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Overexpression of the *Medicago falcata* NAC transcription factor *MfNAC3* enhances cold tolerance in *Medicago truncatula*

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ABSTRACT

Cold stress is the main factor underlying the reduction in productivity of *Medicago*. *Medicago falcata* and *Medicago truncatula* are two subspecies of *Medicago*, whose geographic adaption is limited by water, salinity and temperature. However, the regulatory signaling pathway under cold stress in *Medicago* is unclear. In this study, we identified a gene, *MfNAC3*, induced under salt, drought and cold stress. By generating the overexpression lines of *MfNAC3*, we observed a typical cold-resistant phenotype under both cold-acclimated and non-acclimated conditions, featured by an increased survival rate and significantly higher expression levels of the cold-responsive genes *MtCBFs* and *MtCASs*. Further investigations revealed that this gene encodes a NAC-type transcriptional factor that is localized in the nucleus and exhibits transcription activity. By performing an electrophoretic mobility shift assay, we found that MfNAC3 could bind to the CATGTG and CACG motifs in the promoter region of *MtCBF4*. Taken together, our results demonstrate that MfNAC3 exerts a positive role in cold response and provide evidence that MfNAC3 is a positive regulator of *MtCBF4*.

1. Introduction

Unlike animals, plants are unable to move away to avoid environmental changes, and have to face various biotic and abiotic stresses, such as cold, heat, drought, salinity and high light. These stresses can evidently change the molecular, biochemical, physiological and morphological behaviors of plants (Gehan et al., 2015), affecting their growth, development and productivity. Harsh environmental conditions, especially low temperatures, can lead to significant crop reduction and huge economic losses (Nakabayashi and Saito, 2015; Thomashow, 1999).

Legumes are among the primary global crops, and it is important to study the mechanisms of environmental tolerance in legumes for agricultural production (Wang, 2013). As a leguminous model plant, *M. truncatula* is widely used in molecular and genetic studies (de Lorenzo et al., 2007; Zhao et al., 2010). *M. falcata* is a cultivated, cross-pollinated species with multiple ploidy levels, including diploid and tetraploid, which exhibits strong tolerance against drought, cold, and soil infertility (Zhang et al., 2011). Various transcription factors induced by stress take important roles in regulating and controlling the signaling pathways, which has been generally believed to account for the differences in the stress tolerance among the legumes (Gehan et al., 2015; Tran et al., 2004). For instance, the fact that *M. falcata* has a higher survival rate than *M. truncatula* under the freezing condition can possibly be explained by the fact that more transcripts for CRT binding factor 3 (CBF3) and cold acclimation specific (CAS) genes are present in the former than in the latter (Zhang et al., 2011), such as *CAS15* and *CAS31* (Pennycooke et al., 2008).

As one of the largest families of plant-specific transcription factors (TF), NAC TFs have been well studied in *Arabidopsis*, rice, wheat and soybean (Riechmann et al., 2000). Many members of NAC family have been found to respond to the biotic and abiotic stress-related regulations (Nuruzzaman et al., 2013; Peng et al., 2010; Puranik et al., 2012). In *Arabidopsis*,*NAC019*, *NAC055* and *NAC072* are induced by high salinity, drought and ABA, and the overexpression of these genes enhances the drought tolerance in transgenic plants (Tran et al., 2004). *OsNAC5*, *OsNAC6*, *ONAC045* and *OsNAP* are induced by cold stress, and the overexpression of these genes enhances cold tolerance in transgenic rice (Chen et al., 2014; Nakashima et al., 2007; Ohnishi et al., 2005; Takasaki et al., 2010; Zheng et al., 2009). *SlNAC1* encodes a transcription factor in *Suaeda liaotungensis* K. that is involved in the ABA-dependent pathway and

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enhances cold stress tolerance in transgenic *Arabidopsis* (Li et al., 2014), as do *MlNAC5* in *Miscanthus lutarioriparius* (Yang et al., 2015) and *TaNAC2* in wheat (Mao et al., 2012). In contrast, tobacco overexpressing *GmNAC2* was found to be hypersensitive to cold stress (Jin et al., 2013). Cold stress stimulates the activation of NTL6, and then NTL6 protein induces the expression of cold-responsive pathogenesis-related (PR) genes to enhance disease resistance (Seo et al., 2010). The anthocyanin content increases and the NAC domain protein is induced by brief cold storage in blood oranges (Crifo et al., 2012). In cotton, *GhNAC8* and *GhNAC11* are induced by cold, which may regulate cotton development under abiotic stresses (Huang et al., 2013). In *Medicago*, some of the NAC family transcription factors have also been studied (Shen et al., 2009; Wang, 2013). *MtNAC969* is induced by salt stress and regulated by salt in the roots and nodules in different ways. The overexpression of *MtNAC969* causes the root to be shorter and less branched (de Zelicourt et al., 2012). *MtNST1*, playing an important role in lignification of fibers, is negatively regulated by MYB transcription factors (Wang et al., 2011; Zhao et al., 2010). In *Medicago sativa*, several *NAC* genes have been characterized as responsive to salt and drought stress and induced by exogenous ABA (Wang, 2013). However, it remains unclear how NAC family transcription factors exert their effects and how they are expressed under cold stress in *Medicago*.

