Cloning and Function Analysis of CINAC9 from Chrysanthemum lavandulifolium

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Cloning and Function Analysis of ClNAC9 from Chrysanthemum lavandulifolium

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Abstract: NAC transcription factors have been found to play an important role in several plant development programs and stress responses. In this study, a NAC gene, \textit{ClNAC9} (for \textit{Chrysanthemum lavandulifolium} NAC gene), was isolated from a cDNA library constructed according to the known Expressed sequence tag sequence (EST). The cDNA full-length sequence of \textit{ClNAC9} is comprised of 881 bp, encoding a putative protein of 217 amino acids. \textit{ClNAC9} has a conserved NAC domain in the N-terminus—the NAM domain. \textit{ClNAC9} is highly similar to other NACs, especially SENU5 subgroup members. Transgenic \textit{Arabidopsis} overexpressing \textit{ClNAC9} controlled by the CaMV-35S promoter was generated and subjected to saline, alkaline and drought stresses for morphological and physiological assays. Morphological analyses showed that transgenic plants had enhanced tolerance to saline, alkaline and drought stresses, as indicated by improved physiological traits, including higher SOD

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and POD activities, reduced MDA accumulation. Moreover, overexpression of \textit{ClNAC9} enhanced up-regulation of \textit{RD29A}, \textit{RD26}, \textit{MYB2} and \textit{MYB96} expression, reduced \textit{HAB1} expression under saline, alkaline and drought treatments. Taken together, our results demonstrate that \textit{ClNAC9} is likely related to saline, alkaline and drought resistances and over-expression of \textit{ClNAC9} increases saline, alkaline and drought resistances of the transgenic \textit{Arabidopsis}.

**Keywords:** NAC transcription factor, abiotic stress, transgenic \textit{Arabidopsis}, overexpression

**Introduction**

Drought, salinity, low temperature and other abiotic stresses seriously affect plant growth and development. They are also the main cause of yield and quality decline (Bray et al., 2000). In the long-term evolution, plants have attained series of adaptive changes at physiological, biochemical and molecular levels to increase the chances of survival in adverse conditions (Broun, 2004; Bohnert et al., 2006). Through binding with the cis-acting elements of the promoter region in relevant functional genes, transcription factors regulate the transcription level, enhance the specific role and strengthen gene expression, response to environmental stresses, regulate growth and development (Liu et al., 1998; Uno et al., 2000; Tran et al., 2004). Known transcription factors participating in response to abiotic stresses including NAC, WRKY, AP2/EREBP and MYB families (Singh et al., 2002; Olsen et al., 2005). NAC members are a class of plant-specific transcription factors (Souer et al., 1996; Jin et al., 1999).
with a variety of biological functions. Members of the NAC family have a highly conserved NAC domain of about 150 amino acid residues in the N-terminus. The C-terminus is a transcriptional regulatory region that promotes or inhibits the transcriptional activity (Nakashima et al, 2012; Puranik et al., 2012). After isolation of NAM, the first member of NAC family by Souer et al. (1996), a lot of research has been done. Many NAC members have been found in *Arabidopsis thaliana*, *Oryza sativa*, *Populus L.*, *Nicotiana tabacum L.*, *Glycine max* and other plants (Rushton et al, 2008; Yamasaki et al, 2008; Hu et al, 2010; Nuruzzaman et al, 2010; Le et al, 2011). Many studies showed that NAC transcription factors are capable of activating the abiotic stress response genes on improving plant abiotic stress tolerance (Fujia et al, 2004; Tranlsp et al, 2004). For example, drought, high salinity and other abiotic stresses induce the expression of *SNAC1/2*, *OsNAC5*, *OsNAC10* and *ONAC045* in rice, the expression of *ATAF1*, *ANAC019*, *ANAC055* and *ANAC072/RD26* in *A. thaliana* (Hu et al, 2006; Lu et al, 2007; Nakashima et al, 2007. Wu et al, 2009; Zheng et al, 2009; Jeong et al, 2010; Takasaki et al, 2010). Functional studies showed that overexpression of *SNAC1/2*, *OsNAC1*, *OsNAC5*, *OsNAC045* and *OsNAC63* in plants resulted in strong resistance to drought and high salt (Hu et al, 2006; Hu et al, 2008; Yokotani et al, 2009; Zheng et al, 2009; Takasaki et al, 2010). In addition, *Glycine max*, *Gossypium spp.*, *Triticum aestivum*, *Cicer arietinum* and other plants, there are also reports on the NAC family members in salt, cold and other abiotic stress (Meng et al, 2009; Hao et al, 2011).
Chrysanthemums are popular flowers in Chinese traditional gardening. They have been widely used in landscaping, but their growth can be hindered by low temperatures, drought, high salinity and other abiotic stress conditions, affecting their ornamental value. Thus it is necessary to increase the resilience of chrysanthemums in adverse conditions. Because of the complexity of the genetic background of chrysanthemums, progress in breeding for resistance was slow. *Chrysanthemum lavandulifolium* Fisch. Ex Trautv. Makino is a diploid plant of Asteraceae, having the genetic background of simple and strong salinity resistance features. In this study, based on ESTs of the NAC transcription factor obtained from the transcriptome database of *C. lavandulifolium*, a NAC transcription factor gene *ClNAC9* was cloned and analyzed with bioinformatics tools. Using the Agrobacterium-mediated floral dip method, the gene was introduced to wild-type *A. thaliana* to analyze the overexpression of this gene for their drought resistance, saline-alkali resistance. This study laid the foundation for further study on the biological functions of the gene and its utilization for *chrysanthemum lavandulifolium* resistance genetic engineering improvement.

