

An atypical R2R3 MYB transcription factor increases cold hardiness by CBF-dependent and CBF-independent pathways in apple

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Summary

- Apple (*Malus × domestica*) trees are vulnerable to freezing temperatures. However, there has been only limited success in developing cold-hardy cultivars. This lack of progress is due at least partly to lack of understanding of the molecular mechanisms of freezing tolerance in apple.
- In this study, we evaluated the potential roles for two R2R3 MYB transcription factors (TFs), MYB88 and the paralogous FLP (MYB124), in cold stress in apple and *Arabidopsis*. We found that MYB88 and MYB124 positively regulate freezing tolerance and cold-responsive gene expression in both apple and *Arabidopsis*.
- Chromatin-Immunoprecipitation-qPCR and electrophoretic mobility shift assays showed that MdMYB88/MdMYB124 act as direct regulators of the *COLD SHOCK DOMAIN PROTEIN 3* (*MdCSP3*) and *CIRCADIAN CLOCK ASSOCIATED 1* (*MdCCA1*) genes. Dual luciferase reporter assay indicated that MdCCA1 but not MdCSP3 activated the expression of *MdCBF3* under cold stress. Moreover, MdMYB88 and MdMYB124 promoted anthocyanin accumulation and H₂O₂ detoxification in response to cold.
- Taken together, our results suggest that MdMYB88 and MdMYB124 positively regulate cold hardiness and cold-responsive gene expression under cold stress by C-REPEAT BINDING FACTOR (CBF)-dependent and CBF-independent pathways.

Introduction

Cold stress, including chilling (0–15°C) and freezing (<0°C) temperatures, is one of the adverse environmental conditions plants have to cope with given their sessile nature (Zhu *et al.*, 2007; Shi *et al.*, 2015). Cold stress not only influences the geographical distribution of many important crops, such as apple (*Malus × domestica* Borkh), but also negatively impacts crop productivity and quality. Many temperate plants have the ability to acquire increased freezing tolerance through prior exposure to low, nonfreezing temperatures, a process called cold acclimation (Guy, 1990; Thomashow, 1999; Ruelland *et al.*, 2009; Zhu, 2016). However, most tropical and subtropical crops, such as rice, tomato, maize and soybean, are not capable of cold acclimation and are sensitive to chilling stress.

Apple is one of the most important fruit crops world-wide. Apple cultivation is limited to temperate zones, where winter provides sufficient chilling temperatures for release from

vegetative dormancy, but where temperatures generally do not fall below –30°C. Apple is vulnerable to freezing temperatures, especially during the spring period of floral development before bloom (Feng *et al.*, 2012). Breeding for increased freezing tolerance is important, especially given the prospect for rapid climate change. However, due to the long juvenile period of apple and its high degree of genetic heterozygosity, only limited progress has been made to develop cold-hardy cultivars through traditional approaches. Several studies have shown that biotechnology is a feasible approach to improve cold tolerance in woody perennial plants (Harfouche *et al.*, 2011, 2014; Osakabe *et al.*, 2011; Polle *et al.*, 2013; Strauss *et al.*, 2017). However, genetic engineering requires a thorough understanding of the molecular mechanisms of the response to cold, including cold perception, signal transduction and gene expression.

Numerous studies have revealed that cold exposure results in a variety of alterations in physiology and gene expression patterns (Guy *et al.*, 1994; Thomashow, 1999). Many genes have been

identified that are activated in response to cold, including: those encoding enzymes involved in the metabolism of carbohydrates, lipids, phenylpropanoids and antioxidants; molecular chaperones; antifreeze proteins; signal transduction and regulatory proteins; and many other proteins of unknown function (Guy *et al.*, 1994; Thomashow, 1999).

Studies of cold-regulated gene expression in *Arabidopsis* (*A. thaliana*) led to the identification of the CBF/DREB1 class of transcription factors (TFs) (Guy *et al.*, 1994; Thomashow, 2001, 2010). Cold stress rapidly induces expression of CBF TFs, which then bind to the promoter regions of the *Cold-Regulated* (*COR*) genes to activate their expression. The promoters of *COR* genes contain one or more copies of the so-called dehydration-responsive element (DRE)/C-repeat (CRT) *cis*-element (Yamaguchishinozaki & Shinozaki, 1994; Stockinger *et al.*, 1997). There are three CBF genes (*CBF1/DREB1b*, *CBF2/DREB1c* and *CBF3/DREB1a*) in *Arabidopsis*. CBF expression is controlled at the transcriptional level by several TFs, including INDUCER OF CBF EXPRESSION 1 (ICE1) (Chinnusamy *et al.*, 2003), MYB15 (Agarwal *et al.*, 2006), ICE2 (Fursova *et al.*, 2009), CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3) (Doherty *et al.*, 2009), PSEUDO-RESPONSE REGULATORs (PRRs) (Nakamichi *et al.*, 2009), CIRCADIAN CLOCKASSOCIATED 1 (CCA1) (Dong *et al.*, 2011), PHYTOCHROME INTERACTING FACTOR 4 (PIF4), PIF7 (Lee & Thomashow, 2012), BZR1 (Li *et al.*, 2017b) and ETHYLENE-INSENSITIVE 3 (EIN3) (Shi *et al.*, 2012). In addition, CBF genes are regulated post-transcriptionally by FIERY2 (FRY2) (Chen *et al.*, 2013), STABILIZED 1 (STA1) (Lee, 2006), LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4 (LOS4) (Gong *et al.*, 2005), and post-translationally by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1 (HOS1) (Dong *et al.*, 2006), SAP and Miz 1 (SIZ1) (Miura *et al.*, 2007). In addition to the CBF-dependent pathway, the CBF-independent pathway was also required for plant cold response. Genes involved in the CBF-independent pathway include HOS9 (Zhu *et al.*, 2004), SFR6 (Knight *et al.*, 1999), WRKY6 (Li *et al.*, 2017b), CSP3 (Kim *et al.*, 2009), HAP5A (Shi *et al.*, 2014), etc.

Adverse environmental factors such as cold lead to the overproduction of reactive oxygen species (ROS), which disrupt the electron transport chain and are toxic to cellular processes (Gill & Tuteja, 2010). Plants have developed mechanisms for eliminating ROS, including the production of enzymatic and nonenzymatic antioxidants. Major enzymatic antioxidants include peroxidase (POD), superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) (Gill & Tuteja, 2010). Plants overexpressing *AtPrx22*, *AtPrx39* or *AtPrx69*, encoding class III plant peroxidases, showed increased tolerance to cold (Kim *et al.*, 2012). Nonenzymatic antioxidants include ascorbate, glutathione, carotenoids, tocopherols and flavonoids. Anthocyanins, a main class of flavonoids, serve as protectants against biotic and abiotic stress, in addition to their roles in plant ecology and development (Van den Ende & El-Esawe, 2014). Two *Arabidopsis* glycosyltransferases, UGT79B2 and UGT79B3, responsible for production of anthocyanin, are directly regulated

by CBF1 and play critical roles in plant cold tolerance (Li *et al.*, 2017a).

Since the identification of the first plant MYB TF two decades ago, tremendous amounts of data have accumulated on the diverse functions of MYB TFs in plant species, including *Arabidopsis*, maize, rice (*Oryza sativa*), grapevine (*Vitis vinifera*), poplar (*Populus tremuloides*) and apple. MYB TFs have been found to participate in plant metabolism, development, and response to biotic and abiotic stresses (Dubos *et al.*, 2010). So far, among the 229 MYB TFs identified in the apple genome, only two, MdSIMYB1 and MdoMYB121, have been characterized for their role in cold stress tolerance (Cao *et al.*, 2013; Wang *et al.*, 2014). *Arabidopsis* FOUR LIPS (FLP) and MYB88, two atypical two-repeat R2R3-MYB proteins, are not able to bind to the canonical R2R3 MYB *cis* element ATAACGG or CC [T/A] ACC (Xie *et al.*, 2010a). FLP and MYB88 have been shown to regulate guard mother cell proliferation (Lai, 2005; Xie *et al.*, 2010a), PIN-formed 3 (PIN3)-controlled lateral root development (Chen *et al.*, 2015), female reproductive development (Makkena *et al.*, 2012) and root response to gravity (Wang *et al.*, 2015). Xie *et al.* (2010b) found that *myb88flp-1* double mutant plants were more susceptible to drought and salt stress, indicating that FLP and MYB88 promote tolerance to drought and salinity. The potential involvement of MYB88 and FLP in cold stress response in *Arabidopsis* has not been addressed. There are two genes homologous to FLP and MYB88 in apple genome, MdMYB88 and MdMYB124. Cao *et al.* (2013) performed gene expression analysis of 18 MYBs in apple and found that *MdMYB124* (*MdoMYB148*) expression pattern is slightly upregulated by cold. However, the potential roles for both genes in response to abiotic stress including freezing has not been evaluated. In this study, we show that FLP (MYB124) and MYB88 positively regulate cold stress tolerance and cold-responsive gene expression in apple and *Arabidopsis*. ChIP-seq revealed that MdMYB88 and MdMYB124 target the *MdCSP3* and *MdCCA1* genes respectively, suggesting that MdMYB88 and MdMYB124 modulate cold stress tolerance and cold-responsive gene expression by CBF-dependent and CBF-independent pathways.

Materials and Methods

Plant materials and growth conditions

SALK_068691 (*myb88*), CS65766 (*flp-1*) and SALK_65770 (*myb88flp-1*) were obtained from the Arabidopsis Biological Resource Center (ABRC). Homozygous single and double mutants were verified by PCR. Seeds were surface-sterilized with 70% ethanol followed by 4% (v/v) hypochlorite, cold-treated for 2 d at 4°C for vernalization, and germinated on Murashige and Skoog (MS) medium agar plates (4.43 g l⁻¹ MS salts, 20 g l⁻¹ sucrose, and 6 g l⁻¹ agar, pH 5.7). Plants were grown under long-day photoperiods (16 h : 8 h, light : dark) at 21°C. Seedlings were transplanted to soil 2 wk after germination.

The leaves of apple 'Golden delicious' (*Malus × domestica*) were used for gene cloning. A line with high regeneration capacity isolated from 'Royal Gala' (*Malus × domestica*) named GL-3

(Dai *et al.*, 2013) was used for genetic transformation. GL-3 tissue-cultured plants were subcultured every 4 wk. They were grown on MS medium (4.43 g l⁻¹ MS salts, 30 g l⁻¹ sucrose, 0.2 mg l⁻¹ 6-BA, 0.2 mg l⁻¹ IAA, and 7.5 g l⁻¹ agar, pH 5.8) under long-day conditions (14 h : 10 h, light : dark cycle) at 25°C.

Measurement of chilling and freezing tolerance of *Arabidopsis* seedlings

Chilling stress tolerance was performed as described previously (Guan *et al.*, 2013a).

Electrolyte leakage assays were used to evaluate freezing tolerance. Three-week-old *Arabidopsis* seedlings grown in soil under a long-day photoperiod (16 h : 8 h, light : dark) at 21°C were moved to 4°C for 1 wk for cold acclimation. Fully developed rosette leaves were used for electrolyte leakage measurements as described (Sukumaran & Weiser, 1972; Ristic & Ashworth, 1993; Zhu *et al.*, 2004). Rosette leaves were placed in a low-temperature aqua bath cycle instrument (Thermo Fisher PC200-A40) with the following freezing temperature regime: from 0 to -2°C in 1 h, then hold at -2°C for 1 h (to initiate nucleation); then successive 2°C decreases at 1-h intervals, then hold at -4°C for 1 h; then successive 2°C decreases at 1-h intervals, then hold at -6°C for 1 h. For each temperature (0, -4°C, -6°C), six or eight biological replicates were used. Conductivity was measured 12 h later and this was followed by another measurement after the leaves were autoclaved. Electrolyte leakage was calculated as the percentage of the conductivity before autoclaving relative to that after autoclaving (Agarwal *et al.*, 2006). Whole-plant freezing tests were as described (Guan *et al.*, 2013a) with modifications. Wild-type (WT) and *myb88flp-1* plants were grown in soil under a long-day photoperiod (16 h : 8 h, light : dark) in a growth chamber for 4 wk at 22°C and then at 4°C for 1 wk for cold acclimation. The plants were then treated with freezing stress with a temperature of -12°C for 1 h or 2 h, followed by 22°C under a long-day photoperiod (16 h : 8 h, light : dark) for additional 1 wk.

