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ORIGINAL ARTICLE

Identification and functional characterization of *MdPIF3* in response to cold and drought stress in *Malus domestica*

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Abstract

PHYTOCHROME INTERACTING FACTORs (PIFs) are a subset of helix-loop-helix (bHLH) transcription factors, which play critical roles in plant growth and development, as well as in adaption to ambient environments. However, *PIF* members have not been completely identified in apple (*Malus domestica*), a widely distributed fruit crop with significant economical importance. Here, we characterized *MdPIF3*, the homolog of *AtPIF3*, and determined its role in response to abiotic stresses in apple. We first analyzed its gene and protein structure, and found that it contained bHLH domain, active phytochrome B binding (APB) motif, as well as active phytochrome A binding (APA) motif. Yeast-two-hybrid assays indicated that MdPIF3 formed a homodimer by itself and heterodimers with other MdPIFs. Moreover, *MdPIF3* was responsive to light and cold treatment at both transcriptional and post-translational levels. Overexpression of *MdPIF3* reduced cold tolerance but enhanced drought resistance in both apple callus and Arabidopsis.

Key message

The bHLH-type protein, MdPIF3, plays a key role in cold and drought tolerance in plants.

Keywords Apple · bHLH transcription factor · *MdPIF3* · Cold stress · Drought stress

Introduction

Plant growth requires suitable conditions, however, they always encounter adverse factors, such as drought, low temperature, and high salinity, that restrain the growth of most plants in natural environments (Knight and Knight 2012; Zhu 2002). Drought stress induces a series of adverse effects on plants, including inhibited germination, wilting, decreased chlorophyll content and photosynthesis rate, and results in repressed plant growth, as well as crop yields (Jaleel et al. 2009; Kaya et al. 2006; Li et al. 2015; Manickavelu et al. 2006; Manivannan et al. 2007). Similarly, cold stress affects a series of physiological processes of plants, such as cell membrane permeability, and accumulation of reactive oxygen species (ROS), thus, severely affects the spatial distribution and agricultural productivity of crop plants (Chinnusamy et al. 2007; Gill and Tuteja 2010; Kratsch and Wise 2000).

Apple (*Malus domestica*) is a perennial woody plant that widely distributed worldwide, and its growth and development are suffering from multiple environmental factors after years of cultivation, such as light, temperature, water, and pathogens. Apple trees are usually cultured in the mountain area where water is a limiting factor for plant growth, especially in the Loess Plateau region, which is one of the major area for apple production in China. In addition, apple is vulnerable to low temperatures, especially in the spring when

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floral is in the process of development (Feng et al. 2012; Xie et al. 2018). Therefore, research on how apple plants react to the environmental factors will facilitate to improve their adaptability to ambient complex stress conditions.

Generally, transcription factors (TFs) participate in the regulation of plant gene expression by activating or inhibiting the transcription of downstream genes (Agarwal et al. 2006; Feller et al. 2011; Rehman and Mahmood 2015). As the second largest TFs family, the bHLH TFs play important roles in plant response to multiple biotic and abiotic stresses (Feller et al. 2011; Joshi et al. 2016). For instance, expression of *bHLH122* was induced by drought and salt stresses, and overexpressed *bHLH122* enhanced drought and osmotic tolerances in Arabidopsis (Liu et al. 2014). Overexpression of *EcbHLH57* from *Eleusine coracana* L. promoted resistance to salt and drought in tobacco (Babitha et al. 2015). *PubHLH1* was proved to enhance cold tolerance by regulating stress-related gene expression in tobacco (Jin et al. 2016). However, in the past few decades, only few bHLH TFs have been identified to be involved in stress response in apple (Mao et al. 2017). To date, *MdCIbHLH1* (also named *MdbHLH143*) was reported to enhance the cold tolerance in transgenic Arabidopsis and apple callus (Feng et al. 2012). *MdbHLH104* was shown to enhance iron deficiency tolerance of apple tree (Zhao et al. 2016).

As members of the 15th subfamily of the bHLH TF family, PIFs (PIF1-PIF8) have been identified and deeply investigated in Arabidopsis (Pham et al. 2018; Toledo-Ortiz et al. 2003). In addition to the conserved bHLH domain, all of these PIFs contain an APB motif at the N-terminal, which is necessary for specific binding to phytochrome B (phyB) (Khanna et al. 2004; Shen et al. 2008). Moreover, an APA motif was found in PIF1 and PIF3, which is responsible for binding to phytochrome A (phyA) (Al-Sady et al. 2006; Leivar and Monte 2014; Leivar and Quail 2011).

PIF3, the first identified PIF protein in plant, was initially isolated using the yeast-two-hybrid (Y2H) assay using the C-terminal of phyB as bait (Ni et al. 1998). In the past two decades, extensive researches have been conducted on the functions of *PIFs* in plant growth and development. In Arabidopsis, *PIF1*, *PIF3*, *PIF4*, and *PIF5* act as negative regulators to inhibit hypocotyl elongation and cotyledon expansion (Fujimori et al. 2004; Huq and Quail 2002; Kim et al. 2003; Monte et al. 2004; Oh et al. 2004). Moreover, PIF3 positively regulates anthocyanin biosynthesis by directly binding to the promoter region of anthocyanin biosynthetic genes (Shin et al. 2007). However, *PIF4* and *PIF5* act as negative regulators of anthocyanin biosynthesis (Liu et al. 2015). In addition to be involved in light-mediated plant growth, *PIFs* are also reported to be functional in response to abiotic stresses. For example, ectopic expression of *ZmPIF1* and *ZmPIF3* enhanced drought tolerance in rice (Gao et al. 2015; Gao et al. 2018). *PIF4* and *PIF7* is essential for Arabidopsis to resist high temperature stress (Fiorucci et al. 2020; Kim et al. 2020; Koini et al. 2009; Sun et al. 2019b). Recent studies show that *PIF1*, *PIF3*, *PIF4*, and *PIF5* play a negative role in plant freezing tolerance in Arabidopsis (Jiang et al. 2020; Jiang et al. 2017).

Although *PIFs* has been extensively studied in several plants, its function responding to abiotic stress in apple have not been explored. In this study, we identified a bHLH TF, *MdPIF3*, in apple, and found that it reduced the cold tolerance and enhanced the resistance to drought stress in both apple callus and Arabidopsis.

