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An apple MYB transcription factor regulates cold tolerance and anthocyanin accumulation and undergoes MIEL1-mediated degradation

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Summary

MYB transcription factors (TFs) have been demonstrated to play diverse roles in plant growth and development through interaction with basic helix-loop-helix (bHLH) TFs. MdbHLH33, an apple bHLH TF, has been identified as a positive regulator in cold tolerance and anthocyanin accumulation by activating the expressions of *MdCBF2* and *MdDFR*. In the present study, a MYB TF MdMYB308L was found to also positively regulate cold tolerance and anthocyanin accumulation in apple. We found that MdMYB308L interacted with MdbHLH33 and enhanced its binding to the promoters of *MdCBF2* and *MdDFR*. In addition, an apple RING E3 ubiquitin ligase MYB30-INTERACTING E3 LIGASE 1 (MdMIEL1) was identified to be an MdMYB308L-interacting protein and promoted the ubiquitination degradation of MdMYB308L, thus negatively regulated cold tolerance and anthocyanin accumulation in apple. These results suggest that MdMYB308L acts as a positive regulator in cold tolerance and anthocyanin accumulation in apple by interacting with MdbHLH33 and undergoes MdMIEL1-mediated protein degradation. The dynamic change in MYB-bHLH protein complex seems to play a key role in the regulation of plant growth and development.

Introduction

Cold stress is an adverse environmental factor that limits plants growth and crop production, which may even lead to the death of plants (Thakur *et al*., 2010; Thomashow, 1999). At the cellular level, cold stress damages cell membrane structure and affects metabolism of nutrients. At the tissue level, cold stress causes damages to plant roots, leaves, flowers and fruits. To sense and adapt to adverse cold stress conditions, plants have evolved efficient regulatory mechanisms to increase cold tolerance (Xin and Browse, 2000; Zhu, 2001). Extensive investigations have revealed that numerous genes are activated in response to cold stress, including those involved in the biosynthesis of cold-triggered components, such as soluble sugar, proline, betaine, polyamines, phenylpropanoids and antioxidants (Bajwa *et al*., 2014; Chinnusamy *et al*., 2007; Provart *et al*., 2003; Rivero *et al*., 2001; Thomashow, 1999). C-repeat binding factor (CBF) transcription factors (TFs) play essential roles in the cold stress response by binding to the C-repeat/dehydration-responsive elements (CRT/DRE) in the promoters of the cold-regulated (COR) genes and regulating their expressions (Gilmour *et al*., 2004; Jaglo *et al*., 2001; Stockinger *et al*., 1997). So far, three CBFs in *Arabidopsis* (Medina *et al*., 1999; Medinab *et al*., 2011) and five CBFs in apple (An *et al*., 2018; Wisniewski *et al*., 2014) have been isolated and characterized. Constitutive expression of *CBF* genes improves plant cold stress tolerance (Liu *et al*., 1998; Thomashow, 1999; Wisniewski *et al*., 2014). *CBF* genes are regulated at the transcriptional level by the basic helix-loop-helix (bHLH) TFs ICE1 and ICE2 in *Arabidopsis* (Chinnusamy *et al*., 2003; Fursova *et al*., 2009). In apple, MdClbHLH1, the homolog of *Arabidopsis* ICE1, contributes to the improved cold tolerance by directly activating the *CBF* genes (Feng *et al*., 2012). It is recognized that the ICEs-CBFs-CORs regulatory pathway plays a central role in the regulation of cold stress response. Besides ICEs, the transcriptions of *CBFs* are also regulated by MYB TFs (Agarwal *et al*., 2006; An *et al*., 2018; Xie *et al*., 2018), calmodulin-binding transcription activator 1-3 (CAMTA1-3) (Doherty *et al*., 2009), PSEUDO-RESPONSE REGULATORs (PRRs) (Nakamichi *et al*., 2009), CIRCADIAN CLOCKASSOCIATED 1 (CCA1) (Dong *et al*., 2011), ETHYLENE-INSENSITIVE 3 (EIN3) (Shi *et al*., 2012), phytohormone-interacting factors (PIFs) (Jiang *et al*., 2017; Lee and Thomashow, 2012) and brassinazole-resistant 1 (BZR1) (Li *et al*., 2017a,b). In addition to the CBF cold stress response pathway, many studies have demonstrated that CBF-independent signalling pathway also plays an important role in the regulation of cold stress response (Li *et al*., 2017a,b; Zhu *et al*., 2004).

As the largest TF family, MYB TFs act as important regulators in plant biotic and abiotic stress responses including cold stress response (Chinnusamy *et al*., 2007; Dubos *et al*., 2010; Li *et al*., 2015). MYB TFs involved in the regulation of cold tolerance have been functionally identified in several species. For example, *Arabidopsis* MYB14 and MYB15 negatively regulate cold tolerance by repressing the expression of *CBF* genes (Agarwal *et al*., 2006; Chen *et al*., 2013). In rice, overexpression of *MYB4*, *MYB3R-2*, *MYBS3* and *MYB2* improves plant cold tolerance (Dai *et al*., 2007; Su *et al*., 2010; Vannini *et al*., 2004; Yang *et al*., 2012). Recent investigations in apple show that MdoMYB121, MdSIMYB1, MdMYB4, MdMYB23, MdMYB88 and MdMYB124 are positive regulators of cold tolerance (An *et al*., 2018; Cao

et al., 2013; Wang *et al*., 2014; Wu *et al*., 2017; Xie *et al*., 2018), whereas MdMYB44 and MdMYB15L negatively regulate plant cold tolerance (Wu *et al*., 2018; Xu *et al*., 2018a,b).

In addition to functioning in stress response, MYB TFs have been characterized to play key roles in the regulation of plant secondary metabolism including anthocyanin biosynthesis (Allan *et al*., 2008; Dubos *et al*., 2010; Liu *et al*., 2015). In *Arabidopsis*, MYB75, MYB90, MYB113 and MYB114 contribute to anthocyanin accumulation (Gonzalez *et al*., 2008; Maier *et al*., 2013; Teng *et al*., 2005), whereas MYBL2 represses anthocyanin biosynthesis (Dubos *et al*., 2008; Matsui *et al*., 2008). In apple, MdMYB1 and its allelic genes are recognized as the central regulators in regulating anthocyanin biosynthesis (Ban *et al*., 2007; Espley *et al*., 2007; Takos *et al*., 2006). In addition, MdMYB3, MdMYB9, MdMYB11, MdMYB12, MdMYB16, MdMYB22, MdMYB110a and MdMYBPA1 are also identified to regulate flavonoid biosynthesis in apple (An *et al*., 2014; Umemura *et al*., 2013; Vimolmangkang *et al*., 2013; Wang *et al*., 2017, 2018; Xu *et al*., 2017). A few of MYB TFs are considered to function in association with bHLH and WD-repeat proteins (Dubos *et al*., 2010; Jaakola, 2013; Zimmermann *et al*., 2004). The MYBs-bHLHs-WD-repeat protein complex plays a decisive role in the regulation of anthocyanin biosynthesis.

Ubiquitination is an important post-translational modification that extensively regulates plant growth and development including biotic and abiotic stress responses (Lyzenga and Stone, 2011; Sun and Chen, 2004). Ubiquitination consists of tagging the ubiquitin to target proteins, which leads to the 26S proteasome-mediated degradation of target proteins (Peng *et al*., 2003; Pickart, 2001). Three enzymatic activities are involved in this action including the E1 ubiquitin activating enzymes, the E2 ubiquitin-conjugating enzymes and the E3 ubiquitin ligases (Serino and Xie, 2013; Smalle and Vierstra, 2004). Among these three components, E3 ubiquitin ligases play key roles in determining substrate specificity (Ardley and Robinson, 2005; Buetow and Huang, 2016). According to its conserved structural domain, E3 ubiquitin ligases are divided into four types including HECT, RING, SCF and APC ligases (Deshaies and Joazeiro, 2009; Metzger *et al*., 2012; Morreale and Walden, 2016). Among them, RING domain E3 ubiquitin ligases have been widely studied to modulate abscisic acid signalling (Stone *et al*., 2006; Zhang *et al*., 2007), anthocyanin biosynthesis (An *et al*., 2017a; Li *et al*., 2012; Maier *et al*., 2013), cold (Dong *et al*., 2006), salt (Kim and Kim, 2013; Zhang *et al*., 2015), drought (Kim and Kim, 2013; Qin *et al*., 2008; Ryu *et al*., 2010) and heat tolerances (Liu *et al*., 2016).

In this study, a cold-responsive MYB TF named MdMYB308L was characterized in apple. Overexpression of *MdMYB308L* led to improved cold tolerance and increased anthocyanin accumulation. MdMYB308L physically interacted with MdbHLH33, a bHLH TF that positively regulates cold tolerance and anthocyanin biosynthesis (Xu *et al*., 2017, 2018a,b). Further studies showed that MdMYB308L enhanced the binding of MdbHLH33 to the *MdCBF2* and *MdDFR* promoters through a direct protein interaction. In addition, an apple RING E3 ubiquitin ligase MdMIEL1 interacted with MdMYB308L and promoted the degradation of MdMYB308L through the 26S-proteasome pathway, thus negatively regulating cold tolerance and anthocyanin accumulation. Taken together, we have identified and characterized a novel MYB TF that regulates the cold tolerance and anthocyanin accumulation in association with a bHLH TF and undergoes the 26S proteasome-mediated degradation.

Results

Identification of a cold-responsive MYB TF MdMYB308L in apple

In our previous study, cold stress transcriptome was performed to identify the cold-responsive MYB TFs, in which an MYB TF MdMYB23 was characterized as a positive regulator in cold tolerance (An *et al*., 2018). Similarly, we identified another cold-responsive MYB TF (GenBank accession number: MDP0000950559), which showed the highest cold-induced expression only second to *MdMYB15* and *MdMYB23* (Table S1). Sequence search against the National Center for Biotechnology Information (NCBI) database showed that it was an apple MYB domain 308-like gene, thus designated as MdMYB308L. Sequence alignment and phylogenetic tree analysis demonstrated that MdMYB308L had the highest protein sequence identity with PbMYB308L from pears (*Pyrus x bretschneideri*) and contained a conserved MYB DNA-binding domain and a bHLH binding motif (Figure S1a-d). In addition, we performed another sequence alignment and phylogenetic tree analysis from MdMYB proteins involved in anthocyanin biosynthesis and cold tolerance (Figure S2a-b). The result revealed that MdMYB308L had high genetic relationship with MdMYB3 and it showed a large sequence difference compared with other MdMYB proteins, indicating that MdMYB308L might be a novel MYB protein and it might have unique biological functions.

Next, we tested how the expression of MdMYB308L responded to the cold stress. When apple seedlings were placed at 4°C, the expression of *MdMYB308L* showed increase starting from only 1 h after the treatment and the increase lasted until 9 h-12 h after the treatment (Figure 1a). We also set up a marker gene expression system to further examine the cold stress response of *MdMYB308L*. The promoter sequence of *MdMYB308L* (Figure S3) was cloned into pCAMBIA1391-GUS vector to generate the $Pro_{MdMYB308L}$::GUS construct, which was introduced into apple calli. Transgenic apple calli expressing the $Pro_{MdMYB308L}$::GUS construct clearly exhibited higher GUS activity after cold treatment at 4°C for 9 h compared with the control that was placed at 24°C (Figure 1b), indicating that cold stress triggered the expression of *MdMYB308L*. Furthermore, the MdMYB308L protein was also examined in response to cold treatment (4°C) using an *in vitro* protein degradation system. Purified MdMYB308L-GST fusion proteins were incubated with the total proteins extracted from wild-type apple calli with or without 4°C treatments. It seemed that MdMYB308L-GST protein was slowly degraded since 2 h after the incubation started, which could be blocked by the presence of MG132 (Figure 1c). However, the cold treatment seemed to have slowed down the degradation process of MdMYB308L-GST protein and improved its stability (Figure 1c). Taken together, these data suggest that MdMYB308L is responsive to cold stress at both transcriptional and post-transcriptional levels.