1 MATERIALS AND METHODS

1.1 Materials

*C. lavandulifolium* and seeds of wild-type *Arabidopsis thaliana* (Columbia ecotype, WT) for genetic transformation were gifts from the Beijing Forestry University Landscape College. Wild-type and the transgenic lines of *A. thaliana* were
sown in 1/2MS solid medium, then transplanted into sterilized matrix prepared by mixing nutrient soil: vermiculite in 1: 1 ratio. All the plants were placed indoor in an artificial climate chamber, 16h: 16h light: dark cycle, with light intensity of 120 µmol m\(^{-2}\)s\(^{-1}\), temperature 24/18 °C (day/night). WT A. thaliana was transformed about 7d after bolting. Rosette leaves (5-6 leaves) were used for molecular identification.

*Agrobacterium tumefaciens* GV3101 competent cells and vector pBI121 were gifts from Beijing Forestry University School of Landscape Architecture.

1.2 **Total RNA extraction and reverse transcription**

According to the improved TRIzol method by Huang et al (2009), total RNA was extracted from *C. lavandulifolium* leaves, assessed with 1% agarose gel and UV spectrophotometer for RNA quality and concentration, and reverse transcribed with the M-MLV reverse transcriptase.

1.3 **Cloning of the target gene**

Based on *C. lavandulifolium* EST sequence information, PCR primers shown in Table 1 for 3’ RACE were designed and synthesized. Nested PCR amplification methods were used for 3’ RACE (Hu et al., 2009). The PCR program was 94 °C 5 min; 94 °C 30 s, 59 °C 40 s, 72 °C 1 min, 35 cycles; 72 °C 10 min. PCR products were assessed with 1% agarose gel electrophoresis, recovered, inserted to pGEM-T cloning vector and transformed into E. coli DH5α competent cells. Ampicillin resistant colonies were picked and sequenced at AuGCT (Beijing) Biotech Co.

The full length sequence was obtained by assembly of the 3’ end of the sequence
and known EST sequences of the target gene using DNAMAN software. Subsequently, according to the full-length sequence assembled, the 3' and 5' primers (qcNAC9-s and qcNAC9-x) were designed and used for amplifying, sequencing, verifying the full-length sequence.

1.4 Bioinformatics analysis of sequences

DNAman software was used for gene sequence analysis, stitching, alignment, identifying restriction sites and homology analysis of NAC proteins. MEGA 4.0 software was used construct phylogenetic trees for homologous NAC proteins (neighbor-joining method).

1.5 Plant expression vector and transformation

The target coding sequence was amplified with primers NAC9-BamH I and NAC9-Sac I (Table 1) containing a BamH I site and a Sac I restriction site, respectively. The coding sequence of the target gene was inserted into a plant overexpression vector pBI121. The correct recombinant vector was verified with PCR and restriction enzyme digestion, and transformed to Agrobacterium GV3101, which was used to infect A. thaliana.