Materials and methods

Plant materials and growth conditions

The materials used in this study were apple seedlings (Royal Gala), apple callus (Orin), and *Arabidopsis thaliana* (Columbia). Tissue-cultured apple seedlings (Royal Gala) were maintained on a Murashige and Skoog (MS) medium with 0.5 mg/L 6-benzylaminopurine (6-BA) and 0.5 mg/L 1-naphthylacetic acid (NAA) during a 16-h-light/8-h-dark condition (photon flux density of approximately 60 μmol s^{-1} m^{-2}) at 25 °C for about 3 weeks, then treated with 4 °C and 10% polyethylene glycol 6000 (PEG6000) for the simulation of cold and drought stresses (An et al. 2018a; Zhong et al. 2020). For light treatment, apple seedlings grown in darkness for 72 h were transferred to white light condition for different times. The above apple seedlings set at least three biological replicates. These seedlings (cold-treated, drought-treated, and light-treated apple seedlings) were sampled at 0, 1, 2, 3, 6, and 9 h, immediately cryopreserved by liquid nitrogen, and stored at − 80 °C for gene expression analysis (An et al. 2017b; Zhang et al. 2018).

The apple callus of the 'Orin' cultivar were cultured on MS medium containing 0.8% agar, 0.4 mg/L 6-BA, and 1.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) at 24 °C in the dark (An et al. 2019). *Arabidopsis thaliana* (Columbia) at 22 °C were grown on MS medium under a 16-h-light/8-h-dark photoperiod (photon flux density of approximately 60 μmol s^{-1} m^{-2}).

Multiple sequence alignments and phylogenetic tree construction

The Protein BLAST tool from NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) was used to obtain homologs of Arabidopsis PIF3. Protein sequence of AtPIF3 was obtained from the Arabidopsis database base (https://www.arabidopsis.org/index.jsp). The protein sequences alignment was conducted using the DNAMAN software. The phylogenetic tree

was constructed with MEGA5.1 software using the neighbor-joining method (Sun et al. 2019a).

RNA extraction and quantitative real-time (qRT)-PCR analysis

The total RNAs of plant materials, including apple seedlings, apple callus, and Arabidopsis seedlings, were extracted using the RNA plant plus Reagent (Tiangen, Beijing, China) (Yang et al. 2019). Take 50–100 mg plant tissues and grind them into powder in liquid nitrogen, with at least three replicates for all sample. Reverse transcription was performed using the PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). The qRT-PCR reaction profile was performed under the following procedure: pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 20 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 30 s for 35 cycles (Li et al. 2012). The primers in this study are listed in Table S1. Apple 18S ribosomal RNA was used as control (Ma et al. 2017).

In vitro protein degradation assay of the MdPIF3 protein

Protein degradation assays were conducted as previously described (An et al. 2017c) to test the post-translational regulation of *MdPIF3* protein in response to light or cold treatments in vitro. The incubations of the extraction solution from the apple callus and the purified *MdPIF3-HIS* protein were conducted up to the specific times. For MG132 treatment, the untreated apple callus extract was treated with 100 μMMG132 for 0.5 h before co-incubation with purified *MdPIF3-HIS* protein. The relative *MdPIF3* protein contents were detected by using western blot with anti-HIS monoclonal antibody.

Genetic transformation of apple callus and Arabidopsis

The open reading frame (ORF) and antisense fragment of *MdPIF3* was cloned into pRI 101-AN vector (Takara, Dalian, China) driven by cauliflower mosaic virus 35S promoter to construct overexpression and antisense suppression vectors. For the acquisition of transgenic callus, 14-day-old wild-type apple callus were co-incubation for 20 min with Agrobacterium carrying recombinant constructs of *MdPIF3*, and the apple callus were plated on medium supplemented with antibiotics (Zhang et al. 2019). qRT-PCR analysis was used to identify the successfully transgenic apple callus (*MdPIF3-OE* and *MdPIF3-Anti*) (Fig. S2).

Transgenic Arabidopsis were obtained using the floral dip transformation method (Clough and Bent 1998). And qRT-PCR analysis was used to determine the successfully transformed Arabidopsis plants (*MdPIF3-L1*, *MdPIF3-L2*, and *MdPIF3-L3*) (Fig. S2).

Transcription activation of *MdPIF3* protein experiments

To determine if *MdPIF3* is of autonomous activation and which region is responsible for autonomous activation, transcriptional activity assays were conducted. The *MdPIF3* protein contains conserved bHLH domain. Sequence analysis showed that the bHLH domain is located at in 450–510 aa. Thus, amino acids 443 and 515 were used as breakpoints during segmentation. The full-length *MdPIF3* and five truncated fragments ($MdPIF3^{1-515}$, $MdPIF3^{1-443}$, $MdPIF^{444-515}$, $MdPIF3^{444-708}$, and $MdPIF3^{516-708}$) were amplified and inserted into the pGBKT7 vector (Clontech, USA), forming multiple fusion proteins with GAL4 DNA-bindind domains. Then the fusion constructs were transferred into the yeast strain AH109 (An et al. 2018b). Yeast cells were grown on medium lacking tryptophan (SD/-Trp) at 28 °C for 2 days. For further screening, the colonies were grown on the medium lacking tryptophan, histidine and adenine (SD/-Trp/-His/-Ade) with or without x-α-gal.

Drought and cold stress assays

The 10-day-old apple callus (3 biological replicates, at least 20 samples for each replicate) were treated with 0, 4%, and 6% PEG6000 in the dark for the simulation of drought stress. After 20 days of treatment, the fresh weight was measured. The malondialdehyde (MDA) is a biomarker used to measure damage caused by oxidative stress, and its content was measured by using the methods previously reported (Li and Chow 1994).

For cold stress treatment, the 10-day-old apple callus were transferred to 4 °C in the dark. After 20 days of treatment, the fresh weight of WT and transgenic apple callus were measured using electronic balance (one thousandth). The 5-day-old Arabidopsis seedlings cultured on MS medium were transferred to 4 °C during a 16-h-light/8-h-dark condition (photon flux density of approximately 60 μmol s^{-1} m^{-2}). The root length and electrolyte leakage were measured after 2 weeks.

ROS staining

Nitro blue tetrazolium (NBT) was utilized to determine O_2^- accumulation by using the histochemical staining.