MdMYB308L plays a positive role in cold stress response

To explore the biological role of MdMYB308L in cold stress, its expression was modified by transforming an overexpressing construct (MdMYB308L-OX) or an antisense suppressing construct (MdMYB308L-Anti) into wild-type apple calli (Figure S4a). There was no growth difference observed among these three types of apple calli under normal growth condition at 24°C (Figure 2a-b, control). However, when these 8-day-old wild-type and transgenic apple calli were treated with cold conditions (4°C)

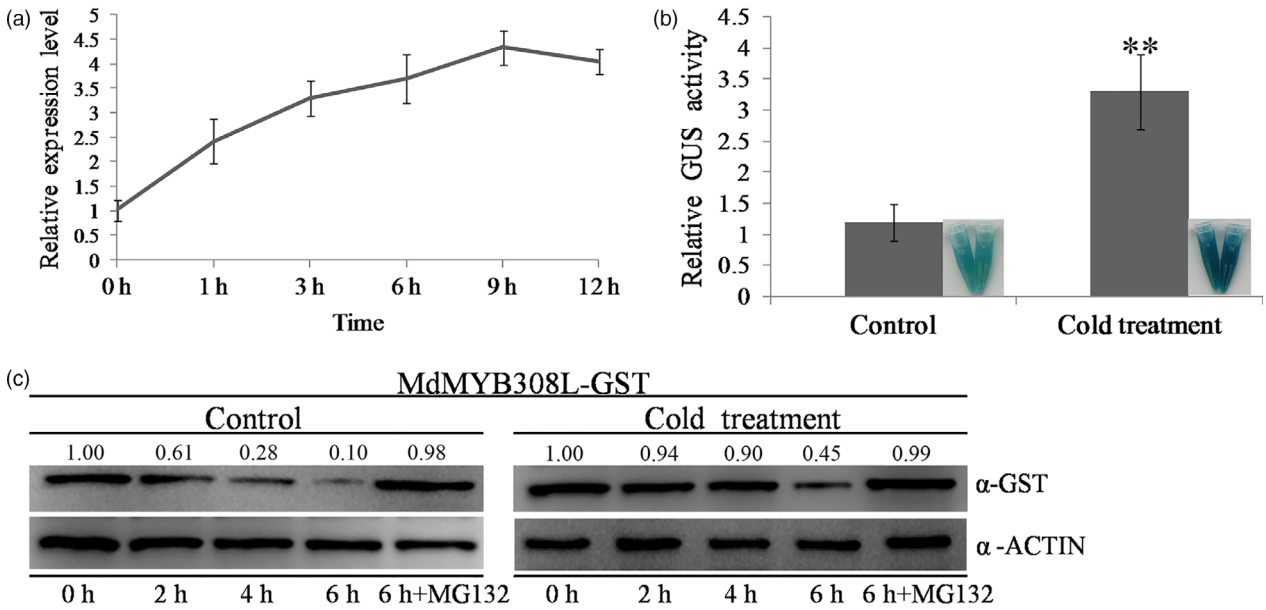

Figure 1 The expression pattern of MdMYB308L in response to cold stress (4°C treatment). (a) *MdMYB308L* gene expression detected by qRT-PCR analysis. The value for 0 h was set to 1. (b) GUS staining and relative GUS activity analysis of the *MdMYB308L* promoter expression construct $Pro_{MdMYB308L}$::GUS in transgenic apple calli. Control: GUS staining and activity analysis at 9 h at 24°C. Cold treatment: GUS staining and activity analysis at 9 h under cold stress. (c) Degradation of the MdMYB308L-GST fusion protein under cold stress. Total proteins extracted from wild-type apple calli with or without 4°C treatments and the inclusion of 100 μM MG132 were incubated with the purified MdMYB308L-GST fusion protein. The samples were collected at the indicated time. Control: 24°C; cold treatment: 4°C. ACTIN was used as internal reference. The relative intensity ratio between the GST and the ACTIN was shown. Each experiment was performed in three replicates. Error bars denote standard deviation. Significant differences were detected by *t*-test (**$P < 0.01$).

for 10 days, apple calli with the overexpression construct (MdMYB308L-OX) showed faster growth (Figure 2a) and higher fresh weight (Figure 2b) compared to the WT control. To the contrary, the apple calli carrying the antisense construct MdMYB308L-Anti showed the opposite phenotypes with slower growth (Figure 2a) and smaller fresh weight (Figure 2b). These observations indicate that MdMYB308L may play a role in the cold stress response. In this experiment, we also observed that the expressions of some cold-responsive genes were modified by the alteration of *MdMYB308L* expression. Among the eight genes tested, *MdCBF2*, *MdKIN1*, *MdRD29A* and *MdCOR47* showed the most dramatic increase of expressions in the MdMYB308L-OX calli and significant decrease of expressions in the MdMYB308L-Anti calli (Figure 2c; An *et al*., 2018). Meanwhile, *MdCBF1* and *MdCBF3* also showed significant increase in expressions in the MdMYB308L-OX calli, although to a much lesser extent (Figure 2c; An *et al*., 2018). These results suggest that MdMYB308L may execute its role in cold tolerance by regulating the expression of cold-responsive genes.

To verify the results in plants, the overexpression construct MdMYB308L-OX was transformed into wild-type *Arabidopsis* Col-0 seedlings and three independent lines with very high expressions of *MdMYB308L* (MdMYB308L-L1, MdMYB308L-L2 and MdMYB308L-L3) were selected (Figure S4b). Together with the Col-0 control, these transgenic *Arabidopsis* seedlings of 12-day-olds were treated at -4°C for 0.5 h by gradient cooling. As shown in Figure 2d and 2e, *MdMYB308L* overexpression seedlings exhibited the higher survival rate compared to the wild type after cold treatments, indicating that *MdMYB308L* plays a positive role in cold stress response.

Overexpression of *MdMYB308L* increases anthocyanin accumulation

Anthocyanin functions in cold tolerance by scavenging reactive oxygen species (Hernández *et al*., 2009; Winkel-Shirley, 2002), and MYB TFs play key roles in the regulation of anthocyanin biosynthesis (Allan *et al*., 2008; Dubos *et al*., 2010). To determine whether the modification of *MdMYB308L* expression has any impact on the biosynthesis of anthocyanin, anthocyanin contents were examined in 15-day-old apple calli treated with high light for 7 days. We found that MdMYB308L-OX calli accumulated significantly more anthocyanin and MdMYB308L-Anti calli accumulated less anthocyanin compared to the wild-type calli (Figure 3a-b). In addition, the anthocyanin biosynthesis-related genes *MdDFR*, *MdUF3GT*, *MdF3H* and *MdCHS* all showed significant increase of expressions in the MdMYB308L-OX calli, and *MdDFR* and *MdUF3GT* also showed decreased expressions in the MdMYB308L-Anti calli (Figure 3c; Figure S4a). These data suggest that MdMYB308L promotes anthocyanin accumulation by modulating the expression of anthocyanin biosynthesis-related genes.

In a parallel experiment, the MdMYB308L-OX and MdMYB308L-Anti constructs were transiently expressed in apple leaves using a vacuum pump (Figure S4c). The apple leaves were treated with high light for 5 days after transformation to induce the anthocyanin deposition. Consistently, overexpression of *MdMYB308L* promoted, but suppression of *MdMYB308L* reduced the accumulation of anthocyanin in these apple leaves (Figure 3d–e), confirming that MdMYB308L contributes to anthocyanin accumulation.

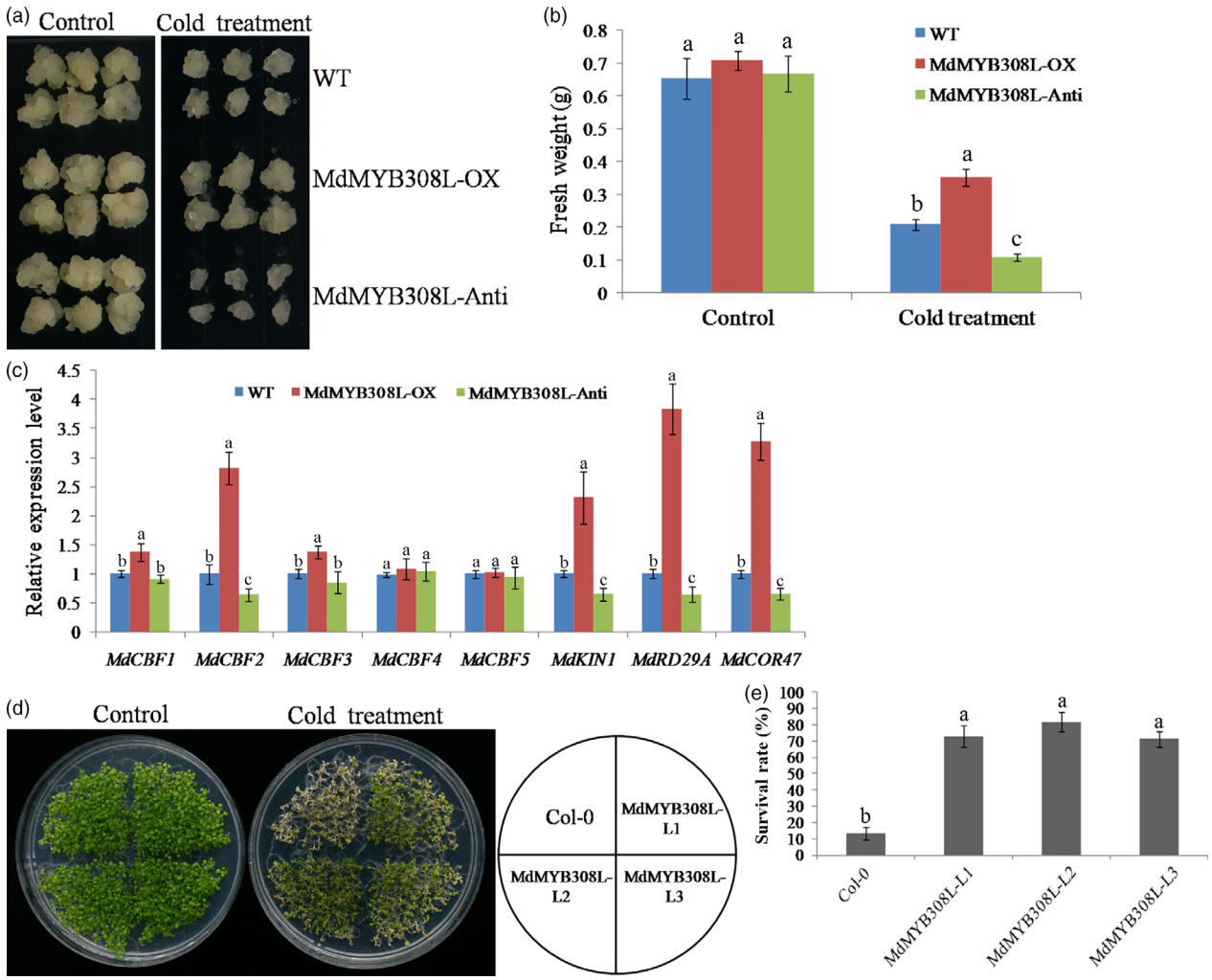

Figure 2 Phenotypes of *MdMYB308L* transgenic apple calli and *Arabidopsis* seedlings under cold stress. (a) Appearance of 8-day-old apple calli under control (24°C) and cold stress (4°C) conditions for 10 days. WT: wild-type; MdMYB308L-OX: *MdMYB308L*-overexpression; MdMYB308L-Anti: *MdMYB308L* antisense suppression. (b) The fresh weight of apple calli shown in (a). (c) Expressions of *MdCBFs* and their target genes in apple calli shown in (a). Quantitative real-time PCR analysis was performed in three biological replicates and three technical replicates. The value for WT was set to 1. (d) Appearance of control (Col-0) and *MdMYB308L* overexpression (MdMYB308L-L1, MdMYB308L-L2 and MdMYB308L-L3) *Arabidopsis* seedlings with or without (control) cold stress treatment. Plants were grown on MS plates at 22°C for 12 days before cold treatment at -4°C for 0.5 h. (e) Survival rate of *Arabidopsis* seedlings shown in (d). Each experiment was performed in three replicates. Error bars denoted standard deviation. Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

