MdMYB4, an R2R3-Type MYB Transcription Factor, Plays a Crucial Role in Cold and Salt Stress in Apple Calli

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ABSTRACT. MYB (v-myb avian myeloblastosis viral oncogene homologs) transcription factors (TFs) are involved in diverse physiological processes, including cell shape determination, cell differentiation, and secondary metabolism, as well as abiotic stress response. In the present study, MdMYB4, an R2R3-MYB protein that is a homolog of *Arabidopsis thaliana* MYB4, was identified and characterized. Quantitative real-time polymerase chain reaction (qRT-PCR) expression analysis demonstrated that *MdMYB4* is extensively expressed in various apple (*Malus domestica*) tissues and that its expression is induced by cold, osmotic, and salt stress. An MdMYB4-GFP fusion protein was localized in the nucleus of transformed onion (*Allium cepa*) epidermal cells and had a certain transcriptional activation activity by yeast one-hybrid assay. Overexpression of the *MdMYB4* gene remarkably enhanced the tolerance of stably transgenic apple calli to severe salt and cold stress, and both the relative conductivity and malondialdehyde (MDA) accumulation of transgenic calli under salt and cold stress were significantly lower than in the wild type control. Taken together, these results suggest that MdMYB4 may play a positive regulatory role in both cold and salt stress responses.

Abiotic stresses, such as cold, drought, and high salinity, are common adverse environmental conditions that can severely limit plant growth and development, as well as crop production (Xie et al., 2010). As sessile organisms, plants have evolved a series of intricate mechanisms that allow them to perceive external signals and respond to complicated stress conditions, and many studies have reported that a large number of genes are responsive to abiotic stresses in higher plants (Kasuga et al., 2004). In general, these stress-induced genes are either directly or indirectly modulated by regulators that are components of signal transduction pathways related to abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 1997). To date, a variety of such genes have been characterized, most of which have been reported to regulate the synthesis of diverse biological components, such as proline (Hmida-Sayari et al., 2005; Hong et al., 2000; Kishor et al., 1995), betaine (Kumar et al., 2004), carbohydrates (Almeida et al., 2007; Avonce et al., 2005), polyamines (Imai et al., 2004), and other osmolytes, all of which are related to environmental stress tolerance. In addition, the genes that encode regulators, such as the late embryogenesis abundant protein HVA1 (Bahieldin et al., 2005; Garay-Arroyo et al., 2000; Park et al., 2005; Sivamani et al., 2000) and calcium-dependent protein kinase (CDPK) genes (Ma and Wu, 2007; Wan et al., 2007; Zhang et al., 2005), can be induced by abiotic stress.

In plants, TFs play vital regulatory roles in abiotic stress responses by binding to the promoters of abiotic stress-responsive

genes (Gujjar et al., 2014; Jung et al., 2008; Umezawa et al., 2006). Plant MYB TFs, which act as the largest family of TFs and are involved in various plant-specific processes, such as cell shape determination, cell differentiation, and secondary metabolism (Gujjar et al., 2014), have been reported to play roles in the response of the model plant *A. thaliana* to abiotic stresses. These genes function via abscisic acid (ABA)-dependent or ABA-independent stress response pathways and include *AtMYB2* and *AtMYB41* (Abe et al., 2003; Lippold et al., 2009); *AtMYB44/AtMYBR1*, *AtMYB60*, *AtMYB96*, *AtMYB13*, *AtMYB15*, *AtMYB33*, and *AtMYB101* (Abe et al., 2003; Cominelli et al., 2005; Gujjar et al., 2014; Lippold et al., 2009); and *AtMYB70*, *AtMYB73*, and *AtMYB77/AtMYBR2* (Park et al., 2011). In addition, stress-related MYB TF genes in rice (*Oryza sativa*) have been successively cloned and transformed into plants using transgenic techniques, and the heterologous expression of such genes has been reported to remarkably enhance the tolerance of transgenic plants to both cold and salt stress (Pasquali et al., 2008). In apple, the MYB TF family includes 229 genes, which were identified using genome-wide analysis, and the effects of abiotic stressors on the expression of 18 MYB genes have also been reported (Cao et al., 2013). Among these genes, the overexpression of apple *MdoMYB121* (Cao et al., 2013) and *MdSIMYB1* (Wang et al., 2014) have been reported to remarkably enhance the tolerance of transgenic apple plants to high salinity, drought, and cold stress. Because apple trees are highly heterozygous and genetically self-incompatible or incompatible with closely related cultivars, they are difficult to modify by conventional breeding techniques; however, with the accomplishment of a high-quality draft genome sequence (Velasco et al., 2010), a variety of genes, such as those encoding MYB TFs, can potentially be used as candidate genes for cultivating resistant and genetically improved varieties.