Harvested T0 generation seeds were disinfected, and spread on ½MS medium (Qingda Hope Bio-Technology CO.,Ltd,Qingdao,China) containing 50 mg L⁻¹ kanamycin for screening of genetically resistant plants. The transgene was assessed using PCR and RT-PCR methods, respectively, at the genome level and the transcription level. T1 seeds were harvested for analysis of physiological parameters.
1.6 Salinity resistance of transgenic A. thaliana

1.6.1 Seed germination

The T\(_1\) transgenic A. thaliana seeds were cold-treated at 4 °C for 48 h, sterilized with 70% alcolol and 2% sodium hypochlorite and rinsed with sterile water, sown on medium containing ½MS 0, 50, 100, 150, 200 mmol/L NaCl or 0, 2, 5, 7.5, 10 mmol L\(^{-1}\) NaHCO\(_3\) for stress, cultured in a long-day phytotron (16 h light/8 h dark cycle at 22 °C for 7 d. Germination of seed was recorded, and the experiment was repeated three times.

1.6.2 Root growth

The T\(_1\) transgenic A. thaliana seeds were cold-treated at 4 °C for 48 h, after disinfection, sown on ½MS medium and transferred to a long-day phytotron, grew for 10 d, then (that is, T2 transgenic plants) were transplanted to ½MS medium containing 0, 50, 100, 150, 200 mmol L\(^{-1}\) NaCl or 0, 2, 5, 7.5, 10 mmol L\(^{-1}\) NaHCO\(_3\) vertical training for 10 d. The root length and growth were recorded.

1.6.3 Salinity tolerance of transgenic seedlings

After cold-treated at 4 °C for 48 h, the T\(_1\) transgenic A. thaliana seeds were sown on ½MS medium. When grew to the stage of 4 true leaves, they were transplanted to pots containing vermiculite: nutritive soil = 1: 1, put in the long-day phytotron. At 50 d (about bolting and flowering), the plants were tested for salt tolerance. First, the plants were fully watered, then treated with 200 mmol L\(^{-1}\) NaCl or 50 mmol L\(^{-1}\) NaHCO\(_3\) solution, once every 12 h, for a total of 6 times. At the conclusion of the
stress treatment, the plants were sampled to detect superoxide dismutase (SOD), peroxidase (POD) activity (Chance et al, 1955; Giannopolitis et al, 1977) and malondialdehyde (MDA) content (Heath et al., 1968). Other plants continue to be cultured and observed for 4 d to determine survival rate of wild-type and transgenic lines.

1.7 Drought resistance of transgenic plants

After cold-treated at 4 ℃ for 48 h, the T₁ transgenic A. thaliana seeds were sown on ½MS medium. When grew to the stage of 4 true leaves, they were transplanted to pots containing vermiculite: nutritive soil = 1: 1, put in the long-day phytotron. Watering was stopped at 50 d (about bolting and flowering). 7 d later, the survival rates of wild-type and transgenic lines were recorded. Samples were measured for activities of SOD and POD, the degree of membrane damage (the content of MDA), with the same detection methods described in section 1.7.3.

1.8 Expression analysis of stress resistance genes in transgenic A. thaliana with qRT-PCR

Leaf RNA was extracted from wild-type A. thaliana and transgenic ones expressing CINAC9, non-stressed or stressed by salt, alkali, or drought. The total RNA was reverse transcribed into cDNA, and analyzed with qRT-PCR to determine the mRNA levels of functional gene of RD29A, RD26, HAB1 and a reference gene ACT2 (AT3G18780). Primer sequence of these genes were shown in Table 1. The reaction program was 95 ℃ 30s; 95 ℃ 5s, 60 ℃ 30s, 40 cycles, using the 2^ΔΔCt method to
calculate the amount of relative expression.

1.9 Statistical Methods

All lines of plants were measured with the above-described method, repeated three times technically and three times biologically. Each parameter was measured and averaged. SPSS13.0 was used for significant difference analysis (Student's t-test).

2 RESULTS

2.1 Cloning and bioinformatics analysis of ClNAC9 from C. lavandulifolium

Use the EST sequence database of C. lavandulifolium (Huang et al., 2012a), candidate NAC sequences with a complete 5' end, after BlastX comparison, were selected to design 3' RACE primers for nested PCR amplification. A specific amplification product of 400 bp (Fig. 1A) was recovered from the gel, purified, cloned and sequenced. A 385-bp sequence was obtained, and stitched with known EST sequences with the DNAMAN software, resulting in a full-length sequence of 881bp. Based on the full-length sequence, amplification primers (Table 1) were design to amplify the full length sequence. The obtained PCR product (Fig. IB) was verified by sequencing, designated as ClNAC9. Use DNAMAN software, the gene was predicted containing a 651-bp open reading frame (ORF), encoding 217 amino acids, with a predicted isoelectric point of 9.14, and a molecular weight of 24.93 KD. An NCBI Conserved Domain Database (CDD) search indicates that the N-terminal amino acid sequence encoded by C. lavandulifolium ClNAC9 contains an NAM domain (Fig. 2), can be presumed a member of the NAC transcription factor family.
2.2 ClNAC9 homology and phylogenetic analysis

