Gene 662 (2018) 10–20

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

The *SlNAC8* gene of the halophyte *Suaeda liaotungensis* enhances drought and salt stress tolerance in transgenic *Arabidopsis thaliana*

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ARTICLE INFO

Keywords:
NAC transcription factor
Transgenic plants
Drought stress
Salt stress
Suaeda liaotungensis K

ABSTRACT

NAC (NAM, ATAF1/2 and CUC) transcription factors play an important role in resisting abiotic stress in plants. In this study, a novel NAC gene, designated *SlNAC8* from *Suaeda liaotungensis* K. was characterized. SlNAC8 protein is localized in the nucleus, and the yeast one-hybrid screening showed that it contains an activation domain in its C-terminus and functions as a transcriptional activator. Gene expression analysis revealed that it is induced by drought and salt stress. *Arabidopsis* plants overexpressing *SlNAC8* demonstrated enhanced tolerance to drought and salt stress, showing significant advantages in seed germination, root growth, shoot growth, and survival rate compared with controls. Moreover, transgenic plants had a significantly higher proline concentration, antioxidant enzyme activity (superoxide dismutase, peroxidase, and catalase), and level of chlorophyll fluorescence than wild-type, and a significantly lower malondialdehyde concentration and electrolyte leakage under drought and salt stress. The overexpression of *SlNAC8* in transgenic plants also enhanced the expression of stress-responsive genes such as *RD20*, *GSTF6*, *COR47*, *RD29A*, *RD29B*, and *NYC1*. In summary, SlNAC8, as a transcription factor, may change the physiological-biochemical characteristic of plants by regulating the expression of stress-responsive genes and enhance the drought and salt stress tolerance of plants. *SlNAC8* can be utilized for developing drought and salinity tolerance in crop plants through genetic engineering.

1. Introduction

Plant growth and development are often constrained by environmental factors. For example, drought and salt stress can lead to the stagnation of plant growth, or even death. The resistance and adaptation of plants to drought and salt stress involve in the perception of stress signals and expression of stress-responsive genes. To date, a large number of stress-responsive genes and transcription factors have been identified in plants, including those from DREB, ERF, zinc finger, WRKY, MYB, bZIP, and NAM/ATAF/CUC (NAC) families. Several transcription factors can be individually or synergistically involved in regulating stress-induced gene expression, together constituting a large gene regulatory network (Ooka et al., 2003).

NAC is a plant-specific transcription factor superfamily that is widely expressed in many plant types such as wheat, rice, and *Arabidopsis* (Saidi et al., 2017; Honghong et al., 2006; Riechmann et al., 2000). NAC proteins have a highly conserved DNA binding domain at their N-terminus, and a highly variable domain at the C-terminus (Olsen et al., 2005). In recent years, much attention has focused on the role of NAC proteins in resisting drought and salt stress. For instance, the overexpression of *ZmNAC55,* a maize stress-responsive NAC transcription factor, in *Arabidopsis* enhanced drought resistance compared with wild-type seedlings (Mao et al., 2016), while drought- and salt stress-induced expression of wheat *TaNAC47* and *TaNAC67* NAC transcription factors in *Arabidopsis* improved drought resistance and salt tolerance (Zhang et al., 2016; Mao et al., 2014). Overexpressing *OsNAC045* in rice was also shown to enhance salt and drought tolerance (Zheng et al., 2009). Resistance to drought stress in rice was increased by overexpressing *OsNAC5* and *OsNAC9*, which significantly up-regulated the expression of genes associated with root development and increased root growth (Jeong et al., 2013; Redillas et al., 2012). More recently, the rice NAC transcription factor, ONAC022, was reported to play a positive role in drought and salt stress tolerance through modulating an ABA-mediated pathway (Hong et al., 2016). *MlNAC9* overexpression in *Arabidopsis* resulted in improved seed germination and root elongation compared with wild-type under salt stress, as well as increased activity