Measurement of chilling and freezing tolerance of apple plants

For chilling tolerance assays, tissue-cultured WT and transgenic plants were grown side-by-side on MS medium containing agar and then placed in a growth chamber at 4°C under a long-day photoperiod (16 h : 8 h, light : dark) for 1 month. Plants were then recovered at a 22°C growth chamber under the same photoperiod for additional 20 d. Reduction in plant height was used as an indicator of sensitivity to chilling stress.

In order to evaluate freezing tolerance of apple plants, we used electrolyte leakage assays similar to the method used in *Arabidopsis* plants. GL-3 and transgenic apples after rooting were transplanted to soil and grown for 4 wk at 25°C under a long-day photoperiod (14 h : 10 h, light : dark), then transplanted into plastic pots (20 cm × 60 cm) and placed outdoors for additional 7 months under natural weather conditions at the experimental

field of Northwest A & F University, Yangling (34°20'N, 108°24'E), China. Standard horticultural practices were followed for disease and pest control. For cold acclimation, plants were subsequently grown for 1 wk at 4°C, also under a long-day photoperiod (14 h : 10 h, light : dark cycle). Fully expanded leaves were punched into leaf discs with diameter of 50 mm. Two to three leaf discs were placed in a test tube containing 300 µl of deionized H₂O, and the tubes were rapidly placed on ice. The tubes were then placed into the low-temperature aqua bath cycle instrument (Thermo Fisher PC200-A40) with the following freezing temperature regime: from 0 to -2°C in 1 h, then hold at -2°C for 1 h (to initiate nucleation); then successive 2°C decreases at 1-h intervals, then hold at -4°C for 1 h; then successive 2°C decreases at 1-h intervals, then hold at -6°C for 1 h. For each temperature (0, -4°C, -6°C), eight biological replicates were used. The measurement of electrolyte leakage was the same as the methods used for *Arabidopsis*. For the whole-plant freezing assay, tissue-cultured GL-3 and transgenic apple plants were grown in soil for additional 2 months under a long-day photoperiod (16 h : 8 h, light : dark) in a growth chamber and then at 4°C for 1 wk for cold acclimation. Plants were then treated at -10°C for 20 min, 25 min, or 30 min, followed by 22°C under a long-day photoperiod (16 h : 8 h, light : dark) for additional 3 wk.

RNA extraction and qRT-PCR analysis

For RNA analysis of *Arabidopsis* seedlings, 2-wk-old seedlings of WT and transgenic plants grown on MS medium were used. For expression of *FLP* and *MYB88*, 14-d-old WT seedlings grown on MS agar plates were exposed to 4°C for 0, 3, 6, 12 or 24 h. For transcript levels of endogenous cold-responsive genes of *CBF1*, *CBF2*, *CBF3*, *RD29A*, *CCA1*, *LHY*, *ZAT12* and *RCF1*, 14-d-old WT and *myb88flp-1* seedlings were grown side by side in MS agar plates and exposed to 4°C for 0, 3 or 24 h. RNA extraction from *Arabidopsis* plants was according to manufacturer's protocol of TRIzol[®] Reagent (#15596018; Invitrogen, Carlsbad, CA, USA) and the extracted RNA was treated with DNase I (#EN0521; Thermo Scientific, Waltham, MA, USA) to remove potential genomic DNA contaminations.

Apple plants grown in soil under long-day conditions (14 h : 10 h, light : dark cycle) were used for RNA isolation. Total RNA of apple leaves was extracted based on the CTAB method (Chang *et al.*, 1993). RNase-free DNase I (Fermentas) was used to remove residual DNA.

The total RNA extracted from *Arabidopsis* plants or apple leaves was subjected to reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (K1622; Thermo Fisher Scientific, Waltham, MA, USA). Quantitative reverse transcription PCR (qRT-PCR) was carried out on a Bio-Rad CFX96[™] instrument (Bio-Rad), using a GoTaq[®] Probe qPCR Master Mix (Promega) according to the user manual. The malate dehydrogenase (*MdMDH*) gene from apple was used as the reference gene in apple, whereas Tubulin8 (*TUB8*) was used as the reference gene in *Arabidopsis*. The specific primer sequences used for expression analysis are shown in Supporting Information

Table S1. The experiments were performed using three biological samples with three technical repeats for each RNA extract. Expression data were presented as relative units after normalization to the reference gene as the internal control, per the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Generation of transgenic *Arabidopsis* and apple plants

In order to generate transgenic *Arabidopsis* plants, pEarleyGate101 vector was used for overexpression of *MYB88* and *FLP* and complementation of *MdMYB88* to *myb88flp-1* double mutants. *MYB88* and *FLP* were cloned into pEarleyGate301 for complementing the *myb88 flp-1* double mutants using their own promoter. The resulting constructs were transferred into *Agrobacterium tumefaciens* (strain GV3101), and *Arabidopsis* plants were transformed by the floral dip method (Clough & Bent, 1998).

In order to generate transgenic apple plants, coding regions (CDS) of *MdMYB88* and *MdMYB124* were cloned and introduced into the pGWB414 vector based on gateway recombination technology (Invitrogen). The vector pHellsgate2 was used as the RNA silencing vector for *MdMYB88/124*. *Agrobacterium*-mediated transformation of apple was carried out as described, using GL-3 as the genetic background (Holefors *et al.*, 1998; Dai *et al.*, 2013) and strain EHA105. The primers used for constructing these vectors were shown in Table S1.

Subcellular localization assays in *Nicotiana benthamiana* leaves

For subcellular localization, the coding regions of *MdMYB88* and *MdMYB124* were amplified by PCR and the PCR products were cloned into the pEarleyGate104 vector. The resulting constructs were transformed into *Agrobacterium* strain C58C1 and co-infiltrated with 35S:p19 (p19 is an RNA silencing repressor protein from Tomato bushy stunt virus (Voinnet *et al.*, 2003)) in *Agrobacterium* strain C58C1 into 5-wk-old leaves of tobacco (*Nicotiana tabacum*) plants. The infiltrated tobacco plants were grown for an additional 3 d in a growth chamber under a 16 h : 8 h, light : dark photoperiod at 21°C. DAPI (4,6-diamidino-2-phenylindole dihydrochloride) was used to identify the nucleus. Yellow fluorescent protein (YFP) signals in transformed tobacco leaves were then detected with a Nikon A1R/A1 confocal microscope (Nikon, Tokyo, Japan).

Chromatin immunoprecipitation followed by Illumina sequencing (ChIP-seq) and qPCR analysis

ChIP-seq assays were performed according to a method described previously (Bowler *et al.*, 2004). Leaves obtained from tissue-cultured apple plants grown for 4 wk at 25°C and then 8 h at 4°C were cross-linked for 15 min in 1% formaldehyde. Chromatin was isolated and sonicated to shear DNA into 0.3–0.7-kb fragments. After centrifugation at 4°C for 5 min at 17 949 g, chromatin was retained in the upper aqueous phase. ChIP-grade protein A/G agarose beads (Thermo Fisher #26159) were used to

pre-clear the chromatin supernatant. The sample was split into two equal aliquots, and anti-MdMYB88/MdMYB124 antibody was added to one aliquot and incubated overnight at 4°C with gentle agitation. The remaining aliquot was used as the no-antibody control. The next day, immune complexes were collected with protein A/G agarose beads pre-equilibrated in ChIP Dilution Buffer for 1 h at 4°C with gentle agitation. Pelleted beads were centrifuged at 514 g and then washed five times. Immune complexes were eluted and resuspended twice. Crosslinking was reversed by overnight incubation at 65°C in 5 M NaCl. Protein was digested with 0.5 M EDTA, 1 M Tris-HCl (pH6.5) and 10 mg ml⁻¹ proteinase K for 1 h at 45°C. The DNA fragments were recovered and sequenced using Illumina TruSeq platform and reagents.

Quality-filtered reads were mapped to an apple (*M. × domestica* cv Golden Delicious) reference genome sequence from NCBI (Velasco *et al.*, 2010) by BOWTIE 2 (Langmead & Salzberg, 2012). The resulting data were analyzed using HOMER (Hypergeometric Optimization of Motif EnRichment), a software suite for ChIP-seq analysis (Heinz *et al.*, 2010) to find potential targets of MdMYB88/MdMYB124. Briefly, a tag directory was created from high-throughput sequencing alignment files, then peak calling and transcript identification analysis were performed to find peaks. Finally, peaks with nearby genes were annotated. Among the three types of peaks (promoter-TSS, exon, TTS), only promoter-TSS peaks were selected for targets and motif finding. Promoter sequences were retrieved from NCBI based on the position of peaks, and MEME-ChIP (Machanick & Bailey, 2011) was used for identifying potential binding motifs of MdMYB88/MdMYB124.

The recovered DNA was also used for ChIP-qPCR analysis to identify enriched DNA fragments. The primers were shown in Table S1.

Electrophoretic mobility shift assay (EMSA)

The coding region of MdMYB88 was amplified by PCR and cloned into the pET-32(+) vector. The resulting construct (MdMYB88-His) was transformed into *Escherichia coli* strain BL21 (DE3). Production and purification of MdMYB88-His fusion protein was performed using Ni-NTA agarose (Millipore). EMSA assays were performed using the LightShift Chemiluminescent EMSA Kit (#89880; Thermo Scientific) according to the manufacturer's protocol. Complementary pairs of 5'-end biotin-labeled and unlabeled oligonucleotides containing 4× (for MdCCA1 probe) or 5× (for MdCSP3 probe) tandem repeats of MdMYB88/MdMYB124 recognition sites (Table S1) were annealed and used as probes for the EMSA studies. The protein-DNA samples were then separated on 6% polyacrylamide gels and signal was captured with a ChemiDoc™ XRS+ (Bio-Rad).

Dual luciferase reporter assays

The *MdCSP3* promoter fragment containing the *cis*-element of AACCG recognized by MdMYB88 was amplified by PCR using genomic DNA extracted from apple (*M. × domestica* cv 'Golden

Delicious'). The PCR product was cloned into the transient expression vector **pGreenII 0800-LUC to serve as a reporter plasmid (MdCSP3: LUC)**. The coding regions of *MdMYB88* were amplified by PCR and cloned into the transient expression vector **pGreenII 62-SK to serve as effector plasmids** (pGreenII 62Sk-*MdMYB88*, abbreviated as MdMYB88). The resulting plasmids (MdCSP3: LUC, *MdMYB88*) and the pSoup helper plasmid (Hellens *et al.*, 2000) were co-transformed into ***Agrobacterium* strain GV3101**. Leaves from 4-wk-old tobacco plants were co-infiltrated with *Agrobacterium* (strain GV3101) harboring the above plasmids in the following combination and ratio: MdCSP3:LUC+MdMYB88 (1:1). The infiltrated tobacco plants were grown for an additional 3 d in a growth chamber under a 16 h:8 h, light:dark photoperiod at 21°C. Proteins were extracted with **Passive Lysis Buffer** (Promega). The activities of firefly luciferase under the control of the *MdCSP3* promoter (MdCSP3: LUC) and Renilla luciferase under the control of the 35S promoter (35S: LUC; both the MdCSP3: LUC and 35S: LUC transgenes are present on the MdCSP3: LUC reporter plasmid, and 35S: LUC served as an internal control) were measured using the dual luciferase assay reagents (Promega) as described (Hellens *et al.*, 2005). For cold treatment, tobacco plants were treated at 4°C for 3 h before protein extraction. Ten independent biological samples were used.

