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

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15 **ABSTRACT**

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

Keywords: *Medicago truncatula*, *Medicago falcata*, abiotic stress, *MfNAC3*, cold tolerance

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. falcate* 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 (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). *SINAC1* encodes a transcription factor in *Suaeda liaotungensis* K. that is involved in the ABA-dependent pathway and enhances cold stress tolerance in transgenic *Arabidopsis* (Li et al., 2014), as do *MINAC5* 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). After cold stress, the NTL6 protein induces the expression of 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/DREB1A* 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 (Chen et al., 2010; Chen et al., 2009; Li et al., 2011; Pennycooke et al., 2008). Among them, *MtCBF3/DREB1C* is a positive regulator in freezing tolerance (Chen et al., 2010). Induced by salt, drought, cold and ABA, *MtCBF4* positively regulates salt stress response genes (Li et al., 2011). However, the relationship between CBFs and NACs in *Medicago* remains unclear.

In a previous work, we have built FalcataBase (<http://bioinformatics.cau.edu.cn/falcata/>), an online database to store the transcriptome data regarding increases or decreases in the transcription levels of genes under salt, drought and cold stress (Miao et al., 2015). In this study, based on the data from FalcataBase, we identified the NAC transcription factor *MfNAC3*, which is induced by cold, drought and salt stress. The overexpression of *MfNAC3* in *M. truncatula* was shown to significantly increase the survival rate under cold stress. To understand the function of *MfNAC3* at low temperatures, we analyzed the expression of cold response genes in transgenic plants and studied the differences between *MfNAC3* and *MtNAC3*.

2. Materials and methods

2.1. Plant materials and growth conditions

One genotype of *M. falcata* PI502449 and one genotype of *M. truncatula* R108 were used in this work. The preparation of seeds and seedling germination were performed as previously described for the assays (de Lorenzo et al., 2007). Seeds were treated for 3 d at 4°C before incubation overnight at 24°C in the dark, after which the germinated seedlings were then transferred into a soil/vermiculite (1:2, v/v) mixture and grown in a greenhouse under a 16 h/8 h day/night photoperiod at 22°C for five weeks.

2.2. freezing treatment and electrolyte leakage assays

A freezing treatment assay was performed as previously described (Pennycooke et al., 2008),

but with some slight modifications. Plants grown in a soil/vermiculite (1:2, v/v) mixture for five weeks were used. After being placed in a freezing chamber (RuMED4001) with a temperature of -4°C for 1 h, plants were moved back to the greenhouse at 22°C for 3 days under a 16 h/8 h day/night photoperiod. Then, the survival rates and electrolyte leakage values were calculated after 3 days. Plants with cold-acclimation were grown at 22°C for 21 days, and then placed in a freezing chamber (RuMED4001) with a temperature of 4°C for 14 days under a 16 h/8 h day/night photoperiod. After freezing treatment at -8°C for 1 h, plants were moved back to the greenhouse at 22°C, and survival rates and electrolyte leakage values were calculated after another 3 days.

The electrolyte leakage assay was measured as previously described (Pennycooke et al., 2008) with some modifications. In brief, four leaf discs of independent plants were placed in 50 mL conical tubes with 25 mL distilled water. After placed under vacuum for 15 min, tubes were shaken for 1 h at 240 rpm, and then conductivity (C1) was measured with an electrical conductivity meter (HI8733 HANNA instruments). The tubes were placed in boiling water for 15 min and recover at 22°C, and total potential conductivity (C2) was measured. The electrical conductivity of distilled water, C0, was measured and used as the reference, and the electrolyte leakage was calculated as: $(C1 - C0) / (C2 - C0)$.

2.3. RNA extraction and RT-PCR

Total RNA was extracted from leaves collected from five-week-old plants using TRIzol reagent (Invitrogen, USA). Then, the cDNA was reverse transcribed from the total RNA (0.5-1 µg) using M-MLV Reverse Transcriptase (Promega).

2.4. Plasmid construction and plant transformation

Full-length gene was cloned from the cDNA of *M. falcata* PI502449 and *M. truncatula* R108 and used to construct the pMDC32-MfNAC3 and pMDC32-MtNAC3 plasmids. The plasmids were transformed into *M. truncatula* R108 through *Agrobacterium tumefaciens* EHA105. Wild-type leaves were used for stable transformation, and the transformed leaves were regenerated via somatic embryogenesis (Cosson et al., 2015). The T1 and T2 generation of the transgenic plants were employed for subsequent experiments.