Abbreviations: GFP, green fluorescent protein; DREB, dehydration-responsive element-binding transcription factor; MYB, myeloblastosis; ERF, epidermal repair factor; DAPI, 4′,6-diamidino-2-phenylindole; PSII, photosystem II

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https://doi.org/10.1016/j.gene.2018.04.012
Received 4 January 2018; Received in revised form 30 March 2018; Accepted 5 April 2018
Available online 06 April 2018
0378-1119/

of the antioxidant enzymes superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) under drought stress. This enhanced the removal of reactive oxygen species (ROS) and increased drought resistance (Zhao et al., 2016). MlNAC5 functions as an important regulator during plant development and responses to salinity and drought stress (Yang et al., 2015). EcNAC67, a NAC transcription factor from finger millet, plays a crucial role in modulating responses against dehydration stress in rice (Rahman et al., 2016), while ANAC069 recognizes C[A/G]CG[T/G] sequences and regulates genes that control salt and osmotic stress tolerance by decreasing the ROS scavenging capability and proline biosynthesis (He et al., 2017).

Halophytes, as an excellent platform for salt responsive genes, have attracted more and more attention. In recent years, our group has been focusing on salt responsive genes in the halophyte *Suaeda liaotungensis*. Many salt-related genes were found in the *S. liaotungensis* transcriptome. Two *SlNAC* genes were cloned and their functions were analyzed. Studies showed that *SlNAC1* improves stress resistance, and that *SlNAC2* might be a good candidate gene for the production of a stress-tolerant plant (Li et al., 2014; Yang et al., 2014). The other members of SlNACs, a big family, are badly in need of being identified.

In this study, we focused on another expressed sequence tag (EST) of NAC transcription factors (named *SlNAC8*) in the *S. liaotungensis* transcriptome. We cloned *SlNAC8* and characterized its function. We found that *SlNAC8* expression was induced by drought and salt stress. SlNAC8 protein was localized in the nucleus and acted as a transcriptional activator. *SlNAC8*-transgenic *Arabidopsis* demonstrated up-regulated expression of some stress-related genes, and significant advantages in morphology and physiology relative to non-transgenic *Arabidopsis* under drought and salt stress. These findings help us to understand the function of the SlNAC8 transcription factor in improving plant stress resistance, and suggest a potential candidate gene for the cultivation of resistant varieties by genetic engineering.

2. Materials and methods

2.1. Plant materials and growth conditions

S. liaotungensis K. leaves were used for genetic cloning and seeds were used for expression analysis. The seeds were grown in pots with soil (humus) and cultured at 25 °C with 12 h light/12 h darkness. Seedlings with seven true leaves were transferred to Murashige and Skoog (MS) (DIFCO, BD) liquid medium for culture.

Arabidopsis thaliana (ecotype: Columbia) was used for genetic transformation and functional analysis. After sowing in MS medium and vernalizing for 3 days, culture dishes were transferred to an incubator (at 22 °C, with 16 h light/8 h darkness, a light intensity of 120–150 μmol $m^{-2} s^{-1}$, and 60% relative humidity). Seedlings with four true leaves were moved into pots with soil, and incubated in a sterile room with 16 h light/8 h darkness, at 60% relative humidity, and at 22 °C.

2.2. Cloning and bioinformatic analysis of SlNAC8

BLAST alignment was performed on EST sequences selected from the *S. liaotungensis* transcriptome database. The EST of the *SlNAC8* sequence contained a complete open reading frame (ORF). A pair of primers, EST-F and EST-R (Table 1), was designed according to the sequence.

Total RNA was extracted from the leaves of wild-type *S. liaotungensis* using RNAiso Plus (TaKaRa). First-strand cDNA was synthesized using the PrimeScript™ RT reagent kit (TaKaRa), amplified by PCR, gel-purified, and sequenced. The obtained sequence was compared with the original EST sequence by Sequencher software to obtain an exact sequence. Multiple sequence alignments of *SlNAC8* together with other stress-responsive NACs were aligned using BioEdit software. NAC transcription factors from *Arabidopsis* and 20 homologous NACs from other plants were collected to construct a phylogenetic tree using the neighbor-joining method with MEGA v. 4.1 software. To verify its stability, 36 NACs were selected to construct another phylogenetic tree using the maximum parsimony method.

