Functional characterization of *Lilium lancifolium* cold-responsive Zinc Finger Homeodomain (ZFHD) gene in abscisic acid and osmotic stress tolerance

Yubing Yong¹,², Yue Zhang¹ and Yingmin Lyu¹

¹ Beijing Key Laboratory of Ornamental Plants Germplasm Innovation & Molecular Breeding, China National Engineering Research Center for Floriculture, Beijing Laboratory of Urban and Rural Ecological Environment, College of Landscape Architecture, Beijing Forestry University, Beijing, Haidian, China
² College of Landscape Architecture, Central South University of Forestry and Technology, Changsha, Hunan, China

**ABSTRACT**

**Background.** We have previously performed an analysis of the cold-responsive transcriptome in the mature leaves of tiger lily (*Lilium lancifolium*) by gene co-expression network identification. The results has revealed that a ZFHD gene, notated as encoding zinc finger homeodomain protein, may play an essential regulating role in tiger lily response to cold stress.

**Methods.** A further investigation of the ZFHD gene (termed as *LlZFHD4*) responding to osmotic stresses, including cold, salt, water stresses, and abscisic acid (ABA) was performed in this study. Based on the transcriptome sequences, the coding region and 5′ promoter region of *LlZFHD4* were cloned from mature tiger lily leaves. Stress response analysis was performed under continuous 4 °C, NaCl, PEG, and ABA treatments. Functional characterization of *LlZFHD4* was conducted in transgenic *Arabidopsis*, tobacco, and yeast.

**Results.** *LlZFHD4* encodes a nuclear-localized protein consisting of 180 amino acids. The N-terminal region of *LlZFHD4* has transcriptional activation activity in yeast. The 4 °C, NaCl, PEG, and ABA treatments induced the expression of *LlZFHD4*. Several stress- or hormone-responsive cis-acting regulatory elements (T-Box, Boxl. and ARF) and binding sites of transcription factors (MYC, DRE and W-box) were found in the core promoter region (789 bp) of *LlZFHD4*. Also, the GUS gene driven by *LlZFHD4* promoter was up-regulated by cold, NaCl, water stresses, and ABA in *Arabidopsis*. Overexpression of *LlZFHD4* improved cold and drought tolerance in transgenic *Arabidopsis*; higher survival rate and better osmotic adjustment capacity were observed in *LlZFHD4* transgenic plants compared to wild type (WT) plants under 4 °C and PEG conditions. However, *LlZFHD4* transgenic plants were less tolerant to salinity and more hypersensitive to ABA compared to WT plants. The transcript levels of stress- and ABA-responsive genes were much more up-regulated in *LlZFHD4* transgenic *Arabidopsis* than WT. These results indicate *LlZFHD4* is involved in ABA signaling pathway and plays a crucial role in regulating the response of tiger lily to cold, salt and water stresses.
INTRODUCTION

Plants have evolved a series of adaptive responses to cope with environmental stresses, such as salinity, drought, and low temperature. Many of these adaptations occur at the molecular level: start with signal perception, move to signal relay, and end with gene expression. Transcription factors (TFs) play pivotal roles in this signaling transduction event, regulating the expression of multiple stress-inducible genes by specifically binding to the corresponding cis-acting elements (Khatun et al., 2017; Huang et al., 2009; Zang et al., 2017). Recently, a homeobox TF, zinc finger-homeodomain (ZFHD), has attracted attention for its functions on mediating plant developmental processes and abiotic stress responses, which can bind to a core consensus sequence of ATTA and form homodimers and heterodimers (Sun et al., 2021).

The ZFHD TFs contain two highly conserved domains: the N-terminal C2H2-type zinc finger (ZF) domain and the C-terminal homeodomain (HD) domain (Wang et al., 2014). The ZF, one of the most critical structural motifs, widely exists in many regulatory proteins. The ZF consists of a single zinc ion in the core surrounded by two pairs of conserved cysteine (Cys) and, or histidine (His) residues, which participates in DNA binding and protein-protein interactions (Krishna, Majumdar & Grishin, 2003; Takatsuji, 1999). According to the presence of Cys and His residues, ZFs are classified into different types, such as Cys3His (C3H), Cys2His2 (C2H2), and Cys2Cys2 (C2C2) (Chen et al., 2020; Engelbrecht, Schoof & Böhm, 2004; Halbach, Scheer & Werr, 2000; Kosarev, Mayer & Hardtke, 2002; Zang et al., 2017). Among them, C2H2-type ZFs act as essential regulators in many plant stress responses or other metabolic pathways (Xie et al., 2019; Zang et al., 2017). The HD, as a well-characterized DNA-binding domain (BD), containing a conserved 60-amino acid motif, is present in TFs in all eukaryotic organisms (Hu, De Pamphilis & Ma, 2008; Mukherjee, Brocchieri & Bürglin, 2009). Most HD-containing proteins are related to additional domains or motifs for various regulatory functions, can also be divided into different subgroups, including ZFHD, leucine zipper-associated HD (HD-ZIP) and Knotted-related homeobox (KNOX) proteins, etc. (Ariel et al., 2007).

The ZFHD gene family was first found in Flaveria trinervia (Windhövel et al., 2001), and was subsequently identified in many other plant species by genome-wide study (Sun et al., 2021; Liu, Yang & Zhang, 2021). Meanwhile, there is increasing evidence indicating that ZFHD genes play vital roles in plant stress response (Khatun et al., 2017; Shalmani et al., 2019; Wang et al., 2016). For example, drought, salt, and cold stresses up-regulated the expression of AtZFHD04 in Arabidopsis thaliana (Barth et al., 2009). AtZFHD1 was induced by drought and salt treatments. Co-overexpression of both the AtZFHD1 and AtNAC gene could activate the expression of dehydration1 (ERD1) and other stress-inducible genes, improve the drought tolerance in transgenic Arabidopsis (Tran et al., 2007). AtZFHD10 was found to interact with TANDEM ZINC-FINGER PLUS3 (TZP) to modulate hormone signaling in stress response (Perrella et al., 2018). Four OsZFHD genes in rice (Oryza sativa) were shown to be involved in cold and drought stress
responses, which can bind to the promoter region of OsDREB1B (Figueiredo et al., 2012). In Chinese cabbage (Brassica rapa ssp. pekinensis), over half of 31 ZFHD genes were up-regulated after heat, cold, and salt treatments but down-regulated after drought (Wang et al., 2016). In tomato (Solanum lycopersicum), the transcript level of SlZFHD19 and SlZFHD20 markedly increased after drought, heat, and cold stress treatments; SlZHD2, SlZHD7, SlZHD8, and SlZHD15 were up-regulated under drought and salt treatments (Khatun et al., 2017). More recent studies showed that four CsZFHDs in cucumber (Cucumis sativus) were significantly down-regulated by drought stress (Lai et al., 2021). In wheat (Triticum aestivum), ten TaZFHD genes were mainly up-regulated under salt, cold and water stress treatments (Liu, Yang & Zhang, 2021). NtZFHD21 from tobacco (Nicotiana tabacum) was highly expressed in response to the drought treatment (Sun et al., 2021).