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However, very little is known about their functions in apple. Therefore, to examine whether the expression of apple *R2R3-MYB* genes could be induced by abiotic stress, we selected 11 other apple *R2R3-MYB* genes from seven *R2R3-MYB* subgroups and used qPCR analysis to analyze their expression in response to NaCl, polyethylene glycol (PEG), and cold treatments (Cao et al., 2013). We subsequently isolated apple *MdMYB4*, owing to its relatively high expression under stress, which suggested that *MdMYB4* plays a role in abiotic stress tolerance.

Materials and Methods

PLANT MATERIALS AND TREATMENTS. Root, stem, leaf, flower, and fruit tissues were collected from a 5-year-old ‘Golden Delicious’ own-rooted apple tree for tissue-specific expression of *MdMYB4* gene under nonstress conditions. In vitro apple shoot cultures of ‘Golden Delicious’ were subcultured on Murashige and Skoog (MS) solid medium with 0.5 mg·L⁻¹ 6-benzylaminopurine and 0.1 mg·L⁻¹ naphthylacetate on a 4-week interval at 25 °C under a 16/8-h light/dark photoperiod ≈25 μmol·m⁻²·s⁻¹ supplied by white fluorescent light, as described by Cao et al. (2013). Four-week-old apple shoot cultures were treated with osmotic (2% PEG), salt (200 mM NaCl), and cold (4 °C) stress, and afterward, young leaves were collected at 0, 3, 6, 9, 12, and 24 h for the analysis of the expression level of 11 *MdR2R3-MYB* genes (Supplemental Table 1), according to the methods described by Yamaguchi-Shinozaki and Shinozaki (1994). The ‘Orin’ apple callus was subcultured on an MS medium with an additional 1.0 mg·L⁻¹ 6-benzylaminopurine and 1.0 mg·L⁻¹ 2–4 d.

QUANTITATIVE PCR ASSAYS. Total RNA was extracted from the tissues using the hot borate method described by Yao et al. (2007), and after treatment with RNase-free DNase, first-strand cDNA was synthesized using a PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). The transcript levels of *MdMYB4* were then examined using qPCR assays with gene-specific primers (Supplemental Table 1). The apple *18S* rRNA gene was used as a loading control, and three technical and three biological replicates were performed for each qPCR reaction.

CLONING AND BIOINFORMATICS ANALYSIS. The full-length *MdMYB4* sequence (MDP0000582174) was obtained from the Apple Genome Database (Jung et al., 2014), and leaves from the ‘Golden Delicious’ shoot culture materials were used for cloning of *MdMYB4* cDNA (Supplemental Table 2). The 20-μL qRT-PCR reactions each contained 10 μL mixture (TaKaRa), 1.0 μL cDNA, 1.0 μL of each primer (10 μM), and 7 μL distilled and deionized water (ddH₂O). The amplification consisted of 34 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, followed by a final extension step of 7 min at 72 °C. The PCR product was purified and subcloned into the pEasy-Simple T1 vector (TaKaRa) and sequenced (UnitedGene, Shanghai, China). After confirmation of the accuracy of the full-length sequence, a homology search of the National Center for Biotechnology Information GenBank database was conducted using protein BLAST, and a phylogenetic tree was constructed using the neighbor-joining method in MEGA 5 (Tamura et al., 2011). Prediction of the number of amino acids and isoelectric point, amino acid sequence alignments, and main domain analysis were completed using DNAMAN V6 (Lynnon Biosoft, San Ramon, CA), and the functional element

analysis of the 1.5-kb promoter region upstream of the start codon was completed using Plant-CARE database (Higo et al., 1999).