Using DNAman, a multiple sequence alignment was carried out for amino acid sequences of ClNAC9, PeNAC010 from populous, cassava MeNAC35, soybean GmNAC3, rice OsNAC3, *A. thaliana* ANAC041, ANAC072, ANAC083. Results showed that ClNAC9 is highly similar to NAC transcription factors of other species, with an N-terminal conserved NAC domain, that is, its DNA binding domain. The amino acid sequences of subdomains A, B, C, D and E were highly conserved (Figure 3A). Based on these sequences, Neighbor-Joining phylogenetic trees were constructed using MEGA4.0 software. ClNAC9 was found on the same clade with *A. thaliana* ANAC041, ANAC083, populus PeNAC010, and cassava MeNAC35, most distant from other NAC members (Figure 3B).

2.3 RT-PCR analysis of transgenic *A. thaliana* overexpressing ClNAC9

Screening with Kan, we obtained a total of 12 T1 plants of *A. thaliana* positively transfected with ClNAC9. In order to show the genetic stability of the exogenous gene in the offspring, Kanamycin resistance T1 plants were assayed with RT-PCR, and the results showed that all 12 lines expressed the transgene ClNAC9, among which two lines had noticeably brighter ClNAC9 bands. N9 had the highest expression, followed by N1, and other lines were the lowest, but expression was not detected in the wild type (Fig. 4). N1 and N9 lines showed the highest ClNAC9 expression, named p-ClNAC9-1, p-ClNAC9-2, were used in the next step for phenotyping.

With the *A. thaliana* ACT2 (AT3G18780) as an internal control, the expression of
the transgene \textit{ClNAC9} was shown to be expressed higher in the transgenic plants than in the wild-type, and the foreign gene was integrated into the chromosome of \textit{A. thaliana} and transmitted to the offspring.

\textbf{2.4 Stress tolerance in plants carrying transgene \textit{ClNAC9}}

Among all transgenic lines of \textit{A. thaliana}, N1 and N9 lines expressed the highest level of the transgene \textit{ClNAC9}, named p-ClNAC9-1, p-ClNAC9-2, thus were used in follow-up stress tolerance studies.

\textbf{2.4.1 Salt tolerance of the trangenic \textit{A. thaliana}}

\textbf{2.4.1.1 Salt stress phenotype}

The wild-type and transgenic plants both showed a high germination rate without significant difference when salt concentrations were below 100 mmol L$^{-1}$; but when the NaCl concentration was raised to 150 mmol L$^{-1}$, the germination rate of wild-type seeds was about 50\%, while the rates of p-ClNAC9-1 and p-ClNAC9-2 were 73\% and 90\%. When the medium contained 200 mmol L$^{-1}$ NaCl, the germination rate of wild-type seeds was only 4\%, while those of p-ClNAC9-1 and p-ClNAC9-2 lines maintained at 12.7\% and 13.3\%, being significantly higher (Figure 5 A, B). These results indicate that high levels of \textit{ClNAC9} expression enhanced salt tolerance during germination.

The vertical growth of roots was measured for the wild type and trangenic plants on the 1/2MS medium containing different levels of NaCl. The root growth of
various strains was shown in Figs 6A, B. Under low NaCl levels, the WT and transgenic plants performed similarly. At higher NaCl concentrations (200 mmol L$^{-1}$), both WT and transgenic plants displayed leaf yellowing, growth inhibition, root length reduction. Under the high NaCl level (200 mmol L$^{-1}$), the transgenic plants showed longer roots, and the difference was significant. These results suggest that overexpression of ClNAC9 increases root growth, and under salt stress, and that overexpression of ClNAC9 increased the tolerance of the transgenic plants to salt.

During the seedling stage (Fig. 7), wild-type plants under salt stress displayed leaf chlorosis, wiltted, and most plants died, and only 27.2% survived, while the transgenic plants maintained mostly vitality, only part of the plant turned yellowish. The survival rates of p-ClNAC9-1 and p-ClNAC9-2 plants were 78.0% and 72.8%. These results indicate that ClNAC9 increased the tolerance to salt during the growth stage of A. thaliana.