We used the same strategy and stain for activation of *MdCBF3* by MdCCA1, and activation of *MdCBFs* by MdCSP3.

Transcriptional activity assay of MdMYB88

A yeast GAL4-based two-hybrid (Y2H) system was used for transcriptional activity assays (Clontech, Mountain View, CA, USA). Full-length MdMYB88 (MdMYB88-FL) or deletions was fused with GAL4-BD and co-transformed with GAL4-AD into yeast strain Y2H. Transcriptional activity was determined by positive colonies on SD medium without tryptophan, adenine, histidine and leucine, supplemented with x- α -gal. MdMYB88-155 contains 155 amino acids and MdMYB88-399 has 399 amino acids.

Determination of anthocyanin, hydrogen peroxide (H₂O₂), peroxidase (POD) activity, catalase (CAT) activity and superoxide dismutase (SOD) activity

For anthocyanin measurement, 4-month-old WT and transgenic plants were placed in a growth chamber at 4°C under a long-day photoperiod (16 h:8 h, light:dark) for 3 wk. Anthocyanins were extracted from mature leaves as described previously (Xie *et al.*, 2012).

H₂O₂ was detected as described previously with minor modifications (Guan *et al.*, 2013b). Briefly, 4-month-old WT and transgenic plants were treated at 4°C or 22°C for 2 h, then mature leaves were vacuum-infiltrated with 1 mg ml⁻¹ 3,3'-diaminobenzidine (DAB) in 50 mM Tris-acetate (pH = 5.0). Plants were incubated for 12 h at room temperature in darkness. Chlorophyll was removed using 70% ethanol in a water bath of 100°C. H₂O₂ content was quantified using the relevant detection kits (#H₂O₂-1-Y, Suzhou Comin Biotechnology Institute, Suzhou, China).

Activity of POD, CAT and SOD were measured as described by Wang *et al.* (2012).

Stomatal aperture measurements

Transgenic apple plants and GL-3 were grown in soil under the same growth conditions (16 h:8 h, light:dark at 21°C) for 2 months. Leaves were detached and submerged in stomatal opening solution (30 mM KCl, 0.1 mM CaCl₂, and 10 mM MES-KOH, pH 6.15) under light (120 μ mol m⁻² s⁻¹) for 2 h as described (Kwak *et al.*, 2001) to induce stomatal opening. Then the leaves in stomatal opening solution were transferred to 4°C under light (120 μ mol m⁻² s⁻¹) for 8 h. Leaf strips were observed and photographed with a EX30 microscope (SDPTOP). The stomatal aperture was measured by IMAGEJ.

Accession numbers

ChIP-seq data have been deposited at NCBI with the project ID PRJNA381840. Sequence data from this article can be found in the *Arabidopsis* Genome Initiative database under the following accession numbers: MYB88 (AT2G02820), FLP (AT1G14350), CBF1 (AT4G25490), CBF2 (AT4G25470), CBF3 (AT4G25480), ZAT12 (AT5G59820), RD29A (AT5G52310), RCF1 (AT1G20920), CSP3 (AT2G17870); in GDR under the following accession numbers: MdCOR47 (MDP0000770493), MdCSP3(MDP0000292743); and in NCBI under the following numbers: MdMYB88 (KY569647), MdMYB124 (KY569648), MdCBF1 (XP_008377096.1), MdCBF2 (ART85558.1), MdCBF3 (NP_001315892.1) MdCCA1 (XP_008343467.1).

Results

MdMYB88 and MdMYB124 positively regulate cold-responsive gene expression and cold-hardiness in apple

When we investigated the gene expression in apple plants exposed to cold stress, we found that expression of *MdMYB88* and *MdMYB124* was slightly (~2-fold) increased upon exposure to low (4°C) temperature (Fig. 1a). As expected given presumed function as TFs, both MdMYB88 and MdMYB124 proteins could direct localization of the YFP marker protein to the nucleus (Fig. 1b). In addition, *MpMYB88* and *MpMYB124* were highly expressed in root and stem tissues of an apple rootstock, *Malus prunifolia* (Fig. 1c). To determine if these genes were important for cold stress tolerance, we evaluated cold stress responses in transgenic apple plants compromised for these genes. We used an RNA interference (RNAi) approach to repress expression of both *MdMYB88* and *MdMYB124* (Fig. 2a). Because sequences of *MdMYB88* and *MdMYB124* are highly similar (Fig. S1), it was not possible to design primers to specifically knock-down either *MdMYB88* or *MdMYB124*. With agrobacterium-mediated transformation methods, we obtained individual transgenic lines where expression level of *MdMYB88* had been reduced to 10–48% and *MdMYB124* to 15–52% of nontransgenic levels (Fig. S2a,b). We found that, irrespective of acclimation, *MdMYB88/124* RNAi plants were more

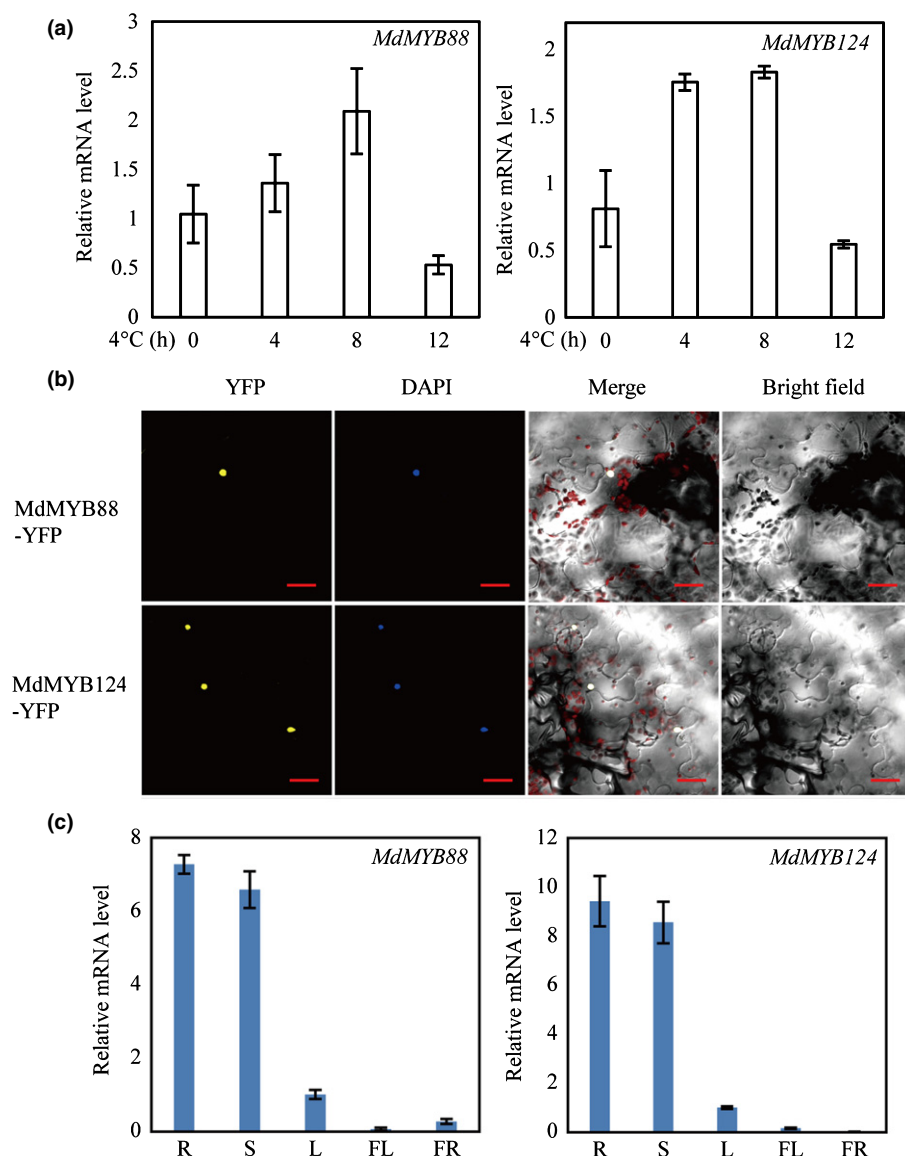


Fig. 1 *MdMYB88* and *MdMYB124* expression in response to cold in apple and protein localization. (a) Time-course expression of *MdMYB88* and *MdMYB124* in *Malus x domestica*. (b) *MdMYB88* or *MdMYB124* is localized in the nucleus of tobacco leaf epidermal cells. YFP, yellow fluorescent protein. Bars, 50 µm. (c) Tissue-specific expression pattern of *MdMYB88* or *MdMYB124* in *M. prunifolia*. R, root; S, stem; L, leaf; FL, flower; FR, fruit. Error bars indicate \pm SD ($n=3$, from three technical replicates). Three biological replicates were performed in (a, c). Data in Fig. 1 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

sensitive to freezing stress (Figs 2a–c, S3a,b). When challenged with chilling temperature for 1 month, followed by 22°C for 20 d, *MdMYB88/124* RNAi apple plants grew significantly more slowly than the nontransgenic plants (GL-3, which is the genetic background for all transgenic apple plants) (Fig. S4). Because apple seedlings are not grown in orchards, so chilling stress is not a serious problem to apple seedlings, we only focused on freezing tolerance of apple plants thereafter. Transgenic apple plants expressing either *MdMYB88* or *MdMYB124* under control of the strong 35S promoter showed increased tolerance to freezing stress (Figs 2d–i, S2c,d, S3c–3f). In addition, when 2-month-old rooted plants were subjected to freezing temperatures, similar results were observed (Fig. S5). These results indicate that *MdMYB88* and *MdMYB124* positively regulate freezing tolerance of apple.

As TFs, MYB88 and FLP are expected to directly regulate expression of target genes (Xie *et al.*, 2010a,b; Chen *et al.*, 2015; Wang *et al.*, 2015). We found that *MdMYB88* has a transcriptional activity in yeast (Fig. S6a,b). To determine if *MdMYB88* or *MdMYB124* may promote cold tolerance through regulation of

downstream genes, we assessed expression of apple homologs of cold-responsive genes in *MdMYB88/124* RNAi or 35S:*MYB88/124* plants that were transferred to soil for 8 months. We found that expression of the apple counterparts of several cold-responsive genes, including *MdCCA1*, *MdCBF1*, *MdCBF3* and *MdCOR47* was decreased in *MdMYB88/124* RNAi plants under cold stress, but was increased in either 35S:*MdMYB88* or 35S:*MdMYB124* plants (Fig. 3). These results are consistent with the observed increased sensitivity of *MdMYB88/124* RNAi plants and tolerance of 35S:*MdMYB88* or 35S:*MdMYB124* plants to freezing, suggesting that *MdMYB88* and *MdMYB124* promote expression of these genes under cold stress in apple.

