

# MaRING2: A Positive Regulator of an Absciscic Acid-dependent Response to Cold Stress from Banana Fruit

Jiao Chen<sup>1</sup>, De-bao Yuan<sup>1</sup>, Chao-zheng Wang, Yi-xing Li, and Fen-fang Li

Hainan Key Laboratory of Banana Genetic Improvement, Haikou Experimental station, Chinese Academy of Tropical Agricultural Sciences, Haikou 570102, China

Ke-qian Hong

Key Laboratory for Postharvest Physiology and Technology of Tropical Horticultural Products of Hainan Province, South Subtropical Crop Research Institute, Chinese Academy of Tropical, Agricultural Sciences, Zhanjiang 524088, China

Wang-jin Lu

State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources/Guangdong Key Laboratory for Postharvest Science, College of Horticultural Science, South China Agricultural University, Guangzhou 510642, China

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**ABSTRACT.** Many reports indicate that an abundance of really interesting new gene (RING) play key roles in regulating defense responses against abiotic and biotic stresses in plants. In this study, the cloning and functional characterization of a RING gene, *MaRING2*, in banana (*Musa acuminata*) fruit are reported. *MaRING2* belongs to the NEP1-interacting protein (NIP) RING-H2 finger protein family. Gene expression profiles revealed that *MaRING2* was cold responsive and induced by abscisic acid (ABA) treatment during cold storage. In this study, the *MaRING2* under control of the *Cauliflower mosaic virus 35S* (CaMV 35S) promoter was transformed to tobacco (*Nicotiana benthamiana*) using agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation. The resultant *MaRING2*-overexpressing transgenic plants (35S:*MaRING2*) exhibited significantly increased tolerance to low temperatures and were hypersensitive to exogenous ABA in terms of germination and early seedling growth. In addition, overexpression of *MaRING2* enhanced the expression of stress-responsive genes under normal (before cold stress) or cold conditions. These results demonstrate the biological role of *MaRING2* in conferring cold tolerance. Taken together, these results suggest that *MaRING2*, a C3H2C3-type RING protein, is a positive regulator of the ABA-dependent stress response.

Plants are frequently subjected to abiotic environmental stresses, such as low temperatures, drought, and high salinity. These conditions have significant detrimental effects on growth and crop yields. To address various environmental stresses, plants execute some physiological and metabolic responses that are normally mediated by the up-regulation of stress-regulated gene expression (Thomashow, 1999; Xiong et al., 2002; Xiong and Zhu, 2002; Zhu, 2001). These induced genes either directly protect plants against stresses by the production of important metabolic proteins (functional proteins) or regulate the genes for signal transduction in the stress response (regulatory proteins). It is of immense importance to elucidate the exact function of genes involved in signal transduction and their regulation to understand their responses to different environmental stresses in plants (Cui et al., 2002; Ronald and Leung, 2002).

Zinc finger protein genes constitute a large and diverse gene family. A number of zinc finger proteins have been found to be involved in abiotic and biotic stress responses (Cui et al., 2002; Huang et al., 2005; Kim et al., 2001; Mukhopadhyay et al.,

2004; Sakamoto et al., 2004). One interesting zinc finger transcription factor is the ring zinc finger domain protein, which was originally named after the acronym for the first RING finger gene (Joazeiro and Weissman, 2000) and was defined by the presence of a consensus sequence harboring cysteine (Cys) and histidine (His) residues (Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys/His-X2-Cys-X4-48-Cys-X2-Cys where X can be any amino acid) that can form a binding site for two zinc atoms. The canonical RING-finger protein family can be subcategorized into two types, RING-H2 and RING-HC, on the basis of the fifth coordination site, Cys or His, respectively. Additional modified types of RINGs, including RING-v, RING-D, RING-S/T, RING-G, and RING-C2, have been previously identified in the arabidopsis (*Arabidopsis thaliana*) genome (Kosarev et al., 2002; Stone et al., 2005). RING finger proteins play important roles in various biological processes (Ciechanover, 1998; Lyzenga and Stone, 2012), including photomorphogenesis (Ang and Deng, 1994), plant growth and development (Li et al., 2011; Zhang et al., 2008), and hormone signaling pathways (Bu et al., 2009; Zhang et al., 2007). In particular, RING finger proteins have been reported to be involved in negatively or positively regulating stress responses of multiple plants (Craig et al., 2009; Dong et al., 2006; Kim and Kim, 2013; Lee and Kim, 2011; Lee et al., 2009; Li et al., 2014; Liu et al., 2008; Ryu et al., 2010; Zhang et al., 2008). For

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<sup>1</sup>Corresponding author. E-mail: chenjiao52@163.com or yuandebao@163.com.

Table 1. Primer sequences used for cloning and expression analysis of banana *MaRING2* and stress-responsive genes in tobacco plants.

Names	Sequences (5'-3')	
	Forward	Reverse
MaRING2-5'RACE <sup>2</sup>	AACTGCTGCAAAGACACAGGT	GAAGAAGGAGACCGAGAGAAGAC
MaRING2-Full	ATGGATGTCTTCTCTCGG	GATATCCCTTCTGCAAAGCG
MaRING2-qPCR	TGGAGAAACAGTGAGGAGATTACC	TCAGAAAGAACCGTGGAGTATCG
MaActin1	GAGGCACCAGTCCGTAGATAGC	CCGCCAGATGAAGGTACAACAT
NbWRKY17-qPCR	CCGCTTTCTACTTCTTCA	GCTGGGACTCTAACAACCTC
NbMYB1-qPCR	GTTACACGAAATGCTTGG	TTTGGAGTTCTGAGGAGG
NbbZIP-qPCR	TAGGTCACGGATGAGGAA	ATTAGCATCAGCCCCAAA
NbUbiquitin	TCCAGGATAAGGAGGGTA	GAGACGGAGGACAAGGTG

<sup>2</sup>RACE = rapid amplification of cDNA ends; qPCR = quantitative polymerase chain reaction.