MdMYB308L interacts with MdbHLH33

A few of MYB TFs have been known to function in association with the bHLH proteins (Dubos *et al*., 2010; Jaakola, 2013; Zimmermann *et al*., 2004). Previous reports have revealed that an apple bHLH TF MdbHLH33 positively regulates both cold stress response and anthocyanin biosynthesis (Xu *et al*., 2017, 2018a,b). To elucidate whether MdMYB308L interacts with MdbHLH33, yeast two-hybrid (Y2H) assays were performed (Figure 4a). MdMYB308L-pGBD and MdbHLH33-pGAD were transformed into yeast cells. And the empty vectors were used as controls. The result showed that yeast cells expressing both MdMYB308L and MdbHLH33 grew normally in the selective medium (Figure 4a), indicating that MdMYB308L interacts with MdbHLH33 in yeast cells. In addition, pull-down assays were carried out using the fusion proteins MdMYB308L-GST and MdbHLH33-HIS (Figure 4b). The protein mixtures were purified using a glutathione purification kit. As shown in Figure 4b, MdbHLH33-HIS was pulled down by MdMYB308L-GST, while HIS alone did not, indicating that MdMYB308L interacts with MdbHLH33 *in vitro*. To provide more evidence for the interaction between MdMYB308L and MdbHLH33, we conducted bimolecular fluorescence complementation (BiFC) assays, by transforming MdMYB308L-YFP^C and MdbHLH33-YFP^N into onion epidermal cells for YFP fluorescence signals (Figure 4c). The result demonstrated that MdMYB308L interacted with MdbHLH33 in the nucleus. In addition, we found that MdMYB308L did not interact with MdbHLH3, a homologous gene of MdbHLH33 (Figure S3c). Taken together, these data reveal that MdMYB308L indeed interacts with MdbHLH33.

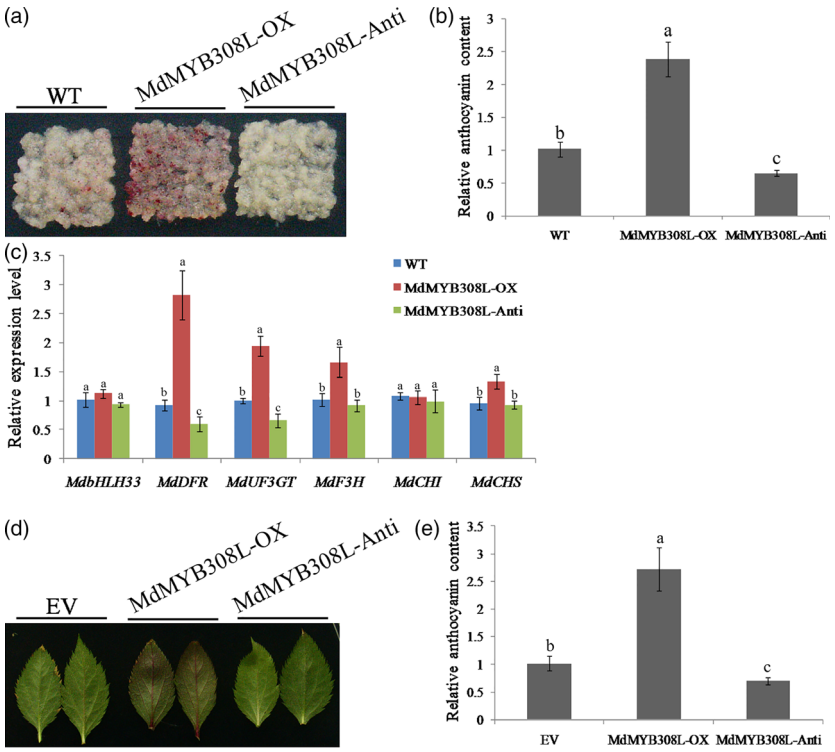

Figure 3 Anthocyanin accumulation in *MdMYB308L* transgenic apple calli and leaves. (a) Appearance of apple calli of 15-day-olds after highlight treatment for 7 days. WT: wild-type; MdMYB308L-OX: *MdMYB308L*-overexpression; MdMYB308L-Anti: *MdMYB308L* antisense suppression. (b) The relative anthocyanin contents of apple calli shown in (a). The value for WT was set to 1. (c) Relative expression levels of anthocyanin biosynthesis-related genes in apple calli shown in (a). Quantitative real-time PCR analysis was performed in three biological replicates and three technical replicates. The value for WT was set to 1. (d) Appearance of apple leaves treated with highlight for 5 days. EV: empty vector; MdMYB308L-OX: *MdMYB308L*-overexpression; MdMYB308L-Anti: *MdMYB308L* antisense suppression. (e) Relative anthocyanin contents of apple leaves shown in (d). The value for EV was set to 1. Each experiment was performed in three replicates. Error bars denoted standard deviation. Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

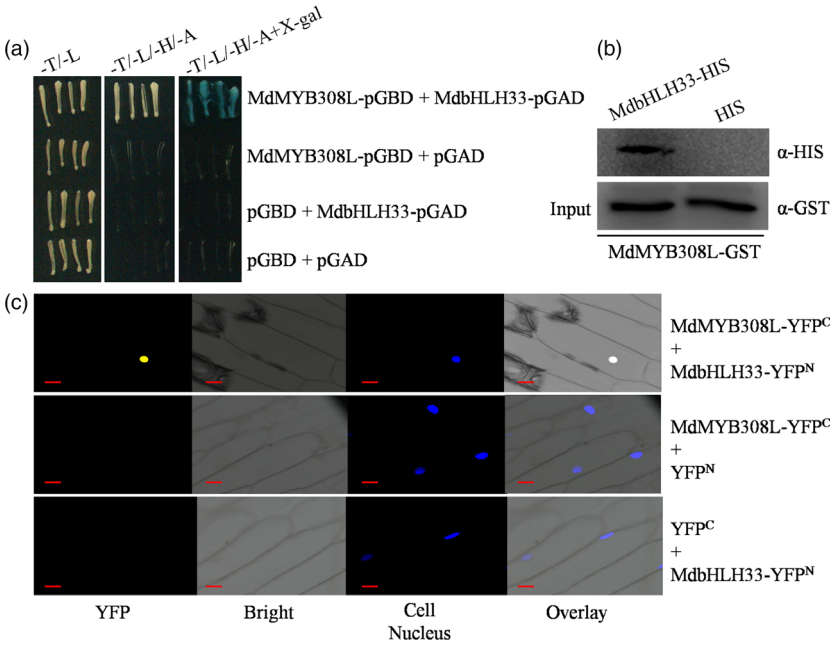

Figure 4 MdMYB308L interacts with MdbHLH33. (a) Yeast two-hybrid assays. The open reading frames of *MdMYB308L* and *MdbHLH33* were fused with pGBD and pGAD vectors, respectively. Transformed yeast cells were grown on SD-Trp/-Leu (-T/-L), SD-Trp/-Leu/-His/-Ade (-T/-L/-H/-A) or SD-Trp/-Leu/-His/-Ade supplementing X-gal (-T/-L/-H/-A+X-gal) media. (b) Pull-down assays. *E. coli*-expressed HIS or MdbHLH33-HIS proteins were incubated with a cobalt chelate affinity resin containing the immobilized glutathione-tagged MdMYB308L protein. The protein mixtures were purified using a glutathione purification kit. (c) Bimolecular fluorescence complementation assays. The open reading frames of *MdbHLH33* and *MdMYB308L* were fused to the N-terminal part of YFP and the C-terminal part of YFP, respectively. Bars = 10 μm.

MdMYB308L enhances the binding of MdbHLH33 to its target genes

In apple calli expressing either MdMYB308L-OX or MdMYB308L-Anti, the expression of *MdbHLH33* did not show any change (Figure 3c). Considering the direct interaction between MdMYB308L and MdbHLH33, we determined to investigate the relationship between these two proteins. Previous studies have demonstrated that MdbHLH33 promotes cold tolerance and anthocyanin biosynthesis by directly activating *MdCBF2* and *MdDFR* genes, respectively (Xu *et al*., 2017, 2018a,b). We verified these results in our study with a gel mobility shift assay using DNA probes carrying the LTR element within the *MdCBF2* promoter and the E-box within the *MdDFR* promoter (Figure 5a-b). Moreover, we found that, with the increased addition of MdMYB308L-GST proteins, the binding intensities of MdbHLH33 to the *MdCBF2* and *MdDFR* promoters increased (Figure 5a-b; Figures S5-S7). Alternatively, we constructed effectors and reporters to perform a luciferase assay (Figure 5c), which showed that the MdbHLH33 contributed to the increased LUC/REN activities of p*MdCBF2* and p*MdDFR*, and the LUC/REN activities further increased when MdMYB308L and MdbHLH33 were co-expressed (Figure 5d-e). These results suggest that MdMYB308L promotes the binding of MdbHLH33 to its target genes.

MdbHLH33 is essential for MdMYB308L-mediated cold tolerance and anthocyanin accumulation

To dissect the genetic interaction between MdMYB308L and MdbHLH33, we generated MdbHLH33 antisense suppressing plasmid (MdbHLH33-Anti) and transformed it into the *MdMYB308L*-overexpressing apple calli and leaves (Figure S4d-e). The transgenic materials were used for the cold tolerance and anthocyanin accumulation assays. Apple calli with altered expressions of MdMYB308L and/or MdbHLH33 did not show any growth difference under control conditions (Figure 6a-b). However, when the calli were placed at 4°C for cold treatments, MdbHLH33-Anti ones showed significantly slower growth (Figure 6a) and smaller fresh weight (Figure 6b), which was overcame by the co-expression of the MdMYB308L-OX construct (Figure 6a-b). Similarly, in both apple calli and apple leaf transient expression systems, the accumulation of anthocyanin was greatly induced by the overexpression of *MdMYB308L* (Figure 6c-f). When the two constructs were co-delivered, the anthocyanin levels were still elevated compared to the controls but significantly lowered than overexpression of *MdMYB308L* alone (Figure 6c-f). These results showed that suppression of *MdbHLH33* decreased MdMYB308L-promoted cold tolerance and anthocyanin accumulation, indicating that MdMYB308L regulates cold tolerance and anthocyanin accumulation partially depending on MdbHLH33.