On the other hand, the phytohormone, abscisic acid (ABA), has been reported to play a predominant role in regulating plant response to multiple environmental stimuli (Chong, Guo & Zhu, 2020); thus, the ABA signaling pathway has been studied extensively (Chen et al., 2020; Dar et al., 2017). ABA can trigger extensive changes in the transcriptome to help plants adapt to abiotic stresses (Verma, Ravindran & Kumar, 2016). So far, many TF genes from ABA-responsive element binding factors (ABF), MYC, MYB and NAC families have been identified as functioning in the transcriptional regulation of ABA-mediated stress-inducible expression. Also, some ZFHD genes were induced by ABA treatment, such as AtZFHD1 OsZHD4, BraZF-HD03 and BraZF-HD05 (Figueiredo et al., 2012; Tran et al., 2007; Wang et al., 2016). However, the involvement of ZFHD genes in the ABA-dependent or -independent signaling pathway is still largely unknown.

Lily is one of the most important flower crops in the world, and it is susceptible to low temperature, high salinity and drought stresses. However, as the most widely distributed wild lily in East Asia, tiger lily (Lilium lancifolium) has been reported to have distinctive molecular mechanisms that confer its superior tolerance to various abiotic stresses (Wang et al., 2014). Based on the cold-responsive (4°C-treated 0, 2, and 16 h) transcriptome in the mature leaves of tiger lily, we have constructed the highest reciprocal rank-based gene co-expression network in our previous study (Yong et al., 2018). Four gene modules were identified due to their GO terms significantly enriched in stress response (Figs. 1A, 1B, 1C, 1D) (Yong et al., 2018). A ZFHD gene was found to be a hub gene in one of these four modules (Fig. 1B). This ZFHD gene co-expressed with some known stress-related genes, including CIPK25 (CBL-interacting protein kinase), ADC (Arginine decarboxylase), SAMDC (S-adenosylmethionine dehydrogenase), and another TF gene from NAC family (Fig. 1E) (Yong et al., 2018). In this study, we cloned and analyzed this ZFHD gene LlZFHD4. The subcellular localization and transcription activation of LlZFHD4 protein were observed. The functions of LlZFHD4 in response to osmotic stress and ABA in tiger lily were detected and discussed further.

**MATERIALS & METHODS**

**Plant materials**
The bulbs of wild tiger lily (Lilium lancifolium) with a size of 3–5 cm collected from Heilongjiang province, China, were used in this study. The bulbs were washed with tap...
water, sterilized with carbendazim, air-dried, covered with a 1-cm layer of moist vermiculite, and stored at 4 °C for 40 days. After vernalization, the bulbs were cultured in the science and technology experimental greenhouse of Beijing Forestry University (116.3° E, 40.0° N).

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used for the ectopic expression study; tobacco Nicotiana benthamiana (NT78) was used for the subcellular localization study. The seeds of tobacco and Arabidopsis were sown in plastic containers filled with the sterile substrate (peat:vermiculite = 1:1), and cultured in a growth chamber with a light/dark regime 16/8 h (100 µmol·m⁻²·s⁻¹), at 22/16 °C, and 65% relative humidity. The containers were covered with plastic wrap under growth chamber condition until seed germination.
Gene cloning and sequence analysis

RNAisomate RNA Easyspin isolation system (Aidlab Biotech, China) was used for total RNA extraction. The RNA quantity was examined by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The First-strand synthesis of cDNA was produced using PC54-TRUEscript RT kit (+gDNA Erase) (Aidlab Biotech, China) using 1 µg total RNA. The complete cDNA sequence of LIZFHD4 was obtained from tiger lily’s RNA-seq data published before (Yong et al., 2018; Yong, Zhang & Lyu, 2019b). Open reading frame (ORF) of LIZFHD4 was amplified using PC09-2×Taq PCR MasterMix (Aidlab Biotech, China) with the forward primer 5′-ATGGATCTCCCCATTTATC-3′ and the reverse primer 5′-TCAGTAGTCACTCTGCAATG-3′. The PCR reaction system and procedure were performed according to the manufacturer’s protocol. The PCR products were ligated into the pEASYT1-Blunt cloning vector (TransGen Biotech, Beijing, China) and sequenced by the Beijing TransGen Biotech Company.

Amino acid sequence alignment analyses were performed by BLASTN in the National Center for Biotechnology Information (NCBI) and DNAMAN (version 7). The unrooted phylogenetic trees were constructed in MEGA5 software using the neighbor-joining method with 1,000 replications. The online database ExPASy (http://expasy.org/tools/protparam.html) was used for the theoretical molecular weight and isoelectric point calculation.