Drought tolerance assay and chlorophyll extraction of Arabidopsis seedlings

Arabidopsis plants are grown in a normal substrate (a mixture of 30% nutrient soil and 70% vermiculite). After

3 weeks of normal growth, the plants were not watered until the leaves began to wilt and turned yellow (Qi et al. 2019). Chlorophyll content was measured following the method (An et al. 2017a). In short, after 3–4 weeks of drought treatment, the transgenic and wild-type Arabidopsis leaves were washed and cut into thin slices. Weigh 0.1 g leaves and immerse them in 95% ethanol for 24 h to extract. The absorbance of extracting solution was measured at 649 nm and 665 nm. All samples are set at least three biological repetitions.

Yeast two-hybrid (Y2H) assays

Y2H assays were performed to investigate whether MdPIF3 can form homodimers and/or heterodimers, and experimental methods were conducted according to the manufacturer's instructions (Clontech, USA). Full-length *MdPIF3*, five truncated segments (*MdPIF3*$^{1-515}$, *MdPIF3*$^{1-443}$, *MdPIF*$^{444-515}$, *MdPIF3*$^{444-708}$, and *MdPIF3*$^{516-708}$), and *MdPIF1/4/8* were cloned into the pGAD (pGAD424) vector. pGBD (pGBT9)-MdPIF3$^{444-708}$ was used as a bait. The mixed plasmids were co-transformed in yeast strain Y2H Gold. The cells were grown on medium lacking tryptophan and leucine (SD/-Trp/-Leu) at 28 °C for 2 days. The cells were then transferred to medium that lacked tryptophan, leucine, histidine, and adenine (SD/-Trp/-Leu/-His/-Ade) with or without x-α-gal for the interactive screening (Xie et al. 2012).

Data analysis

Three biological replicates were conducted for all samples, and the data expressed as the mean ± standard deviation unless noted otherwise. Significant differences were determined using Student's *t* test from DPS software (Enfield, UK) (Hu et al. 2019).

Results

Molecular cloning and phylogenetic relationship analysis of *MdPIF3*

The Arabidopsis *PIF3* (AT1G09530) coding sequence was used as bait to screen out *MdPIF3* by mining the NCBI database. Using synthesized first-strand cDNA from the tissue-cultured apple (*Malus domestica* 'Royal Gala') seedlings as template, *MdPIF3* specific primers were used for PCR amplification (Table S1), and a 2127 bp fragment was obtained and named *MdPIF3* (LOC103450807). Sequence analysis showed that *MdPIF3* gene contains six introns and seven exons (Fig. S1A). The fragment encodes a protein of 708 amino acids with a molecular mass of 75.7 kDa.

We next analyzed the phylogenetic relationship between *MdPIF3* and homologs from other plant species by constructing a neighbor-joining phylogenetic tree of 31 plant *PIF3* proteins using the MEGA 7 software (Fig. 1). The result showed that MdPIF3 was most closely related to PbPIF3 (XP_009366016.1) from *Pyrus bretschneideri,* and they were categorized as a single clade (Fig. 1). Moreover, sequence alignment showed that MdPIF3 and PbPIF3 shared high similarity (86.25%) in amino acid level (Fig. S1B), which further confirmed the high homology between them.

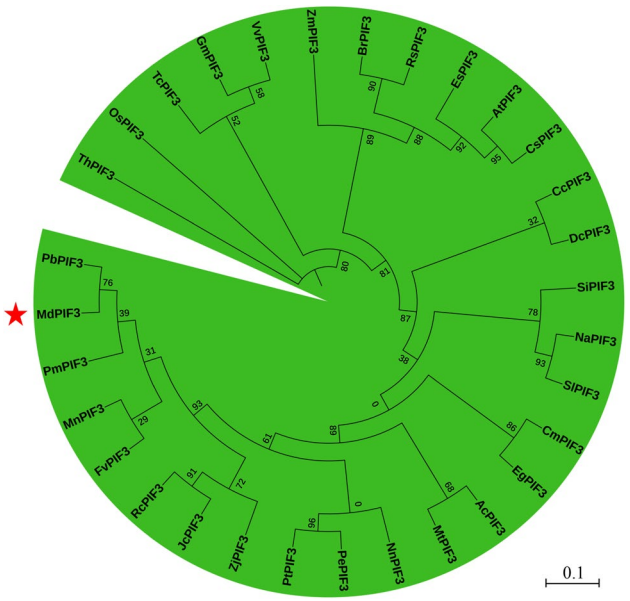

Fig. 1 Phylogenetic relationship analysis of MdPIF3 and 30 other plant PIF3 proteins obtained from the NCBI database. MdPIF3 is denoted by the asterisk. GmPIF3: *Glycine max*, XP_003553685.1; BrPIF3: *Brassica rapa*, XP_009148249.1; RsPIF3: *Raphanus sativus*, XP_018443444.1; ThPIF3: *Tarenaya hassleriana*, XP_010522859.1; PePIF3: *Populus euphratica*, XP_011039053.1; AtPIF3: *Arabidopsis thaliana*, AT1G09530; PbPIF3: *Pyrus bretschneideri*, XP_009366016.1; TcPIF3: *Theobroma cacao*, XP_017982981.1; MdPIF3: *Malus domestica*, LOC103450807; FvPIF3: *Fragaria vesca*, XP_011466209.1; ZjPIF3: *Ziziphus jujube*, XP_015874809.1; PmPIF3: *Prunus mume*, XP_008246329.1; CcPIF3: *Citrus clementina*, XP_006423962.1; EgPIF3: *Eucalyptus grandis*, XP_010070103.1; JcPIF3: *Jatropha curcas*, XP_012088811.1; PtPIF3: *Populus trichocarpa*, XP_024438997.1; CmPIF3: *Cucumis melo*, XP_008453043.1; RcPIF3: *Ricinus communis*, XP_015570501.1; VvPIF3: *Vitis vinifera*, XP_010652329.1; MnPIF3: *Morus notabilis*, XP_024020429.1; SiPIF3: *Sesamum indicum*, XP_020552005.1; NnPIF3: *Nelumbo nucifera*, XP_010240809.1; EsPIF3: *Eutrema salsugineum*, XP_006417565.1; MtPIF3: *Medicago truncatula*, XP_024638901.1; SlPIF3: *Solanum lycopersicum*, XP_025888784.1; AcPIF3: *Ananas comosus*, XP_020086869.1; NaPIF3: *Nicotiana attenuate*, XP_019255455.1; DcPIF3: *Daucus carota*, XP_017226282.1; CsPIF3: *Camelina sativa*, XP_010475789.1; OsPIF3: *Oryza sativa*, XP_015631806.1; ZmPIF3: *Zea mays*, PWZ58728.1

Analysis of MdPIF3 amino acid sequence and regulatory elements in the promoter of its encoding gene

Protein sequence analysis indicated that MdPIF3 contained a highly conserved bHLH domain, just as AtPIF3 (Fig. 2a). Through amino acid sequence alignment with other PIF3, we found that MdPIF3 proteins also contained conserved APB and APA motifs, which were also presented in other PIF proteins (Fig. 2b–c). Moreover, four conserved amino acid residues (ELxxxxGQ), that were reported to be key to the role of APB (Khanna et al. 2004), were found to be present in APB motif of MdPIF3. These results indicated that MdPIF3 had highly conserved functional domains similar to other PIF3 proteins, and may also possess similarly conservative functions.