MdMYB308L interacts with MdMIEL1

Our above results reveal that cold stress influences the stability of the MdMYB308L protein (Figure 1c). To further explore the post-transcriptional regulatory mechanism of MdMYB308L in response to cold stress, a yeast screening assay was performed using MdMYB308L-pGBD as the bait (Table S2). As a result, MdMIEL1 (GenBank accession number: MDP0000185659) was identified. Previous studies have shown that MdMIEL1 encodes a RING E3 ubiquitin ligase in apple and acts as a negative regulator in oxidative and salt stresses (An *et al*., 2017b), as well as anthocyanin accumulation (An *et al*., 2017a). To confirm the interaction between MdMYB308L and MdMIEL1 in Y2H assays, MdMYB308L-pGBD and MdMIEL1-pGAD were transformed into yeast cells. The result showed that yeast cells expressing both MdMYB308L and MdMIEL1 grew normally in the selective medium (Figure 7a), indicating that MdMYB308L interacts with MdMIEL1 in yeast cells. We then performed pull-down assays using fusion proteins MdMYB308L-GST and MdMIEL1-HIS. As shown in Figure 7b, MdMIEL1-HIS was detected with a HIS antibody in eluted solution, indicating that MdMYB308L interacts with MdMIEL1 *in vitro*. Furthermore, BiFC assays were carried out to provide another evidence for the interaction between MdMYB308L and MdMIEL1 by transforming MdMYB308L-YFP^C and MdMIEL1-YFP^N constructs into onion epidermal cells together or individually. The YFP fluorescence signals were only detected with the presence of both MdMYB308L-YFP^C and MdMIEL1-YFP^N constructs, indicating that MdMYB308L interacts with MdMIEL1 in the nucleus (Figure 7c).

MdMIEL1 is a repressor of cold tolerance and anthocyanin accumulation

To study the molecular function of MdMIEL1 in cold stress, we first examined the expression pattern of MdMIEL1 in response to cold stress (4°C). *MdMIEL1* was repressed when apple calli were exposed to cold stress (Figure 8a). In addition, the promoter sequence of *MdMIEL1* was inserted into pCAMBIA1391-GUS vector to generate the Pro$_{MdMIEL1}$::GUS reporter construct (Figure S8). The GUS activity of Pro$_{MdMIEL1}$::GUS transgenic apple calli was reduced significantly after cold treatment (4°C) (Figure 8b), indicating that cold stress repressed the expression of *MdMIEL1*. Furthermore, the MdMIEL1 protein level was examined in response to cold treatment (4°C) using an *in vitro* protein degradation system. The results showed that cold treatment accelerated the degradation of the MdMIEL1 protein (Figure 8c).

To explore the physiological role of MdMIEL1 in cold stress and anthocyanin accumulation, transgenic apple calli (MdMIEL1-OX: overexpression of *MdMIEL1*; MdMIEL1-Anti: suppression of *MdMIEL1*), *Arabidopsis* plants (MdMIEL1-L1, MdMIEL1-L2 and MdMIEL1-L3) and apple leaves (MdMIEL1-OX: overexpression of *MdMIEL1*; MdMIEL1-Anti: suppression of *MdMIEL1*) were generated (Figure S4f-h). In all testing systems, the overexpression of *MdMIEL1* significantly delayed plant material growth (Figure 9a-d) and the anthocyanin accumulation (Figure 9e-h) under cold stress conditions. The overexpression of *MdMIEL1* also decreased the anthocyanin biosynthesis in both apple calli and leaves, while the suppression of *MdMIEL1* expression showed the opposite effect (Figure 9e-h), which is consistent with our previous report (An *et al*., 2017a). These observations suggest that *MdMIEL1* is a repressor of both cold tolerance and anthocyanin accumulation.

MdMIEL1 ubiquitinates the MdMYB308L protein and accelerates its degradation

MIEL1 encodes an E3 ubiquitin ligase, and MYB30, MYB98 and MYB1 have been verified as ubiquitination substrates of MIEL1 (An *et al*., 2017a; Lee and Seo, 2016; Marino *et al*., 2013). Since MdMYB308L and MdMIEL1 proteins interacted with each other directly (Figure 7), and the overexpression of them showed the opposite phenotypes for cold stress response and anthocyanin accumulation (Figures 6 and 9), we examined whether MdMIEL1 regulates the protein stability of MdMYB308L by ubiquitination modification. To test the hypothesis, we performed an ubiquitination detection assay *in vitro*. The ubiquitination pattern of MdMYB308L-GST was detected by anti-GST probing in the

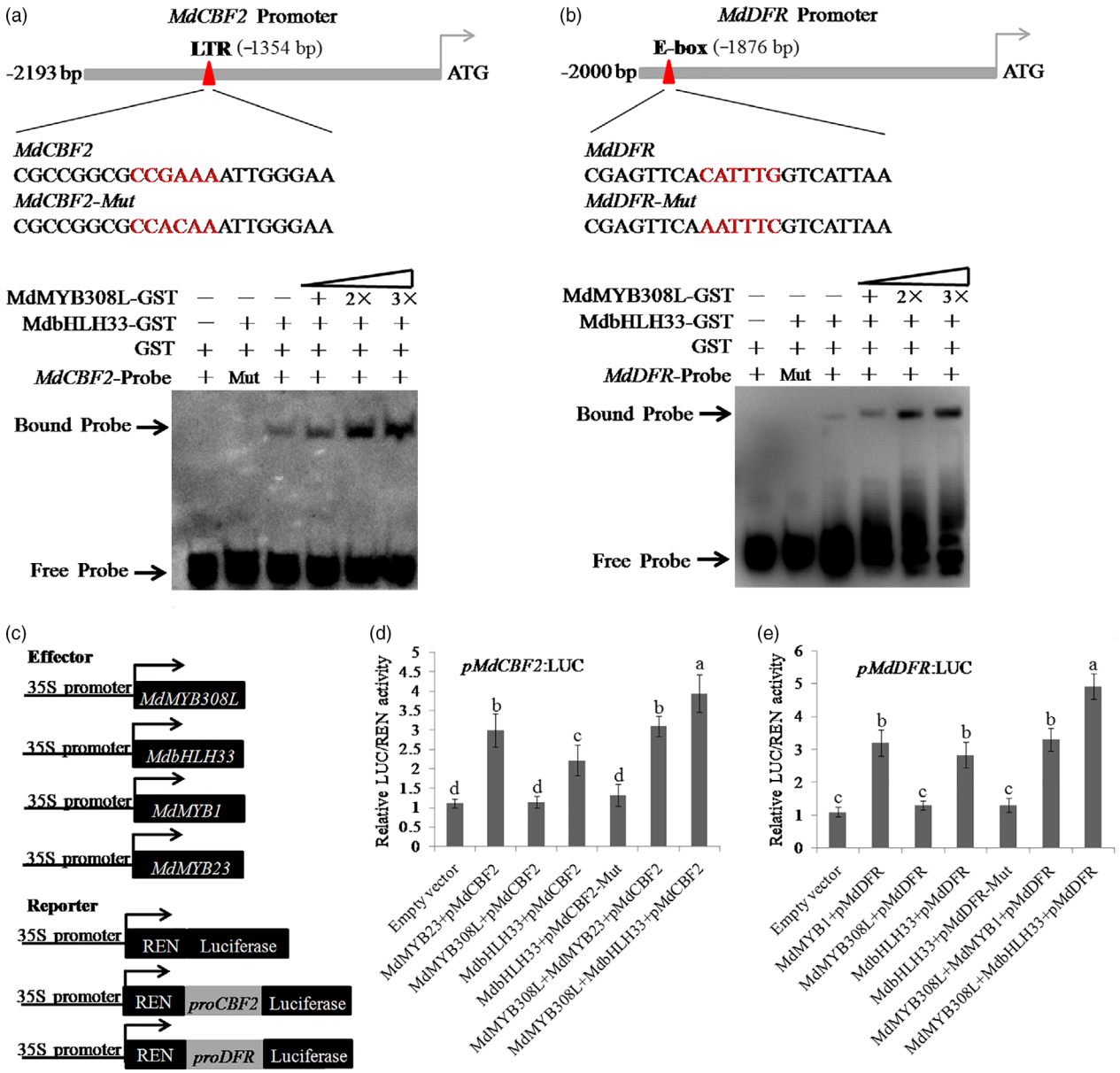

Figure 5 MdMYB308L modifies the binding of MdbHLH33 to its target genes. (a) and (b) Electrophoretic mobility shift assays. The '-' sign represents the absence of relevant proteins; '+' represents the presence of relevant probes or proteins; '2 × ' and '3 × ' represents increased protein levels. *MdCBF2*-Mut is the mutant form of *MdCBF2*, in which the 5′–CCGAAA–3′ motif was replaced with 5′–CCACAA–3′. *MdDFR*-Mut is the mutant form of *MdDFR*, in which the 5′–CATTTG–3′ motif was replaced with 5′–AATTTC–3′. Biotin-labelled probes were incubated with MdMYB308L-GST or MdbHLH33-GST protein, and the free and bound probes were separated on an acrylamide gel. The GST protein was used to ensure an equal quantity of proteins. (c) Schematic representation of the constructs (effector and reporter vectors) used for the luciferase reporter lines. (d) and (e) LUC/REN activities of constructs p*MdCBF2*:LUC (d) and p*MdDFR*:LUC (e) when co-transformed with different constructs. Empty vector was used as the reference. Empty vector: 62SK+LUC; MdMYB23 + p*MdCBF2*: MdMYB23-62SK+p*MdCBF2*-LUC; MdMYB308L+p*MdCBF2*: MdMYB308L-62SK+p*MdCBF2*-LUC; MdbHLH33 + p*MdCBF2*: MdbHLH33-62SK+p*MdCBF2*-LUC; MdbHLH33 + p*MdCBF2*-Mut: MdbHLH33-62SK+p*MdCBF2*-Mut-LUC; MdMYB308L+MdMYB23 + p*MdCBF2*: MdMYB308L-62SK+MdMYB23-62SK+p*MdCBF2*-LUC; MdMYB308L+MdbHLH33 + p*MdCBF2*: MdMYB308L-62SK+MdbHLH33-62SK+p*MdCBF2*-LUC; MdMYB1 + p*MdDFR*: MdMYB1-62SK+p*MdDFR*-LUC; MdMYB308L+p*MdDFR*: MdMYB308L-62SK+p*MdDFR*-LUC; MdbHLH33 + p*MdDFR*: MdbHLH33-62SK+p*MdDFR*-LUC; MdbHLH33 + p*MdDFR*-Mut: MdbHLH33-62SK+p*MdDFR*-Mut-LUC; MdMYB308L+MdMYB1 + p*MdDFR*: MdMYB308L-62SK+MdMYB1-62SK+p*MdDFR*-LUC; MdMYB308L+MdbHLH33 + p*MdDFR*: MdMYB308L-62SK+MdbHLH33-62SK+p*MdDFR*-LUC; each experiment was performed in three replicates. Error bars denoted standard deviation. Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

presence or absence of ATP, ubiquitin, E1, E2 and MdMIEL1-HIS, to test the E3 ubiquitin ligase activity of MdMIEL1-HIS (Figure 10a). The result demonstrated that MdMYB308L-GST could be ubiquitinated by MdMIEL1-HIS in the present of all necessary components (Figure 10a). In addition, an *in vivo* ubiquitination detection assay was performed using MdMYB308L-MYC and