example, a T-DNA insertion mutant of arabidopsis *ATL9* resulted in increased susceptibility to powdery mildew (*Erysiphe cichoracearum*) (Ramonell et al., 2005). Constitutive expression of arabidopsis *ATL2* exhibited the up-regulation and accumulation of defense-related salicylic acid- and jasmonic acid-responsive genes (Serrano and Guzman, 2004). Transgenic tobacco (*Nicotiana tabacum*) plants that constitutively express rice (*Oryza sativa*) *RING* gene *OsBIRF1* exhibited enhanced disease resistance against *Tobacco mosaic virus* and oxidative stress tolerance via up-regulating the expression of oxidative stress-related genes (Liu et al., 2008). The chinese wild grapevine (*Vitis pseudoreticulata*) *RING* finger protein 1 (EIRP1) positively regulated plant disease resistance by mediating proteolysis of the negative regulator VpWRKY11 (Yu

et al., 2013). Constitutive expression of a hot pepper (*Capsicum annuum*) *CaRma1H1* in tomato (*Solanum lycopersicum*) conferred strongly enhanced tolerance to drought and salt stresses (Seo et al., 2012). The *RING* finger gene *NtRHF1* played a positive role in drought stress tolerance possibly through transcriptional regulation of several stress-responsive marker genes in tobacco (Xia et al., 2013). The *RING* finger protein STRF1 was involved in membrane trafficking and modulated salt-stress response in arabidopsis (Tian et al., 2015). The maize (*Zea mays*) *RING*-H2 finger protein gene *ZmXERICO* was up-regulated in salt, drought, abscisic acid, and cold stresses (Gao et al., 2012). A *Brassica rapa* *RING* finger protein gene *BrRZFP1* overexpression conferred increased tolerance to cold, salt, and dehydration stresses (Jung et al., 2013). Suppression of

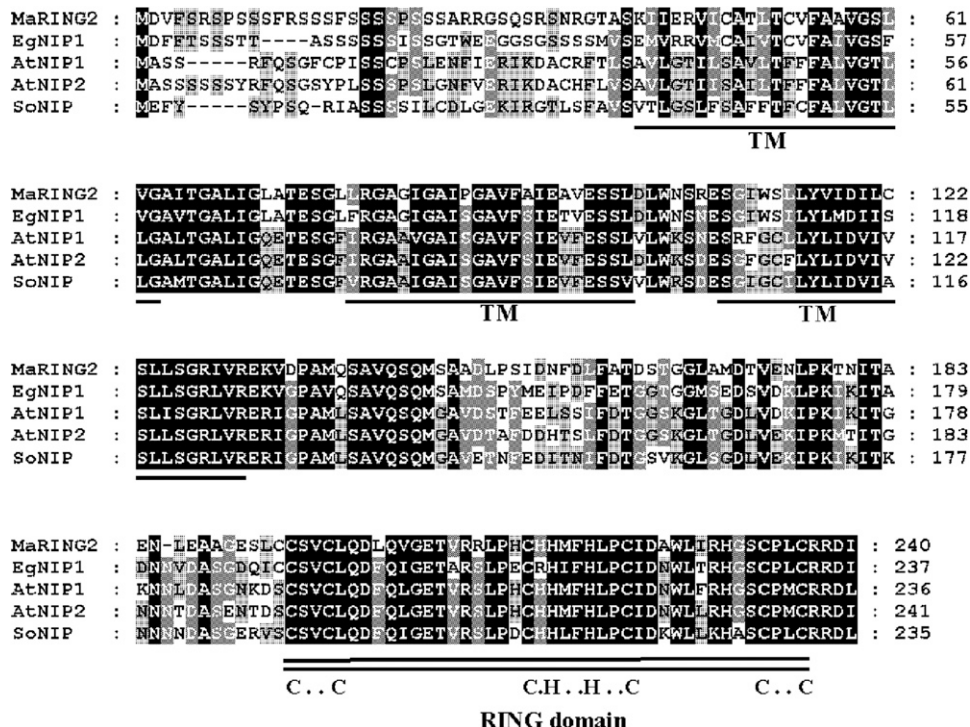


Fig. 1. Amino acid sequence alignment of the banana *MaRING2* with other plant NIP *RING*-H2 finger proteins. *MaRING2* was aligned with oil palm *EgNIP1* (XP\_010918134), arabidopsis *AtNIP1* (At2g17750), arabidopsis *AtNIP2* (Q8GT74), and spinach *SoNIP* (AM883105). Identical and similar amino acids were presented by black and gray shading, respectively. Gaps were introduced to optimize alignment. Simple bars below the sequences indicate the location of the three N-terminal transmembrane domains (TM). The C-terminal *RING* domain is double underlined, and the significant amino acids that determine the *RING* are noted below the line.

and Shinozaki, 2006) and plays important roles in mediating adaptive responses to various environmental stresses, such as high salinity, drought, cold, and other abiotic stresses (Night and Knight, 2001). The pathways leading to stress adaptation can be divided into two major types: ABA-dependent and ABA-independent pathways (Himmelbach et al., 2003; Jakoby et al., 2002; Ladyzhenskaya, 2001; Zhu, 2002). During the ABA-mediated stress response, many stress-related genes, including various kinases and transcription factor genes, have been up-regulated under stress conditions (Chak et al., 2000; Choi et al., 2000; Kang et al., 2002; Koncz and Szabados, 1997; Seki, 2002; Zhu, 2002). Increasing evidence indicates that RING finger proteins play important roles in regulating ABA signaling and in related abiotic stress responses in plants (Lee et al., 2001; Lopez-Molina et al., 2003; Stone et al., 2006; Zhang et al., 2005, 2007). The arabidopsis RING-H2 finger protein gene *XERICO* confers drought tolerance through enhanced ABA biosynthesis (Ko et al., 2006). Salt- and drought-induced ring finger1 (*SDIR1*) is an active E3 ligase and participates in ABA-mediated stress signal transduction (Zhang et al., 2007). Arabidopsis RING finger E3 ligase RHA2b acts additively with RHA2a in regulating ABA signaling and drought response (Li, 2011). These results suggest a link between RING finger proteins and ABA-mediated stress responses in plants. However, the functional relationship between ABA and RING finger proteins in response to chilling stress in banana fruit is far from being clearly understood.