MdMYB88 and MdMYB124 promote anthocyanin accumulation and stimulate H₂O₂ scavenging under cold conditions

The cold induction of *CBF* genes by *MdMYB88* and *MdMYB124* prompted us to investigate their roles in

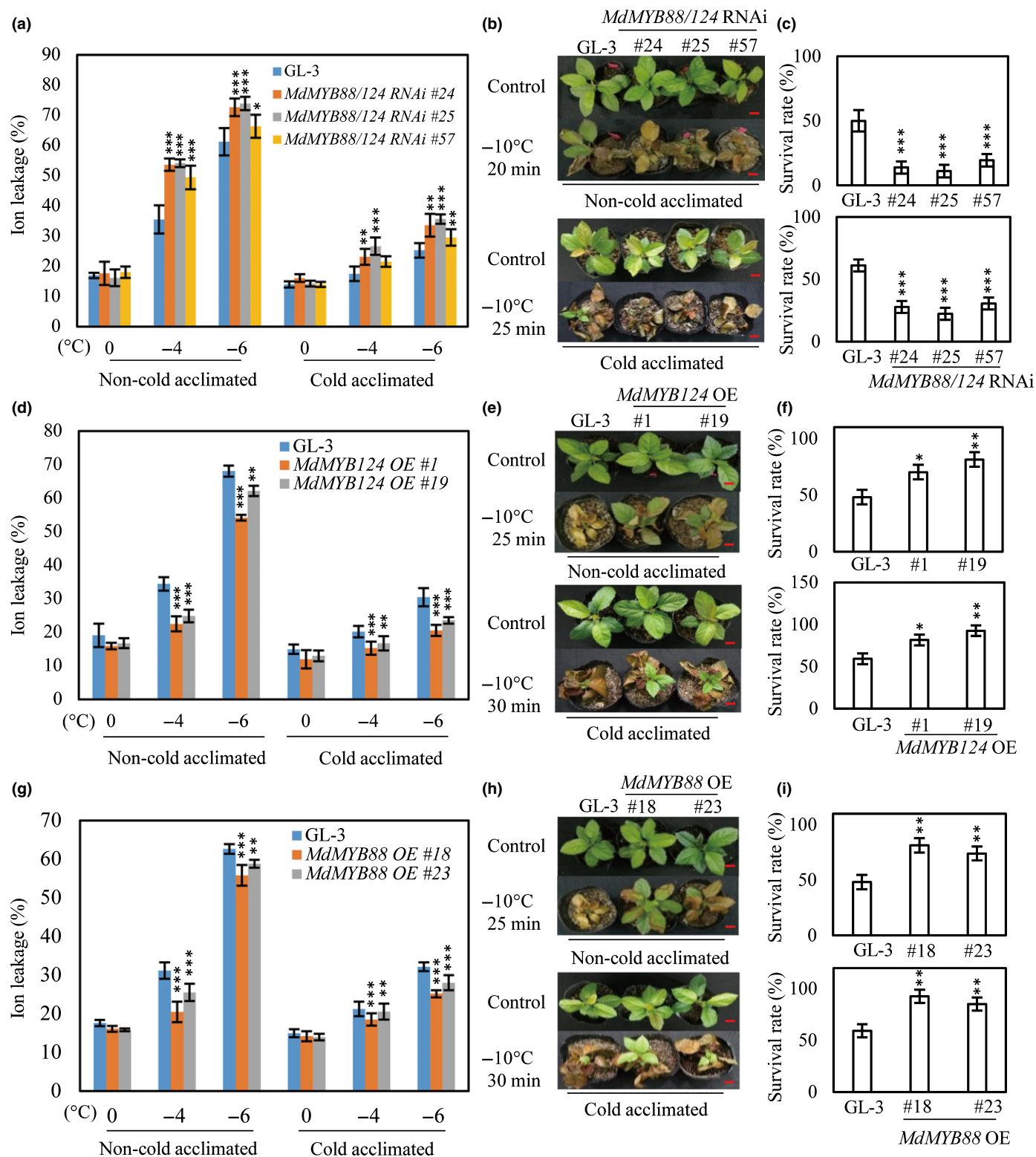


Fig. 2 *MdMYB88* and *MdMYB124* promote cold-hardiness of apple. (a, d, g) Freezing tolerance of *MdMYB88/124 RNAi*, *MdMYB124* over-expression (OE) or *MdMYB88* OE plants determined by leaf electrolyte leakage assays. (b, e, h) Whole-plant freezing tolerance of *MdMYB88/124 RNAi*, *MdMYB124* OE or *MdMYB88* OE plants before or after cold acclimation. Plants were photographed 20 d after freezing treatments. Bars, 2 cm. (c, f, i) Survival rates of *MdMYB88/124 RNAi*, *MdMYB124* OE, or *MdMYB88* OE plants in (b, e, f). Error bars indicate \pm SD ($n = 8$ in (a, d, g), eight plants and three leaf discs from each plant were used for each temperature point; $n = 36$ in (b, e, h), 36 plants were used and each 12 plants was a biological replication). One-way ANOVA (Tukey test) was performed and statistically significant differences were indicated by: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data in Fig. 2 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

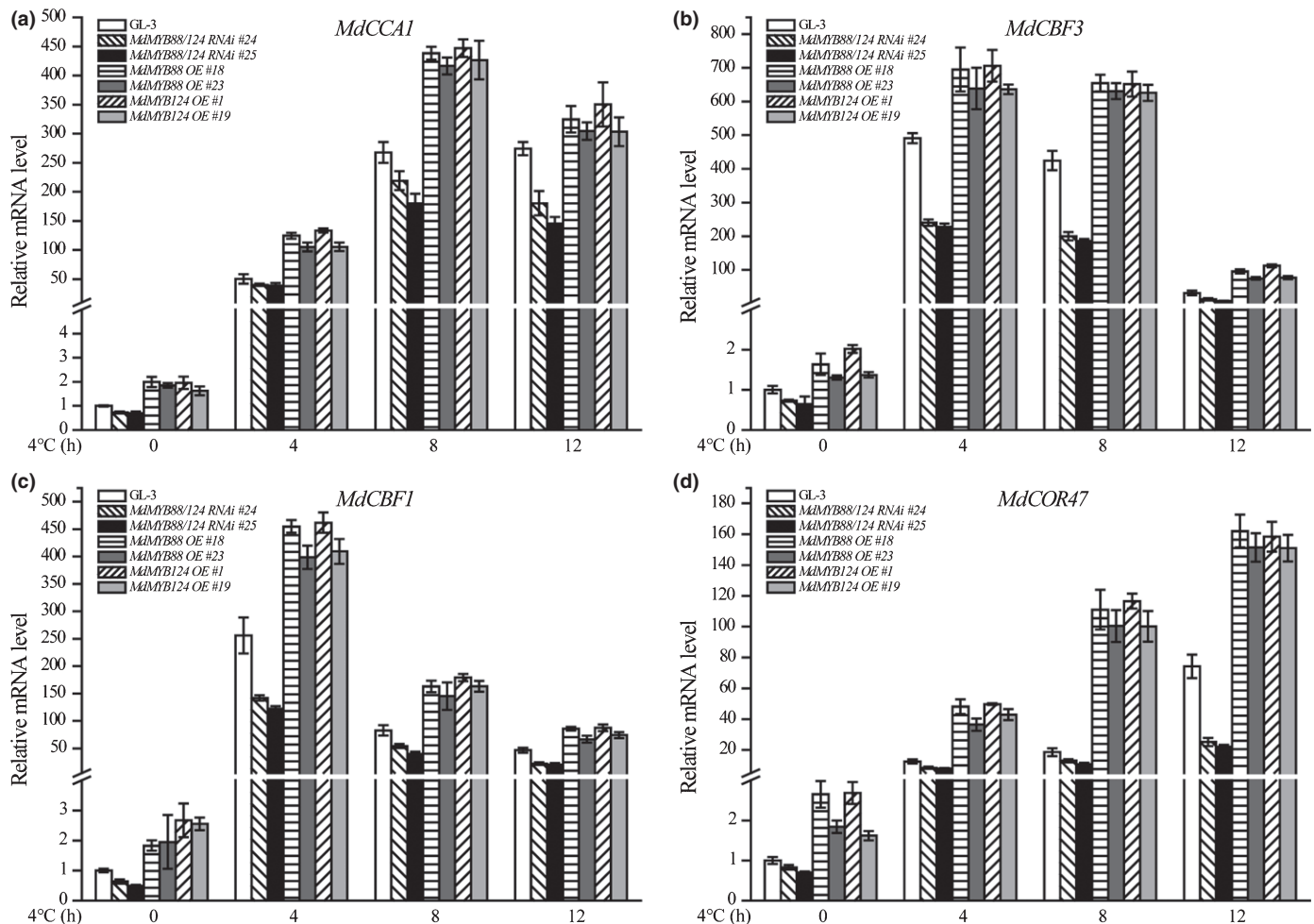


Fig. 3 Transcript levels of cold-responsive genes in transgenic apple plants under cold stress. (a–d) Transcript levels of endogenous cold-responsive genes of *CIRCADIAN CLOCK ASSOCIATED 1* (*MdCCA1*), *C-REPEAT BINDING FACTOR 3* (*MdCBF3*), *C-REPEAT BINDING FACTOR 1* (*MdCBF1*) and *Cold-regulated 47* (*MdCOR47*) in transgenic plants under control or cold conditions. Error bars indicate \pm SD ($n = 3$, from three technical replicates). Values in Fig. 3 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

anthocyanin accumulation, because CBF1 was shown to positively regulate anthocyanin accumulation (Li *et al.*, 2017a). To test this, we examined anthocyanin accumulation in leaves of the transgenic plants after cold treatment. We found that plants overexpressing *MdMYB88* or *MdMYB124* accumulated more anthocyanin than the nontransgenic control under cold stress (Fig. 4a, b, d), whereas *MdMYB88/124* RNAi plants accumulated less (Fig. 4c, d). Consistently, expression of anthocyanin biosynthesis-related genes, which are cold-inducible, was reduced in *MdMYB88/124* RNAi plants (Fig. 4e) but increased in *MdMYB88* or *MdMYB124* overexpressing plants (Fig. 4f). These observations suggest that *MdMYB88* and *MdMYB124* promote anthocyanin biosynthesis in response to cold.

Anthocyanins could protect plants from abiotic stresses including low temperature by serving as antioxidants (Li *et al.*, 2017a). We tested H_2O_2 accumulation of the WT, and *MdMYB88* and *MdMYB124* transgenic plants under cold stress. Staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) suggested that *MdMYB88* or *MdMYB124* overexpressing plants contained less H_2O_2 in response to cold, whereas *MdMYB88/124* RNAi plants

contained more (Fig. 5a, b). In plants, POD and CAT may scavenge H_2O_2 by hydroxylation. We found that activity of POD and CAT was higher in *MdMYB88* or *MdMYB124* overexpressing plants but lower in *MdMYB88/124* RNAi plants under cold stress (Fig. 5c, d), indicating that *MdMYB88* and *MdMYB124* promote H_2O_2 scavenging by modulating activity of antioxidant enzymes, such as POD and CAT. In addition, activity of SOD, another antioxidant enzyme which catalyzes the dismutation of the superoxide ($O_2^{\cdot -}$) radical into H_2O_2 , also showed similar pattern in transgenic plants under cold stress (Fig. 5e).