2.4.1.2 Physiological indexes of transgenic plants under salt stress

The MDA content and activities of SOD, POD in plants reflect the tolerance of plants to abiotic stress to a certain extent. Under normal condition, the MDA content was lower in transgenic plants overexpressing ClNAC9 than in the wild-type plants, while the SOD activities were the same in both the wild-type and transgenic plants(Fig 8). The POD activity varied largely but were higher in the transgenic plants than in the wild type plants. Under 200 mmol L$^{-1}$ NaCl treatment, all plants showed higher MDA contents, SOD and POD activities, but the MDA content, SOD and POD
activities changed differently between the wild-type plants and the transgenic plants. Under salt stress, the increase of the MDA level in the wild type plants was greater than in the transgenic plants overexpressing \textit{ClNAC9}. As a result, overexpression lines contained significantly lower levels of MDA than the wild-type plants; and the SOD activity and the POD activity in transgenic plants increased greater than in the wild-type plants. These results indicate that the wild type plants were less strong in producing SOD and POD, thus produced more MDA, while the transgenic plants produced large amounts of SOD and POD, thus reduced MDA production to avoid stress damage. Thus the transgenic plants tolerate salt better than the wild type plants.

2.4.1.3 Phenotypes of transgenic plants under alkaline stress

\textit{ClNAC9} expression could be induced by NaCl in \textit{C. lavandulifolium} (Huang et al., 2012a), but whether the two could be expressed under NaHCO$_3$ stress. Thus, we studied how transgenic plants overexpressing \textit{ClNAC9} tolerate alkali (NaHCO$_3$). The results were shown in Fig. 9A, B. Seeds of \textit{A. thaliana} were sensitive to NaHCO$_3$ stress. When the concentration of NaHCO$_3$ was 7.5 mmol L$^{-1}$, the germination rate decreased significantly, and being less than 50% for the transgenic plants, 30% or less for the wild type plants. The seeds could not germinate at 10 mmol L$^{-1}$ NaHCO$_3$ (Data not shown). Plants overexpressing \textit{ClNAC9} showed a higher germination rate than the wild type under NaHCO$_3$ stress. The results illustrate that a high level of \textit{ClNAC9} expression in plants can enhance NaHCO$_3$ tolerance during the germination stage.

The root growth of \textit{A. thaliana} was more sensitive to NaHCO$_3$. When the
concentration of NaHCO$_3$ was 5 mmol L$^{-1}$, the root growth was inhibited, particularly significant in the WT. The transgenic plants overexpressing $CINAC9$ had longer roots than the controls. As the NaHCO$_3$ concentration increased, the root growth slowed down; and the leaves were smaller and yellowish. When the NaHCO$_3$ concentration reached 10 mmol L$^{-1}$, the roots almost stopped elongation; plants yellowed even died (Figure 10A, B). Under this stress, the roots of transgenic plants overexpressing $CINAC9$ were longer than those of WT. The results indicate that $A.\ thaliana$ overexpressing $CINAC9$ was more resistant to alkaline.

When stressed by 50 mmol L$^{-1}$ NaHCO$_3$, WT and transgenic plant displayed very obvious leaf chlorosis and yellowing, and only 22.8% of WT survived, while more than 60% of the $CINAC9$ transgenic plants survived, though most plants had leaf chlorosis and yellowed (Figure 11). The results showed that $CINAC9$ improves the tolerance to alkali during $A.\ thaliana$ growth.

2.4.1.4 Physiological indexes of transgenic $A.\ thaliana$ under alkaline stress

Stressed by 50 mmol L$^{-1}$ NaHCO$_3$, the wild-type $A.\ thaliana$ increased significantly its MDA content, while the transgenic plants overexpressing $CINAC9$ showed a lower increase in MDA contents. Although both SOD and POD activities were increased significantly under the NaHCO$_3$ treatment, the wild-type plants showed significantly lower POD and SOD activities than the transgenic plants. These results suggest that wild-type plants had cell membrane damages under NaHCO$_3$ stress, although increased SOD and POD activities, but unable to remove the large amount of
superoxide and oxygen radicals, so more MDA accumulated. The transgenic plants showed a relatively lower MDA content, higher SOD and POD activities than the wild type (Fig. 12). These results indicate that ClNAC9 transgenic plants were more resistant to alkali compared with the wild type.