In this study, a novel *RING* gene, *MaRING2*, was isolated and characterized from banana fruit. The expression patterns of *MaRING2* related to ABA-induced chilling tolerance of banana fruit were investigated. Moreover, in contrast to the wild-type (WT) plants, *MaRING2*-overexpressing transgenic tobacco plants (*35S:MaRING2*) showed hypersensitive phenotypes to exogenous ABA in terms of the germination rate, accumulated higher transcript levels of stress-responsive genes in response to cold tolerance, and were markedly tolerant to cold stress. Overall, the data presented in this study indicate that *MaRING2*, a C3H2C3-type RING protein, plays a role as a positive regulator in the ABA-dependent cold response in banana fruit.

## Materials and Methods

**PLANT MATERIALS AND TREATMENTS.** Prelimacteric banana fruit at the 75% to 80% plump stage were obtained from a commercial plantation near Guangzhou, China. Banana fruit of uniform weight, shape, and maturity that were free

of visual defects were used for this study. ABA (0.1 mM) treatment was performed as described previously (Chen et al., 2008), and the treated fruit were stored at 7 °C (cold stress) and 90% relative humidity for 7 d. For cold stress, fruit without ABA pretreatment were directly stored at 7 °C, while for the non-chilling temperature control, fruit were directly stored at 22 °C. Samples were taken at 0, 6, and 12 h and 1, 3, 5, and 7 d, and banana peels were collected. The sampled peel tissues were frozen in liquid nitrogen immediately and stored at -80 °C for further use. All treatments were repeated three times with three replicates.

The WT tobacco plants were used for phenotypic assays of *35S:MaRING2* plants in all experiments. Unless noted otherwise, plants were grown under sterile conditions on Murashige and Skoog (MS) nutrient agar medium containing 2% sucrose, or on soil in a growth chamber (16 h light/8 h dark) at 23 °C.

**RNA EXTRACTION, GENE ISOLATION, AND SEQUENCE ANALYSIS.** Frozen tissues were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the hot borate method (Wan and Wilkins, 1994). Potentially contaminating DNA was eliminated by treating the RNA with DNase I using an RNase-free kit (Promega, Madison, WI). The DNA-free total RNA was then used as a template for reverse transcription

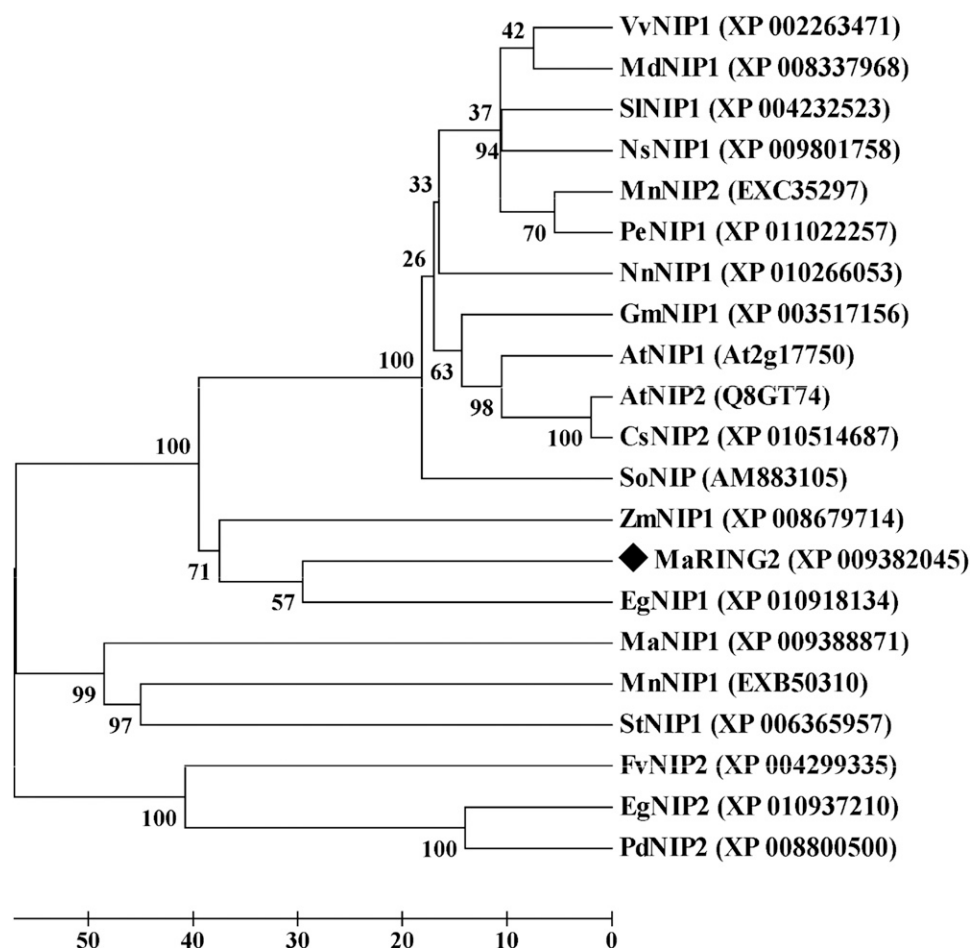


Fig. 2. Phylogenetic tree of banana *MaRING2* with 20 other plant NIP RING-H2 finger proteins. Alignments were made using CLUSTAL X (version 1.83; Toby Gibson, Heidelberg, Germany) and the phylogenetic tree was constructed with MEGA 5.0 (version 5.0, Tokyo Metropolitan University, Tokyo, Japan) using a bootstrap test of phylogeny with minimum evolution test and default parameters. Numbers at the branch points indicated bootstrap values. A scale of distance is shown in the bottom.