PREPARATION OF *Agrobacterium tumefaciens* suspension. A single colony of *A. tumefaciens* strain EHA105 (Hood et al., 1993) was inoculated into liquid Luria-Bertani (LB) medium with 50 mg·L⁻¹ kanamycin and 100 mg·L⁻¹ rifampicin and grown for 16 h at 28 °C on an orbital shaker at 180 rpm. The resulting bacterial solution was diluted to an optical density (OD)₆₀₀ = 0.1–0.2 in 50 mL LB with 100 μM acetosyringone and incubated at 28 °C with shaking for another 5–6 h until a density of OD₆₀₀ = 0.5 was obtained. The bacterial cultures were then centrifuged at 3000 g_n for 5–6 min at 25 °C, after which the supernatant was discarded, and the bacterial pellet was resuspended in 10 mM MgCl₂ to its original titer.

SUBCELLULAR LOCALIZATION OF *MdMYB4*. The complete *MdMYB4* open reading frame was PCR-amplified using primers that contained *EcoRI* and *BamHI* restriction sites (Supplemental Table 2) and cloned into upstream of green fluorescent protein (GFP) of vector pEZS-NL (Song et al., 2012) to create a fusion construct (p35S: MYB4-GFP). Both the fusion construct and the control vector (pEZS-NL) were introduced into *A. tumefaciens* EHA105 and transformed into onion epidermal cells, according to the method described by Li et al. (2002). After being cultured on an MS medium for 2 d at 28 °C in darkness, the transformed cells were visualized using a confocal microscope (LSM 510 META; Zeiss, Jena, Germany).

TRANSCRIPTIONAL ACTIVATION ASSAY OF *MdMYB4* IN YEAST. The complete *MdMYB4* open reading frame was amplified using PCR with primers that contained *EcoRI* and *BamHI* restriction sites (Supplemental Table 2), and subcloned into *EcoRI*- and *BamHI*-digested pGBKT7 vector. The verified recombinant vector was transformed into the yeast strain AH109 and grown on a synthetic dextrose medium without tryptophan or lacking both tryptophan and histidine. Positive clones were assayed for LacZ reporter gene activation, using 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-gal) as a substrate, and yeast cells transformed with empty pGBKT7 vector were assayed as a negative control (Zheng et al., 2009).

***MdMYB4* OVEREXPRESSION VECTOR CONSTRUCTION AND APPLE CALLI TRANSFORMATION.** The complete sequence of the 35S promoter was amplified from the pBI121 vector (Supplemental Table 2) and cloned into the pCAMBIA1304 vector. Then, the complete *MdMYB4* sequence was amplified using PCR with primers containing *KpnI* and *XbaI* restriction sites, and cloned into the *pCAMBIA-35S* vector, which was digested using the same restriction enzymes. The resultant constructs (*pCAMBIA-MYB4*) was introduced into *A. tumefaciens* strain EHA105 and transformed into ‘Orin’ apple calli, as described by Li et al. (2002). After four replicates of hygromycin selection subculture on a 2-week interval, antibiotic-resistant calli were detected using PCR (Supplemental Table 2) and β-glucuronidase (GUS) assays.

MEASUREMENT OF FRESH WEIGHT AND RELATIVE CONDUCTIVITY. The *MdMYB4*-transgenic and control calli were placed on solid subculture media with 0, 100, and 200 mM NaCl treatment or at 4, 15, and 25 °C, and changes in the callus phenotypes and fresh weight were observed after 20 d. To measure relative conductivity, as much as 1.0 g fresh callus from each culture was transferred to an MS solid medium supplemented with 200 mM NaCl or MS solid medium treated

with 4 °C for 4 h and then the relative electronic conductivity of each sample was measured using a conductometer (DDS-308; KangYi, Chengdu, China), and the relative conductivity of each culture was calculated as $R1/R2 \times 100\%$ (Qiu et al., 2002). Three technical and three biological replicates were performed for each measurement.

MEASUREMENT OF MALONDIALDEHYDE CONTENT. Malondialdehyde contents in *MdMYB4*-transgenic and control apple calli under cold (15 °C) and NaCl (100 mM) stress at the indicated treatment time was measured according to Hodges’s method (Hodges et al., 1999). Briefly, the calli were homogenized in 5 mL 10% trichloroacetic acid and centrifuged at 12,000 g_n for 10 min. The clear supernatant (2 mL) from each callus sample was added to 4 mL 0.6% thiobarbituric acid (in 10% trichloroacetic acid), and the reaction mixtures were incubated at 100 °C in a water bath for 15 min. The reactions were ended by cooling them to room temperature, and the absorbance of the supernatants at 450, 532, and 600 nm was determined using an ultraviolet-vis (ultraviolet-vis) spectrophotometer (ultraviolet-2450; Daojin, Chendu, China). Finally, the concentration of MDA (moles per gram) was calculated as $C_{MDA} \times V$, where C_{MDA} (M) = $6.45(OD_{532} - OD_{600}) - 0.56(OD_{450})$ and V (milliliters per gram) is the volume of extracting solution used per gram fresh callus. Three technical and three biological replicates were performed for each measurement.