2.4.1.5 Salt tolerance related gene expression in transgenic A. thaliana

In order to study the transcriptional regulation of ClNAC9 under salt stress (200 mmol L\(^{-1}\) NaCl solution) and alkaline stress (50 mmol L\(^{-1}\) NaHCO\(_3\) solution), functional genes RD26, RD29A, HAB1 were analyzed with qRT-PCR. The results (Figure 13) showed that, without any treatment, transgenic plants expressed RD29A slightly higher than WT; most transgenic plants expressed RD26 higher, though some individuals lower, than WT; and transgenic plants expressed HAB1 higher than WT. After treated with 200 mmol L\(^{-1}\) NaCl solution, and 50 mmol L\(^{-1}\) NaHCO\(_3\) solution, respectively, transgenic lines showed significantly increased expression of RD26 and RD29A. The expression of these genes were significantly higher in the transgenic plants than in WT after treated with 200 mmol L\(^{-1}\) NaCl, and 50 mmol L\(^{-1}\) NaHCO\(_3\). At the same time, after either the NaCl treatment or the NaHCO\(_3\) treatment, the expression of HAB1 was increased, but the increases were lower in the transgenic plants than in WT. This suggests that overexpression of ClNAC9 transcription factors activated the expression of the downstream functional genes in A. thaliana, leading to upward or downward regulation.

2.4.2 Drought tolerance of transgenic A. thaliana
2.4.2.1 Phenotype under drought stress

During the seedling stage (about to blossom), the wild type plants and the transgenic plants responded to drought differently (Fig. 14). When watering stopped, normal growing of the wild type plants first slowed their growth, then exhibited leaf chlorosis, wilted and died, while the transgenic plants overexpressing ClNAC9 appeared only partially yellowed, basically maintained normal vitality. The 7 d survival rates of the transgenic lines were 66.7% and 70.7%, much higher than the survival rate of the wild type (20.0%).

2.4.2.2 Determination of physiological parameters in transgenic A. thaliana under drought stress

Under the drought stress, the MDA contents were increased both in the wild type plants and in the transgenic plants expressing ClNAC9, but the DMA content in the transgenic plants increased to a smaller extent, thus the transgenic plants contained less DMA than the wild-type plants. The SOD and POD activities were significantly increased. These results suggest that ClNAC9 promoted the increase of reactive oxygen species under drought stress (Fig. 15), thereby improved the ability to scavenge superoxide anion radicals and oxygen radicals, to reduce the damage to the plants.

2.4.2.3 Drought-related gene expression in transgenic A. thaliana

In order to study the transcriptional regulation by ClNAC9 under drought stress
conditions, the expression of *MYB2, MYB96, RD29A*, and *HAB1* in the wild type plants and the transgenic plants were quantitatively analyzed using qRT-PCR. The results (Fig. 16) showed that, under normal circumstances, *CiNAC9* overexpressing plants had higher levels of *MYB2, RD29A*, and *HAB1* expression, a slightly lower level of *MYB96* expression than the wild-type plants. After the drought treatment, the expression levels of *MYB2, RD29A, HAB1* and *MYB96* were significantly increased, but levels of *MYB2, RD29A* and *MYB96* expression were much higher in *CiNAC9* overexpression plants than in the wild-type plants, and the expression levels of *HAB1* were lower than in the wild type.

3 DISCUSSION

The NAC family of transcription factors is plant specific. Members of NAC family are reportedly involved in the regulation of plant growth, development, morphogenesis, stress tolerance and other aspects. Researchers continue to focus on NAC transcription factors. A large number of studies have shown that NAC transcription factors regulate the response to abiotic stresses such as salt, alkali, drought, cold, heat (Yokotani et al, 2009; Christianson et al, 2010; Gao et al, 2010; Jensen et al, 2010; Huang et al, 2012b). Currently, there are many reports on NAC genes in response to stress and its mechanisms in *A. thaliana*, rice, soybean and other plants (Xie et al, 2000; Hu et al, 2006; Hao et al, 2010.). Huang and other (2012a) screened some *NAC* genes in *C. lavandulifolium*, analyzed in-depth the resistant mechanism in *C. lavandulifolium* under abiotic stress at the molecular level. They
obtained multiple NAC genes that respond to various stresses from *C. lavandulifolium*, providing a practical base for later transgenic breeding studies. In this study, we cloned *ClnAC9* from *C. lavandulifolium* transcriptome database, analyzed its sequence, and studied its function in salt, alkali and drought stress.

Through the conserved domain database search, it was found that the *ClnAC9* encoded protein is similar to other NAC transcription factors in sequence structure. The N terminus contains an NAM domain, including 5 conserved sub-domains A, B, C, D, E. Therefore, it is reasonable to believe that it is a member of the NAC transcription factor family.