polymerase chain reaction (RT-PCR). The first-strand cDNA of the product was subjected to PCR amplification. The novel *MaRING2* gene induced by cold stress was isolated from a transcriptome database obtained by RNA sequencing (RNA-seq). Briefly, harvested banana fruit at 0 and 1 d of cold storage at 7 °C were chosen and constructed into two different libraries. The library constructions and RNA-seq was performed using a high-throughput Solexa/Illumina sequencing platform, and sequences were assembled and annotated by the Beijing Genome Institute (BGI, Shenzhen, China), with the parameters described by Feng et al. (2012). One *RING* finger gene, induced by cold stress and belonging to the NIP family, was found in the transcriptome database. Thus, this *RING* finger gene, termed *MaRING2*, was selected, and its sequence was verified by recloning and resequencing. Then, the 5'-end sequence of the *MaRING2* was obtained by 5'-rapid amplification of cDNA ends (RACE) using a RACE kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions, and the full sequence of *MaRING2* was further confirmed by resequencing. The specific primers used for PCR amplification and RACE are shown in Table 1.

A randomized complete block design was used with three replications. Identification of nucleotide sequences was performed using the National Center for Biotechnology Information (NCBI) Blast program (Altschul et al., 1997). Alignment and comparison of the sequences were performed with the Clustal W program (Thompson et al., 1994). Open reading frame (ORF) and protein predictions were performed using NCBI ORF Finder (Stothard, 2000). The theoretical isoelectric point (pI) and mass values for mature peptides were calculated using the PeptideMass program (Gasteiger et al., 2003). A phylogenetic tree was constructed using the neighbor-joining method in the MEGA 5 (version 5.0; Tokyo Metropolitan University, Tokyo, Japan) program and was visualized by TreeView software (Page, 1996).

**EXPRESSION ANALYSIS BY QUANTITATIVE REAL-TIME PCR.** The transcript levels of *MaRING2* in banana fruit under various treatments and expression patterns of stress-responsive genes in tobacco (WT and transgenic lines) before and after cold stress or ABA treatment were analyzed by quantitative real-time PCR (qRT-PCR) using the SYBR Green dye method. Synthesis of the first-strand cDNA and all qRT-PCR analyses were performed as described previously (Chen et al., 2011; Zhao et al., 2013). Actin or ubiquitin was used as an internal control to normalize the relative expression level of the analyzed genes in banana or tobacco, respectively. All qRT-PCR reactions were normalized using  $C_t$  values corresponding to the reference gene. The relative expression levels of the target gene were calculated with the formula  $2^{-\Delta\Delta C_t}$  (Livak and Schmittgen, 2001). Three independent biological replicates were used in the analysis. Primer sequences used for qRT-PCR analysis are listed in Table 1.

**PLANT TRANSFORMATION AND GENERATION OF TRANSGENIC PLANTS.** To construct a vector for plant transformation, the full-length cDNA of *MaRING2* was amplified by PCR using an *Xba*I and *Kpn*I primer set: PMV-RING1-UP GCtctagaATG-GATGTCTTCTCTCGG and PMV-RING1-REV GGggtaccC-TAGATATCCCTTCTGCAAAGCG. The PCR products were digested with *Xba*I and *Kpn*I and inserted in the sense orientation into the *Xba*I/*Kpn*I sites of a plant expression vector, PMV (Kan<sup>r</sup>, CaMV 35S promoter, NOS terminator, T DNA), to replace the *b*-glucuronidase (GUS) gene, under control of the CaMV 35S promoter. After sequence confirmation

the construct was introduced into agrobacterium strain EHA105. To produce transgenic tobacco plants, agrobacterium-mediated transformation of leaf discs was carried out according to Horsh et al. (1985). The presence of the transgene in the kanamycin (Kan)-resistant seedlings was verified by PCR using primers: RG-UP: ATGGATGTCTTCTCTCGG; RG-REV: GATATCCCTTCTGCAAAGCG. Overexpression of *MaRING2* in two of the selected putative transgenic plants was examined by semiquantitative RT-PCR using the forward primer 5'-TGGAGAAACAGTGAGGAGATTACC-3' and the reverse primer 5'-TCAGAAGAACCGTGGAGTATCG-3', while the ubiquitin gene was used as an internal control (Table 1). T2 seeds of the overexpressing lines were harvested according to Liu et al. (2009) for the stress tolerance assay; the details are as follows: T0 transgenic plants were verified by PCR, and then T1 seeds were harvested, T1 transgenic plants, in which kanamycin-resistant segregation was 3:1, were considered for T2 seed harvesting.

**ANALYSIS OF COLD TOLERANCE, ELECTROLYTE LEAKAGE AND MALONDIALDEHYDE.** For cold treatment, each 10 seedlings of WT and transgenic plants were planted in the same pot filled with a mixture of soil and sand (1:1) where they were regularly watered for 1 month before the stress was induced. Then, the control (WT) and transgenic plants were chilled for 24 h at 2 °C, after 24 h of treatment, the phenotypes were observed, the plants were photographed, the degree of the leaf blade wilting was observed, and the survival rate was calculated.

Malondialdehyde (MDA) concentration was measured as the indexes of membrane condition according to the previous studies with minor modifications (Chen et al., 2008). Leaf tissues (2.0 g) were homogenized in 6 mL of 10% trichloroacetic acid and then centrifuged for 15 min at 10,000  $g_n$ . The supernatant was collected and 2 mL was mixed with 6 mL of 0.6% thiobarbituric acid. The mixture was heated to 100 °C for 20 min, quickly cooled to 4 °C and centrifuged at 10,000  $g_n$  for 10 min. Absorbance of the supernatant was measured at 532,