A pair of primers, SlNAC8-F and SlNAC8-R (Table 1), was designed to amplify the *SlNAC8* ORF fragment. This was then inserted into the pEASY-T1-Simple vector.

2.3. Stress treatments and expression analysis

S. liaotungensis seedlings were cultured in MS liquid medium supplemented with 200 mM NaCl and 200 mM polyethylene glycol. Leaves (0.1 g) were removed from the seedlings at 0, 0.5, 1, 3, 6, 12, and 24 h post-treatment with NaCl or PEG6000, then used for total RNA extraction with RNAiso Plus (TaKaRa). cDNA was synthesized using the PrimeScript™ RT reagent Kit (TaKaRa), and the transcription profile of *SlNAC8* was examined using quantitative real-time (qRT)-PCR with primer pair SlNAC8-qF and SlNAC8-qR (Table 1). The *S. liaotungensis* actin gene *SlActin* (GenBank no. JX860282.1) was used as the internal reference and amplified with primer pair SlActin-qF and SlActin-qR (Table 1). qRT-PCR was conducted on a Thermal Cycler Dice Real Time System TP800 (Takara, Japan) using SYBR Premix Ex Taq II (Tli RnaseH Plus) (Takara, Japan). Relative transcription was calculated using the $2^{-\triangle\triangle Ct}$ method.

2.4. Transactivation analysis of SlNAC8

The N-terminal domain, C-terminal domain, and full-length ORF of *SlNAC8* were separately inserted into the pGBKT7 vector to create three recombinant vectors. According to the manufacturer's protocol (Stratagene, USA), these were then transferred into the yeast strain AH109 with the negative control empty pGBKT7 vector. Transformed yeast cells were cultured on SD, SD(–Trp), and SD(–Trp/–His/–Ade) plates for 3 days at 30 °C. The transactivation activity of each protein was evaluated according to its growth status and galactosidase filter lift assay (Yeast Protocols Handbook; Clontech, USA).

2.5. Subcellular localization analysis of SlNAC8

The *SlNAC8* ORF was cloned into the pEGAD vector together with the gene encoding green fluorescent protein (*GFP*), then pEGAD-GFP and pEGAD-GFP-*SlNAC8* were transferred into onion epidermal cells by biolistic bombardment with a GeneGun (GJ-1000; China) for transient expression. The expressed proteins were observed under confocal microscopy (Zeiss, Germany) with or without DAPI staining.

2.6. Generation of transgenic Arabidopsis lines

The coding sequence of *SlNAC8* was amplified and cloned into *Xba* I and *Bam*H I sites of the pBI121vector under the control of the CaMV35S promoter. The recombinant vector pBI121-*SlNAC8* and pBI121 vector were then transferred into *Agrobacterium* GV3101. Finally, transgenic *Arabidopsis* plants were generated using the *A. tumefaciens*-mediated floral dipping method (Clough and Bent, 1998). Positive transgenic lines were screened on 100 μg/mL kanamycin plates. Two T3 homozygous *SlNAC8* transgenic lines were selected for further analysis. Total RNA was extracted from these two transgenic lines and WT line, and cDNA was synthesized and used as template for semi-quantitative PCR.

2.7. Analysis of abiotic stress tolerance

Seeds of wild-type (WT), pBI121 (pBI), and two T3 homozygous transgenic *Arabidopsis* plants (L1, L2) were sown in MS medium containing D-mannitol (200 mM) or NaCl (200 mM) to measure the germination rate and primary root length. After germination, WT, pBI, L1, and L2 plants with four true leaves were transferred into soil (humus: