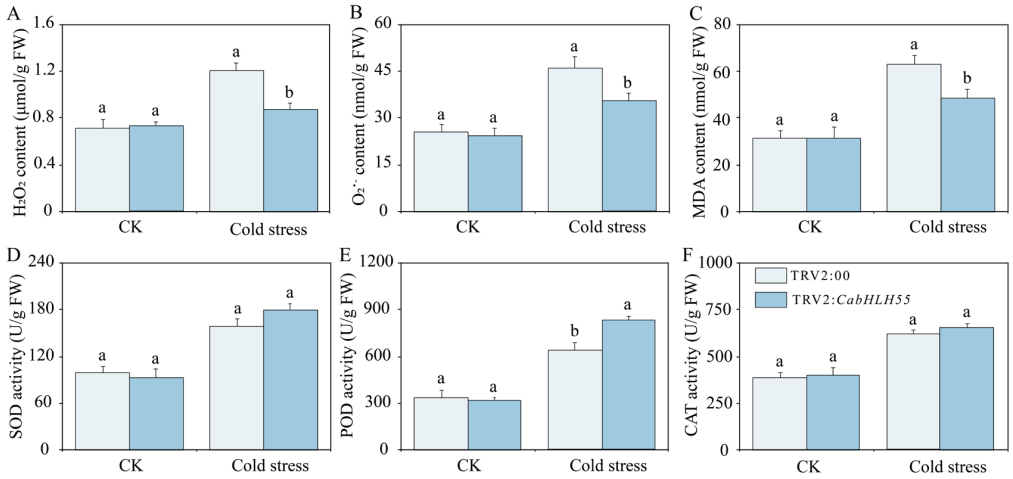


Figure 9. Physiological responses of TRV2:*CabHLH55* plants under cold stress. (**A**) H_2O_2 content; (**B**) $O_2^{\bullet-}$ content; (**C**) MDA content; (**D**) SOD activity; (**E**) POD activity; (**F**) CAT activity. Data are presented as the mean ± SD ($n = 3$). Different lowercase letters indicate significant differences between TRV2:00 and TRV2:CabHLH55 plants under the same temperature condition according to Duncan's multiple range test ($p < 0.05$).

2.10. Silencing of CabHLH55 Promotes the Expression of Several Cold-Responsive Genes

To further elucidate the potential role of *CabHLH55* in cold signaling pathways, RT-qPCR was employed to assess the expression levels of *CaCBF1a*, *CaCBF1b*, *CaKIN*, and *CaCOR47* (Figure 10A–D). Under normal conditions, no significant differences in the expression of these genes were detected between TRV2:00 and TRV2:*CabHLH55* plants. Following exposure to cold treatment, all four genes exhibited induction to varying extents. Notably, the expression levels of *CaCBF1a*, *CaKIN*, and *CaCOR47* were significantly elevated in *CabHLH55*-silenced plants compared to controls, whereas *CaCBF1b* expression did not differ significantly between the two groups.

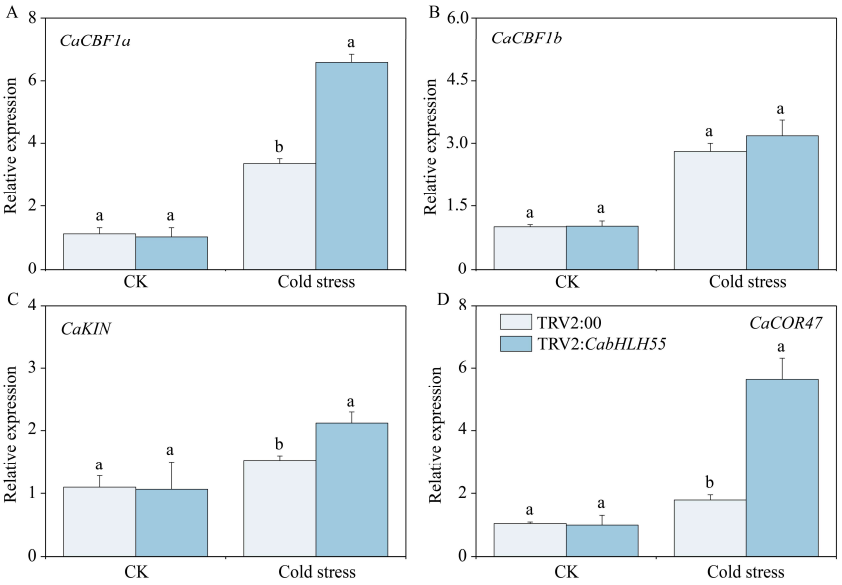


Figure 10. Expression levels of (**A**) *CaCBF1a*, (**B**) *CaCBF1b*, (**C**) *CaKIN*, and (**D**) *CaCOR47* in TRV2:00 and TRV2:*CabHLH55* plants under cold stress. Data are presented as the mean ± SD ($n = 3$). Different lowercase letters indicate significant differences between TRV2:00 and TRV2:*CabHLH55* plants under the same temperature condition according to Duncan's multiple range test ($p < 0.05$).

