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An apple NAC transcription factor negatively regulates cold tolerance via CBF-dependent pathway

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ABSTRACT

Cold stress is an adverse stimulus that affects plant growth and development, and the C-repeat binding factor (CBF) cold-regulatory cascade has been regarded as a master regulator in the plant response to cold stress. Here, we showed that a NAC transcription factor modulated low-temperature tolerance. *MdNAC029/MdNAP*, an apple *NAC* gene was isolated and its role in regulating cold tolerance was investigated. *MdNAC029* was responsive to low-temperature treatment, and over-expression of *MdNAC029* reduced cold tolerance in apple calli and *Arabidopsis*. Furthermore, EMSA assays and transient expression assays demonstrated that MdNAC029 directly repressed the expression of *MdCBF1* and *MdCBF4* by binding to their promoters. Taken together, our data suggest that MdNAC029 functions as a negative regulator in regulating plant cold tolerance in a CBF-dependent manner, providing a deeper understanding of NAC transcription-factor-mediated cold tolerance.

1. Introduction

Multiple abiotic stresses, such as salinity, drought, and low temperature, often cause oxidative damage in plants and severely affect plant growth and development. Among these adverse external stimuli, cold stress has become a major environmental factor limiting crop productivity throughout the world. When exposed to cold stress, a series of changes occurs at the physiological and biochemical levels. For instance, reactive oxygen species (ROS) are dramatically stimulated, causing damage to nucleic acids, proteins, and lipids (Gill and Tuteja, 2010). In addition, low temperature also leads to freezing of the cell membrane, thus resulting in cell death (Thomashow, 1999; Kaplan et al., 2004). To perceive and adapt to cold stress, plants have evolved sophisticated and efficient mechanisms to protect themselves from this adversity. In brief, numerous genes are activated or repressed by low temperature, which results in the accumulation of protective proteins during cold stress (Thomashow, 1999).

Over the past decades, there have been a number of important findings in cold acclimation research, providing further understanding of the low-temperature regulatory mechanism. Many studies have revealed that cold temperature can induce significant changes in the plant transcriptome (Chinnusamy et al., 2007). For example, 4% to 20% of the genome is considered to be responsive to cold treatment in *Arabidopsis* (Hannah et al., 2005; Lee et al., 2005). Among these cold-responsive genes, the CBF (C-repeat binding factor) transcription factors play essential roles in the cold-stress response by directly regulating their target genes (Liu et al., 1998; Gilmour et al., 2004). In *Arabidopsis*, three CBF transcription factors (CBF1, CBF2, and CBF3) have been functionally identified (Stockinger et al., 1997; Gilmour et al., 1998; Medina et al., 1999). As for apple, five *CBF* genes (*MdCBF1*, *MdCBF2*, *MdCBF3*, *MdCBF4*, and *MdCBF5*) are characterized (Wisniewski et al., 2011, 2014), and over-expression of *CBFs* has been shown to result in constitutively enhanced cold tolerance (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Yang et al., 2011).

In the past several years, more attention has been concentrated on the transcriptional regulation of CBF transcription factors. In *Arabidopsis*, a bHLH transcription factor, ICE1, has been identified to bind to the *CBF3* promoter and activate its transcription during cold stress (Chinnusamy et al., 2003), and its homologous gene in apple, *MdCIbHLH1*, has been shown to enhance cold-stress tolerance by activating the *MdCBF2* gene (Feng et al., 2012). Just recently, the key regulator of BR signaling, BZR1, was proven to positively regulate cold-stress tolerance by activating the transcription of *CBF1* and *CBF2* (Li et al., 2017). In addition to the positive regulation of CBFs, several transcription factors have been identified as negative regulators of CBFs. For example, MYB15, a R2R3 MYB protein, was found to negatively modulate the expression of *CBF* genes in *Arabidopsis* (Agarwal et al., 2006). Moreover, ZAT10/12 and PIF4/7 can also bind to the promoters of *CBFs* and repress their expression (Vogel et al., 2005; Mittler et al., 2006; Lee and Thomashow, 2012). Therefore, the

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transcriptional regulation of CBF transcription factors plays a vital role in regulating the cold-stress response.

NAC (NAM, ATAF1/2, CUC2) transcription factors, which are one of the largest transcription factor families, play diverse roles in plant development and stress responses. Although some NAC transcription factors have been identified to be involved in salt and drought tolerance (Nakashima et al., 2012; Puranik et al., 2012), only a few have been characterized to regulate cold tolerance. For example, over-expression of *SNAC2* enhances cold tolerance in rice (Hu et al., 2008). In banana, MaNAC1 is a direct target of MaICE1, and MaNAC1 can also interact with MaCBF1 to regulate cold tolerance (Shan et al., 2014). Ectopic expression of a *Miscanthus lutarioriparius* NAC gene, *MlNAC5*, improves cold resistance in *Arabidopsis* (Yang et al., 2015).

In this study, we analyzed the function of *MdNAC029* (GenBank accession number: MDP0000481448) in the regulation of cold-stress tolerance. Our results demonstrated that MdNAC029 played a negative role in the cold stress response. Furthermore, MdNAC029 negatively regulated the transcription of *MdCBF1* and *MdCBF4* genes by directly binding to their promoters. Taken together, this is the first study showing that MdNAC029 negatively regulates plant cold stress tolerance through a CBF-dependent pathway.