2.5. qRT-PCR measurement

Quantitative real-time PCR analysis was performed using SYBR Premix Ex Taq (TaKaRa) with the CFX-96 Real-Time System (Bio-Rad) and 20µL reaction volumes. The *MtActin* gene was used as an internal control in *M. truncatula*, whose relative expression levels have already been calculated in a previous study (Li et al., 2011). In *M. falcata*, *MfEF1a*, whose expression levels are stable under salt and drought stress, was used as a control under high-salinity and drought conditions. *MfActin*, with stable expression under cold conditions, was used as a control during cold stress (Ooka et al., 2003). All of the specific primers used in this study are shown in Supplementary Table 1.

2.6. Recombinant protein purification and EMSA

The fusion construct *His-MfNAC3* was transformed into *Escherichia coli* BL21. Recombinant fusion protein was purified using Ni-NTA agarose (Invitrogen) and stored at -80°C in 20% glycerol. EMSA was performed essentially as previously reported (Xu et al., 2013). Purified recombinant protein His-MfNAC3 was incubated in a total volume of 20µL. Probes consisting of DNA fragments containing CACG and CATGTG were labeled with biotin (Biotin-p). Probes without biotin were used as competitors (Cold-p). Mutated probes (mp) were labeled with biotin as described in Supplementary Table 2. The reaction mixtures were analyzed on a 12% gel.

2.7. Subcellular localization and transactivation analyses

Subcellular localization analysis was performed as previously described (Li et al., 2011; Xie et al., 2012). The coding sequence of MfNAC3 was subcloned into the pE3025-GFP vector with CaMV dual 35S promoter, and the pE3025-GFP empty vector was used as a control. A gene gun (Bio-Rad, California, USA) was used for transient transformation of the *Arabidopsis* protoplasts and onion (*Allium cepa*) epidermal cells, respectively. A confocal microscope (Nikon) was used for GFP fluorescence observation.

Transactivation analyses were performed as previously described (Shan et al., 2012). A GAL4-responsive reporter system was used in a transient expression assay in yeast AH109. The entire coding sequence of MfNAC3 was subcloned into the pGBKT7-53 (DBD-P53) vector. Transformants with pGBKT7-53 (DBD-P53) + pGADT7-T (T-antigen) were used as positive

control (CK+), whereas transformants with pGBKT7 empty vectors were used as negative control (CK-). Transformed yeast cells were dropped onto SD medium (lacking tryptophan, histidine and adenine) and incubated at 28°C for 3 d. An α -Gal assay was applied to examine the transactivation ability.

3. Results

3.1. *MfNAC3* is induced by cold, drought and NaCl

In a previous work, we have built an online database, FalcataBase (<http://bioinformatics.cau.edu.cn/falcata/>), to store the transcriptome data regarding increases or decreases in the transcription levels of genes under abiotic stresses. Based on the transcriptome data, which was collected after drought (2 h), salt (1M NaCl, 2 h) or cold (0°C, 8 h) treatment in *M. falcata* PI502449 (Miao et al., 2015), we cloned and named a gene, *MfNAC3*, that was significantly induced. *MfNAC3* contained an open reading frame of 990bp, encoding a protein of 329 amino acids with a predicted molecular weight of 36.9 kD and pI of 6.6. The 990-bp sequence was submitted to GenBank (accession no. KT582544).

To study the expression patterns of *MfNAC3* in *M. falcate* under abiotic stresses, qRT-PCR was performed. Plant materials were treated under air drying, 1M NaCl or 0°C from 0 h to 24 h, and then total RNA was extracted. The highest expression level of *MfNAC3* was observed at 1 h under the dehydration condition (Fig. 1A). The expression of *MfNAC3* was significantly increased after 1M NaCl treatment for 2 h and 8 h (Fig. 1B). Under the 0°C treatment, the transcription level hit a peak at 1 h, which then increased accumulatively to a much higher peak at 6-24 h after treatment (Fig. 1C). These results suggest that *MfNAC3* is significantly induced under cold, drought and salt stresses, which is consistent with our previous results from FalcataBase. To understand the expression pattern of *MfNAC3* under cold stress, total RNA was extracted from the roots, stems and leaves independently in PI502449 after cold treatment for 8 h, and qRT-PCR was performed. We found that higher expression levels could be detected in the leaves (Fig. 1D). Taken together, these results demonstrated that *MfNAC3* was involved in the responses to salt, drought and cold stress.