Using the PlantCARE tool, the regulator elements in the promoter region of *MdPIF3* were predicted (Table 1). Among them, typical light-responsive element (G-box) were found. In addition, several stress-responsive elements were also identified, such as the ARE regulatory element that is essential for the anaerobic induction, the MBS regulatory element that is involved in drought-inducibility, and the wound-responsive regulator element (WUN-motif) (Table 1). What's more, some elements that were involved in response to plant hormones, including the ABRE abscisic acid responsive element and gibberellin responsive element (P-box), were also found in the promoter of *MdPIF3* (Table 1).

Transcriptional activation activity of MdPIF3

TFs always bind to the promoter region of their target genes to regulate gene expression, and transcription activity is one the common features of TFs. Here, full-length *MdPIF3* and several truncated fragments were inserted into the pGBKT7 vector. As shown in Fig. 3, all of the transformants grew normally on the SD/-Trp medium. After transferred to SD/-Trp/-His/-Ade medium with or without X-α-gal, the yeast strains containing full-length MdPIF3, the N-terminal fragments $MdPIF3^{1-515}$, and $MdPIF3^{1-443}$ grew normally and turned blue, while the other were unable to grow. These results indicate that MdPIF3 possessed the transcription activating activity and that the N-terminus without bHLH domain is responsible for autonomous activation in yeast cells.

MdPIF3 interacted with itself and other MdPIFs to form homo- and heterodimers

A Y2H assay was performed to investigate if MdPIF3 could form homodimers. The results showed that $MdPIF3^{444-708}$ could interact with the complete MdPIF3 amino acid sequence (Fig. 4a). Moreover, the $MdPIF3^{444-708}$ fragment also interacted with $MdPIF3^{444-708}$. However, $MdPIF3^{1-515}$, $MdPIF3^{1-443}$, $MdPIF^{444-515}$, and $MdPIF3^{516-708}$ did not interact with $MdPIF3^{444-708}$. These data indicate that MdPIF3 can interact with itself and form homodimers.

In addition, we also conducted a Y2H assay to determine whether MdPIF3 could form heterodimers with other MdPIF family members. MdPIF1 (MDP0000289642), MdPIF4 (MDP0000198404), and MdPIF8 (MDP0000439540) were inserted into pGAD vector as prey, whereas C-terminal fragment of $MdPIF3^{444-708}$ was fused to pGBD as bait. The results showed that $MdPIF3^{444-708}$ could interact with the MdPIF1, MdPIF4, and MdPIF8 (Fig. 4b). These data indicate that MdPIF3 can interact with other MdPIFs to form heterodimers, and the bHLH domain is responsible for dimer formation.

MdPIF3 is responsive to light and low-temperature

In this study, the expression of *MdPIF3* was examined using cDNA isolated from Royal Gala apple seedlings treated with light or cold conditions. These results indicated that the expression levels of *MdPIF3* was affected by light and cold treatments. Specifically, *MdPIF3* expression was downregulated in response to light and upregulated by cold treatment (Fig. 5a and d).

In addition, as shown in Fig. 5b–c, the MdPIF3-HIS fusion protein was unstable and rapidly degraded to a lower level within 4 h. Furthermore, the degradation process was significantly repressed when the samples were treated with MG132, confirming that the protein stability of MdPIF3 was regulated by the 26S proteasome. However, the degradation rate of MdPIF3-HIS protein accelerated in response to light treatment (Fig. 5b–c). Unlike the result of light treatment, the degradation rate of MdPIF3-HIS protein slowed down in response to cold stress (Fig. 5e–f). These results suggest that light and cold temperature are involved in the regulation of transcription and post-transcriptional levels of MdPIF3, suggesting that MdPIF3 may play an important role in light signal and cold stress response.

MdPIF3 negatively regulates cold tolerance

In normal conditions, the wild type (WT) callus grew similar with the transgenic lines, and they had similar fresh weight (Fig. 6a–b). When 10-day-old WT and transgenic apple callus were transferred and kept in 4 °C condition for 20 days, the growth of all the three kinds of apple callus were repressed. However, the growth of *MdPIF3-OE* apple callus was worse than that of WT, while the *MdPIF3-Anti* grew better than that of WT; and the fresh weight of the three under cold condition showed consistent results (Fig. 6a–b). The accumulation of ROS in *MdPIF3-OE* apple callus increased significantly, indicating that *MdPIF3-OE*