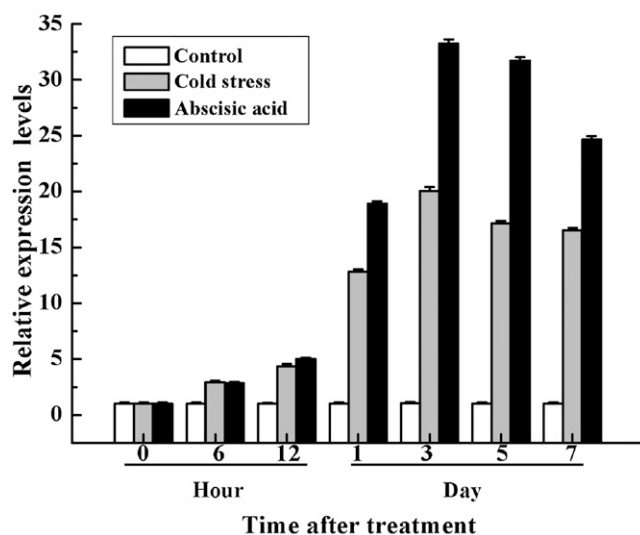


Fig. 3. Expression of *MaRING2* in banana fruit during 7 d of storage. Abscisic acid (ABA)-treated banana fruit were stored at 7 °C for 7 d, for cold stress, fruit without ABA pretreatment were directly stored at 7 °C, while for non-cold stress control, fruit were directly stored at 22 °C. Expression level at different time points was expressed as a ratio relative to the harvest time (0 d of non-cold stress control), which was set at 1. Each value represents the means of three biological replicates, and vertical bars indicate the SE.

600, and 450 nm using an ultraviolet spectrophotometer (Shimadzu, Kyoto, Japan). MDA concentration was calculated according to the formula:  $6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}$ . Three replicates were used for each assessment.

The electrolyte leakage (EL) was measured as the indexes of membrane condition according to the method of Zhang and Ervin (2009) with some modifications. Fresh leaf discs (100 mg) were washed and then placed in Falcon tubes with 20 mL distilled water. The tubes were shaken for 24 h and an initial electrical conductance (C1) was determined using a conductivity meter (model DDSJ\_308A; Shanghai Precision and Scientific, Shanghai, China). The tubes were boiled for 20 min, cooled down to 25 °C, and the final electrical conductance (C2) was measured. The EL was calculated according to the formula:  $EL (\%) = (C1/C2) \times 100$ .

**GERMINATION ASSAY.** Germination assays were performed in triplicate with 25 to 30 seeds. The seeds of WT and 35S: *MaRING2* plants were cleansed with 3% sodium hypochlorite for surface sterilization for 10 min in 2.0 mL centrifuge tube, and were cleansed with sterilized water six times, and then were imbibed in 0.05% agar solution in dark for 2 d at 4 °C for vernalization. Next, seeds were sown in MS medium containing 0, 0.1, 0.5, or 1  $\mu$ M ABA (Sigma-Aldrich, St. Louis, MO) at 22 °C with a 16-h-light/8-h-dark photoperiod, the percentages of radicle emergence and cotyledon greening were determined after 3 and 7 d, respectively.

## Results

**CLONING OF A FULL-LENGTH cDNA OF THE *MaRING2* GENE AND SEQUENCE ANALYSIS.** In this study, a novel putative RING full-length cDNA was isolated from banana fruit and designated as *MaRING2*. *MaRING2* was predicted to encode proteins of 240 amino acids, with a calculated molecular weight of 25.4 kDa and a pI value of 5.72. A BLAST search of GenBank revealed that *MaRING2* had 72% identity at the amino acid level with *Elaeis guineensis* NIP1. The multiple sequence alignment of NIPs showed that the deduced amino acid sequences of *MaRING2* contained all of the structural features of the plant NIP RING-H2 finger proteins, three N-terminal transmembrane domains, and a C-terminal C3H2C3 type RING finger signature; the positions of these domains are underlined (Fig. 1). The phylogenetic tree analysis showed that *MaRING2* clustered with EgNIP1 and ZmNIP1 and had the closest relationship with EgNIP1 (Fig. 2). These results suggest that *MaRING2* encodes

a RING-H2 finger protein with structural features common to the plant-specific NIP family.

***MaRING2* WAS UP-REGULATED BY COLD AND ABA.** To understand the possible role of *MaRING2* in ABA-induced banana fruit chilling tolerance, the expression pattern of *MaRING2* in the peel of cold-stored fruit after ABA treatment was investigated by qRT-PCR. As shown in Fig. 3, compared with the expression of *MaRING2* in non-cold control fruit, the expression of *MaRING2* in the fruit directly stored at 7 °C (cold stress) increased at 6 h and reached  $\approx$ 18-fold on day 3, suggesting that *MaRING2* was responsive to chilling and that the expression levels of *MaRING2* were strongly induced by ABA treatment. The accumulation level in ABA-treated fruit was 13- and 14.5- fold higher than that in the cold stress fruit after 3 and 5 d of storage, respectively. These data indicate that *MaRING2* is cold-inducible and might be related to ABA induced-chilling tolerance of banana fruit.

**GENERATION AND SELECTION OF *MaRING2* TRANSGENIC TOBACCO PLANTS.** *MaRING2* was introduced into tobacco via agrobacterium-mediated transformation, and was constitutively

