Overexpression of a NAC transcription factor enhances rice drought and salt tolerance

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The plant-specific NAC (NAM, ATAF1/2, CUC2) transcription factors play diverse roles in plant development and stress responses. In this study, a rice NAC gene, ONAC045, was functionally characterized, especially with regard to its role in abiotic stress resistance. Expression analysis revealed that ONAC045 was induced by drought, high salt, and low temperature stresses, and abscisic acid (ABA) treatment in leaves and roots. Transcriptional activation assay in yeast indicated that ONAC045 functioned as a transcriptional activator. Transient expression of GFP-ONAC045 in onion epidermal cells revealed that ONAC045 protein was localized in the nucleus. Transgenic rice plants overexpressing ONAC045 showed enhanced tolerance to drought and salt treatments. Two stress-responsive genes were upregulated in transgenic rice. Together, these results suggest that ONAC045 encodes a novel stress-responsive NAC transcription factor and is potential useful for engineering drought and salt tolerant rice.

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Being sessile, plants are frequently up against various environmental stresses such as drought, salt, and low temperature. To cope with these adverse conditions, numerous genes are induced in plant cells, which finally lead to physiological and metabolic changes that increase the chance of plant survival [1,2]. Among these genes, transcription factors play essential roles in stress responses by regulating their target genes through binding to the cognate cis-acting elements [3–5]. Some transcription factors, such as CBF1/DREB1B [6], OsZIP72 [7], AtMYB44 [8] have been reported to be involved in plant stress responses. Transgenic plants overexpressing these genes could enhance their tolerance to various stresses.

NAC family, which is one of the largest plant transcription factor families, is only found in plants to date [9]. Proteins of this family are characterized by a highly conserved DNA binding domain, known as NAC domain in the N-terminal region. In contrast, the C-terminal region of NAC proteins, usually containing the transcriptional activation domain, is highly diversified both in length and sequence [10]. More than 100 members of this family have been identified in both Arabidopsis and rice [10,11]. However, only a few of them have been functionally characterized, especially in rice. NACs play important roles in plant development, including pattern formation of embryos and flowers [12], formation of secondary walls [13], and development of lateral roots [14]. NACs are also reported to participate in abiotic and biotic responses. In potato, StNAC was rapidly and strongly induced by wounding [15]. In Brassica napus, nine NACs were reported to be differently regulated by biotic and abiotic stresses [16]. Expression of GRAB1 and GRAB2 in cultured wheat cells inhibited DNA replication of the wheat dwarf geminivirus [17]. Three Arabidopsis NAC genes, ANAC019, ANAC055, and ANAC072, were shown to bind to the promoter region of ERD1 which was characterized as a stress-responsive gene [5]. Overexpressing these three genes in Arabidopsis resulted in enhanced tolerance to drought stress. Moreover, two other NAC genes in Arabidopsis, ATAF1 and ATAF2, played negative roles in response to drought and pathogen infection respectively [18,19]. Recently, the roles of two rice NAC genes in rice stress adaptation were characterized [20–22]. SnNAC1 was induced mainly in guard cells under drought conditions, and overexpression of this gene in rice resulted in significant increase in drought resistance under field condition at the stage of anthesis [20]. Overexpression of another NAC gene OsNAC6/SNAC2 in rice resulted in enhanced tolerance to drought, salt, and cold during seedling development [21,22].

By analyzing the microarray data (GSE 6901) in public database at NCBI, we found that a NAC gene, designated as ONAC045 according to Ooka et al. [10], was highly induced by abiotic stresses. In this work, the expression profiles of this gene in different organs and under various stress treatments were studied. The transcriptional activation and the subcellular localization of ONAC045 were also investigated. Transgenic rice plants overexpressing ONAC045 showed enhanced drought and salt tolerance, indicating that...
ONAC045 played an important role in abiotic stress response and may serve as a potential target for engineering stress tolerant rice.

Materials and methods

Constructs and transformation. The full-length cDNA of ONAC045 was amplified using a cDNA clone (GenBank Accession No. CT829509) from a cDNA library of Guangluai 4 (Oryza sativa L. ssp. indica) [23] as template (for primers, see Supplementary Table S1). The sequencing-confirmed PCR fragment was ligated into the overexpression vector pCAMBIA1300S. The resultant construct, pCAMBIA1300S-ONAC045, was transformed into Nipponbare (Oryza sativa L. ssp. japonica) by Agrobacterium-mediated transformation method to generate transgenic rice plants [24].