MdMYB88 and *MdMYB124* bind to the promoter regions of cold-responsive genes in apple

In order to identify potential regulatory targets of *MdMYB88* or *MdMYB124*, we performed ChIP analysis coupled with the Illumina sequencing (ChIP-seq). We used an antibody generated against native *MdMYB88* and *MdMYB124* to immunoprecipitate the associated DNAs from tissue-cultured GL-3 plants tissue. After analyzing sequenced reads with HOMER and

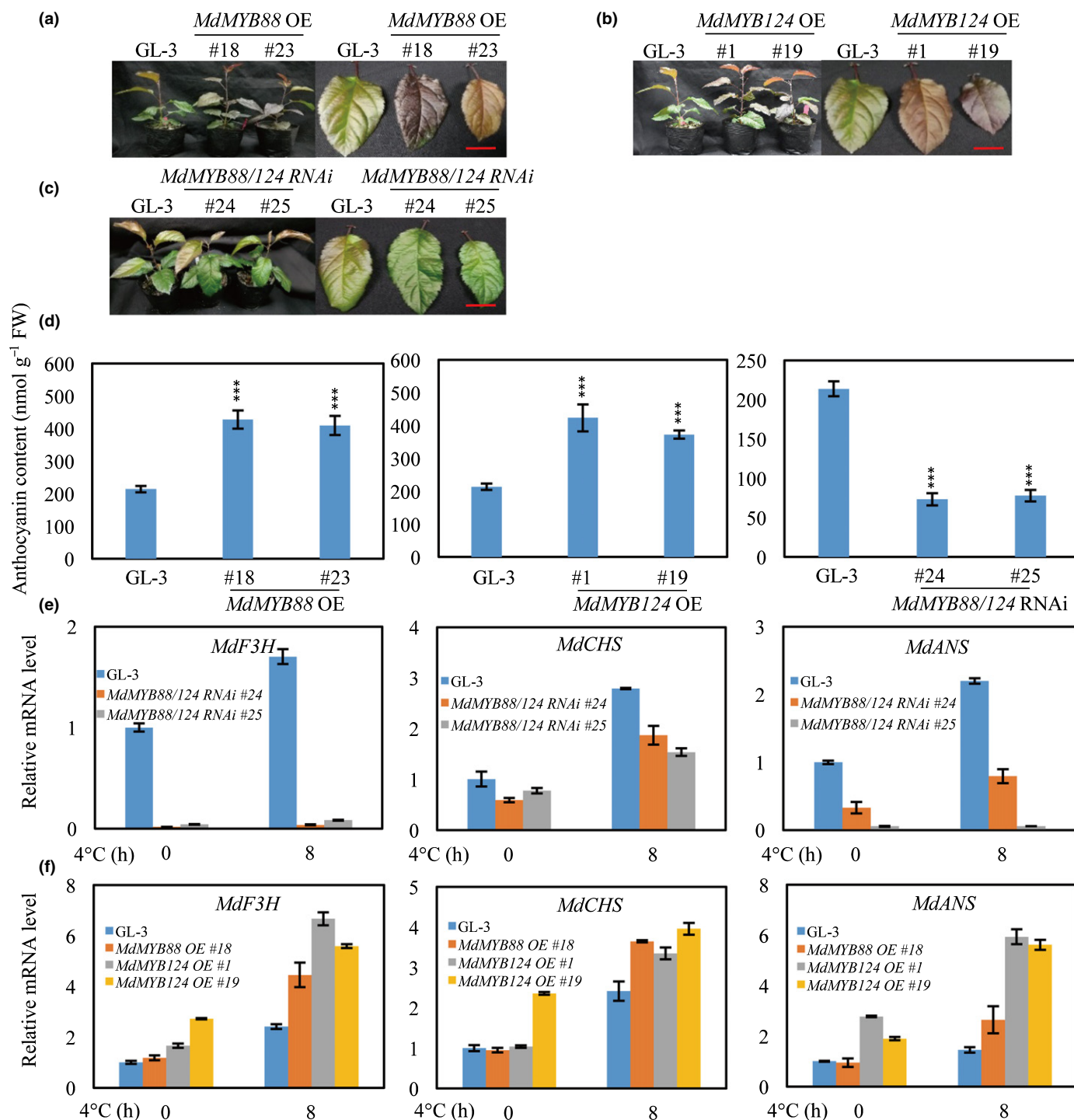


Fig. 4 *MdMYB88* and *MdMYB124* promote anthocyanin accumulation in apple under cold stress. (a–c) Anthocyanin accumulation in *MdMYB88/124* RNAi, *MdMYB88* over-expression (OE) or *MdMYB124* OE plants. Bars, 2 cm. (d) Quantification analysis of anthocyanin content in (a–c). (e, f) Transcript levels of anthocyanin biosynthesis-related genes *flavanone 3-hydroxylase* (*MdF3H*), *chalcone synthase* (*MdCHS*) and *anthocyanidin synthase* (*MdANS*) in *MdMYB88/124* RNAi, *MdMYB88* OE or *MdMYB124* OE plants under control or cold conditions (4°C) for 8 h. Error bars indicate ± SD ($n = 9$ in (d), nine leaves from nine plants were used for each line; $n = 3$ in (e, f), from three technical replicates). One-way ANOVA (Tukey test) was performed for data in (d), and statistically significant differences were indicated by: ***, $P < 0.001$. Data in Fig. 4 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

MEME-ChIP, we identified more than 1000 potential targets for *MdMYB88* and *MdMYB124* in a draft apple genome sequence (Velasco *et al.*, 2010) (Table S2) and two distinct consensus sequences for *MdMYB88* or *MdMYB124* binding (Fig. S7a).

One of the two sequences, AGCCG, is identical to a *cis*-element recognized by MYB88 and FLP in *Arabidopsis* (Xie *et al.*, 2010a), whereas another binding sequence, AGCAG, could not be confirmed by EMSA (Fig. S7b).

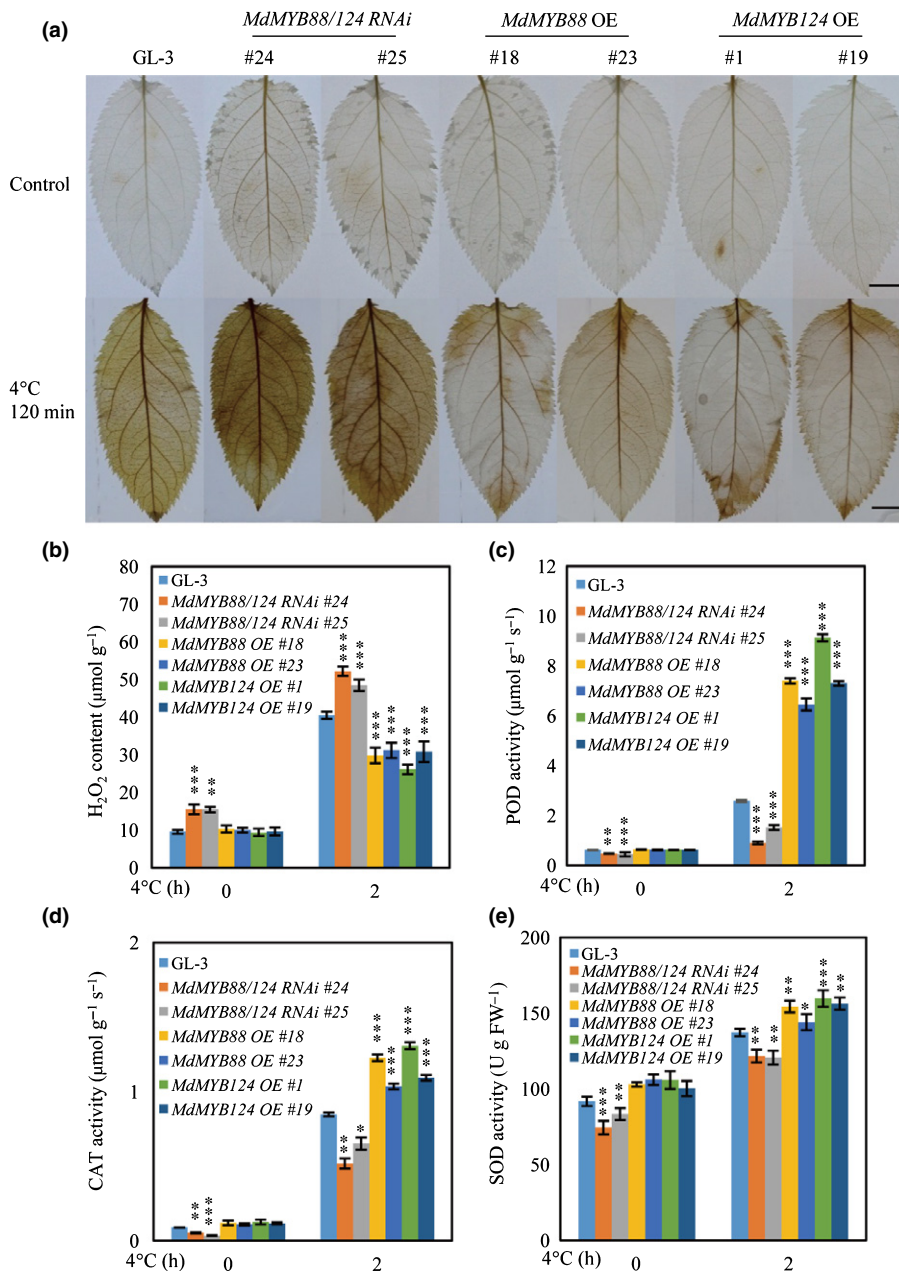


Fig. 5 MdMYB88 and MdMYB124 promote hydrogen peroxide (H₂O₂) scavenging in response to cold stress. (a) H₂O₂ accumulation in MdMYB88/124 RNAi, MdMYB88 over-expression (OE) or MdMYB124 OE plants under cold. H₂O₂ was indicated by DAB staining. Bars, 1 cm. (b–e) H₂O₂ content, peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) activity in MdMYB88/124 RNAi, MdMYB88 OE or MdMYB124 OE plants under cold stress. Error bars indicate ± SD (n = 3). Three leaves from three plants were mixed for each measurement. One-way ANOVA (Tukey test) was performed for data in (b), and statistically significant differences were indicated by: *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data in Fig. 5 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

Among the 1000 potential target genes, one is a homolog of *Arabidopsis* COLD SHOCK DOMAIN PROTEIN 3 (CSP3), a positive regulator of cold stress tolerance in *Arabidopsis* (Kim *et al.*, 2009), which shares 28% similarity with CSP3 (Fig. S8a). The promoter of *MdCSP3* contains a *cis*-element, AACCG, identified by Xie *et al.* (2010a) (Fig. S8b). ChIP-qPCR (Figs 6a,b, S8c) and EMSA (Fig. 6c) verified that MdMYB88 or MdMYB124 can bind to the promoter (–576 bp to –389 bp, contain AACCG site) of *MdCSP3*. We subsequently carried out a dual luciferase reporter assay in tobacco leaves to examine MdMYB88-activated *MdCSP3* promoter activity *in vivo*. We found that MdMYB88 enhanced the promoter activity of *MdCSP3* under both normal and cold conditions (Fig. 6d), further confirming the induction of *MdCSP3* by MdMYB88 and

MdMYB124. In addition, *MdCSP3* is induced and positively regulated by MdMYB88 and MdMYB124 (Fig. S9), suggesting that MdMYB88 and MdMYB124 may regulate cold hardiness of apple by modulating expression of *MdCSP3*.