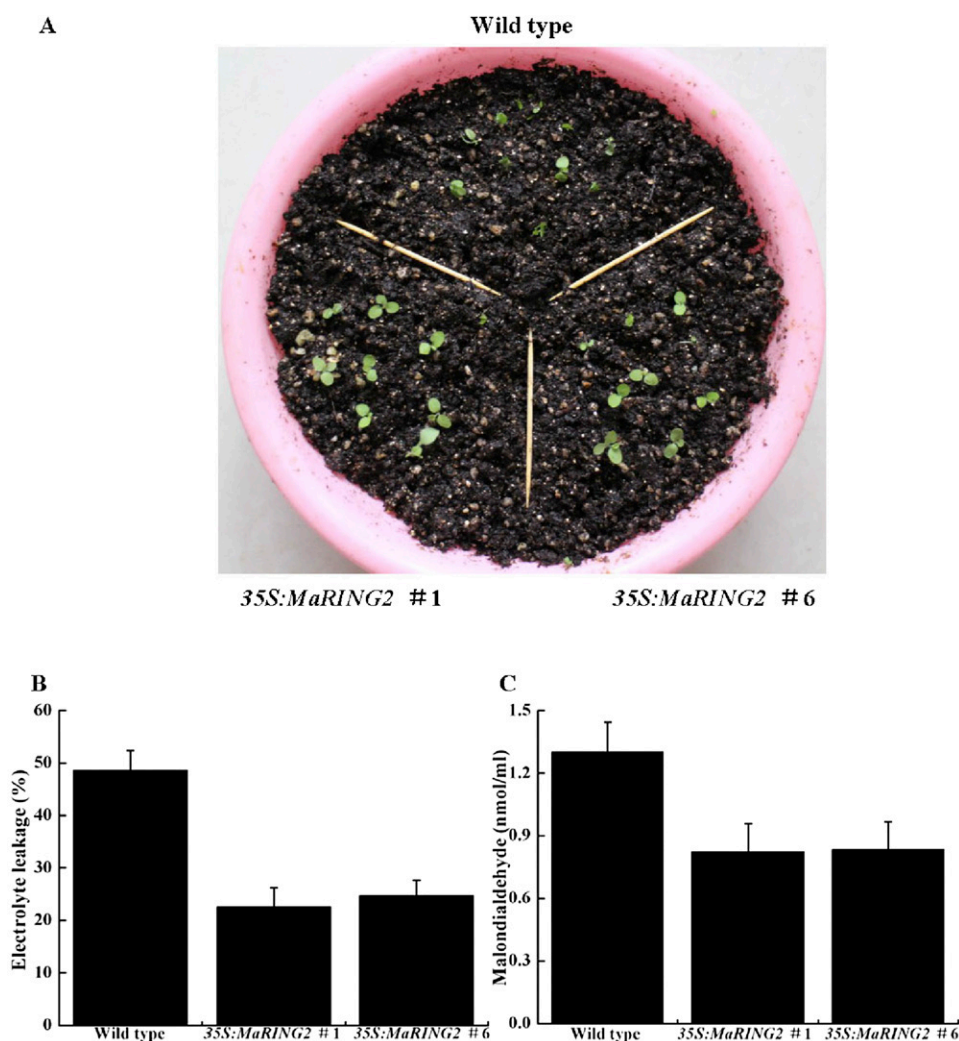


Fig. 4. Overexpression of banana *MaRING2* enhances cold tolerance in transgenic tobacco. (A) The photographs are of representative tobacco wild-type (WT) and transgenic lines (35S: *MaRING2* #1 and 35S: *MaRING2* #6) after chilling for 24 h. (B) Electrolyte leakage (EL) and (C) malondialdehyde (MDA) level in the WT, 35S: *MaRING2* #1, and 35S: *MaRING2* #6 after cold tolerance. Each value represents the means of three biological replicates, and vertical bars indicate the SE.



expressed under the control of the CaMV35S promoter. To verify this integration, kan-resistant transgenic plants were analyzed using PCR. RT-PCR analysis showed that *MaRING2* mRNA was detected in the transgenic plants, but not in the WT (data not shown). We selected nine transgenic lines harboring the *MaRING2* transgene to monitor their expression in 3- to 4-week-old plants (data not shown). Two of the lines (1 and 6), which had a higher *MaRING2* expression level, were used for the stress tolerance test. None of these transgenic plants had any visible alterations from the WT morphology.

**ASSESSMENT OF COLD TOLERANCE IN THE TRANSGENIC LINES.** To assess the effect of *MaRING2* overexpression on cold tolerance, 1-month-old WT and transgenic plants were exposed to 24 h of chilling at 2 °C. The degree of cold resistance differed significantly between the two genotypes.

As shown in Fig. 4A, the transgenic tobacco plant lines 1 and 6 showed enhanced tolerance and the WT plants were extremely sensitive to this stress, WT plants showed a higher degree of the leaf blade wilting compared with the transgenic plants, the WT plants survival rate was 40% (4 of 10), and the survival rate of the transgenic plant lines 1 and 6 were 80% (8 of 10) and 60% (6 of 10), respectively. In addition, EL, an important indicator of membrane injury, was significantly higher in the WT (48.6%) than in line 1 (22.5%) or 6 (24.7%), indicating that the WT suffered from severe membrane damage (Fig. 4B). The MDA level exhibited a profile similar to the EL, which was significantly lower in the transgenic lines relative to the WT (Fig. 4C). These findings demonstrate that the transgenic lines were more resistant to cold stress.

**EXPRESSION ANALYSIS OF STRESS-RESPONSIVE GENES IN THE WT AND TRANSGENIC LINES BEFORE AND AFTER COLD TOLERANCE.** A number of plant transcription factors, such as *bZIP*, *WRKY*, and *MYB*, have been shown to be involved in plant response to environmental stress (Lindemose et al., 2013). To gain further insight into the molecular mechanism underlying the enhanced cold resistance in the transgenic lines, the transcript abundance of stress-responsive genes (*NbWRKY17*, *NbMYB1*, and *NbbZIP*) was examined in the WT and transgenic lines before and after 24 h of cold stress (Fig. 5). Under normal conditions (before cold stress), the mRNA levels of all three genes in lines 1 and 6 were higher than those in the WT, the accumulation level of *NbWRKY17* in lines 1 and 6 was 0.9- and 1.1-fold higher than that in the WT, the expression level of *NbMYB1* in lines 1 and 6 was 1.6- and 2.1-fold higher than that in the WT, and the transcript level of *NbbZIP* in lines 1 and 6 was 1- and 2.2-fold higher than that in the WT. Exposure to chilling caused up-regulation of the transcript levels of the analyzed genes in all lines, but lines 1 and 6 still had a higher expression level in comparison with the WT, the accumulation level of *NbWRKY17* in lines 1 and 6 was 2.3- and 2.2-fold higher than that in the WT, the expression level of *NbMYB1* in lines 1 and 6 was 1.0- and 2.0-fold higher than that in the WT, and the transcript level of *NbbZIP* in lines 1 and 6 was 3.2- and 4.4-fold higher than that in the WT. These results demonstrate that overexpression of *MaRING2* in tobacco enhances the transcript levels of the stress-responsive genes with or without cold stress.