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 (1M 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 $\pm$ SD.

3.2. Overexpression of *MfNAC3* enhances freezing tolerance in *M. truncatula*

To understand the physiological function of *MfNAC3*, we generated *MfNAC3*-transgenic lines (each line from a different callus) on an R108 background and obtained nine *MfNAC3*-transgenic lines (Fig. S1A). Then, qRT-PCR was performed to analyze the expression levels of these transgenic plants, based on which two lines with similar expression levels, *MfNAC3* OE2 (143-fold) and *MfNAC3* OE5 (129-fold), were chosen for the subsequent tests (Fig. 2A). No differences were observed in growth and development between *MfNAC3* transgenic and wild-type (R108) plants under normal conditions. However, when these plants were treated with cold, we found that the survival rates of *MfNAC3* transgenic plants increased to 48% and 41%, which were significantly higher than the 21% survival rate in wild-type plants (Fig. 2B and 2C), and the level of electrolyte leakage in *MfNAC3* transgenic plants was significantly lower than in wild-type plants (Fig. 2C). These results indicated that *MfNAC3* conferred cold tolerance. *MfNAC3*

transgenic plants and wild-type R108 plants were subjected to freezing tolerance (-8°C) after acclimation to low temperature (4°C) for 14 d. The survival rates of *MfNAC3* transgenic plants were significantly higher than that of wild-type plants (Fig. 2E and 2F), while no significant difference of electrolyte leakage was observed (Fig. 2G). These results demonstrated that *MfNAC3* transgenic plants have enhanced freezing tolerance under both cold-acclimated and non-acclimated conditions.

Fig. 2. Freezing phenotypes and expression pattern of *MfNAC3* transgenic lines.

A: Expression analysis of *MfNAC3* by qRT-PCR under normal conditions. Leaves of wild-type (WT) and T1 generation of *M. truncatula* transgenic *MfNAC3* (MfNAC3 OE) were used for total

RNA extraction. The *MtActin* gene was amplified as control. Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, **P<0.01); B: Phenotypes of R108 (WT) and *MfNAC3* transgenic lines in Freezing. T2 generation of *MfNAC3* transgenic lines were used; C: Survival rates of plants in (B). Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, **P<0.01); D: Electrolyte leakage of plants in (B) (Student's t-test, *P<0.05); E: Phenotypes of R108 (WT CA) and *MfNAC3* transgenic lines (MfNAC3 OE CA) in freezing after cold-acclimation. T2 generation of *MfNAC3* transgenic lines were used; F: Survival rates of plants in (E). Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, **P<0.01, *P<0.05); G: Electrolyte leakage of plants in (E).

3.3. *MfNAC3* encodes a NAC family transcription factor

The analysis on full-length protein sequence showed that MfNAC3 contains the NAC domain in the N-terminal region with five subdomains and the TR domain in the C-terminal region. A phylogenetic tree based on amino acid sequences was constructed to investigate the phylogenetic relationships among the NAC proteins in *M. falcata*, rice and *Arabidopsis* (Fig. 3A)(Ooka et al., 2003). Phylogenetic analysis based on the tree indicated that MfNAC3 belongs to the subfamily of NAC3 transcription factors.

To examine the subcellular localization of MfNAC3, MfNAC3 was fused with GFP and transiently expressed in onion (*Allium cepa*) epidermal cells and *Arabidopsis* protoplasts. The results show that MfNAC3 was localized exclusively in the nucleus (Fig. 3B and 3C). To validate the transcriptional activation capability of MfNAC3, a GAL4-responsive reporter system was used in a transient expression assay in yeast cells. The upstream activation sequence of GAL4 was activated and GAL4 was transcribed to promote the growth of transformed yeast cells in histidine-lacking medium. A fusion plasmid containing the full-length coding region of MfNAC3 and GAL4 DBD was constructed and transformed into yeast AH109. Transformants with pGBKT7-53 (DBD-P53) + pGADT7-T (T-antigen) were used as a positive control (CK+), whereas transformants with pGBKT7 empty vectors were used as a negative control (CK-). Transformed yeast cells containing pGBKT7-MfNAC3 and pGBKT7-53 (CK+) grew and showed α -galactosidase activity in SD medium (lacking tryptophan, histidine and adenine), whereas cells containing pGBKT7 empty vectors (CK-) did not (Fig. 3D). These results indicate that MfNAC3 functions as a transcription factor which belongs to the NAC3 subfamily.