CBF/DREB (C-repeat binding factor/dehydration response element binding factor) proteins regulate many stress signaling pathways, especially under cold stress (Park et al., 2015). The CBF/DREB proteins bind to the CRT/DRE element, which is present in the promoters of cold response genes, such as *COR* (Gilmour et al., 1998; Thomashow et al., 2001; Wang and Hua, 2009). Fourteen *GmDREB1* genes have been identified in soybean (*Glycine max*) and shown to be responsive to diverse abiotic stresses of cold, heat, drought and high salinity (Kidokoro et al., 2015). There are six genes encoding DREB1 in *Arabidopsis*. *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* are all induced by low temperature, but not by dehydration or high salinity. *CBF4/DREB1D* is responsive to osmotic stress, whereas *DDF1/DREB1F* and *DDF2/DREB1E* are induced by dehydration and high salinity. CBF2/DREB1C negatively regulates *CBF1/DREB1B* and *CBF3/DREB1A*. The expression of *CBF3/DREB1A* can be regulated by a MYC-like basic helix-loop-helix (bHLH) transcription factor, ICE1 (Chinnusamy et al., 2003; Lee et al., 2005), with its sumoylation mediated by SIZ1 (SUMO E3 ligase) and ubiquitination mediated by HOS1 (High expression of osmotically responsive gene 1) (Dong et al., 2006; Miura et al., 2007). Jasmonate positively regulates the ICE-CBF pathway by repressing JAZ1 and JAZ4, which increases the transcriptional activity of ICE1 (Hu et al., 2013). In *M. truncatula*, *MtCBF1*, *MtCBF2*, *MtCBF3/DREB1C*, *MtCBF4* and *MtDREB2A* have been identified

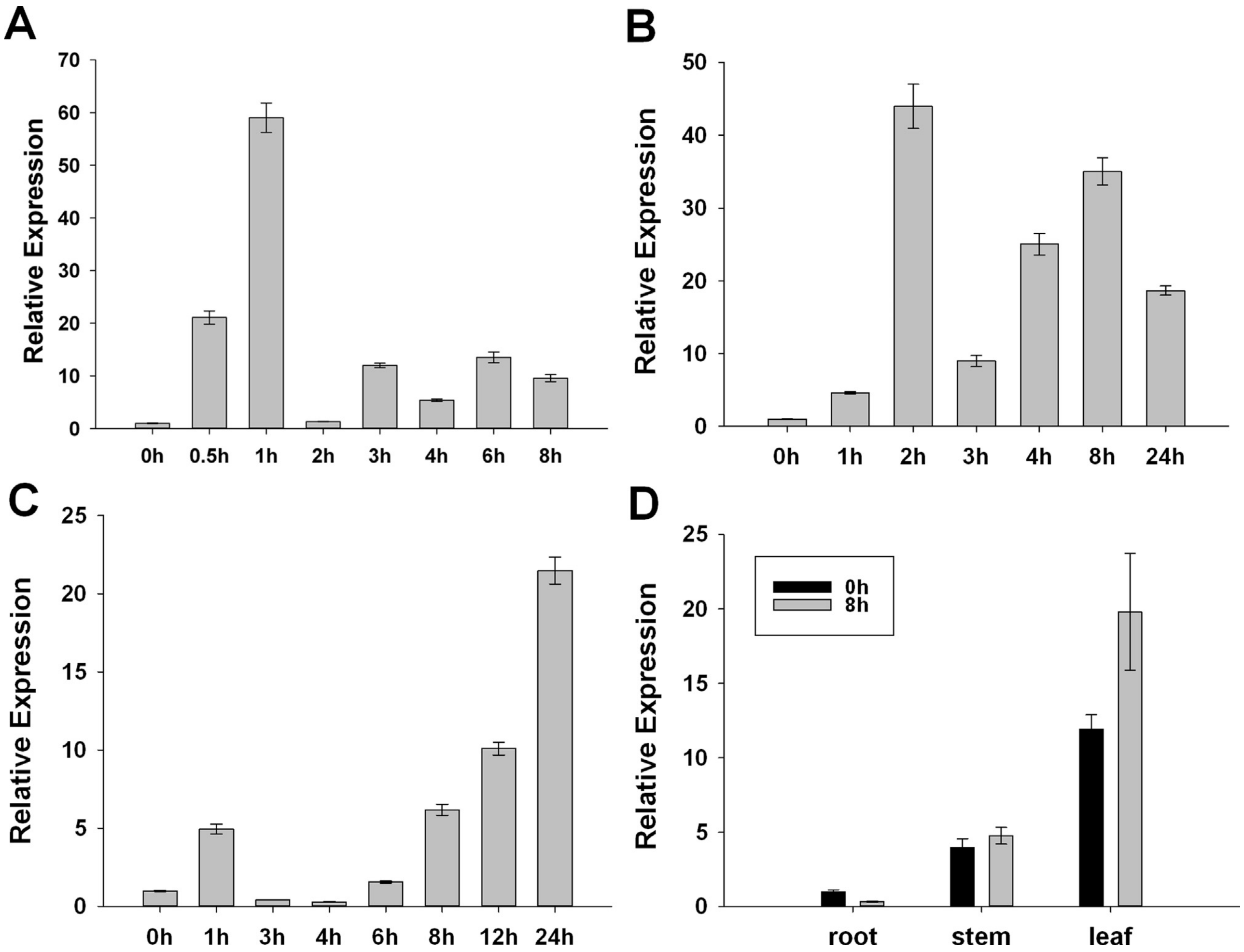

Fig. 1. The expression pattern of *MfNAC3* in *M. falcata* PI502449. (A) Transcript levels of *M. falcata NAC3* in response to drought treatment (air drying); (B) transcript levels of *M. falcata NAC3* in response to salt treatment (1 M NaCl); (C) transcript levels of *M. falcata NAC3* in response to cold treatment (0 °C); (D) expression levels of *MfNAC3* in roots, stems, and leaves. The tissue specific expression of *MfNAC3* is measured by quantitative RT-PCR. Data represent means of three duplicate data and vertical columns are means ± SD.