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

Ruigang Wu, Yi Wang, Ting Wu, Xuefeng Xu, and Zhenhai Han
Institute of Horticultural Plants, China Agricultural University, 2 Yuanmingyuan West Road, Haidian District, Beijing 100193, P.R. China; and Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Nutrition and physiology), Ministry of Agriculture, P.R. China

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/AtMYB41, 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/AtMYB2 (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.
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 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 genespecific 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 (ddH\(_2\)O). 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 acetylsyringone and incubated at 28 °C with shaking for another 5–6 h until a density of OD\(_{600}\) = 0.5 was obtained. The bacterial cultures were then centrifuged at 3000 g for 5–6 min at 25 °C, after which the supernatant was discarded, and the bacterial pellet was resuspended in 10 mM MgCl\(_2\) 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 pGBKTK7 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-b-D-galactopyranoside (X-gal) as a substrate, and yeast cells transformed with empty pGBKTK7 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*) were 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, 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 (mole per gram) was calculated as $C_{MDA} = \frac{6.45(OD_{532}–OD_{600})–0.56(OD_{450})}{V}$ (miliilliters 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). 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 pEZS-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...
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 pGBK7-MdMYB4 fusion protein was transformed into yeast strain AH109, using empty pGBK7 vector as a negative control. The results indicated that AH109/pGBK7 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/pGBK7-MdMYB4 could grow on an SD medium without tryptophan and without both tryptophan and histidine (Fig. 5B and D). In addition, the pGBK7-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 control 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.
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,
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-sunscreens (Formalé 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.

**Literature Cited**

Abe, H., T. Urao, T. Ito, M. Seki, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2003. *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15:63–78.

Almeida, A.M., A.B. Silva, S.S. Araujo, L.A. Cardoso, D.M. Santos, J.M. Torne, J.M. Silva, M.J. Paul, and P.S. Fevereiro. 2007. Responses to water withdrawal of tobacco plants genetically engineered with the *AtTPS1* gene: A special reference to photosynthetic parameters. Euphytica 154:113–126.

Avonce, N., B. Leyman, J. Thevelein, and G. Iturriaga. 2005. Trehalose metabolism and glucose sensing in plants. Biochem. Soc. Trans. 33:276–279.

Bahieldin, A., H.T. Mahfouz, H.F. Eissa, O.M. Saleh, A.M. Ramadan, I.A. Ahmed, W.E. Dyer, H.A. El-Itriby, and M.A. Madkour. 2005. Field evaluation of transgenic wheat plants stably expressing the *HvAI* gene for drought tolerance. Physiol. Plant. 123:421–427.

Cao, Z.H., S.Z. Zhang, R.K. Wang, R.F. Zhang, and Y.J. Hao. 2013. Genome-wide analysis of the apple MYB transcription factor family allows the identification of *MdOMYB121* gene conferring abiotic stress tolerance in plants. PLoS One 8:e69955, doi: 10.1371/journal.pone.0069955.

Chen, Y.H., X.Y. Yang, K. He, M.H. Liu, J.G. Li, Z.F. Gao, Z.Q. Lin, Y.F. Zhang, X.X. Wang, X.M. Qiu, Y.P. Shen, L. Zhang, X.H. Deng,
J.C. Luo, X.W. Wang, Z.L. Chen, H.Y. Gu, and L.J. Qu. 2006. The MYB transcription factor superfamily of *Arabidopsis*: Expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol. Biol. 60:107–124.

Cominelli, E., M. Galbiati, A. Vavasseur, L. Conti, T. Sala, M. Vuylsteke, N. Leonhardt, S.L. Dellaporta, and C. Tonelli. 2005. A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. Curr. Biol. 15:1196–1200.

Dong, Q.L., D.D. Liu, X.H. An, D.G. Hu, Y.X. Yao, and Y.J. Hao. 2011. MdVHPI encodes an apple vacuolar H+-PPase and enhances stress tolerance in transgenic apple callus and tomato. J. Plant Physiol. 168:2124–2133.

Du, H., B.R. Feng, S.S. Yang, Y.B. Huang, and Y.X. Tang. 2011. The R2R3-MYB transcription factor gene family in maize. PLoS One 7:1799–1823.

Du, H., S. Yang, B. Feng, L. Liu, Y. Tang, Y. Huang, and Z. Liang. 2012. Genomic-wide analysis of the MYB transcription factor superfamily in soybean. BMC Plant Biol. 12:1–22.