3. Discussion

Low temperature is a major abiotic stress that adversely affects plant growth and development [18]. Although bHLH transcription factors play important roles in plant cold stress responses, their functions as negative regulators remain poorly understood. In this study, we investigated the role of *CabHLH55* in pepper cold tolerance and found that it functions as a negative regulator of cold tolerance. These findings provide new insights into the regulatory roles of bHLH transcription factors in pepper under cold stress.

A defining structural characteristic of bHLH proteins is the highly conserved bHLH domain, which typically comprises a Basic region, Helix 1, a Loop, and Helix 2. The Basic region primarily facilitates recognition and binding to specific DNA sequences, whereas the two α-helices and the intervening Loop region predominantly mediate protein dimerization. These structural elements are fundamental to the transcriptional regulatory capabilities of bHLH proteins [7,8]. In this study, CabHLH55 was found to contain the canonical Basic region–Helix 1–Loop–Helix 2 configuration. Multiple sequence alignment demonstrated a high degree of sequence conservation within this domain, confirming that CabHLH55 exhibits the characteristic structural features of bHLH family proteins, consistent with previous findings for CabHLH79 in pepper [16]. Furthermore, subcellular localization analysis revealed that CabHLH55 is predominantly localized within the nucleus, thereby supporting its putative role as a nuclear transcription factor involved in regulatory processes.

Cis-acting elements within gene promoters serve as critical mediators linking environmental cues to transcriptional regulation [19–21]. In the present study, the promoter region of *CabHLH55* was found to contain multiple cis-acting elements associated with phytohormone signaling and environmental stress responses, suggesting that its expression may be regulated by both endogenous and environmental factors. Consistently, expression analyses revealed that *CabHLH55* responded to various phytohormone treatments and abiotic stresses. Previous studies have also demonstrated that bHLH transcription factors exhibit diverse expression patterns under different stress conditions. For example, *AtbHLH112* responds to salt and osmotic stresses [22], PmbHLH58 is responsive to both abiotic stresses and phytohormone treatments [23], while *PubHLH1* is induced by cold stress and enhances cold tolerance when heterologously overexpressed in tobacco [24]. These findings indicate that bHLH transcription factors are involved in diverse stress responses, although their regulatory functions may differ among family members. Notably, *CabHLH55* expression was significantly downregulated under cold stress, consistent with the transcriptomic data. Interestingly, although its promoter contains LTR elements associated with low-temperature responsiveness, their presence does not necessarily imply transcriptional activation. Given the negative role of *CabHLH55* in cold tolerance, its downregulation may alleviate its negative regulatory effect, and the underlying regulatory mechanism warrants further investigation.

Previous research has demonstrated that bHLHs can function as negative regulators in plant responses to cold stress. For instance, *HvbHLH132* in hulless barley is upregulated in response to cold stress, and its heterologous overexpression in Arabidopsis results in diminished tolerance to chilling and freezing accompanied by the suppression of COR gene expression [25]. Similarly, heterologous expression of *MdbHLH4* from apple reduces cold tolerance in Arabidopsis by directly binding to the promoters of *MdCBF1* and *MdCBF3*, thereby negatively regulating cold tolerance [26]. In the present study, Arabidopsis plants overexpressing *CabHLH55* exhibited exacerbated injury under cold stress, which was accompanied by a reduction in total chlorophyll content and an increase in relative electrolyte leakage and MDA levels. These findings suggest that overexpression of *CabHLH55* intensifies cold-induced membrane damage. ROS homeostasis is critically linked to plant cold tolerance, as excessive ROS accumulation under cold stress exacerbates membrane