Promoter cloning and sequence analysis

DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and Genome Walker Kit (Clontech, CA, USA) were used for genomic DNA extraction from mature leaves of tiger lily and LIZFHD4 promoter cloning, respectively (Yong, Zhang & Lyu, 2019a, 2019b). The promoter region of LIZFHD4 was amplified by nested PCR methods according to the user manual of Genome Walker Kit (Clontech, CA, USA) with the primary PCR primer GSP1: GGGTGGCTGCTAGGGTCAAACAGTAAT and the secondary PCR primer GSP2: AAGGTGGTTACAATGGGAAGAAGATG. Using touchdown PCR method, the primary PCR reaction procedure was as follows: pre-denaturation at 94 °C for 1 min; seven cycles of denaturation at 94 °C for 25 s, 65–59 °C for 30 s (decrease 1 °C per cycle), 72 °C for 3 min; 32 cycles of denaturation at 94 °C for 25 s, 57 °C for 30 s, 72 °C for 30 s; and extension at 72 °C for 10 min. The 1/50 dilution of the primary PCR product was used as a template in the secondary PCR. The reaction procedure was as follows: pre-denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 40 s, 58 °C for 40 s, 72 °C for 3 min, and extension at 72 °C for 30 s. The prediction of conserved cis-element motifs was performed on PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) databases.

Quantitative real-time PCR (qRT-PCR) analysis

Primer Premier 5.0 was used for primer design: primer annealing temperature 50–65 °C, primer length 18–24 bp, and amplification length 80–250 bp. The primers of LIZFHD4 and AtRD29A, AtRD29B, AtRD20, AtLEA14, AtGolS1, AtAPX2 from Arabidopsis were listed in Table S1. The SYBR® qPCR mix (Takara, Dalian, China) and Bio-Rad/CFX
ConnectTM Real-Time PCR Detection System (Bio-Rad, Irvine, CA, USA) were used in the qRT-PCR experiment. The reaction procedure was as follows: pre-denaturation at 95 °C for 30 s, 40 cycles of denaturation at 95 °C for 5 s, annealing temperature for 30 s, and extension at 72 °C for 30 s. Relative mRNA content was calculated using the 2−ΔΔCT method. Each sample was amplified in biological and technical triplicate. Tiger lily LITIP1 (Wang et al., 2014) and Arabidopsis Atactin (NM_112764) were used as internal reference genes (Table S1).

Subcellular distribution of LIZFHD4
The ORF of LIZFHD4 without stop codon was amplified with the forward primer 5′-CATTACGAAACGATACCTGAG(XhoI)ATGGATCTCCCATTATC-3′ and the reverse primer 5′-CACCATCAGTAGTACGTCGAC(SalI)GTAGTCACTGCAATG-3′. The amplified product was inserted into the XhoI and SalI site of the pBI121-GFP vector driven by a CaMV 35S promoter according to the ClonExpress II kit’s user manual (Vazyme, Nanjing, China). The confirmed recombinant vector pBI121-LIZFHD4-GFP was transformed into Agrobacterium strain GV3101 competent cells by the freeze-thaw method; and introduced in the tobacco leaf epidermal cells by the infiltration method with GV3101 cells. After 32 h incubation, the GFP fluorescence signals were detected in agroinfiltrated tobacco leaves using Leica TCS SP8 Confocal Laser Scanning Platform (Yong, Zhang & Lyu, 2019a, 2019b).

Yeast transcription activation assay of LIZFHD4
Using a ClonExpress II kit, the entire coding region (1–540 bp) and N-terminus (1–270 bp) and C-terminus (271–540 bp) of the LIZFHD4 cDNA region were amplified (primers are shown in Table S1), and cloned into the EcoRI and BamHI site of pGBK77 vector MCS region (Clontech, CA, USA), resulting in recombinant vector pGBK7-LIZFHD4-A (1–180 aa), pGBK7-LIZFHD4-N (1–90 aa) and pGBK7-LIZFHD4-C (91–180 aa). The confirmed recombinant vectors were introduced into the Y2HGold yeast strain (Huayueyang, Beijing, China) according to our previous study (Yong, Zhang & Lyu, 2019a, 2019b). The 1/10 and 1/100 dilution transformed yeast cells were incubated on plates containing the appropriate SD selection medium (Cao et al., 2017) at 30 °C. Yeast colony formation was photographed after three days.

Generation of LIZFHD4 transgenic Arabidopsis
The entire coding region of LIZFHD4 was inserted into the pBI121 vector driven by CaMV 35S for ectopic expression study. The CaMV35S promoter region in the pBI121-GUS vector was replaced by the LIZFHD4 promoter region for promoter activity analysis. After being mobilized to Agrobacterium strain GV3101, the recombinant vectors were transformed into wide-type (WT) Arabidopsis with GV3101 cells through the floral dip method. The MS medium containing 50 mg/L kanamycin was used to screen the progeny transgenic plant seeds. T3-generation of LIZFHD4 transgenic lines were harvested after qRT-PCR confirmation; T2-generation of LIZFHD4 promoter transgenic lines were
collected after PCR confirmation with promoter-specific primers: forward primer 5′-GCTTGATATCGAATTCG-3′ and reverse primer 5′-TCTCAATTTAGGATCCT-3′.

**Stress treatments**

Seedlings during the blooming period were used for stress experiments (Fig. 2). Mature leaves, bulbs, bulb roots, stem roots, stems, and flower petals were sampled, respectively. All tissues and organs were directly frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

Cold, dehydration, salinity, and ABA treatments were performed on tiger lily by treating eight-week-old seedlings with 4 °C, 16.1 % PEG6000 (−0.5 MPa), 100mM NaCl, and 100µM exogenous ABA for 0, 1, 3, 6, 12, 24 h before sampling for expression analysis of *LIZFHD4* (*Yong, Zhang & Lyu, 2019a, 2019b*). Similarly, three-week-old *LIZFHD4* promoter transformant *Arabidopsis* plants were also treated with the above conditions for *GUS* gene expression assay. Three-week-old *LIZFHD4* transgenic *Arabidopsis* and WT seedlings were harvested under normal conditions for expression analysis of *AtRD29A,*
AtRD29B, AtRD20, AtLEA14, AtGolS1, and AtAPX2 genes (Table S1). All experiments were repeated three times in biological triplicates.