Fornalé, S., E. Lopez, J.E. Salazar-Henao, P. Fernández-Nohales, J. Rigau, and D. Caparrós-Ruiz. 2013. AtMYB7, a new player in the regulation of UV-sunscreens in *Arabidopsis thaliana*. Plant Cell Physiol. 55:507–516.

Gao, J.J., Z. Zhang, R.H. Peng, A.S. Xiong, J. Xu, B. Zhu, and Q.H. Yao. 2011. Forced expression of Mdmmyb10, a myb transcription factor gene from apple, enhances tolerance to osmotic stress in transgenic *Arabidopsis*. Mol. Biol. Rpt. 38:205–211.

Garay-Arroyo, A., J.M. Colmenero-Flores, A. Garcia-Rubio, and A.A. Covarrubias. 2000. Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. J. Biol. Chem. 275:5658–5674.

Gujjar, R.S., M. Akhtar, and M. Singh. 2014. Transcription factors in abiotic stress tolerance. Indian J. Plant. Physiol. 19:306–316.

Hmida-Sayari, A., R. Gargouri-Bouzid, A. Bidani, L. Jaoua, A. Gujjar, R.S., M. Akhtar, and M. Singh. 2014. Transcription factors in Arabidopsis screens in expression by AtMYB4 controls production of UV-protecting sunscreen. Mol. Cell. Biol. 24:208–218.

Hong, Z.L., K. Lakkineni, Z.M. Zhang, and D.P.S. Verma. 2000. Removal of feedback inhibition of Δ1-pyrroline-5-carboxylate synthetase results in increased proline production and confers osmotic stress tolerance in transgenic tobacco. Plant Sci. 169:746–752.

Hodges, D.M., J.M. DeLong, C.F. Forney, and R.K. Prange. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta 207:604–611.

Hong, Z.L., L. Kakkineni, Z.M. Zhang, and D.P.S. Verma. 2000. Overexpression of Δ1-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol. 122:1129–1136.

Hood, E.E., S.B. Gelvin, L.S. Melchers, and A. Hoekema. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res. 2:208–218.

Hu, D.G., M. Li, H. Luo, Q.L. Dong, Y.X. Yao, C.X. You, and Y.J. Hao. 2012. Molecular cloning and functional characterization of *MdSOS2* reveals its involvement in salt tolerance in apple callus and *Arabidopsis*. Plant Cell Rpt. 31:713–722.

Imai, A., T. Matsuyama, Y. Hanzawa, T. Akiyama, Y. Jin, H.L., E. Cominelli, P. Bailey, A. Parr, M. Tamaoki, H. Shinozaki, T. Takahashi. 2004. Spermidine synthase genes are induced in abiotic stress tolerance in plants. Physiol. 55:507–516.

Jung, C., J.S. Seo, S.W. Han, Y.J. Koo, C.H. Kim, S.I. Song, B.H. Nahm, Y. Do Choi, and J.J. Cheong. 2008. Overexpression of *AtMYB44* enhances stomatal closure to confer abiotic stress tolerance in transgenic *Arabidopsis*. Plant Physiol. 146:623–635.

Jung, S., S.P. Ficklin, T. Lee, C.H. Cheng, A. Blenda, P. Zheng, and K. Evans. 2014. The genome database for rosaceae (GDB): Year 10 update. Nucleic Acids Res. 42:1237–1244.

Kasuga, M., S. Miura, K. Shinozaki, and K. Yamaguchi-Shinozaki. 2004. A combination of the *Arabidopsis DREB1A* gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiol. 45:346–350.

Katiyar, A., S. Smita, S.K. Lenka, R. Rajwanshi, V. Chinnusamy, and K.C. Bansal. 2012. Genome-wide classification and expression analysis of MYB transcription factor families in rice and *Arabidopsis*. BMC Genet. 13:1–19.

Kishor, P.B.K., Z.L. Hong, G.H. Miao, C.A.A. Hu, and D.P.S. Verma. 1995. Overexpression of Δ1-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Physiol. 108:1387–1394.

Kumar, S., A. Dhingra, and H. Daniell. 2004. Plastic-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol. 136:2843–2854.

Li, D.D., W. Shi, and X.X. Deng. 2002. *Agrobacterium*-mediated transformation of embryogenic calluses of Ponkan mandarin and the regeneration of plants containing the chimeric ribonuclease gene. Plant Cell Rpt. 21:153–156.

Li, Q., C. Zhang, J. Li, L. Wang, and Z. Ren. 2012. Genome-wide identification and characterization of R2R3MYB family in *Cucumis sativus*. PLoS One 7:e47576.