2. Materials and methods

2.1. Plant materials and growth conditions

The apple calli of the 'Orin' cultivar (WT) and *Arabidopsis* (Col-0) were used for genetic transformation. Apomictic crabapple seedlings were used for stress treatments (NaCl, mannitol, ABA, and low temperature) and gene expression analysis.

The apple calli were cultivated on MS medium supplementing 0.5 mg L^{-1} IAA and 1.5 mg L^{-1} 6-BA at 24 °C, and were sub-cultured at 16-d intervals.

Arabidopsis seedlings were grown on MS medium at 22 °C under long-day conditions.

For low-temperature treatment (4 °C), two-week-old apple seedlings (apomictic crabapple) were treated at 4 °C for the indicated time (0 h, 1 h, 3 h, 6 h, and 9 h) under long-day conditions (16-h-light/8-h-dark). For NaCl, mannitol, and ABA treatments, apple seedlings were transferred to medium supplementing 150 mM NaCl, 150 mM mannitol, or 50 uM ABA. Tissues were collected and used for RNA extraction. The expression level of *MdNAC029* was monitored by qRT-PCR or RT-PCR.

2.2. Gene cloning and sequence analysis of MdNAC029

To find the homologs of *AtNAC029/AtNAP* in apple, the NCBI database was searched using the Basic Local Alignment Search Tool (BLAST) program and the apple gene family database (GFDB) was searched using the BLAST program. A gene (GenBank accession number: MDP0000481448) was obtained, which consisted of three exons and two introns and contained an 843-bp open reading frame (ORF) (Fig. S1A).

The amino acid sequence alignment and the conserved domain prediction were performed using the NCBI software and the Simple Modular Architecture Research Tool (SMART) software, respectively. The amino acid sequence alignment of MdNAC029 and *Arabidopsis* NAC029 protein indicated that MdNAC029 contained a conserved NAC domain (A-E) on its N-terminal side (Fig. S1B).

A phylogenetic tree was constructed with MEGA 5.0 software. Phylogenetic analysis between MdNAC029 and NAC029 proteins from other plant species revealed that MdNAC029 had the highest homology with MhNAC029 from *Malus hupehensis* (Fig. S1C).

2.3. PCR analysis

RT-PCR was performed to assess the expression of *MdNAC029* in the response to cold stress. qRT-PCR was conducted to identify the transgenic plant materials and examine the expression levels of cold-responsive genes in transgenic apple calli.

The transcription levels of *MdCBF* genes (MdCBF1: HM992942; MdCBF2: MDP0000198054; MdCBF3: MDC023575.38:2,048.2,752; MdCBF4: MDP0000154764; MdCBF5: MDC001798.196:3,293.4,369) and cold-responsive genes (MdKIN1: MDP0000165526; MdRD29A: MDP0000598443; MdCOR47: MDP0000529003) were tested using specific primers. All of the primers used are shown in Supplemental Table 1.

2.4. Generation of transgenic apple calli and arabidopsis

The over-expression vector *MdNAC029*-pCAMBIA1300 was constructed by inserting the ORF of the *MdNAC029* gene into the transformed vector pCAMBIA1300. The primers used for vector construction are shown in Supplemental Table 1.

Transgenic apple calli and transgenic *Arabidopsis* were generated through *Agrobacterium*-mediated genetic transformation (Clough and Bent, 1998; An et al., 2017).

2.5. Cold-stress tolerance assays

For chilling stress treatment of apple calli, eight-day-old apple calli were transferred to a long-day (16-h-light/8-h-dark) phytotron at 4 °C for another 10 days. The fresh weights of different genotypes of apple calli were measured (An et al., 2017).

For freezing stress treatment of *Arabidopsis* seedlings, 10 day-old *Arabidopsis* seedlings of different genotypes were treated with cold acclimation at 4 °C for three days. Then, they were treated at −4 °C for 0.5 h via gradient cooling, and finally the *Arabidopsis* seedlings were stored at 22 °C for another three days. The survival rates and electrolyte leakages of the *Arabidopsis* seedlings were counted (An et al., 2017; Li et al., 2017).

2.6. EMSA assays

EMSA assays were carried out as previously described (An et al., 2016). Biotin-labeled probes were mixed in a binding solution with MdNAC029-GST fusion protein at 24 °C for 20–25 min. The unlabeled probes were used for competition. The mutated probes (Mut) were generated by replacing the 5′-ACACGT-3′ motif with 5′-ACCCGG-3′. All probe sequences are shown in Supplementary Table 1.

2.7. Transient expression assays in tobacco (Nicotiana benthamiana) leaves

This assay was conducted as described in a previous study (An et al., 2017). The promoter sequences of *MdCBF1* and *MdCBF4* were inserted into pGreenII 0800-LUC vectors to generate the reporters. The effector was obtained by inserting the ORF of the *MdNAC029* gene into the pGreenII 62-SK vector. In the mutated sequences (M), the 5′-ACACGT-3′ motif was replaced by 5′-ACCCGG-3′. The following tests were carried out as described by An et al. (2017). The quantitative analysis of luminescence activities was conducted as described by Meng et al. (2017).

2.8. Statistical analysis

Statistical analysis was carried out using R (3.0.2) software with the R Commander package. Asterisks denote Student's *t*-test significance: $*P < 0.05$ and $**P < 0.01$. The results were analyzed in triplicate.