lipid peroxidation and cellular injury [5,18]. Antioxidant enzymes mitigate this damage by scavenging surplus ROS, thereby maintaining cellular homeostasis. For example, in trifoliate orange, overexpression of *PtrbHLH* enhances cold tolerance by decreasing ROS and MDA accumulation under cold conditions and by elevating the activities of antioxidant enzymes such as CAT, POD, and SOD [27]. Additionally, bHLHs can modulate cold tolerance through regulation of CBFs and their downstream cold-responsive genes. In Arabidopsis, heterologous expression of grapevine *VabHLH1* and *VvbHLH1* enhances cold tolerance, an effect associated with modulation of the CBF pathway and downstream cold-responsive genes [28]. In this study, TRV2:*CabHLH55* plants exhibited reduced injury under cold stress, accompanied by lower levels of H_2O_2, $O_2^{\bullet-}$, and MDA, as well as increased POD activity. Furthermore, the expression levels of *CaCBF1a*, *CaKIN*, and *CaCOR47* were significantly upregulated in *CabHLH55*-silenced plants. Given the important role of the CBF signaling pathway in plant cold stress responses, together with the reduced ROS accumulation and MDA levels observed in *CabHLH55*-silenced plants, these findings suggest that *CabHLH55* may negatively regulate cold tolerance by influencing cold-responsive gene expression and membrane lipid peroxidation. However, whether *CabHLH55* directly binds to the promoters of these genes or acts through other regulatory factors remains unclear. Further studies are required to identify its direct target genes and elucidate the underlying transcriptional regulatory mechanisms.

4. Materials and Methods

4.1. Plant Materials and Treatments

The pepper cultivar 'Chuannong Paojiao' was used in this study. Initially, seeds were immersed in warm water at 55 °C for 15 min, followed by soaking at 25 °C for 6 h to enhance germination. Subsequently, the seeds were placed on moist filter paper within Petri dishes and allowed to germinate at ambient room temperature. Upon germination, seedlings were transplanted into nutrient-rich soil and cultivated within a controlled growth chamber maintained at 25 °C under a 16 h photoperiod and 20 °C during an 8 h dark period. When seedlings developed between six and eight true leaves, they were segregated into three distinct groups for further analysis.

The first group was designated for tissue-specific expression profiling. Samples including cotyledons, roots, stems, and leaves were harvested at the seedling stage. The remaining seedlings were transplanted into 28 cm diameter pots. At the flowering stage, floral tissues were collected, while green fruits, red fruits, roots, stems, and leaves were sampled during the fruiting stage, resulting in a total of ten tissue types collected. The second group underwent phytohormone treatments. The second group was subjected to phytohormone treatments. Seedling leaves were sprayed with 0.1 mmol/L ABA, SA, MeJA, or GA solutions at 9:00 a.m. for two consecutive days. Each treatment included 30 seedlings, and the solutions were uniformly sprayed onto the leaf surfaces until evenly wetted without excessive runoff. Leaf samples from the apical region of the seedlings were collected at 3, 6, 12, and 24 h after treatment. Seedlings sprayed with ddH_2O served as the control (0 h). The third group was subjected to various abiotic stress treatments. For cold stress, seedlings were exposed to 4 °C, with leaf samples collected at 0, 3, 6, 12, and 24 h. For heat stress, seedlings were maintained at 39 °C, and leaves were harvested at the same time intervals. Drought stress was induced by withholding irrigation, with the third day following the last watering of 30 mL defined as day 0. Leaf samples were subsequently collected on days 1, 2, 3, and 4 post-treatment. Salt stress was imposed by irrigating seedlings with 250 mmol/L NaCl solution at a volume of 30 mL per seedling. Leaf samples were collected at 0, 6, 12, 24, and 48 h after the initiation of salt treatment, All collected samples were immediately flash-frozen in liquid nitrogen and stored at −80 °C until further analysis.

Wild-type *Arabidopsis thaliana* Columbia (Col-0) was employed for genetic transformation experiments. Arabidopsis seeds were initially imbibed in water and subjected to vernalization at 4 °C for a duration of two days. Subsequently, the seeds were sown in 7 × 7 cm nutrient containers filled with high-pressure sterilized 'Pindstrup' substrate (particle size 0–6 mm) and cultivated within a controlled light incubator for a period of 24 d. Growth conditions were maintained at 24 °C with a 16 h photoperiod, followed by 22 °C with an 8 h dark period. Following this growth phase, Arabidopsis seedlings were exposed to a cold treatment at 4 °C for 24 h. After cold stress, leaf samples used for all physiological and biochemical analyses were flash-frozen in liquid nitrogen and stored at −80 °C until further use.