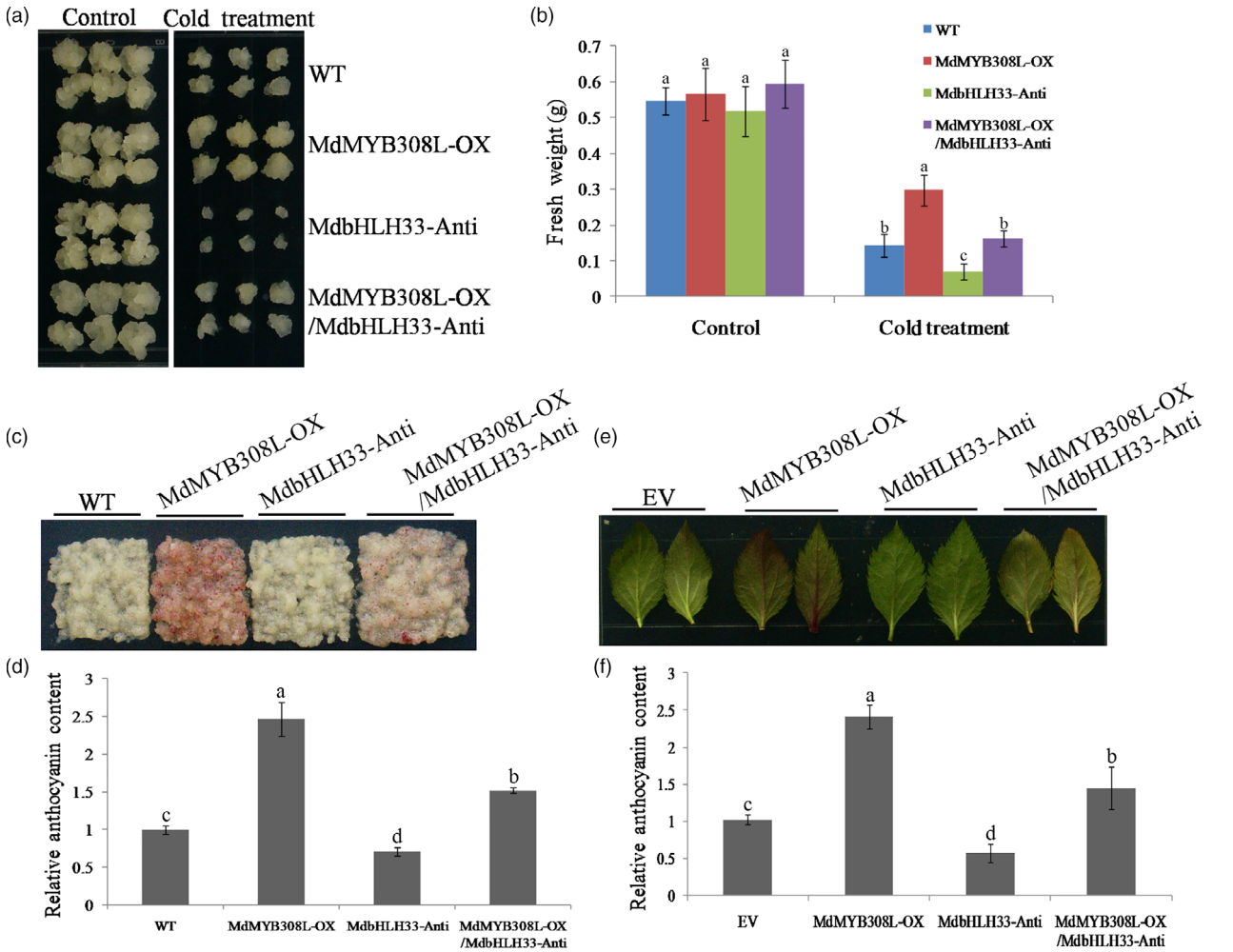

Figure 6 Phenotypes of apple calli and leaves with modified expressions of *MdMYB308L* and *MdbHLH33*. (a) Appearance of 8-day-old apple calli grown at 24°C (control) or 4°C (cold treatment) for 10 days. WT: wild-type; MdMYB308L-OX: *MdMYB308L*-overexpression; MdbHLH33-Anti: *MdbHLH33* antisense suppression; MdMYB308L-OX/MdbHLH33-Anti: suppression of *MdbHLH33* in the background of *MdMYB308L*-overexpression. (b) The fresh weights of apple calli shown in (a). (c) Appearance of apple calli (15-day-old) used in (a) when treated with highlight for 5 days. (d) The relative anthocyanin content of apple calli shown in (c). The value for WT was set to 1. (e) Appearance of apple leaves expressing different constructs after highlight treatment for 5 days. EV: empty vector; MdMYB308L-OX: *MdMYB308L*-overexpression; MdbHLH33-Anti: *MdbHLH33* antisense suppression; MdMYB308L-OX/MdbHLH33-Anti: suppression of *MdbHLH33* in the background of *MdMYB308L*-overexpression. (f) The relative anthocyanin content of apple leaves shown in (e). The value for EV was set to 1. Each experiment was performed in three replicates. Error bars denoted standard deviation. Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

MdMYB308L-MYC/MdMIEL1-OX apple calli. Protein gel blot analysis showed that MdMIEL1 enhanced the ubiquitination modification of the MdMYB308L-MYC protein (Figure 10b). These findings suggest that MdMIEL1 ubiquitinates the MdMYB308L protein *in vitro* and *in vivo*.

We then carried out *in vitro* protein degradation assays to assess whether MdMIEL1 affects the stability of the MdMYB308L protein. Total proteins extracted from wild-type and *MdMIEL1* transgenic apple calli were incubated with the purified MdMYB308L-GST fusion protein. As expected, overexpression of *MdMIEL1* accelerated the degradation of the MdMYB308L-GST protein, while suppression of the *MdMIEL1* expression showed the opposite effect (Figure 10c). Collectively, these results illustrated that MdMIEL1 accelerates MdMYB308L protein degradation by modulating the ubiquitination modification of MdMYB308L.

MdMIEL1 negatively regulates MdMYB308L-promoted cold tolerance and anthocyanin accumulation

To provide genetic evidence for the above conclusion, the *MdMIEL1* overexpressing plasmid (MdMIEL1-OX) and antisense suppressing plasmid (MdMIEL1-Anti) were transformed into *MdMYB308L*-overexpressing apple calli, *Arabidopsis* seedlings and apple leaves (Figure S4f-h). We then examined the cold tolerance and anthocyanin accumulation of co-transformed plant materials. In apple calli, the cold-induced suppression of growth was smaller in the *MdMYB308L* overexpression materials compared to the WT and the calli overexpressing both *MdMYB308L* and *MdMIEL1* (Figure 11a-b). However, the calli with both the *MdMYB308L* overexpression construct and the *MdMIEL1* suppression construct showed the best growth under cold condition among the four different plant materials (Figure 11a-b). In

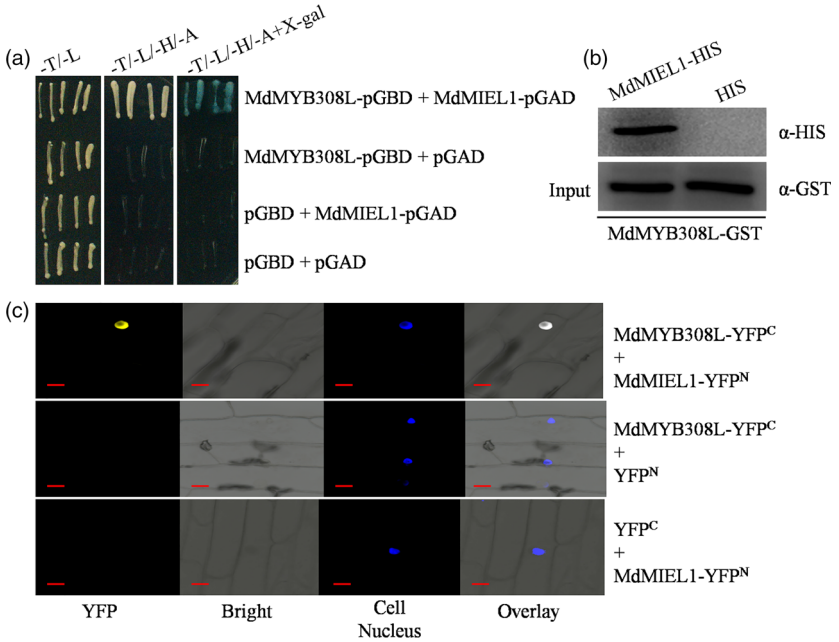

Figure 7 MdMYB308L interacts with MdMIEL1. (a) Yeast two-hybrid assays. The open reading frames of *MdMYB308L* and *MdMIEL1* were fused with pGBD and pGAD vectors, respectively. Transformed yeast cells were grown on SD-Trp/-Leu (-T/-L), SD-Trp/-Leu/-His/-Ade (-T/-L/-H/-A) or SD-Trp/-Leu/-His/-Ade supplementing X-gal (-T/-L/-H/-A+X-gal) media. (b) Pull-down assays. *E. coli*-expressed HIS or MdMIEL1-HIS proteins were incubated with a cobalt chelate affinity resin containing the immobilized glutathione-tagged MdMYB308L protein. The protein mixtures were purified using a glutathione purification kit. (c) Bimolecular fluorescence complementation assays. The open reading frames of *MdMIEL1* and *MdMYB308L* were fused to the N-terminal part of YFP and the C-terminal part of YFP, respectively. Bars = 10 μm.

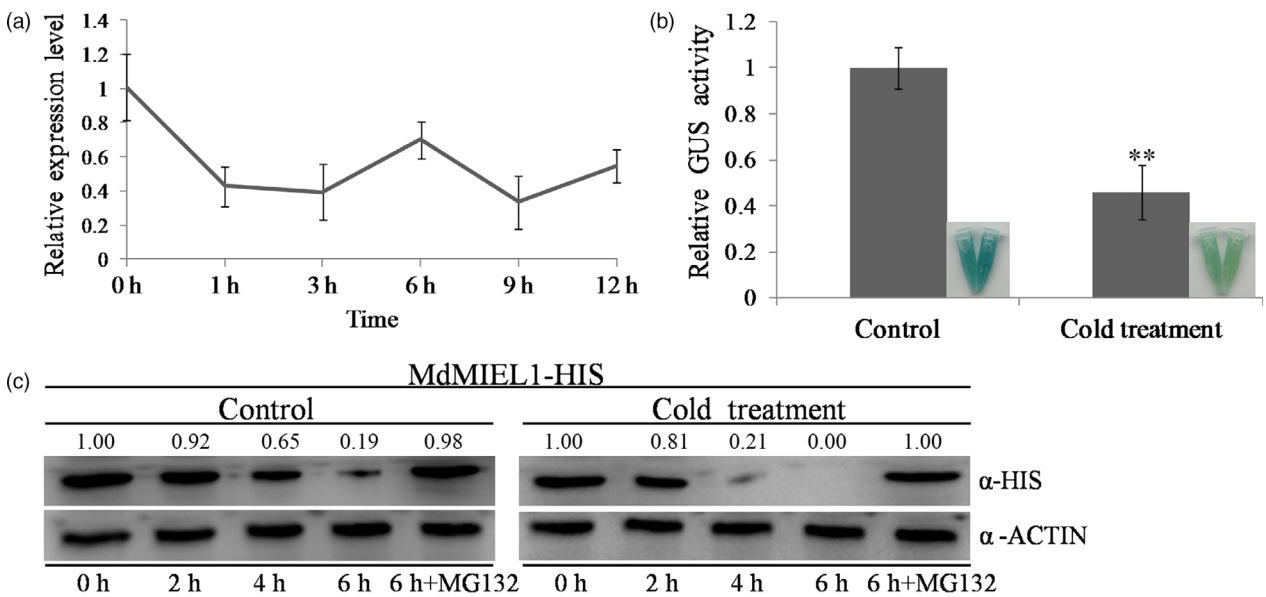

Figure 8 The expression pattern of MdMIEL1 in response to cold stress (4°C treatment). (a) *MdMIEL1* gene expression detected by qRT-PCR analysis. Experiments were performed in three biological replicates and three technical replicates. The value for 0 h was set to 1. (b) GUS staining and relative GUS activity analysis of the *MdMIEL1* promoter expression construct $Pro_{MdMIEL1}$::GUS in transgenic apple calli. Control: GUS staining and activity analysis at 9 h at 24°C. Cold treatment: GUS staining and activity analysis at 9 h under cold stress. (c) Degradation of the MdMIEL1-HIS fusion protein under cold stress. Total proteins extracted from wild-type apple calli with or without 4°C treatments and the inclusion of 100 μM MG132 were incubated with the purified MdMIEL1-HIS fusion protein. The samples were collected at the indicated time. Control: 24°C; cold treatment: 4°C. ACTIN was used as internal reference. The relative intensity ratio between the HIS and the ACTIN was shown. Each experiment was performed in three replicates. Error bars denoted standard deviation. Significant differences were detected by *t*-test (**$P < 0.01$).