Lippold, F., D.H. Sanchez, M. Musialak, A. Schlereth, W.R. Scheible, D.K. Hincha, and M.K. Udvardi. 2009. AtMYB41 regulates transcriptional and metabolic responses to osmotic stress in *Arabidopsis*. Plant Physiol. 149:1761–1772.

Ma, S.Y. and W.H. Wu. 2007. AtCPK23 functions in *Arabidopsis* responses to drought and salt stresses. Plant Mol. Biol. 65:511–518.

Park, B.J., Z.C. Liu, A. Kanno, and T. Kameya. 2005. Increased tolerance to sal- and water-deficit stress in transgenic lettuce (*Lactuca sativa*) by constitutive expression of LEA. Plant Growth Regul. 45:165–171.

Park, M.Y., J.Y. Kang, and S.Y. Kim. 2011. Overexpression of *AtMYB52* confers ABA hypersensitivity and drought tolerance. Mol. Cells 31:447–454.

Pasquali, G., S. Bircolti, F. Locatelli, E. Baldoni, and M. Mattana. 2008. Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples. Plant Cell Rpt. 27:1677–1686.

Preston, J., J. Wheeler, J. Heazlewood, S.F. Li, and R.W. Parish. 2004. AtMYB32 is required for normal pollen development in *Arabidopsis thaliana*. Plant J. 40:979–995.

Qiu, Q.S., Y. Guo, M.A. Dietrich, K.S. Schumaker, and J.K. Zhu. 2002. Regulation of SOS1, a plasma membrane Na+/H+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc. Natl. Acad. Sci. USA 99:8436–8441.

Shinozaki, K. and K. Yamaguchi-Shinozaki. 1997. Gene expression and signal transduction in water-stress response. Plant Physiol. 115:327–334.

Sirvainen, E., A. Bahieldin, J.M. Wraith, T. Al-Niemi, W.E. Dyer, T.D. Shinozaki, K. and K. Yamaguchi-Shinozaki. 2004. A combination of the *Arabidopsis DREB1A* gene and stress-inducible rd29A promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. Plant Cell Physiol. 45:346–350.

Song, S., Y. Chen, M. Zhao, and W.H. Zhang. 2012. A novel *Medicago truncatula* HD-Zip gene, *MdHB2*, is involved in abiotic stress responses. Environ. Exp. Bot. 80:1–9.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGAS: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739.

Umezawa, T., M. Fujita, Y. Fujita, K. Yamaguchi-Shinozaki, and K. Shinozaki. 2006. Engineering drought tolerance in plants: Discovering and tailoring genes to unlock the future. Curr. Opin. Biotechnol. 17:113–122.
Vannini, C., F. Locatelli, M. Bracale, E. Magnani, M. Marsoni, M. Osnato, M. Mattana, E. Baldoni, and I. Coraggio. 2004. Overexpression of the rice Osmyb4 gene increases chilling and freezing tolerance of Arabidopsis thaliana plants. Plant J. 37:115–127.

Velasco, R., A. Zharkikh, J. Affourtit, A. Dhingra, A. Cestaro, A. Kalyanaraman, P. Fontana, S.K. Bhatnagar, M. Troggio, D. Pruss, S. Salvi, M. Pindo, P. Baldi, S. Castelletti, M. Cavaiuolo, G. Coppola, F. Costa, V. Cova, A. Dal Ri, V. Goremykin, M. Komjanc, S. Longhi, P. Magnago, G. Malacarne, M. Malnoy, D. Micheletti, M. Moretto, M. Perazzolli, A. Si-Ammour, S. Vezzulli, E. Zini, G. Eldredge, L.M. Fitzgerald, N. Gutin, J. Lanchbury, T. Macalma, J.T. Mitchell, J. Reid, B. Wardell, C. Kodira, Z. Chen, B. Desany, F. Niazi, M. Palmer, T. Koepke, D. Jiwan, S. Schaeffer, V. Krishnan, C. Wu, V.T. Chu, S.T. King, J. Vick, Q. Tao, A. Mraz, A. Stormo, K. Stormo, R. Bogden, D. Ederle, A. Stella, A. Vecchietti, M.M. Kater, S. Mastero, P. Lasserre, Y. Lespinasse, A.C. Allan, V. Bus, D. Chagne, R.N. Crowhurst, A.P. Gleave, J.A. Fawcett, S. Proost, P. Rouze, L. Sterck, S. Toppo, B. Lazzari, R.P. Hellens, C.E. Durel, A. Gutin, R.E. Bumgarner, S.E. Gardiner, M. Skolnick, M. Egholm, Y. Van de Peer, F. Salamini, and R. Viola. 2010. The genome of the domesticated apple (Malus × domestica Borkh.). Nat. Genet. 42:833.