STATISTICAL ANALYSIS. All data were analyzed using analysis of variance (ANOVA) by SPSS Statistics (version 18.0; IBM Corp., Armonk, NY), and the graphics were produced in SigmaPlot (version 10.0; Systat Software, San Jose, CA).

Results

EXPRESSION OF 11 R2R3-MYB GENES IN APPLE UNDER ABIOTIC STRESS. To examine if the expression of apple *R2R3-MYB* genes is induced by abiotic stress including NaCl, PEG, and cold treatments, 10 apple *R2R3-MYB* gene models (*MdMYB4*, 42, 59, 93, 113, 131, 143, 151, 194, and 214) were chosen from stress-related subgroups, whereas another (*MdMYB8*) was chosen from the other subgroups (Cao et al., 2013). The results of the qRT-PCR analysis indicated that the transcription of each of the *MdMYB* genes was responsive to at least one of the three stress treatments (Fig. 1; Supplemental

Fig. 1. Effect of abiotic stress on the expression levels of 11 *MdR2R3-MYB* genes in leaves of ‘Golden Delicious’ apple. Expression levels were measured after 12 h or exposure to 200 mM NaCl, 2% polyethylene glycol (PEG), or cold (4 °C) treatments. Ten of the *MdR2R3-MYB* genes were chosen from stress-related subgroups, and *18S* rRNA expression was used as an internal standard.

Fig. 1). Interestingly, the *MdMYB4* transcripts were strongly induced by all stresses tested, which indicated that the stresses induced the expression of *MdMYB4*.

THE EFFECT OF ABIOTIC STRESS ON *MdMYB4* EXPRESSION. To further study the *MdMYB4* gene as a TF, the regulatory regions of *MdMYB4* were analyzed in detail, and the effects of abiotic stress at different time spans on the expression of *MdMYB4* was analyzed using qRT-PCR. We identified a variety of hormone-related response elements (Supplemental Table 3), including an ABA-responsive element (ABRE), CGTCA and TGACG motifs, TGA and TCA elements, stress-induced elements, such as HSE, and TC-rich repeats in the promoter region, which suggested that *MdMYB4* expression may be induced by both abiotic stress and stress hormones. Furthermore, the expression of *MdMYB4* in apple leaves cultured under salt (200 mM NaCl), cold (4 °C), and osmotic stress (2% PEG) indicated that *MdMYB4* transcription was markedly induced by all three abiotic stresses at different times (Fig. 2B–D).

In addition, the qRT-PCR results also indicated that *MdMYB4* was widely expressed in apple tissues, including in root, stem, leaf, flower, and fruit tissue, with the lowest level of *MdMYB4* expression observed in the leaves (Fig. 2A). The results suggested that *MdMYB4* may play an important role in responding to cold and salt stress in apple.

IDENTIFICATION OF THE *MdMYB4* GENE. To further investigate functional characterization of *MdMYB4*, the gene was isolated from apple cDNA template. Cloning and sequencing indicated that the full-length *MdMYB4* cDNA was 495 bp (Fig. 3A), and further analyses predicted that the gene encoded a protein with 164 amino acid residues, a molecular mass of 18.3 kDa, and a pI of 10.34. In relation to other R2R3-MYB TFs in *A. thaliana* and apple, phylogenetic analysis indicated that *MdMYB4* formed a cluster with *AtMYB3*, *AtMYB4*, *AtMYB7*, *AtMYB32*, *MdMYB16*, and *MdMYB17* (Fig. 3B and C; Supplemental Fig. 2).