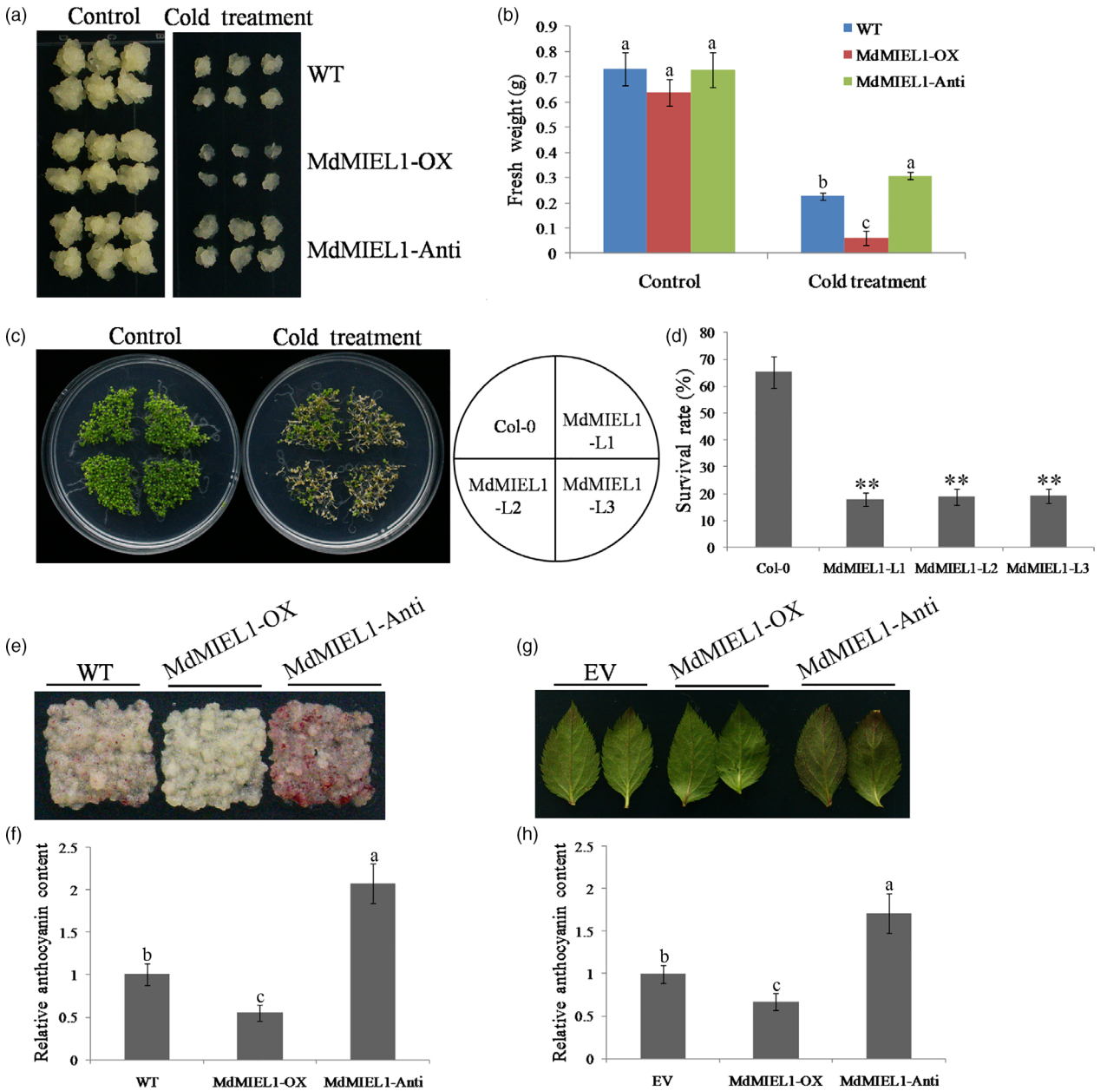

Figure 9 Phenotypes of *MdMIEL1* transgenic apple calli, *Arabidopsis* seedlings and apple leaves. (a) Appearance of 8-day-old apple calli under control (24°C) and cold stress (4°C) conditions for 10 days. WT: wild-type; MdMIEL1-OX: *MdMIEL1*-overexpression; MdMIEL1-Anti: *MdMIEL1* antisense suppression. (b) The fresh weight of apple calli shown in (a). (c) The appearance of control (Col-0) and *MdMIEL1* overexpression (MdMIEL1-L1, MdMIEL1-L2 and MdMIEL1-L3) *Arabidopsis* seedlings with or without (control) cold stress treatment. Plants were grown on MS plates at 22°C for 12 days before cold treatment at −4°C for 10 min. (d) Survival rate of *Arabidopsis* seedlings shown in (c). (e) Appearance of apple calli (15-day-old) used in (a) when treated with highlight for 7 days. WT: wild-type; MdMIEL1-OX: *MdMIEL1*-overexpression; MdMIEL1-Anti: *MdMIEL1* antisense suppression. (f) The relative anthocyanin content of apple calli shown in (e). The value for WT was set to 1. (g) Appearance of apple leaves expressing different constructs after highlight treatment for 5 days. EV: empty vector; MdMIEL1-OX: *MdMIEL1*-overexpression; MdMIEL1-Anti: *MdMIEL1* antisense suppression. (h) The relative anthocyanin content of apple leaves shown in (g). The value for EV was set to 1. Each experiment was performed in three replicates. Error bars denoted standard deviation. Significant differences were detected by *t*-test (**$P < 0.01$). Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

Arabidopsis young seedlings, a similar pattern was observed. While overexpression of *MdMYB308L* helped the plant growth under cold condition, seedlings overexpressing both *MdMYB308L* and *MdMIEL1* behaved similarly to the Col-0 control with cold stress (Figure 11c-d).

In a similar manner, apple calli overexpressing *MdMYB308L* showed significant increase in anthocyanin accumulation, but co-expression of both *MdMYB308L* and *MdMIEL1* at the same showed less increase in anthocyanin accumulation (Figure 11e-f). However, suppressing the expression of *MdMIEL1* in the

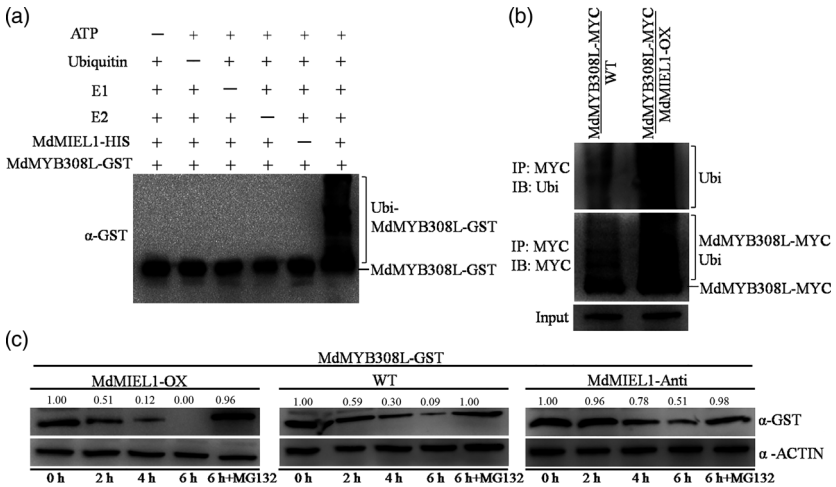

Figure 10 MdMYB308L is an ubiquitination substrate of MdMIEL1. (a) MdMIEL1 ubiquitinates MdMYB308L *in vitro*. MdMIEL1-HIS was tested for E3 ubiquitin ligase activity in the presence and absence of ATP, ubiquitin, E1, E2, MdMIEL1-HIS and MdMYB308L-GST. The protein gel blot was analysed using a GST antibody. (b) MdMIEL1 ubiquitinates MdMYB308L *in vivo*. MdMYB308L-MYC was immunoprecipitated using MYC antibody from the two transgenic apple calli (MdMYB308L-MYC and MdMYB308L-MYC/MdMIEL1-OX). Immunoblotting using an ubiquitin antibody was shown on the top and using a MYC antibody at below. (c) MdMIEL1 promotes the degradation of MdMYB308L-GST protein *in vitro*. Total proteins extracted from wild-type and transgenic apple calli with or without 100 μM MG132 treatment were incubated with the purified MdMYB308L-GST fusion protein. The samples were collected at the indicated time. ACTIN was used as internal reference. The relative intensity ratio between the GST and the ACTIN was shown.

MdMYB308L overexpression background increased the anthocyanin deposition even more dramatically (Figure 11e-f). The same trend of anthocyanin accumulation was observed in apple leaves (Figure 11g-h). These findings demonstrate that MdMIEL1 negatively regulates MdMYB308L-promoted cold tolerance and anthocyanin accumulation.

Discussion

Cold stress affects plant growth and development. In agricultural production, cold stress can cause crop freezing damage and large area of yield reduction and even lead to crop failure under severe circumstances (Thakur *et al*., 2010). As an important economic crop, apple production is also affected by cold stress. Therefore, it is of great significance to study the cold stress response mechanism of apple for improving apple yield and farmers' income. We previously performed transcriptome analyses to identify the cold-responsive MYB TFs, in which an apple MYB TF MdMYB23 was characterized to positively regulate cold tolerance (An *et al*., 2018). Here, we investigated the cold response mechanism of another MYB TF MdMYB308L (GenBank accession number: MDP0000950559), whose induction by cold was second only to MdMYB23 (Table S1; An *et al*., 2018). Our present data demonstrate that MdMYB308L is a positive regulator of cold tolerance and anthocyanin accumulation and functions in association with MdbHLH33 in apple. In addition, MdMYB308L is confirmed as a target protein of MdMIEL1 and undergoes MdMIEL1-mediated ubiquitination degradation.

MdMYB308L interacts with MdbHLH33 to regulate cold tolerance and anthocyanin accumulation

Cold stress induces the reactive oxygen species production and anthocyanin accumulation (Jaakola, 2013; Suzuki and Mittler, 2006). In return, anthocyanin contributes to reactive oxygen species scavenging and improved cold tolerance (Hernández *et al*., 2009; Winkel-Shirley, 2002). Multiple types of TFs play key roles in the regulation of both cold stress response and anthocyanin accumulation including MYB TFs (Allan *et al*., 2008; Chinnusamy *et al*., 2007; Dubos *et al*., 2010; Li *et al*., 2015; Liu *et al*., 2015). For example, in apple, MdMYB23, MdMYB88 and MdMYB124 positively regulate both cold tolerance and flavonoids accumulation (An *et al*., 2018; Xie *et al*., 2018), whereas MdMYB15L is a negative regulator of both cold tolerance and anthocyanin accumulation (Xu *et al*., 2018a,b). In the present study, a cold-induced MYB TF MdMYB308L was isolated and overexpression of *MdMYB308L* improved cold tolerance and anthocyanin biosynthesis (Figures 1–3). It is acknowledged that MYBs-bHLHs-WD-repeat protein complex play a central role in the regulation of anthocyanin biosynthesis (Jaakola, 2013; Xu *et al*., 2015). Previous reports have revealed that an apple bHLH TF MdbHLH33 regulates both cold stress response and anthocyanin biosynthesis (Xu *et al*., 2017, 2018a, b). A series of physiological and biochemical data indicated that MdMYB308L physically interacted with MdbHLH33 (Figure 4), and MdMYB308L improved cold tolerance and anthocyanin accumulation by enhancing the binding of MdbHLH33 to its downstream target genes (Figures 5, 6). These findings indicate that MdMYB308L is a novel cold-responsive gene, which functions through a 'MYB-bHLH' module.