In the phylogenetic tree, the *ClnAC9* protein formed a clade with *A. thaliana* ANAC041 and ANAC083 belonging to the SENU5 subfamily of NAC family proteins (Ooka et al, 2003;.. Huang et al, 2012a). Analyzed with qPCR, *ClnAC9* was found to be inducible by drought, salt, heat stress and exogenous ABA. After induction by these stresses, *ClnAC9* expression increased sharply at the initial stage. *ClnAC9* expression did not respond to cold and salicylic acid (Huang et al., 2012a ). The chickpeas *CarNAC1* also belongs to the SENU5 subfamily. CarNAC1 was found responsive to salt, drought and cold, but ABA, indicating that it plays an important role in non-ABA-dependent signal transduction pathways (Peng et al., 2010). Under ABA stress for 12 h, *CarNAC1* expression was increased 19.85 times (Huang et al., 2012a), indicating that members of this subfamily are significantly different in response to stress. The diverse mechanisms of this family in response to regulatory
mechanisms are to be studied. Based on the expression patterns of CINAC9 under ABA stress, we speculate that the SENU5 subfamily may exert its function in an ABA-dependent/independent pathway.

NAC genes participate in a wide range of biotic and abiotic stresses and the growth process in plants. Their pleiotropic functions are extremely beneficial in transgenic applications, that is, it is possible to achieve multiple improvements by altering the expression of one NAC gene. Studies have shown that, in transgenic rice, the overexpression of OsNAC10 improves root growth and the drought tolerance by activating some of its downstream genes involved in stress response functions (Jeong et al., 2010). TaNAC69 isolated from wheat regulates gene expression in order to adapt to drought stress environment (Xue et al., 2011). Transgenic rice overexpressing either OsNAC6, SNAC1, or ONAC045 showed increased drought and salt tolerance (Hu et al, 2006; Nakashima et al, 2007; Zheng et al, 2009). A. thaliana AtNAC2 overexpression reduced the salt tolerance of transgenic plants (Balazadeh et al., 2010).

In this study, transgenic A. thaliana expressing CINAC9 were tested for its tolerance. Results showed that, under drought and high salt conditions, most WT died, with a survival rate being only 20% -30%, while the majority of transgenic plants expressing CINAC9 grew normally, with the survival rate maintained at 60%-80%. These data, together with the germination rate and root length data, confirmed that overexpression of CINAC9 enhances tolerance to drought and high salinity stress in A. thaliana.

High concentrations of NaCl affect the physiology and metabolism of plants to
various degrees, including water deficit, ion toxicity, nutritional imbalance and oxidative damages (Vinocur and Altaian, 2005). Physiological and biochemical tests showed that, \textit{ClNAC9} overexpressing plants contained significantly less MDA, had higher SOD and POD activities than WT plants under drought and salinity stresses. \textit{ClNAC9} overexpressing plants have better antioxidation capability. These data indicate that \textit{ClNAC9} expression reduces the ROS content under alkali stress, improves the ability of ROS clearance, thus reduces stress damage to the plant. The improvement of stress tolerance in \textit{ClNAC9} overexpression lines may be due to the improved anti-oxidation capability.

In abiotic stress responses in plants, transcription factors play an important function through regulating the expression of the downstream genes. Drought can induce the expression of \textit{MYB96} to improve drought tolerance by inducing the synthesis plant cuticle wax (Seo et al., 2011). \textit{MYB2} can regulate gene expression induced by ABA and JA, thereby regulate the ability of plants against drought stress (Abe et al, 2003; Shinozaki et al, 2007). \textit{RD29A} belongs to stress-inducible promoters, inducible by drought, high salt, cold and ABA (Yamaguchi-Shinozaki and Shinozaki, 1994). \textit{RD26} is a key gene in plant ROS clearance mechanisms (Fujia et al., 2004), and is involved in plant responses to drought stress. In the transgenic plants expressing \textit{ClNAC9} under stresses, the expression of \textit{RD26}, \textit{RD29A}, \textit{MYB96}, \textit{MYB2} increased significantly, much higher in transgenic plant than in WT. \textit{HAB1} is a member of the PP2C family. It is a negative regulator of the ABA pathway. \textit{HAB1} expression
increased under stress, but was significantly lower in the transgenic plants than in the wild type. Thus presumably, CINAC9 overexpression promotes the expression of downstream stress genes RD26, RD29A, MYB96 and MYB2, inhibited the expression of the negative regulator HAB1, thereby enhanced the stress tolerance in CINAC9 transgenic A. thaliana.