Wan, B., Y. Lin, and T. Mou. 2007. Expression of rice Ca2+-dependent protein kinases (CDPKs) genes under different environmental stresses. FEBS Lett. 581:1179–1189.

Wang, R.K., Z.H. Cao, and Y.J. Hao. 2014. Overexpression of a R2R3-MYB gene MdSIMYB1 increases tolerance to multiple stresses in transgenic tobacco and apples. Physiol. Plant. 150:76–87.

Xie, Z., D. Li, L. Wang, F.D. Sack, and E. Grotewold. 2010. Role of the stomatal development regulators FLP/MYB88 in abiotic stress responses. Plant J. 64:731–739.

Yamaguchi-Shinozaki, K. and K. Shinozaki. 1994. A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought. Plant Cell 6:251–264.

Yao, Y.X., M. Li, Z. Liu, Y.J. Hao, and H. Zhai. 2007. A novel gene, screened by cDNA-AFLP approach, contributes to lowering the acidity of fruit in apple. Plant Physiol. Biochem. 45:139–145.

Zhang, M., S.P. Liang, and Y.T. Lu. 2005. Cloning and functional characterization of NtCPK4, a new tobacco calcium-dependent protein kinase. Biochim. Biophys. Acta 1729:174–185.

Zheng, X., B. Chen, G. Li, and B. Han. 2009. Overexpression of a NAC transcription factor enhances rice drought and salt tolerance. Biochem. Biophys. Res. Commun. 379:985–989.
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, 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. 2. MdMYB4 phylogenetic tree with R2R3-MYB proteins in *Arabidopsis thaliana.*
Supplemental Table 1. Primer sequences used for expression analysis of 11 MdR2R3-MYB genes under abiotic stress treatments (Cao et al., 2013).

| Gene    | Subgroup | Forward  | Reverse                           |
|---------|----------|----------|-----------------------------------|
| MdMYB8  | S23      | GGCCTTGAGAGTAATGGGATAAG  | TCTAGGCACCTGGGTTGATA             |
| MdMYB42 | S14      | TGGGAAGCAGGTGGTCAATAA    | GTGTTGCTTCCTGGTATGA              |
| MdMYB59 | S22      | CTCTGCTGACTCTGTCATTACC  | CCTCCATGTAACCTCCACTTC            |
| MdMYB93 | S4       | GGGAGAGACTGTAACCGAAGATT  | GGGAGAGACTGTAACCGAAGATT          |
| MdMYB113| S2       | GGTGGCTGGAATGGGAGATCG    | CAACTGGAGCTGGTCTCCTTAC           |
| MdMYB131| S1       | GGTCCTGGTAAATGGGAGATCG   | CAACTGGAGCTGGTCTCCTTAC           |
| MdMYB143| S4       | GGGAAACCTGGTCGGAATAA     | TTTGCTGGCTGGTCTGAGATT            |
| MdMYB151| S20      | CACCAGCACTGACTATGGTCTC   | GGTGCTTCCTGAGAGTCCAA             |
| MdMYB194| S22      | CAGAGTGACAGCGAGAAAGA     | TTTGCTGGCTGGTCTGAGATT            |
| MdMYB214| S1       | CTGGCGAATGACTGCAATGG     | CAGACGACCTCCTCCTC                |
| MdMYB4  | S4       | TGGTAAAGGCTCAACCAACCAAAG| TTGGTCAATGATGACGATCTAGTGAAG      |
| 18S     | —        | CCTCCATGGAATGCTCTGTTA    | ACACGGGGAGAGTATGGAAC             |

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

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

---

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

| Primers                                    | Sequences (5′–3′)                  |
|--------------------------------------------|------------------------------------|
| Subcellular localization                   | CCGGAATTCATGGGAAGATCTCCTTGCTG      |
| Self-activating yeast capability            | CCGGAATTCCCTCAGAATCTACCAGCAC       |
| Construction of overexpression vector       | CCGGTACCATGGGAAGATCCTCTTGCTGAAA     |
| Quantitative RT-PCR analysis               | TGTTAAAAAGGCTCACACCAACAAG          |
| 18S                                        | CCTCCAAATGGATCTCTGTGTA              |
| 35S promoter                               | AGATTAGCCTTTTCAATTTTCAGAAA         |
| Construction of overexpression vector       | ATGGGAAGATCTCCTTGCTGAAA             |
| PCR detection of antibiotic-resistant calli| AGGTGGCGAAACCCGACAGGACTA           |

EcoRI site is underlined, BamHI site is underlined.