4.2. Cloning and Sequence Analysis of CabHLH55

Total RNA was isolated from pepper leaves utilizing the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme, Nanjing, China). cDNA synthesis was then carried out using the HiScript III RT SuperMix for qPCR (+gDNA wiper) Kit (Vazyme, Nanjing, China). Gene-specific primers targeting *CabHLH55* (LOC107855876) from pepper were designed with SnapGene software (version 6.0.2). The sequences of these primers are provided in Supplementary Table S2. PCR amplification was conducted using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The resulting PCR products were purified using the FastPure Gel DNA Extraction Mini Kit (Vazyme, Nanjing, China) and cloned into the pEASY-Blunt Simple vector (TransGen Biotech, Beijing, China). The recombinant plasmids were subsequently submitted to Sangon Biotech for sequencing.

4.3. Bioinformatics Analysis of CabHLH55 in Pepper

The molecular weight, theoretical isoelectric point, and instability index of the CabHLH55 protein were determined using the ExPASy server (http://web.expasy.org/protparam/, accessed on 1 June 2026). The gene structure of CabHLH55 was graphically represented utilizing GSDS (version 2.0, https://gsds.gao-lab.org/, accessed on 1 June 2026). Secondary structure prediction of the protein was conducted via SOPMA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html, accessed on 3 June 2026), while the tertiary structure was modeled employing the SWISS-MODEL platform (https://swissmodel.expasy.org/interactive, accessed on 1 June 2026). A 2000 bp sequence upstream of the CabHLH55 start codon was extracted using TBtools (version 2.485) [29] and analyzed for cis-acting elements using PlantCARE (https://bio.tools/plantcare, accessed on 7 June 2026). Homologous protein sequences of CabHLH55 from various species were obtained from the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/, accessed on 15 June 2026). Multiple sequence alignment and phylogenetic analyses were performed using MEGA (version 11), with conserved domains annotated within the aligned sequences. A comprehensive list of all web-based tools employed in this study is provided in Table S3.

4.4. Subcellular Localization of CabHLH55

The coding sequence of CabHLH55, excluding the stop codon, was initially amplified. Subsequently, the p1300-CabHLH55-eGFP fusion expression vector was generated utilizing the Ready-to-Use Seamless Cloning Kit (Sangon Biotech, Shanghai, China). This fusion construct was then transformed into *Agrobacterium* strains GV3101, which was used to infiltrate the 3-week-old leaves of *Nicotiana benthamiana*. Following a 72 h incubation in darkness, fluorescence signals were detected using a confocal laser scanning microscope (Nikon Instruments, Tokyo, Japan). The empty p1300-eGFPA vector was included as the

negative control in this assay, and an RFP nuclear localization marker was used to delineate nuclear boundaries.

4.5. Analysis of CabHLH55 Expression Patterns

To investigate the expression profile of *CabHLH55* under various treatment conditions, publicly accessible pepper RNA-seq datasets were downloaded from the NCBI database. Data pertaining to phytohormone treatments were sourced from BioProject PRJNA634831 [30], while datasets related to abiotic stress were obtained from BioProject PRJNA525913 [31]. The RNA-seq data were processed utilizing HISAT2 (version 2.2.1), StringTie (version 2.1.7), and Ballgown (version 4.4.0) software packages. Gene expression levels were quantified as fragments per kilobase of transcript per million mapped reads (FPKM).

Total RNA was extracted from pepper leaves, and cDNA synthesis was conducted following the protocols outlined in the gene cloning section. RT-qPCR assays were performed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The *CaUbi3* gene (LOC107873556) served as the internal reference control [32]. Relative expression levels of *CabHLH55* were calculated employing the $2^{-\Delta\Delta Ct}$ method [33]. The primer sequences utilized for RT-qPCR are provided in Table S2.

4.6. Genetic Transformation of Arabidopsis

The ORF of *CabHLH55* was inserted into the pBI121 vector to create the recombinant construct pBI121-*CabHLH55*. This recombinant plasmid was then introduced into *Agrobacterium* strain GV3101. Transformation of Arabidopsis plants was performed utilizing the floral dip technique as described by Clough and Bent [34]. Briefly, T1 seeds were harvested and surface-sterilized, then sown on 1/2 MS solid medium supplemented with 50 mg/L kanamycin for positive transformant selection. Homozygous transgenic Arabidopsis lines were subsequently established following the protocol outlined in a prior study [35]. In the T3 generation, homozygous lines were further validated by genomic PCR amplification and fluorescence observation, and three independent homozygous transgenic lines exhibiting the highest levels were selected for subsequent phenotypic analysis.