There was no report about function of NAC genes in C. lavandulifolium so far. In this study, we studied the function of CINAC9 under drought, salt, alkali stresses. Results further indicated the pleiotropic effect of NAC genes. These genes play an important role in plant abiotic stress response, are excellent candidates for genetic engineering of plant.

In conclusion, through cloning and functional studies of CINAC9 from C. lavandulifolium, we found that CINAC9 gene increase resistance to drought and salinity, played a role in the plants’ response to salinity and drought stress.

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<th>Primers</th>
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<td>RD26-X</td>
<td>GCATCGTAAACCACGGTA</td>
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<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<td>CCTGTCCATCATCCGAGCTAGA</td>
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</table>
Fig. 1 The electrophoresis analysis of 3’RACE and full-length cDNA of *C/NA9*

**A**: The fragment of 3’RACE  **B**: The PCR product of full-length

Fig. 2 Domain analyses of *C/NA9* protein
Fig. 3 Amino acid sequence analysis of NACs. A: Alignment of the amino acid
sequence derived from \textit{ClNAC9} with those of other NACs. B: phylogenetic analysis of \textit{ClNAC9} homologene in different plant species. The sequences were populous \textit{PeNAC010} (XP_011020822.1), cassava \textit{MeNAC35} (ALC79012.1); soybean \textit{GmNAC3} (NP_001238234.1); rice \textit{OsNAC3} (BAA89797.1); cotton \textit{GhNAC52} (AHJ79193.1); \textit{Brassica napus} \textit{BnNAC5} (AFI56995.1); \textit{Morus notabilis} \textit{MnNAC29} (EXB40352.1); \textit{Jatropha curcas} \textit{JcNAC011} (AGL39667.1); chickpea \textit{CarNAC1} (ACA96935.1); tomato \textit{SENU5} (CAA99760.1); \textit{A. thaliana} \textit{ANAC041} (NP_001118435.1); \textit{ANAC072} (NP_567773.1); \textit{ANAC083} (NP_196822.1).

\begin{table}
\begin{tabular}{ccccccccccccc}
\textit{ClNAC9} & & & & & & & & & & & & \\
\textit{ACT2} & & & & & & & & & & & & \\
\end{tabular}
\end{table}

Fig. 4. RT-PCR of \textit{ClNAC9} fragments in wild type and \textit{T1} transgenic \textit{A. thaliana}

WT: Wild type; N1-N12: Transgenic lines of \textit{T1} generation

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{The germination of \textit{T1} transgenic \textit{A. thaliana} treated with NaCl}
\end{figure}

WT: Wild type; 1: \textit{p-ClNAC9-1}; 2: \textit{p-ClNAC9-2}. *: \textit{P<0.05}, **: \textit{P<0.01}, t-test
Fig. 6. Root length of T2 transgenic A. thaliana treated with NaCl.

WT: Wild type; 1: p-ClNAC9-1; 2: p-ClNAC9-2. *: P<0.05, **: P<0.01, t-test

Fig. 7. Phenotype of T2 transgenic A. thaliana treated with high amount of NaCl
Fig. 8. Effect of salt stress on MDA, SOD and POD in T2 transgenic *A. thaliana*.

*: P <0.05, **: P <0.01, t-test.

Fig. 9. Germination of T1 transgenic *A. thaliana* treated with NaHCO₃

WT: Wild type; 1: p-CINAC9-1; 2: p-CINAC9-2. *: P <0.05, **: P <0.01, t-test
Fig. 10. Root length of T$_2$ transgenic *A. thaliana* treated with NaHCO$_3$

WT: Wild type; 1: p-ClNAC9-1; 2: p-ClNAC9-2. *: P <0.05, **: P <0.01, t-test

Fig.11. Phenotype of T$_2$ transgenic *A. thaliana* treated with high amount of NaHCO$_3$

WT  p-ClNAC9-1  p-ClNAC9-2

22.8%  64.3%  68.7%
(23/101)  (65/101)  (68/99)
Fig. 12. MDA content, SOD and POD activities in T2 transgenic *A. thaliana* treated with a high amount of NaHCO$_3$ (50 mmol/L). *: $P<0.05$, **: $P<0.01$, t-test
Fig. 13. Expression of stress-responsive genes in WT and CINAC transgenic plants

Fig. 14. The phenotype of T$_2$ transgenic A. thaliana treated with drought
Fig. 15. MDA content, SOD and POD activities in T2 transgenic A. thaliana under drought stress.

*: P<0.05, **: P<0.01, t test
Fig. 16. Expression of stress-responsive genes in WT and ClNAC transgenic plants under drought stress.