**Stress tolerance assays in LIZFHD4 transgenic Arabidopsis**

Three-week-old T3-generation transgenic Arabidopsis lines and WT seedlings were pretreated under 4 °C for 3 h, and then treated under −4, −6, or −8 °C for 12 h as cold treatment (Yong, Zhang & Lyu, 2019a, 2019b). The water intake of seedlings was withheld for 30 days as drought treatment (Yong, Zhang & Lyu, 2019b). After stress treatments, the stress-treated Arabidopsis seedlings were recovered in the growth chamber under normal growth conditions for seven days. The survival rate of Arabidopsis seedlings was scored. The relative electrolyte leakage and soluble sugar content were determined before (control) and after 3 h 4 °C and 16.1% PEG6000 (−0.5 MPa) treatments by the thermal conductivity measurement method and the anthrone method, according to Zhang et al. (2015). The measuring method of water loss rate was described in previous study of Cao et al. (2007).

Seeds of selected T3-generation homozygous transgenic lines and WT were sowed on MS medium containing 2 µM ABA or 50 mM NaCl for seven days (Yong, Zhang & Lyu, 2019a); and then the germination was scored and photographed.

**RESULTS**

**Gene isolation and sequence analysis of LIZFHD4**

LIZFHD4 gene comprises 543 bp open reading frame (ORF) corresponding to a protein of 180 amino acids with a calculated molecular mass of 20.08 kDa and a pI of 8.91 (Fig. S1). The LIZFHD4 contained a DNA-binding homeodomain in the C-terminus, and a conserved zinc finger domain in the N-terminus; two segments Ia and Ib were located in the zinc finger domain (Fig. 3A). Amino acid sequence alignment results showed that LIZFHD4 shared 67%, 47%, 60%, 59%, 54%, 53% identities with AtZHD4 and AtZHD3 from Arabidopsis, PdZHD4 from Phoenix dactylifera, EgZHD4-like from Elaeis guineensis, OsZHD3 from rice, GmZHD4-like from Glycine max (Fig. 3A). A phylogenetic tree was constructed based on the known amino acid sequences of ZFHD genes in the model plant (Arabidopsis) and crop (rice), showing that the LIZFHD4 sequence clustered closely with Arabidopsis AtZFHD4 and rice OsZHD4 shared 67% and 55% identities, respectively (Fig. 3B).

**LIZFHD4 is a nucleus-localized transcriptional activator**

The LIZFHD4 ORF without stop codon was connected with the N-terminal of the GFP gene driven by a CaMV 35S promoter. The 35S-LIZFHD4-GFP fusion protein was observed in the subcellular localization of LIZFHD4. A nuclear localization signal was detected in 35S-LIZFHD4-GFP transformed tobacco leaf epidermal cell; in contrast, a ubiquitous distribution signal in the whole cell was detected in control (35S-GFP) (Fig. 4A), suggesting LIZFHD4 is a nucleus-localized protein.

To investigate whether the LIZFHD4 has transactivation activity, recombinant pGBKT7 vector LIZFHD4-A (entire coding region), LIZFHD4-N (N-terminal region), LIZFHD4-C
Figure 3 Conserved domain and phylogenetic analysis of LlZFHD4 protein. (A) Alignment of LlZFHD4 with Arabidopsis AtZHD3 and AtZHD4, rice OsZHD3, Elaeis guineensis EgZHD4-like, Glycine max GmZHD4-like and Phoenix dactylifera PdZHD4. (B) Phylogenetic tree analysis of LlZFHD4 with Arabidopsis AtZHD3 (NP_178358.1), AtZHD4 (Q9M980.1), AtZFDH2 (NP_201344.1), AtZHD14 (NP_563956.1), AtHB21 (OAP10319.1), AtZHD6 (NP_565436), AtZHD9 (NP_189534), AtZHD7 (NP_190658), AtHB22 (NP_850266.1), AtZFDH3 (NP_197025.1), AtHB23
(C-terminal region), and pGBDKT7 vector (control) were introduced into the Y2HGold yeast strain. The LIZFHD4-A and LIZFHD4-N contained yeast strain grew well on the SD/-Trp/-His/-Ade medium, and the colonies are blue on SD/-Trp/-His-x-α-gal medium. These results indicate the LIZFHD4 has transcriptional activity, and the deletion of the C-terminal region (from the position of 91 to 180 aa) did not affect the activation (Fig. 4B), which means that LIZFHD4 is a transcription activator with transactivation in the N-terminus.

Expression patterns of **LIZFHD4** under stresses

Under normal conditions, **LIZFHD4** was expressed in all detected organs of tiger lily, including mature leaf, bulb, bulb root, stem root, stem, and flower petal. The transcript level of **LIZFHD4** was shown to be the highest in flower petal followed by bulb, while it was low in leaf and stem (Fig. 5A). Under ABA (100 μM) treatment, the expression of **LIZFHD4** was highly induced within 2 h with a threefold to fourfold increase, and then peaked at 24 h (Fig. 5B). Similarly, salt (NaCl 100 mM) treatment also induced the expression of **LIZFHD4** within 2 h showing a twofold to threefold increase (Fig. 5E). However, treatment of plants with cold (4 °C) and drought (16.1% PEG6000) stresses could not up-regulate the expression of **LIZFHD4** until 24 h with a fivefold to sixfold increase (Figs. 5C, 5D). These results showed that **LIZFHD4** is cold, drought, and salt-responsive (Table S2 lists all the raw data of Fig. 5).

Promoter analysis of **LIZFHD4** in response to stresses

A 789 bp upstream of the ATG start codon of the **LIZFHD4** gene was cloned and used as the **LIZFHD4** promoter sequence (Fig. S2). Putative cis-acting regulatory elements annotated as stress- or hormone-responsive elements (T-Box, Box1, and ARF elements) were identified (Table 1). Three binding sites (MYC, DRE, and W-box) of MYC, DREB, and WRKY TFs were also found in the promoter region of **LIZFHD4**. The expression of the GUS gene driven by the **LIZFHD4** promoter was detected by qRT-PCR in transgenic *Arabidopsis* seedlings. The qRT-PCR results showed that treatment of transgenic *Arabidopsis* with cold (4 °C), drought (16.1% PEG6000), salt (NaCl 100 mM), and ABA (100 μM) could induce GUS gene with a maximal transcript level at 12 h, leading to a fivefold to eightfold increase (Fig. 6); suggesting the promoter activity of **LIZFHD4** can be induced by these stresses in some degree (Table S3 lists all the raw data of Fig. 6).