***MdMYB4* LOCALIZATION.** To observe the subcellular localization of the *MdMYB4* protein, the open reading frame of *MdMYB4* was fused to the N-terminus of green fluorescence protein (GFP) in the pEVS-NL vector, and its expression was driven by a constitutive 35S CaMV promoter. In onion epidermis cells transformed with the *MdMYB4*-GFP fusion construct, green fluorescence was exclusively observed in the

Fig. 2. Expression of *MdMYB4* in apple. (A) Tissue-specific expression under nonstress conditions. (B–D) Expression in leaves of apple shoot cultures induced by salt (200 mM NaCl), osmotic (2% polyethylene glycol), and cold (4 °C) stress treatments, respectively. The expression of *18S* rRNA was used as an internal reference. Values are means $\pm$ SE ($n = 3$).

nucleus (Fig. 4A and B), whereas fluorescence was detected in both the nucleus and the cytoplasm of the cells transformed with the control vector (Fig. 4C and D), thus indicating that MdMYB4 is localized to the nucleus.

TRANS-ACTIVATION OF MdMYB4 IN YEAST. To determine whether MdMYB4 protein had transcriptional activation activity, the pGBKT7-MdMYB4 fusion protein was transformed

into yeast strain AH109, using empty pGBKT7 vector as a negative control. The results indicated that AH109/pGBKT7 could grow on an SD medium without tryptophan but not on an SD medium lacking both tryptophan and histidine (Fig. 5A and C); whereas AH109/pGBKT7-MdMYB4 could grow on an SD medium without tryptophan and without both tryptophan and histidine (Fig. 5B and D). In addition, the pGBKT7-MdMYB4 fusion protein also promoted the activity of the LacZ reporter gene (Fig. 5E). Therefore, MdMYB4 possesses the ability to self-activate.

EFFECT OF MdMYB4 OVEREXPRESSION ON

STRESS TOLERANCE IN APPLE CALLI.

To confirm the functions of *MdMYB4* in apple, MdMYB4-overexpressed transgenic apple calli were obtained and named MdMYB4-OVX1-, MdMYB4-OVX2-, and MdMYB4-OVX3-transgenic apple calli. To confirm that the calli were free from contamination by transgenic *A. tumefaciens*, specific primers were designed according to the sequences, excluding the vector T-DNA regions. However, no fragments of the expected size were PCR amplified. Therefore, the transgenic calli were free from potential contamination by *A. tumefaciens*. Those transgenic apple calli were subsequently detected using GUS staining assays (Fig. 6A). Based on the results of GUS staining and the growth status of calli, MdMYB4-OVX2 was used for further studies on abiotic stress tolerance. There was no significant difference in the growth patterns of *MdMYB4*-transgenic and control apple calli grown on an MS medium. When transferred to cold treatment (15 °C) or an MS medium supplemented with 100 mM NaCl, the *MdMYB4*-transgenic calli exhibited greater tolerance to salt and cold stress than the control calli, as indicated by a greater increase in fresh weight. However, when transferred to cold treatment (4 °C) or an MS medium supplemented with 200 mM NaCl, it was impossible to obtain the same result, as the plant growth was arrested (Fig. 6B and C).

In addition, we also measured the relative conductivity and MDA content of *MdMYB4*-transgenic and control calli, since both are known indicators of membrane damage (Dong et al., 2011). The *MdMYB4*-transgenic and control calli exhibited nearly identical relative conductivity on an MS medium, whereas the relative conductivities of salt- and cold-stressed transgenic calli were lower than those of salt- and cold-stressed control calli (Fig. 6D); and both the *MdMYB4*-transgenic and control calli produced similar levels of MDA under normal conditions, whereas the MDA contents of salt- and cold-stressed transgenic calli were lower than those of salt- and cold-stressed control calli (Fig. 7A–C). To confirm if the phenotypic alterations were due to the overexpression of *MdMYB4*, second line MdMYB4-OVX1-transgenic apple calli were used to perform experiments presented in Figs. 6 and 7, and similar results were obtained (Supplemental Fig. 3). Therefore, the expression level of *MdMYB4* was negatively associated with both relative conductivity and MDA content,

Fig. 3. Phylogenetic analysis of myeloblastosis (MYB) proteins. (A) Clone of *MdMYB4* from apple leaf. (B) Phylogenetic relationship of predicted amino acid sequences from *MdMYB4* and other MYB genes from *Arabidopsis thaliana* and apple. (C) Alignment of MYB proteins from *A. thaliana* and apple subgroup 4. The R2 and R3 domains are underlined.