**THE 35S:MaRING2 PLANT IS HYPERSENSITIVE TO ABA.** We compared the seed germination rates of WT and 35S:MaRING2 plants in the presence or absence of ABA. To measure the germination rates, ≈25 seeds were placed on full-strength solid MS medium supplemented with different concentrations (0, 0.1, 0.5, 1, or 2 μM) of ABA. After 3 d of stratification, the

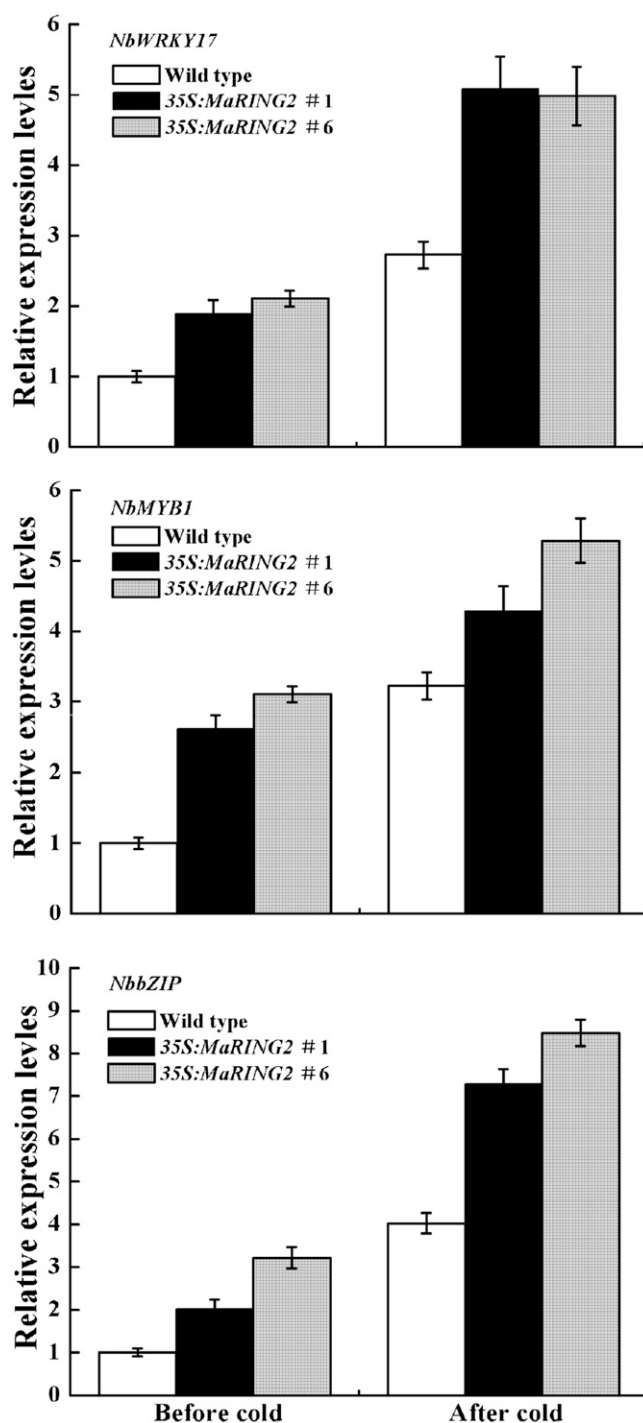


Fig. 5. Quantitative real-time polymerase chain reaction (PCR) analysis of expression levels of stress-responsive genes in the wild-type (WT) and the transgenic tobacco plants (35S:MaRING2 #1 and 35S:MaRING2 #6) under normal (before cold stress) and cold conditions (after 24 h of cold stress). Each value represents the means of three biological replicates, and vertical bars indicate the SE.

germination rates were determined as the percentage of radicle emergence (Fig. 6A). WT and 35S:MaRING2 (lines 1 and 6) seeds fully germinated on MS medium without ABA. On the ABA-containing growth medium, the germination rates of WT seeds were concomitantly reduced as the concentration of ABA increased. In the presence of 0.5 μM ABA, 56% of WT seeds failed