Overexpressing **LIZFHD4** alters the abiotic stress tolerance of transgenic *Arabidopsis*

Two T2 generations **LIZFHD4** transgenic *Arabidopsis* lines, Line 6 and Line 7 (L6, L7), with relatively high LIZFHD4 transcript levels, were chosen by qRT-PCR (Fig. S3). The T3 generation of L6 and L7 were used for subsequence stress tolerance analysis. Under normal
Figure 4  Subcellular localization and transactivation assay of LIZFHD4. (A) Subcellular localization of free GFP and LIZFHD4-GFP under the control of CaMV 35S promoter by transient expression assay in tobacco leaf epidermal cells (scale bars=50 μm). Green fluorescence represents GFP fluorescence. Chlorophyll auto-fluorescence is in red. Bright-field images show the equivalent field observed under white light. All of the signals were monitored by confocal microscopy. (B) Recombinant pGBKTT vector LIZFHD4-A (entire coding region), LIZFHD4-N (N-terminal region), LIZFHD4-C (C-terminal region) and empty pGBKTT vector (control) were introduced into the Y2HGold yeast strain. The 1/100 dilution transformed yeast cells were incubated on plates containing the appropriate SD selection medium.

DOI: 10.7717/peerj.11508/fig-4
growth conditions, L6, L7, and WT Arabidopsis seedlings all grew well. No difference in plant morphology between L6, L7, and WT plants was noticed (Figs. 7A, 7B). However, under cold and water stress treatments, the less damaging effects were observed on L6
and L7, compared to WT. After exposing to freezing temperatures (especially under $-6 \degree C$) for 12 h or withholding water for 30 days, L6 and L7 seedlings displayed better growth status with larger leaf area, and significantly higher survival rate as compared to WT plants (Figs. 7A, 7B). Additionally, some essential physiological parameters, including water-loss rate, relative electrolyte leakage, and soluble sugar content, were measured before (control) and after 3 h $-4 \degree C$ and 16.1% PEG6000 ($-0.5$ MPa) treatments. We found that L6 and L7 plants showed lower water-loss rates and electrolyte leakage amounts, higher soluble sugar levels than WT plants (Figs. 7C, 7D, 7E). These results indicate the LlZFHD4 transgenic plants are more tolerant to cold and water stresses than WT plants.

Under salt treatment, however, lower germination ratios measured by radicle protrusion rate were observed in L6 and L7, especially in L7, than in WT on the MS agar plates supplemented with 50 mM NaCl (Figs. 8A, 8B). We also found L6 and L7, especially L6, displayed lower germination ratios than WT under ABA (2 µM) treatment measured by both radicle protrusion and cotyledon greening (Figs. 8A, 8B). These results indicate the LlZFHD4 transgenic plants are less tolerant to salinity and more hypersensitive to ABA than WT plants.

**Overexpressing LlZFHD4 increases the expression of stress- and ABA-responsive genes**

To further explore the molecular mechanism underlying LlZFHD4 in response to osmotic stresses, we assessed the expression levels of some known stress- and ABA-responsive genes in LlZFHD4 transgenic Arabidopsis under normal growth conditions. The results showed that the transcripts of AtRD29A, AtRD20, AtGolS1, AtLEA14, AtAPX2, and AtRD29B genes (Table S1) accumulated significantly higher in L6 and L7 than WT seedlings (Fig. 9). More importantly, the expression level of AtRD29A, AtRD29B, and AtAPX2 in L6 and L7 was even more than 20 folds of that in WT (Fig. 9), implying

| Site name | (Strand) position | Sequence | Function |
|-----------|-------------------|----------|----------|
| MYCRS     | (+)145,502;       | CAA(A/G)TG | MY recognition site involved in cold and drought-inducibility |
|           | (-)46             | CAGCTC   |          |
| BoxI      | (-)318            | TTTCAAA  | light responsive element |
| T-Box     | (-)307            | ACTTTG   | light responsive element |
| SORLIP    | (+)55             | GGGCC    | Sequences Over-Represented in Light-Induced Promoters (SORLIPs) |
| G-BOX     | (-)399            | CACGTC   | cis-acting regulatory element involved in light responsiveness |
| ARE       | (+)234            | TGGTTT   | cis-acting regulatory element essential for the anaerobic induction |
| DRE       | (+)759; (-)458    | GTCGAC   | dehydration-responsive element (DRE) |
|           |                   | ACCGAC   |          |
| ARF       | (-)535            | TGTCTC   | ARF (auxin response factor) binding site |
| GARE-motif| (+)238            | TCTGTTG  | Gibberellin-responsive element |
| W-box     | (+)328; (-)305    | TTGAC(C/T) | WRKY proteins bind specifically to the DNA sequence motif (T)(T)TGAC(C/T) |
LlZFHD4 may confer cold and water stress tolerances by effectively up-regulating stress- and ABA-responsive genes.

**DISCUSSION**

In this study, we identified a novel stress-responsive ZFHD TF gene, *LlZFHD4*, from the wild lily species *Lilium lancifolium*. Sequence analysis showed that *LlZFHD4* contains conserved domains in both the N-terminal and the C-terminal regions. The N-terminal domain contains five conserved cysteine and two conserved histidine residues, whereas the C-terminus harbors a conserved DNA-binding homeodomain. *LlZFHD4* protein is likely to be involved in stress responses and may play a role in the adaptation to cold and water stress conditions.
also shown to be a nucleus-localized transcriptional activator with transactivation activity in the N-terminus.