Fig. 4. Subcellular localization of MdMYB4-GFP fusion proteins transiently expressed in onion epidermal cells. Bright light (A and C) and fluorescence (B and D) images of cells transformed with p35S:MdMYB4-GFP (A and B), and p35S:GFP plasmids (C and D).

Fig. 5. Transcription activation ability of MdMYB4 in yeast cells. (A and C) Growth of yeast cells transfected with pGBKT7 on SD/-Trp or SD/-Trp-His medium, respectively. (B and D) Growth of yeast cells transfected with the pGBKT-MdMYB4 fusion protein on SD/-Trp or SD/-Trp-His medium, respectively. (E) Analysis of LacZ reporter gene activation, using X-gal as the substrate.

which indicated that *MdMYB4* is a positive regulator of tolerance to salt and cold stress.

Discussion

The MYB TF family is large and functionally diverse in higher plants and members have been identified in *A. thaliana*, rice, apple, and various other species (Du et al., 2011, 2012; Li et al., 2012). Substantial evidence suggests that the R2R3-MYBs play important roles in abiotic stress tolerance in various plant species. These stress-induced genes are mostly distributed in some subfamilies, such as S1, 2, 4, 11, 14, 20–23, and H5 (Cao et al., 2013); however, research regarding MYB TFs in apple has remained sparse. Subsequently, 229 apple MYB genes were identified through genome-wide analysis and divided into 45 subgroups and the expression of 18 genes in response to various abiotic stresses was examined (Cao et al., 2013). To further find the desired genes for genetic manipulation to enhance abiotic stress tolerance in fruit trees and other crops, 11 other apple R2R3-MYB genes were chosen in the present study. Among them, 10 genes were from those stress-related subgroups, whereas another one from other subgroup, and the expression of 11 apple *R2R3-MYB* genes from different subgroups (Fig. 1; Supplemental Fig. 2) was induced by multiple abiotic stresses, suggesting its involvement in the response and tolerance to abiotic stress, which is consistent with the fact that the expression of many MYB TF genes can be induced by abiotic stress in model plants (Chen et al., 2006). Besides, *MdMYB4* transcripts were produced in all tested apple tissues, including in root, stem, leaf, flower, and fruit tissue (Fig. 2A), and markedly induced by all three abiotic stresses at different times (Fig. 2B–D), and it should be chosen as a candidate stress-inducible gene for further research.

When compared with other R2R3-MYB TFs, phylogenetic analysis indicated that *MdMYB4* formed a close cluster with *A. thaliana* subgroup S4, which includes *AtMYB3*,

Fig. 6. Effect of *MdMYB4* overexpression on salt and cold stress tolerance in *MdMYB4*-transgenic apple calli. (A) *MdMYB4* expression. (B) Tolerance of calli to salt stress (MS medium with 0, 100, or 200 mM NaCl). Photographs taken at 20 d after treatment. (C) Fresh weight of salt- and cold-stressed apple calli. Values and error bars indicate means $\pm$ SE ($n = 9$). (D) Relative electronic conductivity of apple calli after 4 h of water (H_2O), salt (NaCl), and cold ($4^\circ C$) stress. Values and error bars indicate means $\pm$ SE ($n = 3$). Asterisks indicate values that are significantly different from those of the control group (* = $P < 0.05$, ** = $P < 0.01$), according to ANOVA.

Fig. 7. Effects of cold (15 °C) and NaCl (100 mM) stress on the malondialdehyde (MDA) contents of *MdMYB4*-transgenic and control apple calli. Values and error bars indicate means $\pm$ SE ($n = 3$). Asterisks indicate values that are significantly different from those of the control group (* = $P < 0.05$, ** = $P < 0.01$), according to ANOVA.

AtMYB4, AtMYB7, and AtMYB32 (Fig. 3B). In reported studies, AtMYB4 has been shown to regulate sinapate ester biosynthesis in ultraviolet-exposed plants (Jin et al., 2000), and AtMYB7 is a new player in the regulation of ultraviolet-screens (Fornalé et al., 2013), whereas AtMYB32 is required for pollen development (Preston et al., 2004). Meanwhile, the present study also determined that many hormone-related and stress-induced response elements were present in *MdMYB4* regulatory sequences, which suggests that the expression of the *MdMYB4* protein may be induced by hormones and stresses.