4.7. VIGS of CabHLH55 in Pepper

The tobacco rattle virus (TRV) vectors TRV1, TRV2, and the recombinant TRV2:*PDS* vector were kindly provided by Professor Huanxiu Li from the College of Horticulture at Sichuan Agricultural University. To enhance the efficiency of gene silencing, a specific fragment targeting *CabHLH55* was designed utilizing the SGN VIGS tool [36]. The TRV2:00 vector was digested with the restriction enzymes *BamHI* and *XbaI*, after which the target fragment was inserted into TRV2:00 via seamless cloning to construct the TRV2:*CabHLH55* recombinant vector. This construct was subsequently transformed into *Agrobacterium* strain GV3101. The gene silencing procedure was conducted following the protocol established by Velasquez et al. [37]. In brief, bacterial solution inoculation is performed when the cotyledons of pepper seedlings are fully expanded and the true leaves have not yet emerged. Subsequently, the seedlings are maintained in a dark environment for a duration of 2 d, under controlled conditions of 25 °C for 16 h during the light period and 22 °C for 8 h in darkness. Four weeks post-infiltration, the silencing efficiency of *CabHLH55* in TRV2:*CabHLH55* plants was assessed through RT-qPCR.

To investigate the role of *CabHLH55* in response to cold stress, plants infiltrated with TRV2:00 and TRV2:*CabHLH55* were exposed to 4 °C for 24 h. Phenotypic analyses were then performed. Leaves from the apical region of the seedlings were used for tissue staining

procedures. Additional leaves from the same region were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent physiological and biochemical analyses.

4.8. Measurement of Physiological Parameters

Histochemical detection of H_2O_2 and $O_2^{\bullet-}$ was conducted utilizing 1 mg/mL DAB and 0.5 mg/mL NBT, respectively. Pepper leaves were submerged in 150 mL of the respective staining solutions and incubated in darkness for 12 h. Following incubation, the leaves were subjected to boiling in 95% ethanol to achieve decolorization, after which photographic documentation was performed. Safranin O-Fast Green staining procedures were carried out by Servicebio Technology Co., Ltd. (Wuhan, China).

Total chlorophyll content was quantified through pigment extraction using an equal-volume mixture of acetone and absolute ethanol, as described in reference [38]. Relative conductivity was assessed via the conductivity method, with calculated as (A/B) × 100%, where A and B denote conductivity measurements taken prior to and following boiling, respectively [39]. The contents of MDA (AKFA013C), H_2O_2 (AKAO009C), and $O_2^{\bullet-}$ (AKAO008C)and the activities of SOD (AKAO001C-50s), POD (AKAO005c), and CAT (AKAO003-2c) were measured using biochemical assay kits supplied by Beijing Boxbio Science & Technology Co., Ltd. (Beijing, China). All assays were performed in strict accordance with the manufacturers' protocols.

4.9. Statistical Analysis

All physiological parameters were assessed using three biological replicates, while RT-qPCR analyses were conducted with three biological replicates, each comprising three technical replicates. Data are presented as the mean ± SD. Statistical comparisons were conducted using SPSS version 20 (IBM Corporation, Armonk, NY, USA). Differences among treatments were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for multiple comparisons. Differences were considered statistically significant at $p < 0.05$, and different lowercase letters indicate significant differences among treatments. Graphs were produced using Origin 2019b software (OriginLab Corporation, Northampton, MA, USA).

5. Conclusions

In this study, *CabHLH55* was cloned and characterized from pepper. Sequence analysis and subcellular localization confirmed that CabHLH55 contains a conserved bHLH domain and is localized in the nucleus. Expression analysis revealed that *CabHLH55* responds to various abiotic stresses, with its expression significantly downregulated under cold stress. Functional analyses demonstrated that heterologous overexpression of *CabHLH55* in Arabidopsis reduced cold tolerance, whereas VIGS-mediated silencing in pepper alleviated cold-induced injury. Furthermore, *CabHLH55* silencing reduced ROS and MDA accumulation, increased POD activity, and enhanced the expression of cold-responsive genes. Collectively, these findings suggest that *CabHLH55* functions as a negative regulator of cold tolerance in pepper, potentially by influencing ROS homeostasis and cold-responsive gene expression. This study provides new insights into the regulatory roles of bHLH transcription factors in pepper cold stress responses.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/plants15223513/s1, Table S1: Details of the CabHLH55 gene in pepper; Table S2: Sequences of the primers used in this study; Table S3: Website for identification and bioinformatics analysis of CabHLH55; Figure S1: PCR amplification of the *CabHLH55* gene from pepper; Figure S2: Expression level analysis of CabHLH55 in transgenic Arabidopsis; Figure S3: Phe-

notypes of gene-silenced pepper seedlings 3–4 weeks after inoculation; Figure S4: Analysis of silencing efficiency of CabHLH55.

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