Fig. 3. Phylogenetic tree, subcellular localization and transactivation activity of MfNAC3.

A: Phylogenetic relationship between MfNAC3 and NAC-domain proteins of other plants; B: Nuclear localization of MfNAC3. p35S:: MfNAC3-GFP plasmids were transiently expressed in onion epidermal cells. Bright field images show cell morphology from MfNAC3-GFP and dark field fluorescence images show GFP fluorescence. Merged images are shown at the bottom; C: Nuclear localization of MfNAC3. p35S:: MfNAC3-GFP plasmids were transiently expressed in *Arabidopsis thaliana* mesophyll protoplast cells. Bright field images show cell morphology from MfNAC3-GFP and dark field fluorescence images show GFP fluorescence. Merged images are shown at right; D: Transactivation analysis of MfNAC3 in yeast. Transformants with pGBKT7-53 (DBD-P53) + pGADT7-T (T-antigen) were used as positive control (CK+), whereas transformants with pGBKT7 empty vectors were used as negative control (CK-).

3.4. *MfNAC3* is a positive regulator of *MtCBF4*

Based on our previous data (Fig 2), *MfNAC3* transgenic plants demonstrated a notable enhancement in cold tolerance under both cold-acclimated and non-acclimated conditions, which motivated us to study the cold-responsive genes in both wild-type and *MfNAC3* transgenic plants. We found that the expression levels of *MtCBF1-4* were significantly increased in *MfNAC3* transgenic plants (Fig. 4A), suggesting that MfNAC3 can positively regulate the expression of cold-responsive genes.

The promoter sequences in *MtCBF1-4* were analyzed, and the results showed that the promoter region of *MtCBF4* contains the binding motifs, CACG and CATGTG, of NAC family transcription factors. To determine whether *MtCBF4* is a target of MfNAC3, an electrophoretic mobility shift assay (EMSA) was performed. The probes used in this assay were a 59-bp

biotin-labeled fragment containing the CACG and CATGTG motifs (Biotin-p) and the same DNA fragment without labeling as a cold competitor probe (Cold-p). A probe consisting of biotin and the 59-bp DNA fragment with the CACG and CATGTG replaced by the sequences CCCC and CCCCCC, respectively, was used as a mutant probe (Biotin-mp), and the same DNA without biotin was used as a cold mutant competitor probe (Cold-mp). As shown in Fig. 4B, a clear gel shift was observed when adding the Biotin-p, and the signal was reduced by adding the Cold-p but not by adding the Cold-mp, whereas no shift was observed when adding the Biotin-mp. These results demonstrate that MfNAC3 proteins specifically bind to the promoter region of *MtCBF4* carrying the CACG and CATGTG motifs.

To determine whether *MfCBF4* is induced under cold stress in *M. falcata*, plant materials of *M. falcata* PI502449 were treated in 4°C from 0 h to 24 h, and qRT-PCR was performed. As shown in Fig. 4c, the expression of *MfCBF4* was increased after 0.5 h cold treatment, and a peak expression level was observed at 3 h, and another peak after 12 h treatment. The observed expression pattern showed an increased expression level of *MfCBF4* right after the increase in the expression level of *MfNAC3* under cold treatment (Fig. 1C), which suggests that the transcription of *MfCBF4* may be regulated by MfNAC3 under cold stress in *M. falcata*.

According to previous studies, ICEs are important transcription factors in regulating the expression of *CBFs*. To determine whether MfNAC3 affects the expression of *MtICEs* in *MfNAC3* transgenic plants, we further tested the expression levels of *MtICE1-5* through qRT-PCR in transgenic plants under normal conditions, and found a significant increase in the expression level for *MtICE3* (Fig. 4D). Some other works showed that *CASs* are important regulons of *CBFs*. We performed qRT-PCR to test the expression levels of *MtCAS15* and *MtCAS31* and found that they were significantly increased (Fig. 4E). According to these results, we speculated that MfNAC3 regulates *MtCBF4* under cold stress.