MdMIEL1 negatively regulates cold tolerance and anthocyanin accumulation by degrading MdMYB308L

Since the MdMYB308L protein stability was regulated by cold treatments (Figure 1c), the post-transcriptional regulatory mechanism of MdMYB308L was explored here. An apple RING ubiquitin ligase MdMIEL1 was identified as an MdMYB308L-interacting protein (Figure 7), whose function was characterized previously (An *et al*., 2017a,b). *Arabidopsis* MIEL1 negatively regulates plant defence and ABA signalling by degrading MYB30 and MYB96 proteins, respectively (Lee and Seo, 2016; Marino *et al*., 2013). *Arabidopsis* MIEL1 also inhibits the biosynthesis of stem cuticular wax (Gil *et al*., 2017). In rice, *OsSRFP1*, the

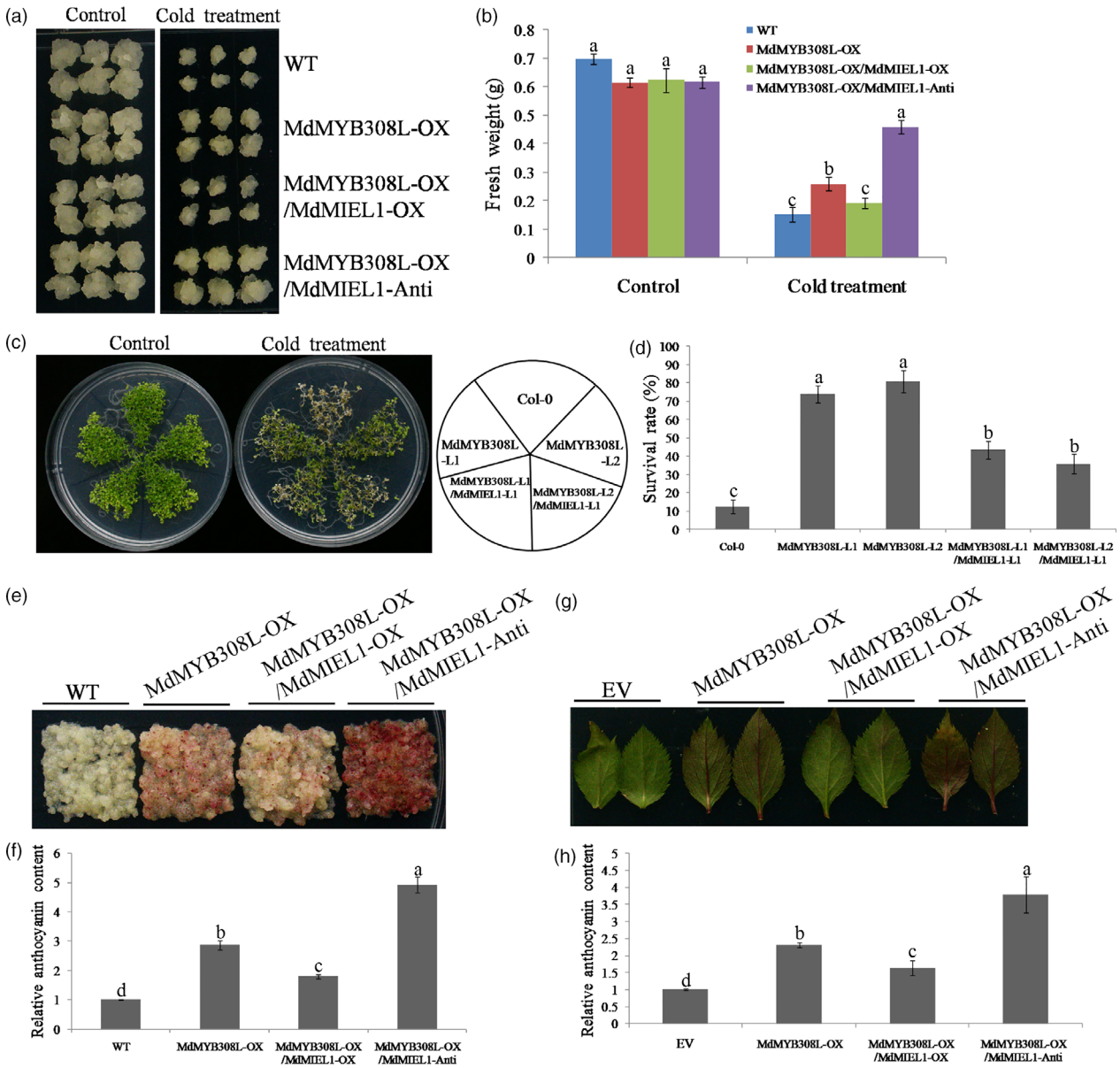

Figure 11 MdMIEL1 decreases MdMYB308L-promoted cold tolerance and anthocyanin accumulation. (a) Appearance of apple calli of 8-day-olds expressing different constructs under control condition (24°C) or cold treatment condition (4°C) for 10 days. WT: wild-type; MdMYB308L-OX: *MdMYB308L*-overexpression; MdMYB308L-OX/MdMIEL1-OX: overexpression of *MdMIEL1* in the background of *MdMYB308L*-overexpression; MdMYB308L-OX/MdMIEL1-Anti: suppression of *MdMIEL1* in the background of *MdMYB308L*-overexpression. (b) The fresh weight of apple calli shown in (a). (c) Appearance of wild-type (Col-0) and transgenic *Arabidopsis* seedlings under control condition or cold treatment condition. *Arabidopsis* seedlings were grown on MS plates at 22°C for 12 days before being treated at −4°C for 0.5 h. MdMYB308L-L1 and MdMYB308L-L2: *MdMYB308L*-overexpression *Arabidopsis* seedlings. MdMYB308L-L1/MdMIEL1-L1 and MdMYB308L-L2/MdMIEL1-L1: *MdMIEL1* and *MdMYB308L* co-transformed *Arabidopsis* seedlings. Control: no treatment; cold treatment: grow at 4°C for 2 days, followed by cold treatment at −4°C for 0.5 h. (d) Survival rate of *Arabidopsis* seedlings after cold stress treatment. (e) Appearance of 15-day-old apple calli treated with highlight for 5 days. (f) The relative anthocyanin content of apple calli shown in (e). The value for WT was set to 1. (g) Appearance of apple leaves treated with highlight for 5 days. EV: empty vector; MdMYB308L-OX: *MdMYB308L*-overexpression; MdMYB308L-OX/MdMIEL1-OX: overexpression of *MdMIEL1* in the background of *MdMYB308L*-overexpression; MdMYB308L-OX/MdMIEL1-Anti: suppression of *MdMIEL1* in the background of *MdMYB308L*-overexpression. (h) The relative anthocyanin content of apple leaves shown in (g). The value for EV was set to 1. Each experiment was performed in three replicates. Error bars denoted standard deviation. Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

homologous gene of *MIEL1*, is involved in multiple abiotic stress tolerance responses to salt, cold and oxidative stresses (Fang *et al.*, 2015). Our previous studies in apple have shown that MdMIEL1 negatively regulates oxidative and salt stresses (An *et al.*, 2017b) and inhibits the anthocyanin accumulation by degrading MdMYB1 (An *et al.*, 2017a). Here, we found that cold stress inhibited the expression of MdMIEL1 (Figure 8), and MdMIEL1 acted as a repressor of cold tolerance and anthocyanin accumulation by degrading the MdMYB308L protein (Figures 9–11). Taken together, our data reveal that MYB308L is a specific

ubiquitination target of MIEL1, and MIEL1 may mediate plant growth and development by regulating different MYB proteins.

In addition, MdMIEL1 seems to undergo an ubiquitination modification in response to cold stress (Figure 8c), similar as MdMYB308L (Figure 1c), which indicates that E3 ligases may also undergo the ubiquitination modification (Serino and Xie, 2013). And this observation may be indicative of the connector role of the E3 ligases in response to external stresses and regulating their downstream substrates. However, which E3 ubiquitin ligase regulates the ubiquitination of the MdMIEL1 protein remains unknown and it will be an interesting research topic in the future.

'MIEL1-MYB308L-bHLH33' module is a novel dynamic cold stress response mechanism

Post-translational modifications, such as ubiquitination, sumoylation and phosphorylation, affect the plant cold stress response. The E3 ubiquitin ligase HOS1 negatively regulates plant cold response by degrading the ICE1 protein in *Arabidopsis* (Dong *et al*., 2006). An apple BTB protein MdBT2 mediates the ubiquitination and degradation of MdMYB23 to suppress cold tolerance (An *et al*., 2018). SIZ1 modulates sumoylation of ICE1 to modify the *Arabidopsis* freezing tolerance (Miura *et al*., 2007). In phosphorylation modification, *Arabidopsis* OPEN STOMATA 1 (OST1) and MAP kinases regulate the stability of ICE1 to mediate plant cold tolerance (Ding *et al*., 2015; Li *et al*., 2017a,b; Liu and Zhou, 2018; Zhao *et al*., 2017). Our current results show that apple MdMIEL1 promotes the ubiquitination and degradation of the MdMYB308L protein, which leads to decreases in MdMYB308L-activated expressions of *MdCBF2* and *MdDFR*, thus negatively regulates cold tolerance and anthocyanin accumulation. When exposed to cold stress, the transcription of *MdMYB308L* is induced, but the expression of MdMIEL1 is repressed at both transcriptional and post-transcriptional levels. This in turn releases the inhibition effect of MdMIEL1 on MdMYB308L. As a result, the accumulation of MdMYB308L promotes the expressions of *MdCBF2* and *MdDFR* through its interaction with MdbHLH33, which leads to increased cold tolerance and anthocyanin accumulation (Figure 12). The dynamic regulatory module of 'MIEL1-MYB308L-bHLH33' shows the flexibility of cold stress response mechanism. We propose that 'MIEL1-MYB308L-bHLH33' signalling regulatory mechanism may play an important role in coordinating the cold stress response with other response mechanisms.

Experimental procedures

Plant materials and growth conditions

Apple calli (*Malus domestica*, 'Orin'), leaves of apple tissue culture seedlings (*Malus domestica*, 'GL3') and *Arabidopsis* seedlings (*Arabidopsis thaliana*, 'Col-0') were used for the present studies. Apple calli were grown at 24°C under dark conditions, and subcultured at a 20-day interval. Apple tissue culture seedlings were grown at 24°C for a 16-h light/8-h dark cycle and subcultured at a 30-day interval. *Arabidopsis* seedlings were grown at 22°C for a 16-h light/8-h dark cycle.

Phylogenetic tree and sequence alignment

The MYB308L protein sequences of 16 different species and MYB proteins from different species were obtained from the NCBI database. Phylogenetic tree was constructed with MEGA 5.0. Sequence alignment was performed using DNAMAN.