Members of the ZFHD TF family in Arabidopsis, wheat, barley, Chinese cabbage, Tartary buckwheat (Fagopyrum tataricum), cucumber and tobacco are reported to function as transcriptional regulators in floral development or stress response processes (Abu-Romman, 2014; Abu-Romman & Al-Hadid, 2017; Lai et al., 2021; Khatun et al., 2017; Liu et al., 2019; Sun et al., 2021; Tan & Irish, 2006). In this study, we found that LlZFHD4 may play similar roles in tiger lily. The qRT-PCR results showed that LlZFHD4 has the highest expression levels in flower petals. Cold, drought, salt, and ABA treatments can also significantly up-regulate the expression level of LlZFHD4. Furthermore, the expression of the GUS gene driven by the LlZFHD4 promoter could be up-regulated by cold, salt, and water stresses, and ABA. Thus, we suppose the LlZFHD4 promoter has higher transcript

Figure 8 Overexpression of LlZFHD4 in Arabidopsis reduced salinity and ABA tolerance. (A) Observation and (B) seed germination counts of T3 generation LlZFHD4 transgenic Arabidopsis lines (L6, L7) and WT seeds on MS medium supplemented with 2 µM ABA or 50 mM NaCl after 7 d of incubation at 22 °C. The bars show the mean ±SD of three biological replicates. Significant differences between the L6, L7 and WT plants are indicated as 0.01 < p-value < 0.05 and **p-value < 0.01. DOI: 10.7717/peerj.11508/fig-8
activation activity under cold, drought, salt, and ABA conditions. On the other hand, the expression of *LlZFHD4* in response to stresses may also be regulated by some upstream regulatory factors like DREB, MYC, and WRKY TFs for their binding sites (MYC, DRE, and W-box) are located in the *LlZFHD4* promoter region. This may be the reason why the expression patterns of *LlZFHD4* were different from the expression patterns of *GUS* driven by *LlZFHD4* promoter under stress treatments.

Recent studies on *ZFHD* genes in tomato and tobacco has revealed that the silencing of the *SL-ZH13* gene exhibited reduced drought and salt tolerance of transgenic tomato (*Zhao et al.*, 2019), and silencing of *NtZFHD21* decreased the drought tolerance of transgenic tobacco (*Sun et al.*, 2021). Additionally, many researchers have reported that C2H2-type zinc finger proteins play crucial roles, acting both positively and negatively in abiotic stress signaling in *Arabidopsis*. For instance, overexpression of *Zat12* in *Arabidopsis* could not only result in enhanced tolerance of freezing, osmotic, salinity, oxidative and light stresses and iron deficiency (*Davletova et al.*, 2005; *Vogel et al.*, 2005);
but also conferred enhanced heat sensitivity in contrast (Le et al., 2016). Similarly, constitutive expression of Zat10 in transgenic plants was found to improve drought stress tolerance; but suppress defense responses that enhance osmotic and salinity stress tolerance (Mittler et al., 2006). Moreover, AZF2 and STZ function as transcriptional repressors to increase drought, cold, and salinity tolerance by negatively regulating ABA-repressive and auxin-inducible genes (Kodaira et al., 2011; Sakamoto et al., 2004).

In this study, we found that LIZFHD4 plays both positive and negative roles in plant stress responses. Under cold and water stress conditions, compared to WT seedlings, better growth status, higher survival rate, higher soluble sugar level, lower electrolyte leakage amount, and lower leaf water-loss ratios were observed in LIZFHD4 transgenic lines. Meanwhile, the expression levels of 6 well-known stress-related genes from Arabidopsis (AtRD29A, AtRD20, AtGolS1, AtLEA14, AtAPX2, and AtRD29B) were significantly higher (approximately ten to thirtyfold) in LIZFHD4 transgenic lines than in WT. Therefore, we assume LIZFHD4 TF can directly or indirectly regulate these genes at the transcriptional level to activate the plant adaptation to cold and drought stresses. In contrast, overexpression of LIZFHD4 had resulted in reduced salt tolerance in Arabidopsis, suggesting the LIZFHD4 may play a negative role in the plant adaptive response to salt stress. However, the molecular mechanism underlying this phenotype needs to be further illustrated.

On the other hand, some ZFHD genes from Arabidopsis, rice, and Chinese cabbage have been reported to involve in ABA signaling pathway responding to stresses (Tran et al., 2007; Figueiredo et al., 2012; Wang et al., 2016). In this study, the expression level and promoter activity of LIZFHD4 can be induced by ABA treatment; the LIZFHD4 transgenic Arabidopsis showed enhanced ABA sensitivity than WT plants. Considering the induced stress-related genes in LIZFHD4 transgenic lines are also known to be ABA-responsive genes, these results suggest LIZFHD4 may somewhat rely on ABA signaling to function in plant adaptation to abiotic stresses.

**CONCLUSIONS**

LIZFHD4 is a stress-responsive gene induced under cold, drought, salt, and ABA conditions. The expression of LIZFHD4 responding to osmotic stresses may be activated by the stress-inducible promoter; and, or regulated by the upstream regulatory factors like DREB, MYC, and WRKY TFs, for their binding sites are located in the LIZFHD4 promoter region. Overexpression of LIZFHD4 can up-regulate the expression of some stress- and ABA-responsive functional genes in Arabidopsis; thus, under freezing and drought conditions, LIZFHD4 transgenic Arabidopsis showed better growth status, higher survival rates, and higher osmotic adjustment capacity than WT. Meanwhile, reduced salt and ABA tolerance were observed in LIZFHD4 transgenic Arabidopsis. Our findings provide a novel ZFHD gene that may play positive role in cold and drought stress response, whereas function negatively in salinity tolerance, through the ABA signaling pathway.
ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This research was supported by China National Key Research & Development Project (grant no. 2019YFD1000400) and China National Natural Science Foundation (grant nos. 31672190, 31872138, 31071815, 31272204). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
China National Key Research & Development Project: 2019YFD1000400.
China National Natural Science Foundation: 31672190, 31872138, 31071815 and 31272204.

Competing Interests
The authors declare that they have no competing interests.

Author Contributions
• Yubing Yong conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
• Yue Zhang conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
• Yingmin Lyu conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability
The following information was supplied regarding data availability:
Raw measurements are available in the Supplemental Figures and Tables.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.11508#supplemental-information.

REFERENCES
Abu-Romman S. 2014. Molecular cloning and expression analysis of zinc finger-homeodomain transcription factor TaZFHD1, in wheat. South African Journal of Botany 91:32–36 DOI 10.1016/j.sajb.2013.11.014.
Abu-Romman S, Al-Hadid K. 2017. Novel zinc finger-homeodomain gene from barley (HvZFHD1) is differentially regulated during spike development and under hormonal treatments and abiotic stresses. Notulae Botanicae Horti Agrobotanici Cluj-Napoca 45(1):89–96 DOI 10.15835/nbha45110612.
Ariel FD, Manavella PA, Dezar CA, Chan RL. 2007. The true story of the HD-Zip family. *Trends in Plant Science* 12(9):419–426 DOI 10.1016/j.tplants.2007.08.003.

Barth O, Vogt S, Uhlemann R, Zschiesche W, Humbeck K. 2009. Stress induced and nuclear localized HIPP26 from Arabidopsis thaliana interacts via its heavy metal associated domain with the drought stress related zinc finger transcription factor ATHB29. *Plant Molecular Biology* 69(1–2):213–226 DOI 10.1007/s11103-008-9419-0.

Cao WH, Liu J, He XJ, Mu RL, Zhou HL, Chen SY, Zhang JS. 2007. Modulation of ethylene responses affects plant salt-stress responses. *Plant Physiology* 143(2):707–719 DOI 10.1104/pp.106.094292.

Cao H, Wang L, Nawaz MA, Niu M, Sun J, Xie J, Kong Q, Huang Y, Cheng F, Bie Z. 2017. Ectopic expression of pumpkin NAC transcription factor CmNAC1 improves multiple abiotic stress tolerance in Arabidopsis. *Frontiers in Plant Science* 8:2052 DOI 10.3389/fpls.2017.02052.

Chen K, Guojun L, Bressan R, Song CP, Zhu JK, Zhao Y. 2020. Abscisic acid dynamics, signalling and functions in plants. *Journal of Integrative Plant Biology* 62(1):25–54 DOI 10.1111/jipb.12899.

Chong L, Guo P, Zhu Y. 2020. Mediator complex: a pivotal regulator of ABA signaling pathway and abiotic stress response in plants. *International Journal of Molecular Sciences* 21(20):7755 DOI 10.3390/ijms21207755.

Dar N, Amin I, Wani W, Wani S, Shikari A, Wani S, Masoodi K. 2017. Abscisic acid: a key regulator of abiotic stress tolerance in plants. *Plant Gene* 11(4):106–111 DOI 10.1016/j.plgene.2017.07.003.

Englbrecht CC, Schoof H, Böhm S. 2004. Conservation, diversification and expansion of C2H2 zinc finger proteins in the Arabidopsis thaliana genome. *BMC Genomics* 5(1):39 DOI 10.1186/1471-2164-5-39.

Figueiredo DD, Barros PM, Cordeiro AM, Serra TS, Lourenco T, Chander S, Oliveira MM, Saibo NJM. 2012. Seven zinc-finger transcription factors are novel regulators of the stress responsive gene OsDREB1B. *Journal of Experimental Botany* 63(10):3643–3656 DOI 10.1093/jxb/ers035.

Halbach T, Scheer N, Werr W. 2000. Transcriptional activation by the PHD finger is inhibited through an adjacent leucine zipper that binds 14-3-3 proteins. *Nucleic Acids Research* 28(18):3542–3550 DOI 10.1093/nar/28.18.3542.

Hu W, De Pamphilis CW, Ma H. 2008. Phylogenetic analysis of the plant-specific zinc finger-homeobox and mini zinc finger gene families. *Journal of Integrative Plant Biology* 50(8):1031–1045 DOI 10.1111/j.1744-7909.2008.00681.x.

Huang J, Sun SJ, Xu DQ, Yang X, Bao YM, Wang ZF, Tang HJ, Zhang H. 2009. Increased tolerance of rice to cold, drought and oxidative stresses mediated by the overexpression of a gene that encodes the zinc finger protein ZFP245. *Biochemical and Biophysical Research Communications* 389(3):556–561 DOI 10.1016/j.bbrc.2009.09.032.

Khatun K, Nath UK, Robin AHK, Park JI, Lee DJ, Kim MB, Kim CK, Lim KB, Nou IS, Chung MY. 2017. Genome-wide analysis and expression profiling of zinc finger homeodomain (ZHD) family genes reveal likely roles in organ development and stress responses in tomato. *BMC Genomics* 18(1):695 DOI 10.1186/s12864-017-4082-y.

Kodaira KS, Qin F, Tran LS, Maruyama K, Kidokoro S, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K. 2011. Arabidopsis Cys2/His2 zinc-finger proteins AZF1 and AZF2
negatively regulate abscisic acid-repressive and auxin-inducible genes under abiotic stress conditions. *Plant Physiology* **157**(2):742–756 DOI 10.1104/pp.111.182683.

**Kosarev P, Mayer KFX, Hardtke CS. 2002.** Evaluation and classification of RING-finger domains encoded by the Arabidopsis genome. *Genome Biology* **3**(4):research0016.0011 DOI 10.1186/gb-2002-3-4-research0016.

**Krishna SS, Majumdar I, Grishin NV. 2003.** Structural classification of zinc fingers: survey and summary. *Nucleic Acids Research* **31**(2):532–550 DOI 10.1093/nar/gkg161.

**Lai W, Zhu C, Hu Z, Liu S, Wu H, Zhou Y. 2021.** Identification and Transcriptional Analysis of Zinc Finger-Homeodomain (ZF-HD) Family Genes in Cucumber. *Biochemical genetics* DOI 10.1007/s10528-021-10036-z.

**Le CTT, Brumbarova T, Ivanov R, Stoff C, Weber E, Mohrbacher J, Fink-Straube C, Bauer P. 2016.** ZINC FINGER OF ARABIDOPSIS THALIANA12 (ZAT12) interacts with FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) linking iron deficiency and oxidative stress responses. *Plant Physiology* **170**(1):540–557 DOI 10.1104/pp.15.01589.

**Liu M, Wang X, Sun W, Ma Z, Zheng T, Huang L, Wu Q, Tang Z, Bu T, Li C, Chen H. 2019.** Genome-wide investigation of the ZF-HD gene family in tartary buckwheat (*Fagopyrum tataricum*). *BMC Plant Biology* **19**(1):248 DOI 10.1186/s12870-019-1834-7.