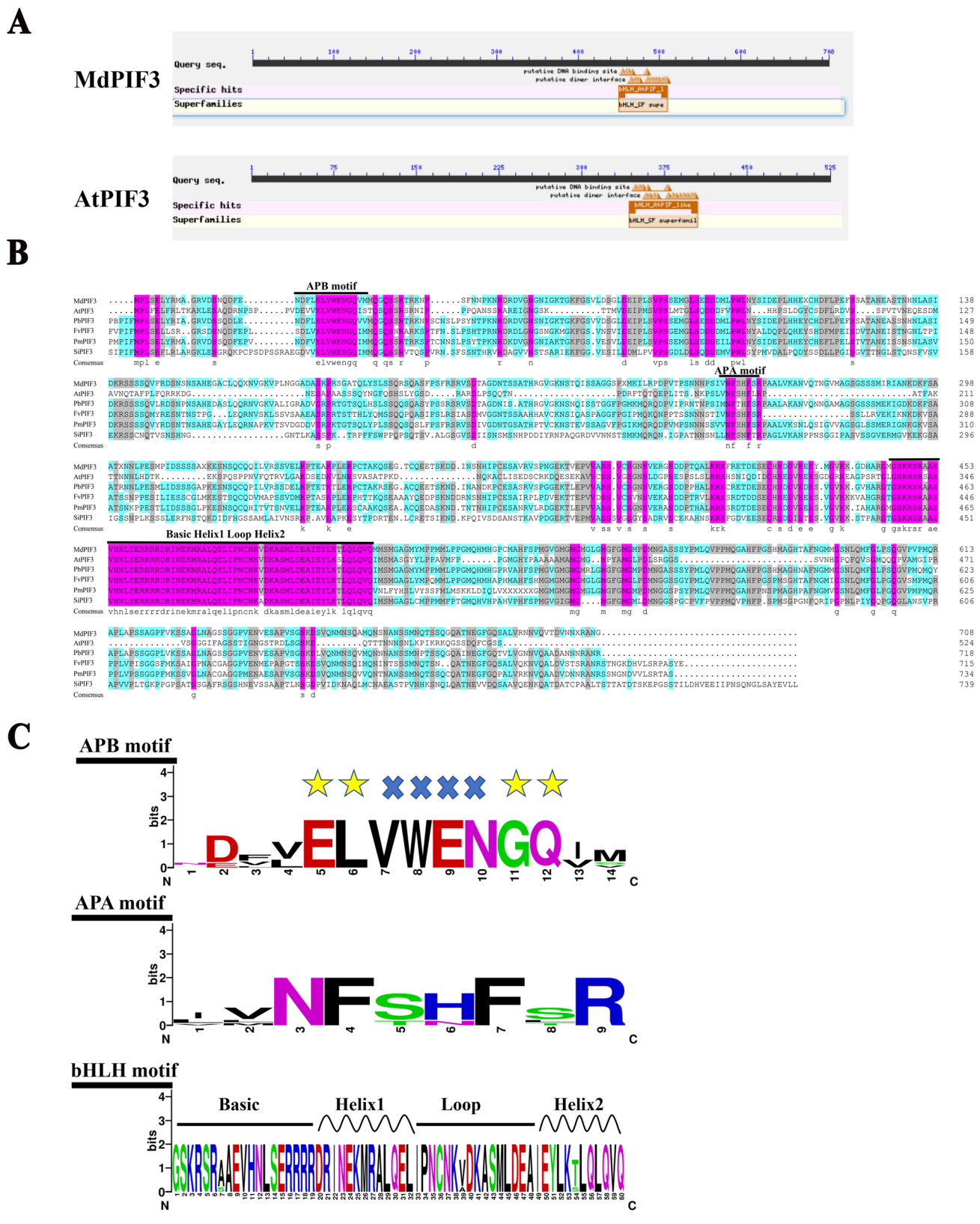

Fig. 2 The sequence analysis of MdPIF3 protein. **a** Conserved bHLH domain in MdPIF3 and AtPIF3 proteins. **b** Alignment of amino acid sequences of MdPIF3 and other PIF3 proteins. Locations of the three conserved motifs are marked with black lines. **c** Conservation of residues across MdPIF3 and other PIF3 proteins by the height of each letter. The most conserved amino acid residues in bHLH domain are indicated by yellow asterisks. The bit scores show the information for each conserved motif in the sequence

Table 1 *Cis*-element analysis in the *MdPIF3* promoter regions

Regulatory sequence	Sequence	Function of site	Location
ABBE	ACGTG	Cis-acting element involved in the ahscisic acid responsiveness	− 917
ARE	AAACCA	Cis-acting regulatory element essential for the anaerobic induction	+ 14
G-box	CACGTT	Cis-acting regulatory element involved in light responsiveness	+ 917
MBS	CAACTG	MYB binding site involved in drought-inducibility	+ 53
P-box	CCTTTTG	Gibberellin-responsive element	+ 932
TC-rich repeats	GTTTTCTTAC	Cis-acting element involved in defense and stress responsiveness	− 313
WUN-motif	AAATTTCCT	Wound-responsive element	− 1015

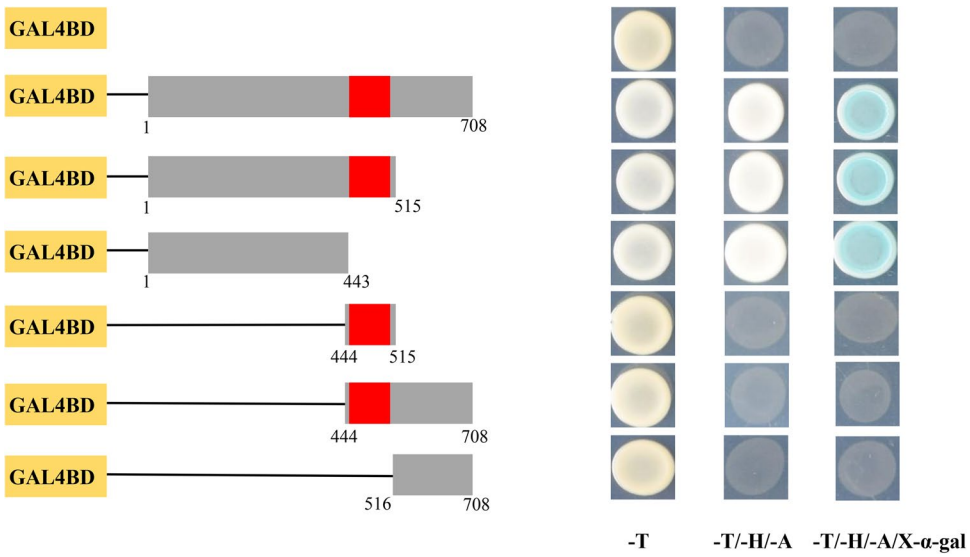

Fig. 3 Analysis of transcriptional activation activity of MdPIF3 in yeast. The bHLH domain is indicated in red. Yeast cells were screened on SD/-Trp, SD/-Trp/-His/-Ade, and SD/-Trp/-His/-Ade/X-α-gal medium