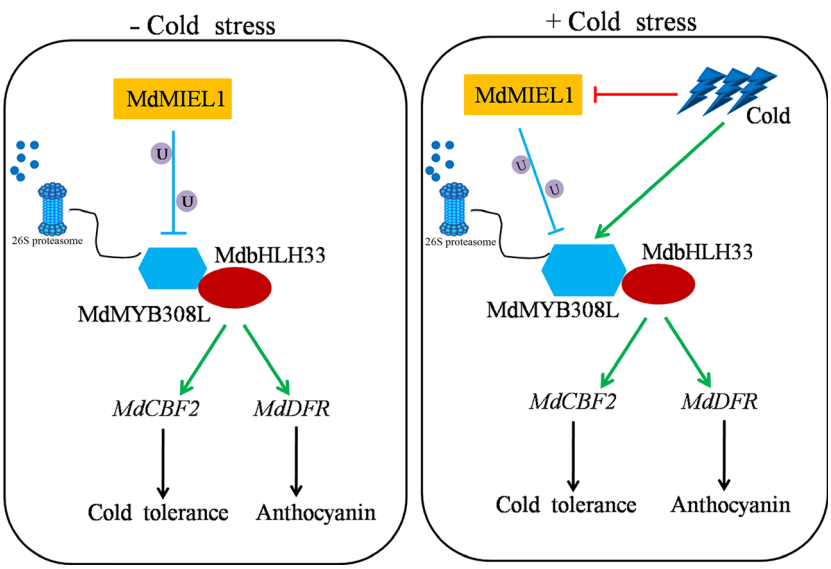

Figure 12 A working model illustrating that MdMYB308L functions in cold stress response and anthocyanin accumulation. MdMYB308L interacts with MdbHLH33 to increase its transcriptional activity and enhance its binding to the *MdCBF2* and *MdDFR* promoters, thus promoting cold tolerance and anthocyanin accumulation. In the absence of cold stress, MdMIEL1 interacts with MdMYB308L to ubiquitinate and degrade it, thus negatively regulating MdMYB308L-promoted cold tolerance and anthocyanin accumulation. On one hand, cold stress up-regulates the transcription of *MdMYB308L*, which promotes the cold tolerance and anthocyanin accumulation. On the other hand, cold stress inhibited MdMIEL1 expression, thus to release the MdMIEL1-enhanced protein degradation of MdMYB308L, which also contributes to cold tolerance and anthocyanin accumulation. 26S proteasome represents that MdMYB308L undergoes 26S proteasome-mediated degradation by MdMIEL1. The green line represents transcriptional regulation. The blue line represents post-translational regulation. The red line represents transcriptional and post-translational regulations.

Plasmid construction and genetic transformation

The promoter sequences of *MdMYB308L* and *MdMIEL1* genes were inserted into pCAMBIA1391-GUS vector to generate $Pro_{MdMYB308L}$::GUS and $Pro_{MdMIEL1}$::GUS. The promoter sequences of *MdCBF2* and *MdDFR* genes were inserted into pGreen0800-LUC to generate p*MdCBF2*-LUC and p*MdDFR*-LUC. The open reading frame (ORF) of *MdMYB308L*, *MdbHLH33* or *MdMIEL1* was cloned into pGAD or pGBD vector to generate MdMYB308L-pGBD, MdbHLH33-pGAD and MdMIEL1-pGAD. The ORF of *MdMYB308L*, *MdbHLH33* or *MdMIEL1* was cloned into pGEX4T-1 or pET32a vector to generate MdMYB308L-pGEX4T-1, MdbHLH33-pET32a, MdbHLH33-pGEX4T-1 and MdMIEL1-pET32a. The ORF of *MdMYB308L*, *MdbHLH33* or *MdMIEL1* was cloned into YFP^C or YFP^N vector to generate MdMYB308L-YFP^C, MdbHLH33-YFP^N and MdMIEL1-YFP^N. The ORF of *MdMYB308L*, *MdbHLH33*, *MdMYB23* or *MdMYB1* was cloned into pCXSN-MYC or pGreen 62-SK to generate MdMYB308L-MYC, MdMYB308L-62SK, MdbHLH33-62SK, MdMYB23-62SK and MdMYB1-62SK. The ORF of *MdMIEL1* was cloned into pRI101 to generate MdMIEL1-OX. The fragments of *MdMYB308L*, *MdbHLH33* and *MdMIEL1* were cloned into pCXSN to generate MdMYB308L-Anti, MdbHLH33-Anti and MdMIEL1-Anti. Primers used in this study are listed in Table S3.

The transgenic apple calli were obtained as described previously (An *et al*., 2018). To generate transient transgenic apple leaves, leaves were incubated with *Agrobacterium* carrying overexpression or antisense suppression plasmids of *MdMYB308L*, *MdbHLH33* and *MdMIEL1*, and vacuumed using a vacuum pump for about 15 minutes. qRT-PCR was performed to examine the expression levels of transient transgenic apple leaves (An *et al*., 2019). The transgenic *Arabidopsis* seedlings were generated as described previously (Clough and Bent, 1998).

Quantitative real-time-PCR (qRT-PCR) analysis

Apple calli, apple leaves and *Arabidopsis* seedlings were collected, and full ground with liquid nitrogen and total RNAs were extracted using a RNA extraction kit (TIANGEN, Beijing, China). Single-stranded cDNA was obtained using a reverse transcription kit (TaKaRa, Shiga, Japan). All qRT-PCR analyses were performed with three biological repeats and three technical repeats. All primers used in this study are listed in Table S3.

GUS staining and activity analysis

$Pro_{MdMYB308L}$::GUS and $Pro_{MdMIEL1}$::GUS transgenic apple calli were used for cold stress treatment and GUS staining. GUS staining and activity analysis were performed as described previously (An *et al*., 2018).

Cold tolerance assays

Cold tolerance assays were performed as described previously (An *et al*., 2018). For apple calli, 8-day-old apple calli were treated at 4°C for 10 days. Apple calli growing at 24°C for 10 days were used as controls. The fresh weights of apple calli under normal and cold stress conditions were recorded. For the cold stress treatment of *Arabidopsis* seedlings, 12-day-old *Arabidopsis* seedlings grown at 22°C for 12 days were treated at 4°C for 2 days for cold acclimation. And then, seedlings were transferred to −4°C for indicated time (10 min or 0.5 h) by gradient cooling and finally grown at 22°C for 2 days. Gradient cooling was carried out in the incubator. In the incubator, the temperature can reduce slowly from 22°C to −4°C and can also increase slowly to 22°C from −4°C. And the processing time can be adjusted according to the actual situation. Survival rate of *Arabidopsis* seedlings after cold stress treatment was recorded.

Anthocyanin accumulation assays

Apple calli and leaves of 15-day-olds were transferred in a highlight phytotron (photon flux density: 100 μmol/m^2/s) for indicated time (5 days or 7 days). Anthocyanin was extracted using anthocyanin extraction buffer. Anthocyanin content was determined as described previously (An *et al*., 2018).

Screening the potential interacting proteins of MdMYB308L

Yeast two-hybrid screening assays were performed to identify the interacting proteins of MdMYB308L using MdMYB308L-pGBD as the bait. MdMYB308L-pGBD and an apple library (Shanghai OE Biotech. Co., Ltd.) were mixed and transformed into yeast cells 'Y2H Gold'. The transformed yeast strains were grown in the SD-Trp/-Leu/-His/-Ade (-T/-L/-H/-A) medium for 4 days. The cDNA fragments of positive yeast strains were identified by sequencing. As a result, 19 potential interacting proteins were obtained (Table S2).

Due to the false-positive phenomenon of yeast two-hybrid screening, we further confirmed the interactions through a one-to-one yeast two-hybrid assays. The results showed that only MDP0000185659 (MdMIEL1) and DQ266451 (MdbHLH33) were the direct interacting proteins of MdMYB308L.

Y2H, pull-down and BiFC assays

Y2H, pull-down and BiFC assays were performed as described previously (An *et al*., 2018). In brief, for Y2H assays, MdMYB308L-pGBD, MdbHLH33-pGAD, MdbHLH3-pGAD and MdMIEL1-pGAD were transformed into yeast 'Y2H Gold'. The transformed yeast strains were grown in SD medium. For pull-down assays, the fusion proteins of MdMYB308L-GST, MdbHLH33-HIS and MdMIEL1-HIS were prepared. The protein mixtures were purified using a glutathione purification kit (Thermo Fisher Scientific, Waltham, MA, USA). The eluted solution was detected using HIS or GST antibodies (Abmart, Shanghai, China). For BiFC assay, MdMYB308L-YFP^C, MdbHLH33-YFP^N and MdMIEL1-YFP^N were transformed into onion epidermal cells by *Agrobacterium*-mediated genetic transformation.

Electromobility shift assays (EMSA)

EMSAs were performed as described previously (An *et al*., 2018). MdMYB308L-GST, MdbHLH33-GST and biotin-labelled probes (*MdCBF2*-probe and *MdDFR*-probe) were prepared. Biotin-labelled probes were incubated with MdMYB308L-GST or MdbHLH33-GST protein in the binding buffer for 25 minutes, and the free and bound probes were separated on an acrylamide gel. Unlabelled probes were used as competitors. The GST protein was used to ensure an equal quantity of proteins.

LUC/REN activity analysis

LUC/REN activity was determined as described previously (An *et al*., 2018). MdbHLH33-62SK, MdMYB308L-62SK, MdMYB23-62SK, MdMYB1-62SK p*MdCBF2*-LUC and p*MdDFR*-LUC were prepared. *Agrobacterium* carrying indicated plasmids was injected into the back of tobacco leaves. MdMYB23-62SK and MdMYB1-62SK were used as positive controls.

Ubiquitination detection and protein degradation assays

Ubiquitination detection was performed as described previously (An *et al*., 2017a). For *in vitro* ubiquitination detection, MdMIEL1-HIS was tested for E3 ubiquitin ligase activity in the presence or absence of ATP, ubiquitin, E1, E2, MdMIEL1-HIS or MdMYB308L-GST. The protein gel blot was analysed using a GST antibody. For *in vivo* ubiquitination detection, MdMYB308L-MYC was immunoprecipitated using MYC antibody from the MdMYB308L-MYC and MdMYB308L-MYC/MdMIEL1-OX apple calli. The protein gel blot was analysed using MYC and Ubi antibodies. Total proteins extracted from apple calli were incubated with the purified MdMYB308L-GST or MdMIEL1-HIS fusion protein. The samples were collected at the indicated periods. The protein gel blot was analysed using GST and HIS antibodies.

Statistical analysis

Each experiment was performed in three replicates. Experimental results were analysed using GraphPad Prism 6.02 or DPS software. Error bars denote standard deviations. Significant differences were detected by *t*-test: $**P < 0.01$. Different letters above the bars indicated significant difference ($P < 0.05$) as obtained by one-way ANOVA and LSD test.

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Author contributions

Y.J.H. and J.P.A. conceived and designed the experiments. J.P.A. performed the research. J.P.A., X.X.W., X.W.Z., H.F.X., S.Q.B. and C.X.Y. analysed the data. J.P.A. and Y.J.H. wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Sequence analysis of MYB308L proteins.
Figure S2 MdMYB308L is a novel MYB protein in apple.
Figure S3 The promoter sequence of *MdMYB308L*.
Figure S4 Gene expression analysis of transgenic plant materials using qRT-PCR.
Figure S5 The promoter sequence of *MdCBF2*.
Figure S6 The promoter sequence of *MdDFR*.
Figure S7 MdMYB308L does not affect MYB-related genes expression and not bind to the *MdCBF2* and *MdDFR* promoters.
Figure S8 The promoter sequence of *MdMIEL1*.

Table S1 Cold-inducible MYB transcription factors identified from a cold stress transcriptome study using cDNA samples extracted from apple seedlings treated with or without cold stress (4°C) for 9 h.
Table S2 Screening the potential interacting proteins of MdMYB308L.
Table S3 Primers used for gene expression analysis and vector construction.