**Liu H, Yang Y, Zhang L. 2021.** Zinc-finger-homeodomain transcriptional factors (ZF-HDs) in wheat (*Triticum aestivum* L.): identification, evolution, expression analysis and response to abiotic stresses. *Plants* **10**(3):593 DOI 10.3390/plants10030593.

**Mittler R, Kim Y, Song L, Coutou J, Coutou A, Ciftci-Yilmaz S, Lee H, Stevenson B, Zhu J-K. 2006.** Gain- and loss-of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. *FEBS Letters* **580**(28–29):6537–6542 DOI 10.1016/j.febslet.2006.11.002.

**Mukherjee K, Brocchieri L, Bürglin TR. 2009.** A comprehensive classification and evolutionary analysis of plant homeobox genes. *Molecular Biology and Evolution* **26**(12):2775–2794 DOI 10.1093/molbev/msp201.

**Perrella G, Davidson M, O’Donnell L, Nastase A-M, Herzyk P, Breton G, Pruneda Paz J, Kay S, Chory J, Kaiserli E. 2018.** ZINC-FINGER interactions mediate transcriptional regulation of hypocotyl growth in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **115**(19):E4503–E4511 DOI 10.1073/pnas.1718099115.

**Sakamoto H, Maruyama K, Sakuma Y, Meshi T, Iwabuchi M, Shinozaki K, Yamaguchi-Shinozaki K. 2004.** Arabidopsis Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiology* **136**(1):2734–2746 DOI 10.1104/pp.104.046599.

**Shalmani A, Muhammad I, Sharif R, Caiping Z, Ullah U, Zhang D, Jing X-Q, Amin B, Jia P, Tahir M, Xu Z, Chen K-M, Na A. 2019.** Zinc finger-homeodomain genes: evolution, functional differentiation, and expression profiling under flowering-related treatments and abiotic stresses in plants. *Evolutionary Bioinformatics* **15**:117693431986793 DOI 10.1177/1176934319867930.

**Tan QKG, Irish VF. 2006.** The Arabidopsis zinc finger-homeodomain genes encode proteins with unique biochemical properties that are coordinately expressed during floral development. *Plant Physiology* **140**(3):1095–1108 DOI 10.1104/pp.105.070565.
Tran LS, Nakashima K, Sakuma Y, Osakabe Y, Qin F, Simpson SD, Maruyama K, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K. 2007. Co-expression of the stress-inducible zinc finger homeodomain ZFHD1 and NAC transcription factors enhances expression of the ERD1 gene in Arabidopsis. *The Plant Journal* 49(1):46–63 DOI 10.1111/j.1365-313X.2006.02932.x.

Verma V, Ravindran P, Kumar P. 2016. Plant hormone-mediated regulation of stress responses. *BMC Plant Biology* 16(1):1–10 DOI 10.1186/s12870-016-0771-y.

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF. 2005. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. *The Plant Journal* 41(2):195–211 DOI 10.1111/j.1365-313X.2004.02288.x.

Wang J, Wang Q, Yang Y, Liu X, Gu J, Li W, Ma S, Lu Y. 2014. De novo assembly and characterization of stress transcriptome and regulatory networks under temperature, salt and hormone stresses in Lilium lancifolium. *Molecular Biology Reports* 41(12):8231–8245 DOI 10.1007/s11033-014-3725-1.

Wang W, Wu P, Li Y, Hou X. 2016. Genome-wide analysis and expression patterns of ZF-HD transcription factors under different developmental tissues and abiotic stresses in Chinese cabbage. *Molecular Genetics and Genomics* 291(3):1451–1464 DOI 10.1007/s00438-015-1136-1.

Windhövel A, Hein I, Dabrowa R, Stockhaus J. 2001. Characterization of a novel class of plant homeodomain proteins that bind to the C4 phosphoenolpyruvate carboxylase gene of *Flaveria trinervia*. *Plant Molecular Biology* 45(2):201–214 DOI 10.1023/A:1006450005648.

Xie M, Sun J, Gong DP, Kong Y. 2019. The roles of Arabidopsis C1-2i subclass of C2H2-type zinc-finger transcription factors. *Genes* 10(9):653 DOI 10.3390/genes10090653.

Yong YB, Li WQ, Wang JM, Zhang Y, Lu YM. 2018. Identification of gene co-expression networks involved in cold resistance of Lilium lancifolium. *Biologia Plantarum* 62(2):287–298 DOI 10.1007/s10535-017-0767-y.

Yong Y, Zhang Y, Lyu Y. 2019a. A MYB-related transcription factor from *Lilium lancifolium* L. (LIMYB3) is involved in anthocyanin biosynthesis pathway and enhances multiple abiotic stress tolerance in Arabidopsis thaliana. *International Journal of Molecular Sciences* 20(13):3195 DOI 10.3390/ijms20133195.

Yong YB, Zhang Y, Lyu YM. 2019b. A stress-responsive NAC transcription factor from tiger lily (LINAC2) interacts with LIDREB1 and LIZHFD4 and enhances various abiotic stress tolerance in Arabidopsis. *International Journal of Molecular Sciences* 20(13):3225 DOI 10.3390/ijms20133225.

Zang D, Wang L, Zhang Y, Zhao H, Wang Y. 2017. ThDof1.4 and ThZFP1 constitute a transcriptional regulatory cascade involved in salt or osmotic stress in Tamarix hispida. *Plant Molecular Biology* 94(4–5):495–507 DOI 10.1007/s11103-017-0620-x.

Zhang L, Zhang L, Xia C, Zhao G, Jia J, Kong X. 2015. The novel wheat transcription factor TaNAC47 enhances multiple abiotic stress tolerances in transgenic plants. *Frontiers in Plant Science* 6:1174 DOI 10.3389/fpls.2015.0151.

Zhao T, Wang Zy, Bao YF, Zhang XC, Yang HH, Zhang DY, Jiang JB, Zhang H, Li JF, Chen QS, Xu XY. 2019. Downregulation of SL-ZH13 transcription factor gene expression decreases drought tolerance of tomato. *Journal of Integrative Agriculture* 18(7):1579–1586 DOI 10.1016/S2095-3119(19)62621-3.