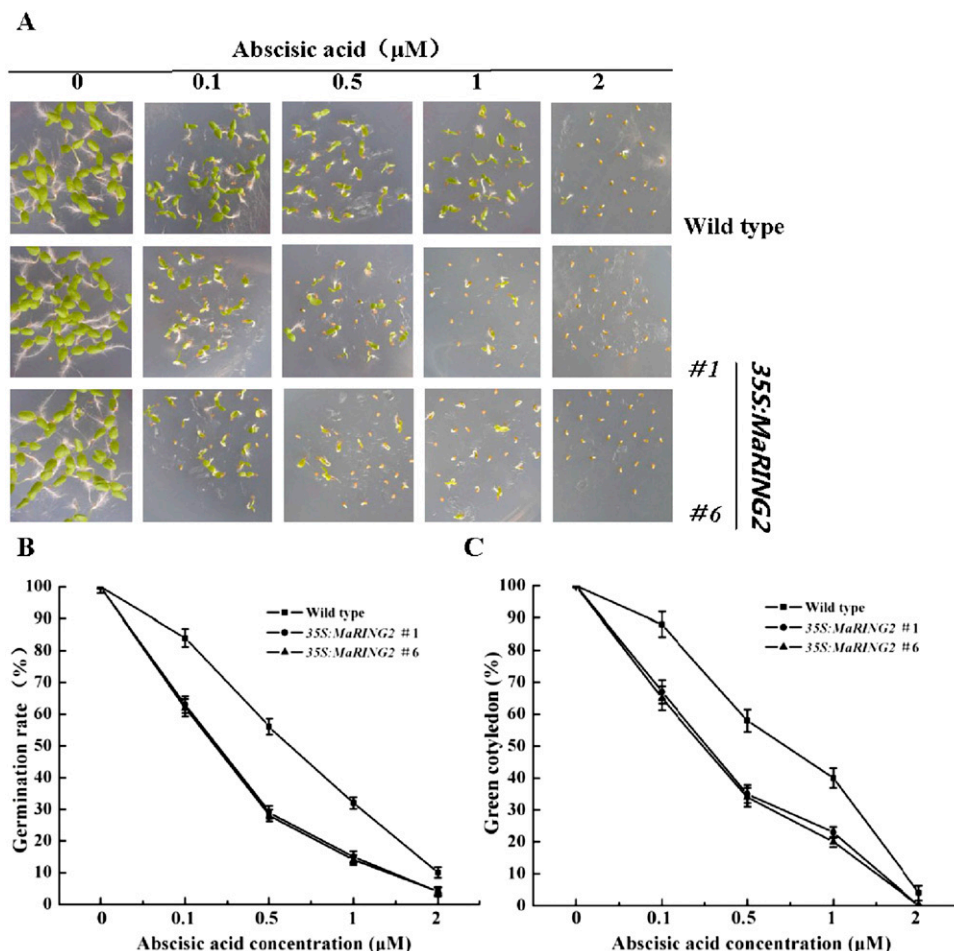


Fig. 6. Phenotypes of tobacco wild-type (WT) and transgenic lines (*35S:MaRING2* #1 and *35S:MaRING2* #6) in response to abscisic acid (ABA) during seed germination and early seedling growth. (A) ABA sensitivity of WT, *35S:MaRING2* #1, and *35S:MaRING2* #6 plants in the germination stage. Surface-sterilized seeds were imbibed in 0.05% agar solution for 2 d at 4 °C for vernalization, sown on Murashige and Skoog (MS) medium supplied with ABA (0, 0.1, 0.5, 1, and 2  $\mu\text{M}$ ), and then incubated for 7 d at 22 °C under a 16-h-light/8-h-dark photoperiod; bars = 0.5 cm. (B) Quantification of radicle emergence in response to ABA. (C) Quantification of cotyledon greening in response to ABA. Average and SD values were determined from three biological replicates ( $n = 25\text{--}30$ ).

to germinate at 3 d after culture (Fig. 6B). In contrast, *35S:MaRING2* transgenic seeds exhibited hypersensitivity to ABA. More than 50% of these transgenic seeds were unable to germinate in the presence of 0.5  $\mu\text{M}$  ABA, and less than 5% of the transgenic seeds germinated with 2  $\mu\text{M}$  ABA (Fig. 6B). In addition, at 7 d after germination,  $\approx 40\%$  of WT seedlings developed true green cotyledons in the presence of 1  $\mu\text{M}$  ABA (Fig. 6C). In contrast, under the same concentration of ABA, the growth of 77% and 80% of *35S:MaRING2* (lines 1 and 6) transgenic seedlings was completely arrested and true green cotyledons failed to develop at 7 d after germination (Fig. 6C). Thus, *MaRING2*-overexpressing plants were hypersensitive to ABA in terms of both radicle emergence and cotyledon development.

### Discussion

A wide variety of *RING* finger genes were isolated from multiple plant species, such as arabidopsis, rice, and poplar (*Populus tremula*), which contain 469, 378, and 399 *RING* finger genes, respectively (Du et al., 2009; Kraft et al., 2005). Some *RING* finger proteins are involved in the regulation of hormone signaling pathways, including ABA (Bu et al., 2009;

Stone et al., 2006; Zhang et al., 2005, 2007), auxin (Xie et al., 2002), and brassinosteroid (Molnar et al., 2002). In particular, ABA is an essential mediator in triggering plant responses to common abiotic stresses, including drought, salt, heat, cold, and oxidative stresses (Finkelstein et al., 2002; Xie et al., 2002). However, up to now, the report of the function of NIPs was that *AthATL25/NIP2* mediated the intraplasmic trafficking of *RPOTmp* at thylakoid membranes (Guzmán, 2012). Whether NIP *RING*-H2 proteins have additional functions related to ABA-mediated abiotic stress responses has never been explored. Therefore, in this study, we attempted to investigate the functional relationship between ABA and NIP *RING*-H2 finger protein in response to cold stress in banana fruit.

We report here the role of the *MaRING2* gene that encoded a NIP *RING*-H2 finger protein (Figs. 1 and 2) and played a positive role in regulating ABA signaling and stress responses. *MaRING2* was cold-inducible, and its transcript levels increased rapidly following the ABA treatment during cold storage (Fig. 3). In addition, overexpression of *MaRING2* led to ABA-associated phenotypes such as ABA hypersensitivity in terms of radicle emergence and cotyledon development (Fig. 6), reduced EL and the MDA level (Fig. 4B and C), enhanced the

transcript levels of the ABA-mediated abiotic stress response genes (Galbiati et al., 2011; Jiang et al., 2014; Suzuki et al., 2003; Xiang et al., 2008; Yan et al., 2014; Ying et al., 2012), *NbWRKY17*, *NbMYB1*, and *NbbZIP* (Fig. 5), and, therefore, increased cold tolerance (Fig. 4A). These are supported by previous studies which reported that the maize *RING*-H2 finger protein gene *ZmXERICO* was up-regulated in salt, drought, abscisic acid, and cold stress (Gao et al., 2012); overexpression of *RING* finger protein gene *BrRZF1* conferred increased tolerance to cold, salt, and dehydration stresses (Jung et al., 2013); and overexpression of *SDIR1* and *RHA2b* led to ABA hypersensitivity and ABA-associated phenotypes, such as salt hypersensitivity in germination, enhanced ABA-induced stomatal closing, and enhanced drought tolerance (Li, 2011; Zhang et al., 2007). These data support that *MaRING2* is a positive regulator of an ABA-dependent response to cold stress.

It is noteworthy that our analyses suggest that *MaRING2* may mediate the degradation of substrates that are negative regulators of ABA signaling. Alternatively, *MaRING2* may mediate the stabilization and activation of substrates that are positive regulators of ABA signaling. Future identification of *MaRING2* substrates will further our understanding of the ABA signaling pathway.

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