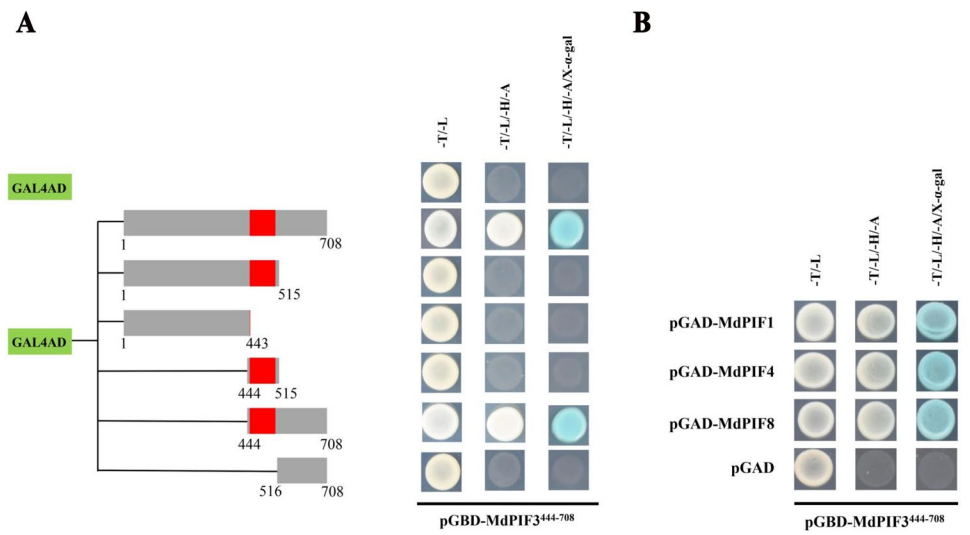

Fig. 4 Y2H assay to test the interaction among the MdPIF proteins. **a** MdPIF3 formed homodimers by interacting with itself. **b** MdPIF3 formed heterodimers by interacting with MdPIF1, MdPIF4, and MdPIF8. The bHLH domain is indicated in red. Yeast cells were screened on SD/-Trp/-Leu, SD/-Trp/-Leu/-His/-Ade, and SD/-Trp/-Leu/-His/-Ade/X-α-gal medium

apple callus was damaged heavier than the WT by cold stress (Fig. 6c). Consistent with the results in apple callus, ectopic expression of *MdPIF3* in Arabidopsis significantly inhibited the root length under cold condition (Fig. 6d–e). Furthermore, we tested the relative electrolyte leakage in wild type (col) and transgenic Arabidopsis. The relative electrolyte leakage of *MdPIF3* transgenic Arabidopsis lines were higher than col, indicating that the membrane lipids

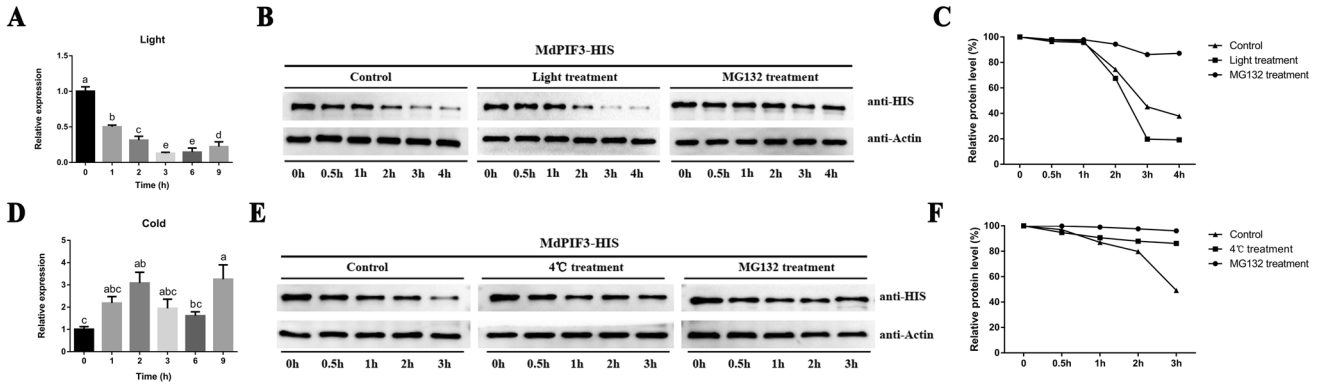

Fig. 5 Effects of light and cold treatments on the transcript level and protein stability of MdPIF3. **a** Expression analysis of *MdPIF3* gene in response to light. **b** Degradation of the MdPIF3-HIS protein and its stabilization by light or MG132. **c** Relative protein level of (**b**) is shown. **d** Expression analysis of *MdPIF3* gene in response to low temperature. **e** Degradation of the MdPIF3-HIS protein and its stabilization by low temperature or MG132. **f** Relative protein level of (**e**) is shown