In the present study, we also determined that *MdMYB4* is localized to the nucleus in a subcellular manner (Fig. 4), which is consistent with the characteristics of TFs (Katiyar et al., 2012). Furthermore, our yeast one-hybrid results indicated that *MdMYB4* functions as a transcriptional activator in plants and that it might regulate the expression of stress-related genes independently.

Previous results have also suggested that cold temperature (4 °C) and 200 mM NaCl are suitable treatments for gene expression analysis of in vitro apple shoot cultures (Cao et al., 2013; Hu et al., 2012; Wang et al., 2014). In the present study,

when in vitro 'Golden Delicious' apple shoot cultures were exposed to 12-h cold (4 °C) and salt (200 mM NaCl) stress treatments, the plants still grew well, and gene expression was strongly induced. Therefore, cold (4 °C) and salt (200 mM NaCl) treatments were suitable for *MdMYB4* expression analysis. When the long-term transgenic and control callus cultures were transferred to an MS solid medium that was supplemented with NaCl (200 mM) or exposed to cold stress (4 °C), growth was completely suppressed. However, when the transgenic and control calli were kept at 15 °C or treated with 100 mM NaCl, the calli still grew well. Meanwhile, fresh weight exhibited significant differences. Therefore, the 15 °C and 100 mM NaCl treatments were used to measure MDA content and were deemed suitable for further functional analysis.

In addition, abiotic stress can result in membrane damage, as indicated by electrolyte leakage and MDA concentrations (Dong et al., 2011). In previous studies, enhanced tolerance to freezing and osmotic stress in *OsMYB4*- and *MdMYB10*-transgenic plants, respectively, has been accompanied by lower MDA contents (Gao et al., 2011; Vannini et al., 2004). In addition, *AtMYB2*-transgenic *A. thaliana* exhibited enhanced tolerance to osmotic stress, as indicated by reduced electrolyte leakage (Abe et al., 2003). In the present study, transgenic-*MdMYB4* calli produced less MDA and exhibited less electrolyte leakage than the control calli, which indicates that *MdMYB4* enhances plant tolerance to multiple stresses.

Although overexpression of a novel apple *R2R3-MYB* gene (*MdMYB4*) enhanced the tolerance of transgenic apple calli to salt and cold stress, whether field-grown transgenic apples will respond to stressors in the same way remains to be determined.

Conclusion

In conclusion, the overexpression of a novel apple *R2R3-MYB* gene (*MdMYB4*) enhanced the tolerance of apple calli to salt and cold stress. Therefore, *MdMYB4* should be considered a target gene for genetic manipulation aimed to enhance salt and cold stress tolerance in fruit trees, as well as in other crops.

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Supplemental Fig. 1. Effect of abiotic stress on the expression levels of 10 *MdR2R3-MYB* genes in leaves of 'Golden Delicious' apple. Apple shoot cultures were treated with osmotic (2% polyethylene glycol), salt (200 mM NaCl), and cold (4 °C) stress, and then young leaves were collected at 0, 0, 3, 6, 9, and 12 h respectively for the expression levels of 10 *MdR2R3-MYB* genes, and *18S* rRNA expression was used as an internal standard.

Supplemental Fig. 3. Effect of *MdMYB4* overexpression on salt and cold stress tolerance in *MdMYB4*-transgenic apple calli. (A and B) Tolerance of calli to salt stress (MS medium with 0, 100, or 200 mM NaCl) or cold stress (25, 15, or 4 °C). Photographs taken at 20 d after treatment. (C and D) Fresh weight of salt- and cold-stressed apple calli. Values and error bars indicate means $\pm$ SE ($n = 9$). (E) Relative electronic conductivity of apple calli after 4 h of water (H₂O), salt (NaCl), and cold (4 °C) stress. Values and error bars indicate means $\pm$ SE ($n = 3$). (F) Effects of cold (15 °C) and NaCl (100 mM) stress on the malondialdehyde (MDA) contents of *MdMYB4*-transgenic and control apple calli. Values and error bars indicate means $\pm$ SE ($n = 3$). Asterisks indicate values that are significantly different from those of the control group (* = $P < 0.05$, ** = $P < 0.01$), according to ANOVA.

Supplemental Table 1. Primer sequences used for expression analysis of 11 *MdR2R3-MYB* genes under abiotic stress treatments (Cao et al., 2013).