These results indicated that MfNAC3 was involved in the cold stress response and played a positive role in cold tolerance.

Fig.4. Positive regulation of *MfNAC3* on *MtCBF4*.

A: Expression analysis of cold responsive genes of *MfNAC3* transgenic lines under normal conditions. The expression levels of T2 generation of *MfNAC3* are measured by quantitative RT-PCR. Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, ** $P < 0.01$); B: EMSA assay for testing *MfNAC3* binding to the CACA and CATGTG motifs in the promoter of *MtCBF4*. *In vitro* DNA binding reactions were performed with the wild-type DNA probes containing the CACG and CATGTG motifs (Biotin-p and Cold-p) and the base-substituted DNA probes in which the CACG and CATGTG motifs were replaced by CCCC and CCCCCC respectively (Biotin-mp and Cold-mp); C: Transcript levels of *M. falcata CBF4* in response to cold treatment (4°C); D: Expression analysis of *MfNAC3* and *MtICEs* by qRT-PCR under normal conditions. Leaves of wild-type (WT) and T2 generation of *M. truncatula* transgenic *MfNAC3* (*MfNAC3* OE) were used for total RNA extraction. The *MtActin* gene was amplified as control. Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, ** $P < 0.01$); E: Expression analysis of *MtCAS15* and *MtCAS31* by qRT-PCR under normal conditions. Leaves of wild-type (WT) plants and T2 generation of *MfNAC3* transgenic plants (*MfNAC3* OE) were used for total RNA extraction. The *MtActin* gene was

amplified as control. Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, **P<0.01).

3.5. *MfNAC3* function is different with *MtNAC3*

As shown in previous studies, that *M. falcata* possesses a stronger resistance than *M. truncatula* under cold stress. To determine whether *MfNAC3* enables *M. falcata* to have stronger tolerance against cold stress, a homologous gene was cloned from *M. truncatula* and named *MtNAC3*. Containing an open reading frame of 990bp, *MtNAC3* encodes a protein of 329 amino acids with a predicted molecular weight of 36.9 kD and pI of 6.3. The 990-bp sequence was submitted to GenBank (accession no. KT582543). Transformants with pGBKT7-MtNAC3 grew and showed α -galactosidase activity in SD medium (lacking tryptophan, histidine and adenine), which means that MtNAC3 has transcriptional activation ability (Fig. 5B).

ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) were used for multiple sequence alignment, which showed a high similarity between *MfNAC3* and *MtNAC3* (Fig. 5A), and thus we assumed that the functions of these two proteins are similar. To verify this assumption, *MtNAC3* overexpression transgenic lines were generated on an R108 background, and four transgenic lines were obtained (Fig. S1B). Based on qRT-PCR analysis, the expression levels of *MtNAC3 OE1* and *MtNAC3 OE2* were increased to 89-fold and 37-fold, respectively (Fig. 5C), which were similar to those of the *MfNAC3* transgenic lines (Fig. 2A). These lines were chosen for freezing treatment. Growth and development were the same between *MfNAC3* overexpression transgenic plants and wild type (R108) under normal or cold conditions. Compared with R108 wild-type plants, no significant difference was found in the survival rate of *MtNAC3* overexpression transgenic plants (Fig. 5D and 5E). Moreover, no significant increase was observed when qRT-PCR was performed to test the expression of *MtCBFs* in *MtNAC3* overexpression transgenic plants under the normal condition (Fig. 5F). These results indicate that *MfNAC3*, but not *MtNAC3*, confers cold tolerance.

Fig. 5. Amino acid sequences of MtNAC3 and freezing phenotypes of *MtNAC3* overexpression transgenic lines.

A: Alignment of the deduced amino acid sequences of MtNAC3 and MfNAC3. Identical amino acids are highlight in black, and similar amino acids are shown in gray; B: Transactivational analysis of MtNAC3 in yeast. Transformants with pGBKT7-53 (DBD-P53) + pGADT7-T (T-antigen) were used as positive control (CK+), whereas transformants with pGBKT7 empty vectors were used as negative control (CK-); C: Expression analysis of *MtNAC3* gene by qRT-PCR. Leaves of wild-type (WT) and T1 generation of *M. truncatula* overexpressing *MtNAC3* (MtNAC3 OE) were used for total RNA extraction. The *MtActin* gene was amplified as control.

Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, **P<0.01); D: Survival rates of plants in (E). Data represent means of three duplicate data and vertical columns are means $\pm$ SD (Student's t-test, **P<0.01); E: Phenotypes of R108 (WT) and *MtNAC3* transgenic plants in freezing. T2 generation of *MtNAC3* transgenic plants were used for this assay; F: Expression analysis of *MtNAC3* and *MtCBFs* by qRT-PCR under normal conditions. Leaves of wild-type (WT) and T2 generation of *M. truncatula* overexpression transgenic *MtNAC3* (*MtNAC3* OE) were used for total RNA extraction. The *MtActin* gene was amplified as control. Data represent means of three duplicate data and vertical columns are means $\pm$ SD.

4. Discussions

In this study, we identified *MfNAC3*, a gene which encodes a NAC-type transcriptional factor, and manifested its positive role in response to cold stress in *M. falcata*. We also showed that *MfNAC3* can bind directly to the promoter of *MtCBF4*, providing a plausible explanation for why the overexpression of *MfNAC3* can enhance freezing tolerance.

NAC proteins contain a consensus NAC domain (NAM, ATAF and CUC) in the N-terminal region and a highly diverse TR domain (transcriptional regulation) in the C-terminal region (Ernst et al., 2004). The NAC domain, with DNA and/or protein binding function, contains five subdomains (A-E) consisting of approximately 150 amino acids. The C-terminal TR region undergoes transcription activation or repression to constrain the functions of NAC proteins (Ooka et al., 2003). Our experimental result suggests that transgenic plants overexpressing *MtNAC3* does not lead to any enhancement in cold tolerance. To understand the differences between *MfNAC3* and *MtNAC3*, we analyzed the amino acid sequences of these two proteins, and found that the two sequences are the same in the NAC domain in the N-terminal region, but several amino acids in the TR domain in the C-terminal are different. It has been reported in previous works that two typical domains, NAC and TR, exist in NAC-type transcription factors. The five sub-domains contained in the NAC domain are conserved, whereas the protein sequence in the TR region is diverse. The TR domains in transcription factors either activate or repress gene transcription. For instance, the TR domain in *SiNAC* in *Setaria italic* and that in *AtNAC2* in *Arabidopsis* both function in activating gene transcription (He et al., 2005; Puranik et al., 2011). In *Brassica napus*, nine NAC-type transcription factors show similarities in protein sequence in the TR domain; however, their functions in terms of transcription activity are different. Biochemical analysis in BnNAC5-8 and BnNAC485 indicated that the key region responsible for this activity is the C-terminal region of the protein (Hegedus et al., 2003). Four members of the ANAC001

subfamily, namely, ANAC001, ANAC003, ANAC004 and ANAC005, share similar TR regions, but have different functions (Ooka et al., 2003). The variety in protein conformations in the TR region is attributed to the divergence in protein sequence, which may also result in different functions. Based on these studies and our experiment results, we speculate that the differences in the TR region between MfNAC3 and MtNAC3 lead to different protein functions, thus affecting their transcription activity.

On the other hand, our results indicate that the expression levels of *MtCBF1-4*, which are typical cold-responsive genes, are altered in *MfNAC3* transgenic plants. As CACG- and CATGTG-binding motifs are all responsible for NAC-type transcription factor binding, we further analyzed the promoter regions in these four genes and found that the promoter region of *MtCBF1* contains a CACG-binding motif and both the CACG- and CATGTG-binding motifs occur in the promoter region of *MtCBF4*. However, neither the CACG nor the CATGTG motif occurs in the *MtCBF2* and *MtCBF3* promoters, suggesting that only *MtCBF1* and *MtCBF4* may be regulated by *MfNAC3*. Furthermore, *MfCBF4* was induced by cold stress in *M. falcata* PI502449 (Fig. 4C), but *MfCBF1* was not (Fig. S2). Therefore, we speculate that only *MtCBF4* is regulated by MfNAC3 in *M. truncatula*. Our results show that MfNAC3 can bind to the promoter of *MtCBF4*, suggesting that *MtCBF4* is a target gene of MfNAC3.

Taken together, our results demonstrate that MfNAC3 plays a positive role in cold tolerance in *Medicago*, providing grounds for obtaining breeds with strong freezing tolerance.

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