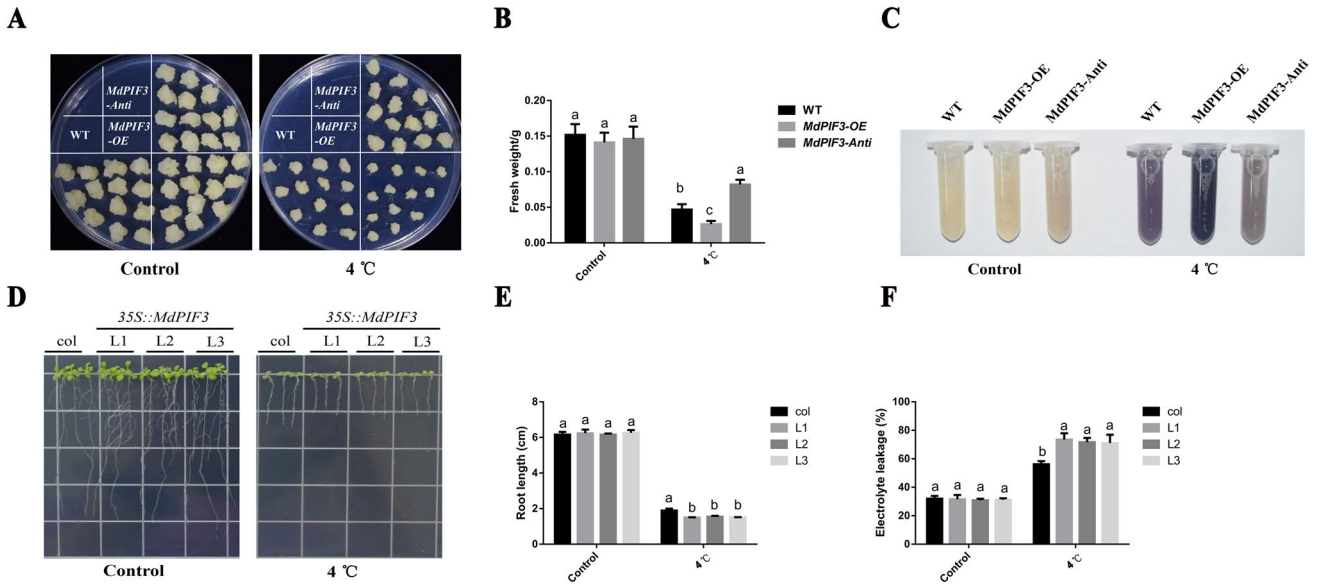

Fig. 6 Cold stress assays of *MdPIF3* transgenic lines. **a** Cold stress phenotypes of *MdPIF3* transgenic apple callus. The wild-type (WT) and transgenic apple callus (*MdPIF3-OE* and *MdPIF3-Anti*) were grown on medium at 24 °C for 10 days and then treated at 4 °C for another 20 days. **b** Fresh weight of wild-type and transgenic apple callus after cold treatment. **c** O_2^- accumulation in wild-type and transgenic apple callus by histochemical staining with NBT after cold treatment. **d** Freezing stress phenotypes of *MdPIF3*-overexpressing Arabidopsis under low temperature condition. The wild-type (Col) and transgenic Arabidopsis (*MdPIF3-L1*, *MdPIF3-L2*, and *MdPIF3-L3*) were grown on MS medium at 22 °C for 5 days and then treated at 4 °C for 2 weeks. **e** Root length and **f** electrolyte leakages of the col and *MdPIF3*-overexpressing Arabidopsis after cold treatment

showed a higher degree of damage in *MdPIF3* transgenic Arabidopsis lines (Fig. 6f). Thus, we conclude from these data that *MdPIF3* functions as a negative regulator in resistance to cold tolerance in both apple callus and Arabidopsis.

MdPIF3 positively regulates drought tolerance

When analyzing the regulatory elements, a potential drought responsive sequence (MBS regulatory element) was found to be present in the promoter of *MdPIF3* (Table 1 and Fig. S3). We then performed RT-qPCR to determine the expression pattern of *MdPIF3* under drought stress, and the result showed that drought significantly induced the expression of *MdPIF3* (Fig. 7a).

To further explore the function of *MdPIF3* in drought response, the WT and transgenic apple callus (*MdPIF3-OE* and *MdPIF3-Anti*) were treated with different concentrations of PEG 6000 to mimic the drought stress. No clear

differences were observed between WT and transgenic callus under normal condition, and they all had similar fresh weight (Fig. 7b–c). However, when treated with different concentration of PEG 6000, *MdPIF3-OE* apple callus grew better but *MdPIF3-Anti* apple callus grew worse compared to that of WT (Fig. 7b). The fresh weight was consistent with the phenotype (Fig. 7c). This suggested that *MdPIF3* played a positive role in resistance to drought stress. Malondialdehyde (MDA) is the major product of lipid peroxidation and its content has been developed to be an important tool to measure the degree of damage caused by stress (Dey et al. 2019; Yamane et al. 2009). Here, the MDA content in *MdPIF3-Anti* apple callus was higher, while that was lower in *MdPIF3-OE* apple callus compared to that of WT (Fig. 7d), which further indicated the positive role of *MdPIF3* in drought resistance. We next performed the drought-resistant experiments in Arabidopsis. The three transgenic lines of Arabidopsis grew similar with the WT before drought treatment, however, they showed better growth than that of WT under water shortage condition (Fig. 7e), suggesting ectopic expression of *MdPIF3* enhanced the drought tolerance in Arabidopsis. The chlorophyll content was also consistent with the phenotype (Fig. 7f). Thus, these data indicate that *MdPIF3* enhanced drought tolerance in both apple callus and Arabidopsis.

Discussion

With the development of genome sequencing technology, critical TF families have been identified in more and more plant species. Among them, PIF TFs have been isolated and investigated in many species, such as *Oryza sativa* (Nakamura et al. 2007), *Solanum lycopersicum* (Rosado et al. 2016), *Arabidopsis thaliana* (Lee and Choi 2017; Pham et al. 2018), *Moss Physcomitrella patens* (Possart et al. 2017), and *Zea mays L* (Gao et al. 2019; Wu et al. 2019). However, PIFs have not been studied in depth in woody plant apple, except for the report of *MdPIF1* on light response (Zhou et al. 2017). Here, we identified *MdPIF3*, the apple homolog of *AtPIF3*, and revealed its role in plant cold and drought tolerance.

As predicted, MdPIF3 protein is highly conserved with PIF3 from other reported species. The MdPIF3 protein has a typical bHLH domain (Fig. 2a), a conserved APB motif (Fig. 2b–c), as well as APA motif (Fig. 2b–c). Among them, the bHLH domain promoted the formation of homo- and/or heterodimers, which is necessary for PIFs to perform multiple functions (Toledo-Ortiz et al. 2003). Previous reports showed that PIF1, PIF3, and PIF4 could form homodimers with themselves (Leivar et al. 2008). PIF3 forms a heterodimer with PIF1 or PIF4, and then bind the G-box element in the promoter of the target genes to regulate their transcription (Bu et al. 2011; Hao et al. 2012; Hornitschek et al.