Gene	Subgroup	Sequence (5'–3')	
		Forward	Reverse
<i>MdMYB8</i>	S23	GGCCTTGAGAGTAATGGGATAAG	TCTAGGCACCTGGGTTGATA
<i>MdMYB42</i>	S14	TTGGAAGCAGGTGGTCAATAA	GTGTTGCTTCCTCGGTATGA
<i>MdMYB59</i>	S22	CTCTGCTGACTCTGTCATTACC	CCTCCATGTAACCTCCTCACTTC
<i>MdMYB93</i>	S4	GGGAAGAACCAGATAACGAGATT	GTGGTTGTTGACGGGTTTATG
<i>MdMYB113</i>	S2	GGTCGCACAGACAATGAAATAAA	GAAACAGGCACACAATCCAAG
<i>MdMYB131</i>	S1	GGTCCTGGTAATTGGAGATCAG	CAAGTGGGTGTTCCAGTAGTT
<i>MdMYB143</i>	S4	GGAAACCCTGCTGCGATAA	TTGAGGTCTGGTCGTAGATAGT
<i>MdMYB151</i>	S20	CACCGCCACTAATTCCTCTATC	GGTGATACCTGAGTCCCAAAC
<i>MdMYB194</i>	S22	CAGCAGTAACAGCGAGAAGAA	TCCTTATCATCCCTTGCATCAC
<i>MdMYB214</i>	S1	CTGTGGCAAATACTGCGAATAG	CAGCAGCACCTTCATCAAAC
<i>MdMYB4</i>	S4	TGTGAAAAGGCTCACACCAACAAAG	TTGCCATGATTGAGATCAGGTCTAAGGT
<i>18S</i>	—	CCTCCAATGGATCCTCGTTA	ACACGGGGAGGTAGTGACAA

Supplemental Table 2. Primers used for cloning, subcellular localization analysis, vector construction, transgenic confirmation, and expression analysis.

Primers	Sequences (5'–3')	
	Forward	Reverse
Subcellular localization	CCGGAATTCATGGGAAGATCTCCTTGCTG EcoRI site is underlined	CGCGGATCCAATCATAGATTGAAAGAAGG BamHI site is underlined
Self-activating yeast capability	CCGGAATTCCTCAGAATCTACCAGCAC EcoRI site is underlined	CGCGGATCCACGCCTTCATCGGACTAT BamHI site is underlined
Construction of overexpression vector	CGGGGTACCATGGGAAGATCTCCTTGCTGTGAAA KpnI site is underlined	GCTCTAGATCAATCATAGATTGAAAGAAGGTGA XbaI site is underlined
Quantitative RT-PCR analysis	TGTGAAAAGGCTCACACCAACAAAG	TTGCCATGATTGAGATCAGGTCTAAGGT
18S	CCTCCAATGGATCCTCGTTA	ACACGGGGAGGTAGTGACAA
35S promoter	AGATTAGCCTTTTCAATTTAGAAA	CGTGTCTCTCCAAATGAAATGAAC
Cloning of <i>MdMYB4</i> gene	ATGGGAAGATCTCCTTGCTGTGAAA	TCAATCATAGATTGAAAGAAGGTGA
PCR detection of antibiotic-resistant calli	AGGTGGCGAAACCCGACAGGACTA	TATGAAGATGAACAAAGCCCTGAA

Supplemental Table 3. *cis*-acting elements identified in the *MdMYB4* sequence.

Site name	Strand	Position	Sequence	Function
ABRE	+	81/1126	CACGTG	<i>cis</i> -acting element involved in the abscisic acid responsiveness
ACE	–	862	AAAACGTTTA	<i>cis</i> -acting element involved in light responsiveness
I-Box	+	513/663/596	CTCTTATGCT	<i>cis</i> -acting regulatory element involved in light responsiveness
HSE	+	911	AGAAAATTCG	<i>cis</i> -acting element involved in heat stress responsiveness
TC-rich repeats	–	908	ATTTTCTTCA	<i>cis</i> -acting element involved in defense and stress responsiveness
TGA element	–	9/280/1054/1325	AACGAC	auxin-responsive element
TCA element	+	441/1486	GAGAAGAATA	<i>cis</i> -acting element involved in salicylic acid responsiveness
TGACG motif	+	414/662/1459	CCATCTTTTT	<i>cis</i> -acting regulatory element involved in the MeJA responsiveness
		17/1041/1062	TGACG	