Another potential target of MdMYB88 and MdMYB124 from our ChIP-seq results is *MdCCA1* (Table S2), a homologous gene of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and a positive regulator for cold tolerance in *Arabidopsis* (Dong *et al.*, 2011). *MdCCA1* was positively regulated by MdMYB88 and MdMYB124 (Fig. 3a,b). We analyzed promoter sequence of *MdCCA1* and found that it contains five potential MdMYB88 and MdMYB124 binding sites including CGCGG, AACCG, and AACGG (Figs 7a, S10a). To examine if MdMYB88 and MdMYB124 could directly bind to these motifs, we carried out a

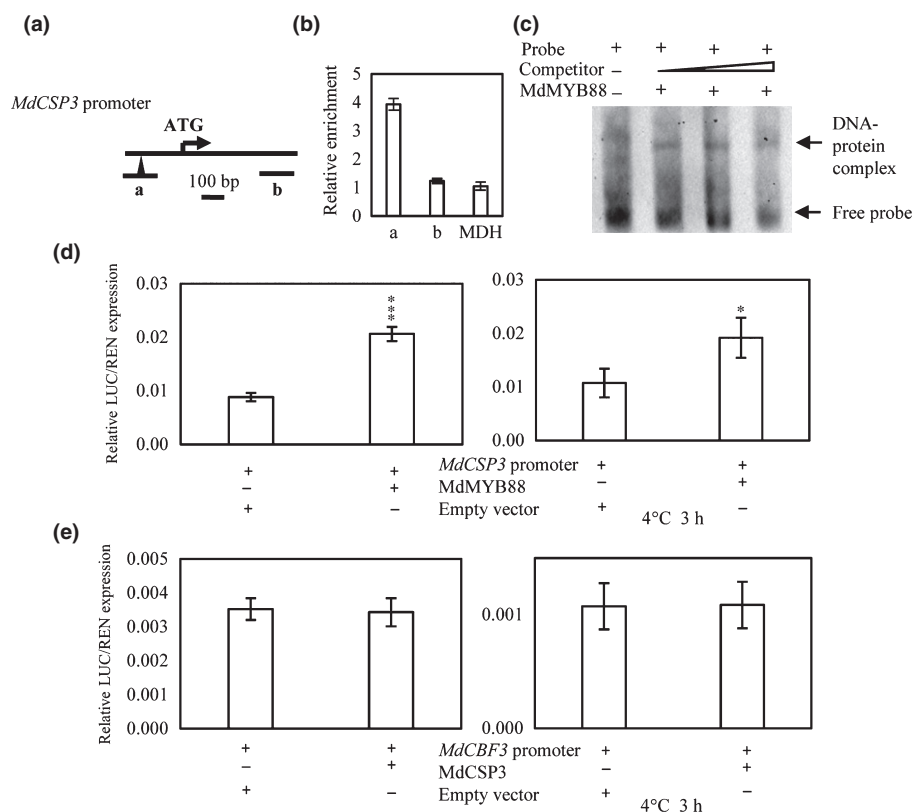


Fig. 6 MdMYB88 directly binds to the promoter region of *MdCSP3*. (a) Diagram of *MdCSP3* promoter regions. Fragment a contains a *cis*-element of AACCG. Fragment b serves as a negative control. (b) Chromatin immunoprecipitation - quantitative PCR (ChIP-qPCR) analysis of *MdCSP3* using cold-treated GL-3 at 4°C for 8 h. *MDH* is the negative control, also serves as the reference gene. (c) MdMYB88-His is able to bind to the promoter region of *MdCSP3* determined by EMSA analysis. Arrows indicate protein-DNA complex or free probe. *MdCSP3* probe contains AACCG element. (d, e) Relative luciferase activity from the dual luciferase reporter assays in *Nicotiana benthamiana* leaves. Pro35S: REN (pGreenII 62-SK) was used as an internal control. Quantification was performed by normalizing firefly luciferase activity to that of renilla luciferase. Left panels in (d, e), leaves of *N. benthamiana* were co-infiltrated and grown under normal conditions for 72 h. Right panels in (d, e), leaves of *N. benthamiana* were co-infiltrated and grown under normal conditions for 69 h, then 4°C growth chamber for additional 3 h before harvest. Error bars indicate \pm SD ($n = 3$ in (b), from three technical replicates; 10 in (d, e), three leaf discs from one plant and 10 plants were used for relative luciferase activity). One-way ANOVA (Tukey test) was performed for data in (d, e), and statistically significant differences were indicated by: *, $P < 0.05$; ***, $P < 0.001$. Data in Fig. 6 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

ChIP-qPCR assay. Our results showed that MdMYB88 and MdMYB124 bind to three motifs in the promoter of *MdCCA1* (Figs 7b, S10b). We subsequently performed an EMSA assay to further confirm the direct binding, and found that MdMYB88 and MdMYB124 directly bind to the motif of AACCG in the *MdCCA1* promoter (Fig. 7c). Dual luciferase assay also supported that MdMYB88 induces expression of *MdCCA1* (Fig. 7d).

MdMYB88 and MdMYB124 modulate expression of target genes and cold tolerance by CBF-dependent and CBF-independent pathways

Arabidopsis CSP3 is not required for *CBFs* expression under cold stress (Kim *et al.*, 2009), implying that it promotes freezing tolerance through a CBF-independent pathway. To investigate if apple *MdCSP3* also functions in a similar way, we performed a dual luciferase assay. We found that under both normal and cold conditions, MdCSP3 was not able to activate expression of

MdCBF genes (Figs 6e, S11), indicating its roles in cold responses via a CBF-independent pathway in apple.

Because *Arabidopsis* CCA1 acts upstream of *CBF* genes and activates *CBFs* expression under cold stress (Dong *et al.*, 2011), we speculated that apple MdCCA1 might induce expression of *MdCBF* genes. With a dual luciferase assay, we found that MdCCA1 could induce expression of *MdCBF3* under both normal and cold conditions, whereas cold stress enhanced this induction (Fig. 7e). The results here suggest that MdMYB88 and MdMYB124 modulate cold-responsive gene expression and cold hardiness of apple by a CBF-dependent pathway.

Arabidopsis MYB88 and FLP are two positive regulators for cold stress tolerance and expression of cold-responsive genes

MdMYB88 and *MdMYB124* are conserved among diverse plant species and typically are found as an orthologous pair (Fig. S12a,b). The MdMYB88 and MdMYB124 proteins share 56% and 55%

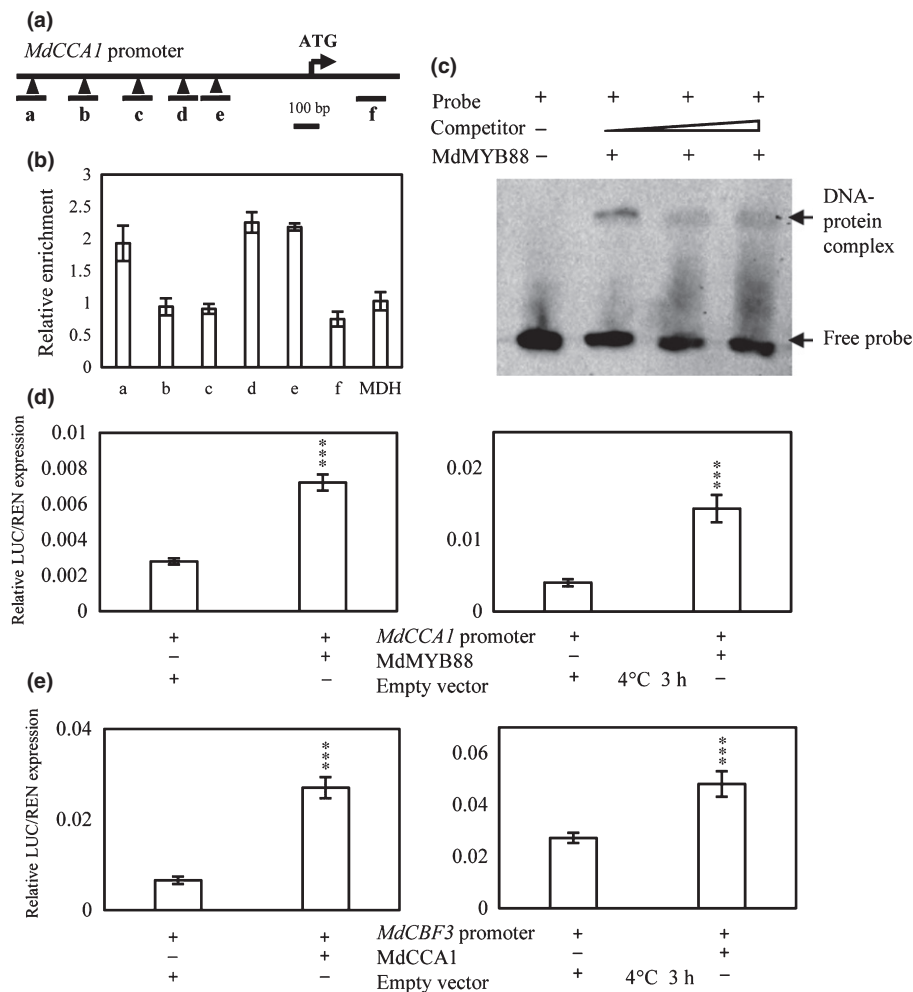


Fig. 7 MdMYB88 directly binds to the promoter region of *CIRCADIAN CLOCK ASSOCIATED 1* (*MdCCA1*). (a) Diagram of *MdCCA1* promoter regions. Fragment a contains a *cis*-element of CGCGG, fragments b, c and d contain *cis*-elements of AACCG, fragment e contains a *cis*-element of AACCG. Fragment f serves as a negative control. (b) Chromatin immunoprecipitation – quantitative PCR (ChIP-qPCR) analysis of *MdCCA1* using cold-treated GL-3 at 4°C for 8 h. *MDH* is the negative control, also serves as the reference gene. (c) MdMYB88-His is able to bind to the promoter region of *MdCCA1* determined by EMSA analysis. Arrows indicate protein-DNA complex or free probe. *MdCCA1* probe contains AACCG element. (d, e) Relative luciferase activity from the dual luciferase reporter assays in *Nicotiana benthamiana* leaves. Pro35S: REN (pGreenII 62-SK) was used as an internal control. Quantification was performed by normalizing firefly luciferase activity to that of renilla luciferase. Left panels in (d, e), leaves of *N. benthamiana* were co-infiltrated and grown under normal conditions for 72 h. Right panels in (d, e), leaves of *N. benthamiana* were co-infiltrated and grown under normal conditions for 69 h, then 4°C growth chamber for additional 3 h before harvest. Error bars indicate \pm SD ($n = 3$ in (b), from three technical replicates; 10 in (d, e), three leaf discs from one plant and 10 plants were used for relative luciferase activity). One-way ANOVA (Tukey test) was performed for data in (d, e), and statistically significant differences were indicated by: ***, $P < 0.001$. Data in Fig. 7 were derived from experiments that were performed at least three times with similar results, and representative data from one repetition were shown.

similarity with MYB88 and FLP, respectively (Fig. S13a). Similar to their counterparts in apple, expression of *MYB88* and *FLP* genes was slightly (~two-fold) increased in *Arabidopsis* under cold stress (Fig. 8a,b). To determine if, as in apple, MYB88 and FLP play positive roles in *Arabidopsis* cold tolerance, we examined the chilling tolerance (measured as the elongation of the hypocotyl at 4°C) and freezing tolerance (ion leakage) of *myb88*, *flp-1* and *myb88flp-1* mutant plants. We found that *myb88flp-1* mutant plants were more sensitive to chilling stress than the WT, whereas *myb88* or *flp-1* single mutant plants showed a response indistinguishable from the WT (Fig. S14a–c). Likewise, *myb88flp-1* double mutant plants were more sensitive to freezing under both acclimated and nonacclimated conditions, whereas the two single

mutants showed a degree of freezing tolerance similar to that of WT plants (Fig. 8c–g).

We also generated transgenic *myb88flp-1* plants expressing *MYB88* or *FLP* driven by their native promoter. We found that introduction of transgenes expressing *FLP* or *MYB88* from their native promoters into the *myb88flp-1* double mutant rescued the cold-sensitive defect (Fig. S15a,b). In addition, overexpression of both *MYB88* and *FLP* in Col-0 plants resulted in increased freezing tolerance (Fig. S15c,d). These results suggest that MYB88 and FLP function redundantly to promote cold stress tolerance in *Arabidopsis*.

In order to evaluate the possibility that MYB88 and FLP promote cold stress tolerance through directing gene expression, as

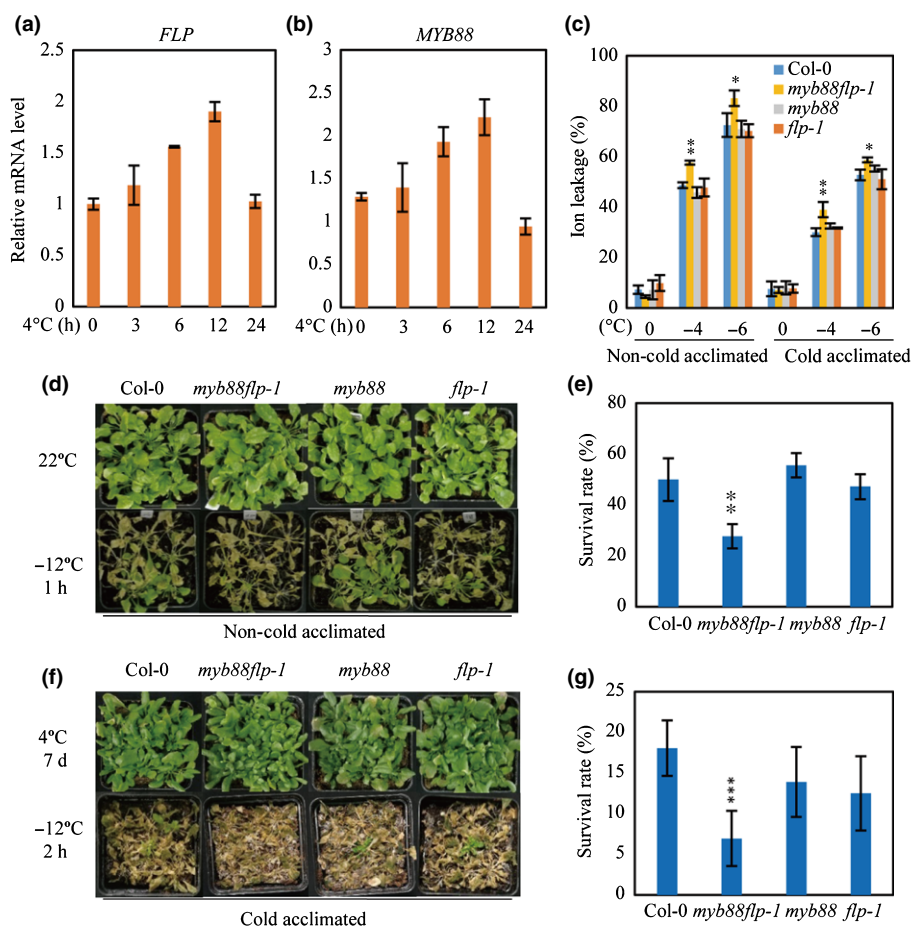


Fig. 8 *FOUR LIPS* (*FLP*) and *MYB88* expression in *Arabidopsis* and freezing tolerance of transgenic plants. (a, b) Expression of *FLP* and *MYB88* in 14-d-old wild-type (WT) seedlings exposed to cold (4°C) for up to 24 h. (c) Freezing tolerance of Col-0, *myb88flp-1*, *myb88* or *flp-1* seedlings determined by electrolyte leakage assay. (d, f) Whole-plant freezing tolerance of Col-0, *myb88flp-1*, *myb88* or *flp-1* plants. Plants were photographed 10 d after freezing treatments. (e, g) Survival rates of Col-0, *myb88flp-1*, *myb88* or *flp-1* shown in (d) and (f). Error bars indicate \pm SD ($n = 3$ in (a, b), from three technical replicates; six in (c), and 72 in (e) and (g)). One-way ANOVA (Tukey test) was performed for data in (c, e, g), and statistically significant differences were indicated by: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values in Fig. 8 were derived from experiments that were performed at least three times with similar results, with representative data from one repetition were shown.

in apple, we evaluated the effect of loss of these two genes on cold-responsive gene expression. We found that the expression levels of several cold-responsive genes were significantly reduced in the *myb88flp-1* mutant plants compared to WT plants under cold stress conditions; these included *CBF1/2/3*, *RD29A*, *ZAT12*, *CCA1*, *LHY*, *CSP3* and *REGULATOR OF CBF GENE EXPRESSION1(RCF1)* (Fig. 9a–h) (Thomashow, 1999; Vogel *et al.*, 2005; Guan *et al.*, 2013a). Thus, *MYB88* and *FLP* are important for proper expression of a diversity of cold-responsive genes.

Apple *MdMYB88* restores the phenotype of *Arabidopsis myb88flp-1* under freezing stress

In order to further confirm the positive roles of *MYB88* and *FLP* (*MYB124*) in apple and *Arabidopsis*, we generated transgenic *myb88flp-1* expressing *MdMYB88* from the strong 35S promoter. An ion leakage assay showed that expression of *MdMYB88* in *myb88flp-1* plants could restore the freezing defects of *myb88flp-1* plants (Fig. S13b), suggesting that *Arabidopsis MYB88* has a similar function to *MdMYB88*, at least in freezing tolerance.

Discussion

We have shown that *MYB88* and *FOUR LIPS* (*FLP*) (*MYB124*) are cold-inducible genes required for cold tolerance and

cold-responsive gene expression in apple and *Arabidopsis*. We cloned the two homologs of *MYB88/FLP* from *Malus × domestica*. Cao *et al.* (2013) performed an analysis of MYB proteins from *M. × domestica* cataloged by the Genome Database for Rosaceae (GDR) and named them *MdoMYB203* and *MdoMYB148* (Evans *et al.*, 2013; Jung *et al.*, 2014). *MdMYB88* and *MdMYB124* transcripts characterized in this study were slightly shorter than those of *MdoMYB203* and *MdoMYB148*, resulting from the inclusion of an alternative exon in the latter sequences (Fig. S16a,b). We designated our transcripts as *MdMYB88* and *MdMYB124* to distinguish them from the previously identified sequences.

Apple *MdMYB88/MdMYB124* are functionally conserved with *Arabidopsis MYB88/FLP*, at least for cold tolerance, because expression of *MdMYB88* in transgenic *myb88flp-1* plants could restore freezing tolerance (Fig. S13b). Two lines of evidence support positive roles for *MdMYB88* and *MdMYB124* in cold hardiness of apple. First, transgenic apple plants expressing either *35S:MdMYB88* or *35S:MdMYB124* were more tolerant to freezing stress, either with or without cold acclimation under 4°C for 1 wk (Fig. 2d–i). Second, *MdMYB88/124* RNAi plants were more sensitive to freezing stress, before or after cold acclimation (Fig. 2a–c). Besides their positive roles in cold acclimation of apple, we also observed that *MdMYB88* and *MdMYB124* influenced the basal freezing tolerance of apple plants (Fig. 2). This basal tolerance may be

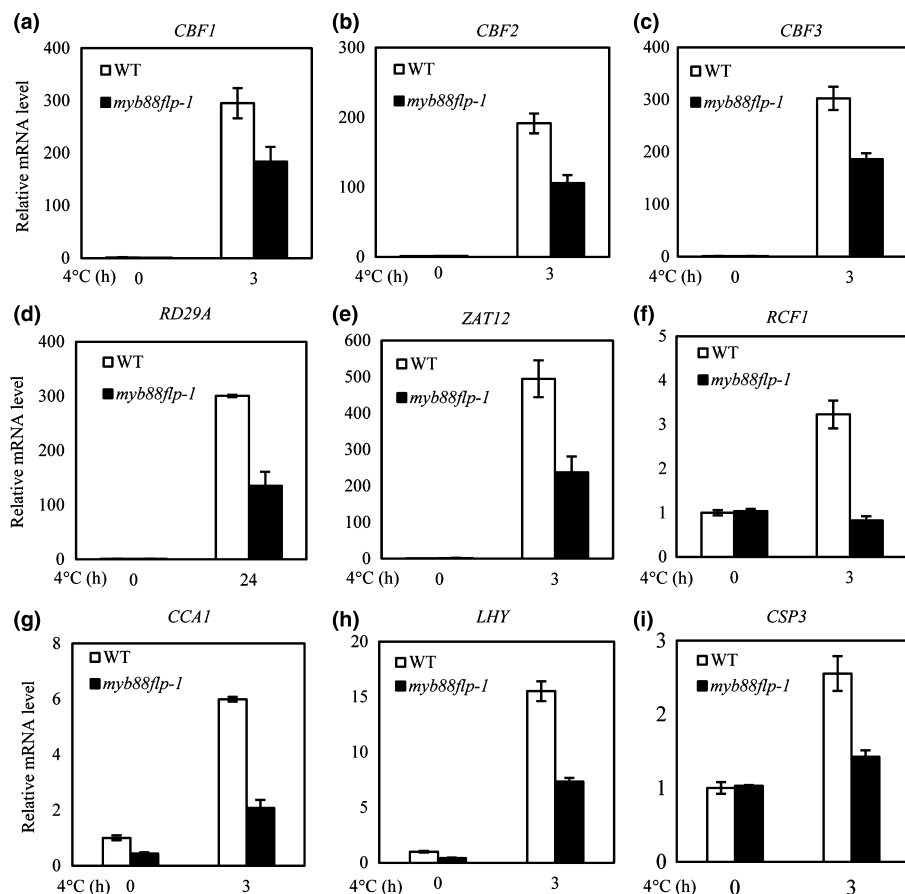


Fig. 9 Transcript levels of cold-responsive genes in *Arabidopsis myb88flp-1* mutant plants under cold stress. (a–i) Transcript levels of endogenous cold-responsive genes of *CBF1*, *CBF2*, *CBF3*, *RESPONSIVE TO DESICCATION 29A* (*RD29A*), *ZAT12*, *REGULATOR OF CBF GENE EXPRESSION 1* (*RCF1*), *CCA1*, *LATE ELONGATED HYPOCOTYL* (*LHY*), and *COLD SHOCK DOMAIN PROTEIN 3* (*CSP3*) in 14-d-old wild-type (WT) and *myb88flp-1* seedlings under control or cold conditions. Error bars indicate \pm SD ($n = 3$, from three technical replicates). WT and *myb88flp-1* plants were grown side by side in the same plate. Values in Fig. 9 were derived from experiments that were performed three times with similar results, and representative data from one repetition were shown.

due to the basal expression of cold-responsive genes in transgenic plants, including *MdCCA1*, *MdCBFs*, *MdCOR47* (Fig. 3) and *MdCSP3* (Fig. S9).

Anthocyanins are fundamental compounds for plant tolerance to drought, cold, ultraviolet-B, heavy metals, insects and pathogens. Numerous studies have shown that cold-induced anthocyanin biosynthesis is required for reactive oxygen species (ROS) scavenging and cold acclimation (Havaux & Kloppstech, 2001). For example, the anthocyanin biosynthesis genes *UGT79B2* and *UGT79B3* were shown to positively regulate anthocyanin production and ROS scavenging under cold (Li *et al.*, 2017a). *pfd* mutant plants are more tolerant to low temperature by accumulating more anthocyanin after cold acclimation (Perea-Resa *et al.*, 2017). *HY5*, a transcription factor (TF) and a PFD interacting partner, plays a key role in cold acclimation by controlling anthocyanin biosynthesis (Catala *et al.*, 2011). In this work, we found a lower concentration of anthocyanin and transcripts of anthocyanin biosynthesis genes in *MdMYB88/124* RNAi plants and a higher concentration in *MdMYB88* or *MdMYB124* over-expression (OE) plants under cold stress, suggesting that *MdMYB88* and *MdMYB124* positively regulate anthocyanin biosynthesis under cold. Antioxidants, such as peroxidase (POD) and catalase (CAT), play critical roles in scavenging hydrogen peroxide (H_2O_2). *PtHbHLH* mediates ROS scavenging under cold stress by directly regulating a POD gene (Huang *et al.*, 2013). We also found that activity of POD and

CAT was higher in *MdMYB88* or *MdMYB88/124* OE plants but lower in *MdMYB88/124* RNAi plants, suggesting an H_2O_2 scavenging role of *MdMYB88* and *MdMYB124* in response to cold. Our data indicated that *MdMYB88* and *MdMYB124* positively regulate cold tolerance of apple, at least in part by controlling anthocyanin biosynthesis and, therefore, by attenuating ROS amounts in response to cold.

UGT79B2 and *UGT79B3*, responsible for overproduction of anthocyanin, also were found to be directly regulated by C-REPEAT BINDING FACTOR (CBF)1 and play critical roles in plant cold tolerance (Li *et al.*, 2017a). Ectopic expression of *PpCBF1* in M26, a rootstock of apple, accumulated more anthocyanin in major and minor veins in transgenic lines after cold acclimation (Wisniewski *et al.*, 2011). In addition, transgenic lines of *PpCBF1* but not the wild-type (WT) control had a higher concentration of anthocyanin when plants were transferred from the glasshouse to the field in fall after 2 wk, as well as after a mild frost (-2.1°C) (Artlip *et al.*, 2014). These results indicate that ectopic expression of *PpCBF1* can promote anthocyanin synthesis under cold stress in apple rootstock. Because apple CBFs share a very high similarity with pear CBFs (91–97%), it is possible that apple CBFs might have a similar function with pear CBFs in anthocyanin accumulation under cold. However, due to lack of *CBF* transgenic plants, we cannot rule out the possibility that *MdMYB88* and *MdMYB124* regulate anthocyanin accumulation by regulating genes other than *CBFs*.

Using the approach of SELEX (systematic evolution of ligands by exponential enrichment), Xie *et al.* (2010a) identified a distinct binding motif for MYB88/FLP, [A/T/G] [A/T/G] C [C/G] [C/G]. Because MdMYB88 and MdMYB124 share a relatively high sequence similarity with MYB88 and FLP (55.98% and 54.69%, respectively), and MdMYB88 could rescue the freezing-sensitive phenotype of *myb88flp-1* (Fig. S13a,b), we hypothesized that MdMYB88/MdMYB124 may also recognize this atypical sequence. Our ChIP-seq analysis using anti-MdMYB88/MdMYB124 antibodies resulted in the identification of >1000 genes potentially targeted by MdMYB88/MdMYB124. These genes encode proteins participating in a variety of processes, including cold/oxidative stress, heat stress, drought stress, control of cell cycle and cell division, root development, circadian gene regulation, RNA splicing, growth, DNA binding, auxin signaling and abscisic acid (ABA) signaling. Promoter analysis identified two potential binding motifs in apple plants: AGCCG, and AGCAG (Fig. S6a). AGCCG is one of the binding motifs identified by Xie *et al.* (2010a), whereas AGCAG cannot be verified by EMSA (Fig. S7b). One target gene of MdMYB88 and MdMYB124, a homolog of *Arabidopsis* *TIME FOR COFFEE* (*TIC*), which contain a AGCCG *cis*-element in the promoter region, was verified by ChIP-seq analysis (Fig. S17a,b). Our ChIP-qPCR and EMSA experiments revealed that besides AGCCG, MdMYB88 and MdMYB124 were able to bind to AACCG, another sequence identified by Xie *et al.* (2010a). Xie *et al.* (2010a) also carried out ChIP-chip analysis in *Arabidopsis* with anti-MYB88/FLP antibodies and identified *c.* 226 potential targets, encoding proteins involved in cell cycle, cell division, DNA binding, oxidative stress, cold stress tolerance, splicing, etc. Many of the target genes of MdMYB88 and MdMYB124 identified in our study overlap in function with those identified by Xie *et al.* (2010a). However, we identified additional genes with roles in hormone response and circadian gene regulation, suggesting that the function of MdMYB88 and MdMYB124 might be more diverse than their paralogous genes in *Arabidopsis*.

MdMYB88 and MdMYB124 positively regulate expression of *MdCCA1* by directly binding to promoter regions of *MdCCA1* under cold stress (Figs 3a,b, 7). In *Arabidopsis*, we also found that MYB88 and FLP positively regulate *CCA1* transcripts (Fig. 9). Moreover, the promoter of *CCA1* in *Arabidopsis* also contains a potential binding site for MYB88 and FLP (AACCG), indicating that MYB88 and FLP may directly regulate *CCA1* expression under cold by binding to its promoter. However, the ChIP-chip assay of MYB88 and FLP by Xie *et al.* (2010a) did not identify *CCA1* as their direct target gene. Further studies such as EMSA need to be performed to verify this direct binding in *Arabidopsis*. Furthermore, we speculate that MYB88 and FLP might bind to of the *CCA1* promoter only under cold stress.

FLP and MYB88 redundantly regulate stomatal proliferation and *flp-1myb88* mutant plants display stomatal defects in *Arabidopsis* (Xie *et al.*, 2010a). We also found that some stomata were clustered in *MdMYB88/124* RNAi plants (Fig. S18a). Wilkinson *et al.* (2001) reported that low temperature could induce stomatal closure of cold-tolerant *Commelina communis* but not of cold-sensitive tobacco. We examined stomatal aperture of transgenic

apple plants under cold stress. We found that the stomatal aperture of *MdMYB88/124* RNAi, *MdMYB88* or *MdMYB124* overexpressing plants was indistinguishable from that of WT plants after cold stress (4°C) for 8 h (Fig. S18b,c), suggesting that MdMYB88 and MdMYB124 did not regulate stomatal opening in response to cold.

Based on the data presented in this work, we propose a model for the function of MdMYB88 and MdMYB124 in response to cold in apple (Fig. 10). Under cold stress, induced MdMYB88 and MdMYB124 act upstream of *MdCCA1* and directly regulate its expression by binding to the AACCG motif of its promoter. Activated *MdCCA1* then modulates expression of *MdCBF* genes which induce expression of *COR* genes and may promote anthocyanin accumulation, the latter contributes to H₂O₂ scavenging under cold stress. It is possible that MdMYB88 and MdMYB124 regulate anthocyanin accumulation under cold via a pathway independent of MdCBFs. MdMYB88 and MdMYB124 also directly regulate expression of *MdCSP3* which promotes cold tolerance of apple via a CBF-independent pathway.

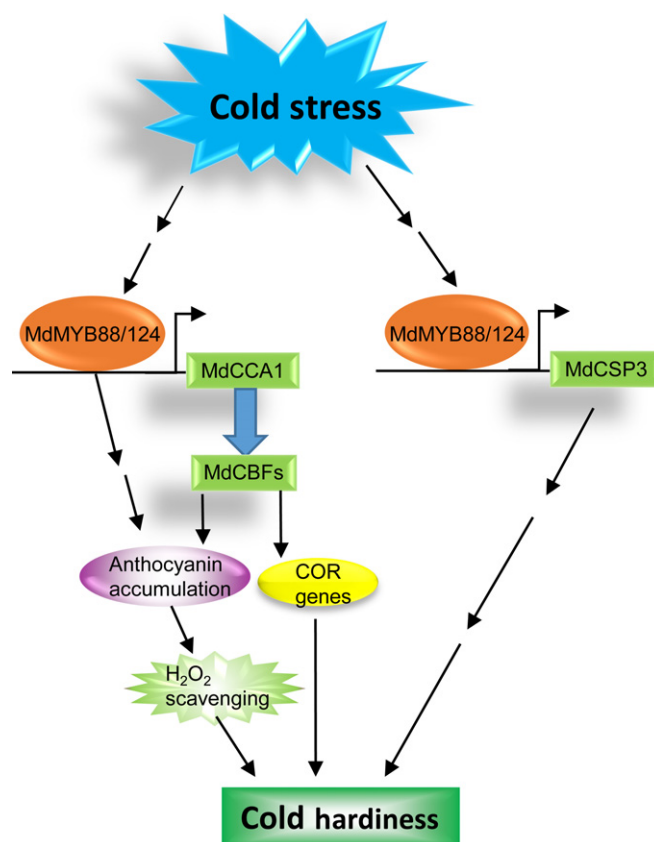


Fig. 10 A working model for MdMYB88 and MdMYB124 in response to cold in apple. Under cold stress, induced MdMYB88 and MdMYB124 act upstream of *CIRCADIAN CLOCK ASSOCIATED 1* (*MdCCA1*) and directly regulate its expression by binding to the AACCG motif of its promoter. Activated *MdCCA1* then modulates expression of *MdCBF* genes which induce expression of *COR* genes and may promote anthocyanin accumulation, the latter contributes to hydrogen peroxide (H₂O₂) scavenging under cold stress. It is possible that MdMYB88 and MdMYB124 regulate anthocyanin accumulation under cold stress via a pathway independent of MdCBFs. MdMYB88 and MdMYB124 also directly regulate expression of *MdCSP3* which promotes cold tolerance of apple via a CBF-independent pathway.

Apple is a perennial plant. Unlike annual plants, full acclimation response of apple plants may depend on seasonal cues, for example short day length and prolonged cold. In this study, we identified the upstream TFs of *MdCCA1* and *MdCSP3*, *MdMYB88* and *MdMYB124*, and characterized their role in freezing tolerance of apple before or after cold acclimation. Our findings provided new information for transcriptional regulation of cold response in apple, as well as the possible strategy to improve apple freezing tolerance by genetic engineering in the future.

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Author contributions

Q.G., Y.X., L.W., P.C. and Y.Y. designed the experiments; Y.X., L.W., P.C., C.B., Y.Y., X.S., X. Li, C.N., X.Liu, H.L., C.Z. and N.F. performed experiments; J.Y., T.Z., Y.S., J.Z., L.X., S.V.N. and F.M. analyzed data; and Q.G., L.W., Y.Y., P.C., S.V.N. and Y.X. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 Comparison of nucleotide sequences of *MdMYB88* with *MdMYB124*.

Fig. S2 Expression level of *MdMYB88* or *MdMYB124* in transgenic plants.

Fig. S3 Whole-plant freezing tolerance of *MdMYB88/124* RNAi, *MdMYB124* OE or *MdMYB88* OE plants before or after cold acclimation.

Fig. S4 *MdMYB88* and *MdMYB124* promote plant growth under chilling stress.

Fig. S5 Whole-plant freezing tolerance of GL-3, *MdMYB88/124* RNAi, *MdMYB88* OE or *MdMYB124* OE plants before or after cold acclimation.

Fig. S6 *MdMYB88* transcriptional activity assay.

Fig. S7 Two predicted binding sequences of *MdMYB88*/*MdMYB124* by ChIP-seq analysis.

Fig. S8 Sequence of *MdCSP3*, *MdMYB88*/*MdMYB124* core binding sites (AACCG) in the promoter region of *MdCSP3*, and ChIP-qPCR analysis.

Fig. S9 Transcript levels of *MdCSP3* under cold stress.

Fig. S10 Recognition sites in promoter of *MdCCA1* and ChIP-qPCR analysis.

Fig. S11 *MdCSP3* cannot active expression of *MdCBF1* and *MdCBF2*.

Fig. S12 Comparison of *MdMYB88* and *MdMYB124* with their close homologs in plant species.

Fig. S13 Apple *MdMYB88* is able to complement the freezing sensitive phenotype of *myb88flp-1*.

Fig. S14 Hypocotyl elongation of WT, *myb88*, *flp-1* or *myb88flp-1* under chilling stress and expression of *MYB88* or *FLP* in transgenic plants in *Arabidopsis*.

Fig. S15 FLP and MYB88 promote freezing tolerance in *Arabidopsis*.

Fig. S16 Nucleotides alignment of *MdMYB88* with *MdoMYB203* from GDR, and *MdMYB124* with *MdoMYB128* from GDR.

Fig. S17 *MdMYB88*/*MdMYB124* may be able to bind to the *cis*-element of AGCCG in the promoter region of *MdTIC*.

Fig. S18 Stomatal development and movement of GL-3, *MdMYB88/124* RNAi, *MdMYB88* OE, *MdMYB124* OE plants under cold.

Table S1 Primers used in this study

Table S2 Potential targets of *MdMYB88* and *MdMYB124* by using ChIP-seq analysis

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