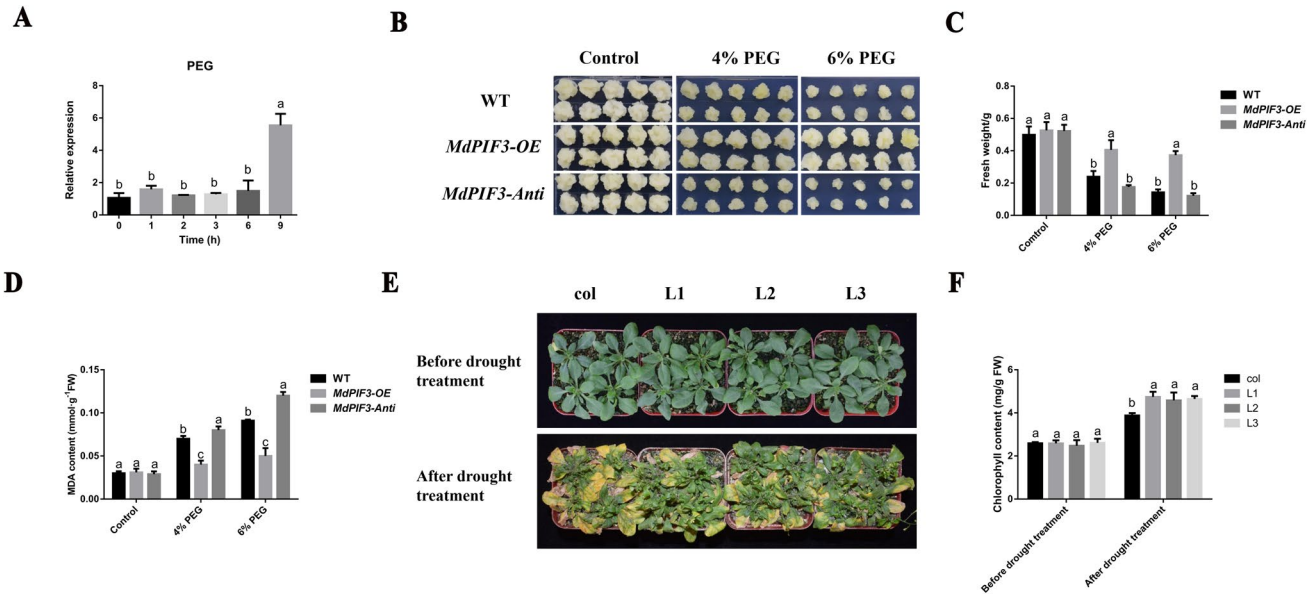

Fig. 7 Drought tolerance assays of *MdPIF3* transgenic lines. **a** The expression analysis of *MdPIF3* under PEG treatment. **b** Drought stress phenotypes of *MdPIF3* transgenic apple callus containing 0, 4% and 6% PEG6000. **c** Fresh weight of wild-type and transgenic apple callus after PEG treatment. **d** Determination of MDA content in wild- type and transgenic apple callus. **e** Drought stress phenotypes of *MdPIF3*-overexpressing Arabidopsis seedlings in the absence of water. **f** Determination of chlorophyll content of the col and *MdPIF3*-overexpressing Arabidopsis seedlings

2009). Moreover, PIFs could form heterodimers with atypical PIF proteins such as LONG HYPOCOTYL IN FAR-RED1 (HFR1) and participate in far-red and blue signal transductions (Shi et al. 2013; Shin et al. 2009). Therefore, we designed Y2H assays to explore whether MdPIF3 interacts with related proteins. Due to transcriptional autonomous activation activity of MdPIF3 (Fig. 3), the C-terminal fragment of MdPIF3$^{444-708}$ containing the bHLH domain was selected to perform the experiment. The results showed that MdPIF3 could homodimerize with itself and heterodimerize with MdPIF1 (the homolog of AtPIF1), MdPIF4 (the homolog of AtPIF4), and MdPIF8 (the homolog of AtPIF8) (Fig. 4). The formation of these homodimer or heterodimer types enriched signal transduction pathways and regulatory networks, indicating that PIFs also have similar working mode in apple. In addition to bHLH domain, MdPIF3 also contained conserved APB and APA motifs, which were necessary for the binding to phyB and phyA, respectively, and participate in the phy-signaling pathway in Arabidopsis (Khanna et al. 2004; Leivar and Quail 2011; Ni et al. 1998; Shen et al. 2008). The presence of APB and APA motifs in MdPIF3 protein implies its potential binding ability with phyA and phyB in apple, and further experiments are needed to determine its interactions.

In recent years, more and more researches have focused on the post-transcriptional regulation of PIFs. The phyB/phyA-PIF interaction mentioned above has been proved to promote the phosphorylation and degradation of PIF proteins (Al-Sady et al. 2006; Leivar and Quail 2011; Ni et al. 2013; Park et al. 2004; Shen et al. 2008). In addition, it has been identified that other factors are involved in the ubiquitination and degradation of PIFs, such as light-response bric-a-brack/tramtrack/broad (LRB), DELLAs, BRASSINOSTEROID-INSENSITIVE 2 (BIN2), and CONSTITUTIVELY PHOTOMORPHOGENIC 1 (COP1) (Li et al. 2016; Ling et al. 2017; Ni et al. 2014; Oh et al. 2020). To investigate the post-translational regulation of MdPIF3 protein, we performed protein degradation assay in vitro. The results revealed that, like PIF3, the MdPIF3 protein was unstable and degraded through the 26S proteasome system (Fig. 5b–c and e–f). Moreover, the degradation rate accelerates when exposed to light, similar to previous reports of PIF3 in Arabidopsis (Al-Sady et al. 2006; Shen et al. 2008). Since MdPIF3 contains APB motif, we speculate that the photo-activated apple phyB may interact with MdPIF3 to degrade it. When exposed to low temperature, the degradation rate of MdPIF3 protein slows down (Fig. 5e–f). Previous studies have showed that EIN3-BINDING F BOX PROTEIN 1 (EBF1) and EBF2, two F-box proteins, mediate PIF3's ubiquitination degradation via 26S proteasome pathway. At the same time, cold stress stabilizes the protein level of PIF3 by promoting the degradation of EBFs (Jiang et al. 2017). A recent study showed that C-REPEAT BINDING FACTOR 1 (CBF1) interact with PIF3 and stabilize PIF3 and phyB protein under cold stress. Intriguingly, PIF1, PIF4, and PIF5 do not interact with CBFs, and their protein stability were down-regulated under cold stress (Jiang et al. 2020). However, whether the above-mentioned molecular mechanisms regulate the protein stability of MdPIF3 under cold stress remains unknown, and further research is needed.

Previous studies have indicated that *PIF3* plays an important role in response to multiple abiotic stresses, such as drought, salt, and cold (Gao et al. 2015; Jiang et al. 2020; Jiang et al. 2017). Regulatory elements of stress response also are present within the promoter of *MdPIF3*, indicating that it might participate in the abiotic stress response (Table 1). qRT-PCR analysis showed that the expression of *MdPIF3* was induced by cold and drought to varying degrees (Fig. 5d and Fig. 7a). To further explore the function of *MdPIF3* in apple, we obtained transgenic apple callus and Arabidopsis of *MdPIF3*. Stress tolerance assays revealed that *MdPIF3* positively regulates plant drought resistance but negatively regulates plant cold resistance (Figs. 6 and 7). These data indicated that *MdPIF3* also plays a vital role in stress tolerance and has different regulatory functions in response to different stresses.

In summary, our work identified a new stress-responsive bHLH factor in apple, *MdPIF3*, which positively regulates the drought resistance of plants and negatively regulates the cold resistance of plants. This provides a new gene reserve for genetic engineering technology to improve apple's adaptability in different environments in the future.

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Author contribution Y-JH, Z-LZ, and P-FZ conceived and designed the experiments; P-FZ and Z-LZ performed most of the experiments; Y-YY, SZ and C-XY analyzed the data; P-FZ, Z-LZ and Y-JH wrote the paper.

Compliance with ethical standards

Conflict of interest No conflict of interest declared.

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