Plant materials, growth condition and stress treatments. The rice seeds of Guangluai4 were germinated at 37 °C for 2 days, and then grown in a growth chamber (14 h light 30 °C/10 h dark 28 °C cycles) to harvest the young leaves and young roots. Mature leaves, stems, and panicles after heading were prepared from the same staged plants. For expression analysis, seedlings at the three-leaf stage were subjected to various stress treatments. For salt and ABA treatments, seedlings were incubated in solutions containing 200 mM NaCl and 100 μM ABA, respectively. For drought treatment, whole seedlings were exposed to air under dim light. For cold treatment, seedlings were treated at 8–10 °C under dim light. Leaves and roots were harvested at different time points as shown in Fig. 1. For the marker gene analysis, leaves of wild type rice and transgenic rice at the three-leaf stage were harvested for RNA isolation.

Real-time RT-PCR analysis. Total RNA was extracted using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The DNase-treated RNA was reverse-transcribed using M-MLV reverse transcriptase (TaKaRa). Real-time RT-PCR was performed on the Applied Biosystems 7500 real time PCR System using SYBR Premix Ex Taq™ (TaKaRa). The PCR thermal cycle conditions were as following: denature at 95 °C for 10 s and 40 cycles for 95 °C, 5 s; 60 °C, 34 s. Two rice genes used as internal references for calculating relative transcript levels were UBQ5 (GenBank Accession No. AK061988) and eEF-1z (GenBank Accession No. AK061464) [25]. The primer efficiency used for calculating the relative quantification was 2.0 [26].

Drought and salt tolerance assay. For drought tolerance test, transgenic seedlings were grown hydroponically for 15–18 days to three-leaf stage, and parallelly grown WT rice plants of the same stage were used as control. Whole plants were exposed to air for 9.5 h, and then rehydrated and recovered for an additional 10 days. The survival rates of transgenic lines and the WT control were calculated.

Transcriptional activation analysis in yeast. For transcriptional activation assay, the sequencing-confirmed PCR fragment of full ORF, N-terminal ORF with the NAC domain (1–159 amino acids), and C-terminal ORF with the potential activation domain (160–359 amino acids) were fused in frame with GAL4 DNA binding domain in pGBK7 to construct pGBK7-ONAC045, pGBK7-ONAC045ΔC, and pGBK7-ONAC045ΔN, respectively (for primers, see Supplementary Table S1). pGBK7 was used as a negative control. These different constructs were transformed into yeast strain AH109. The transformants were streaked on the SD/Trp- and SD/Trp-/His-/Ade- medium. After incubated at 28 °C for 3 days, the growth status of the transformants was evaluated. The β-galactosidase filter assay was carried out according to the manufacturer’s instructions (Clontech).

Subcellular localization analysis. The full open reading frame (ORF) of ONAC045 was amplified using the cDNA clone mentioned above as template (for primers, see Supplementary Table S1). The PCR product was ligated into the pA7-GFP vector, resulting in an in-frame fusion protein of GFP gene and the ONAC045 ORF. The construct (p35S:GFP-ONAC045) and the control vector (p35S:GFP) were transformed into onion epidermal cells by particle bombardment using a Biologic PDS-1000/He gene gun system (Bio-RAD). After 24 h incubation of transformed onion epidermal cells, GFP signal was detected by a confocal fluorescence microscope (Zeiss, LSM510 Meta, Germany).

Results

Expression profile of ONAC045

Expression pattern of ONAC045 in young leaves, young roots, mature leaves, stems, and panicles was investigated using real-time RT-PCR. It was shown that the expression level was higher in young roots than in other organs examined (Fig. 1A).

The expression pattern of ONAC045 under various stress treatments in leaves and roots was also investigated (Fig. 1B). Under ABA treatment, the expression level was peaked at 2 h in leaves and then decreased, while it could only be observed until 4 h after ABA treatment in roots and maintained at the similar level until 8 h. Under drought treatment, the expression of ONAC045 was peaked at 2 h in both roots and leaves, and then decreased at 4 and 8 h, respectively. When treated with 200 mM NaCl, the expression of ONAC045 was only slightly induced in leaves; however, the induction reached a much higher level in roots at 4, 12, and 24 h after NaCl treatment. Upon cold conditions, the most dramatic
induction of ONAC045 was observed in both leaves and roots. The induction was peaked at 12 h in leaves and decreased at 24 h while it was continuously increased and peaked at 24 h in roots.

**ONAC045 had transcriptional activation and was localized in the nucleus**

Yeast two-hybrid system was used to investigate the transcriptional activation of ONAC045. As shown in Fig. 2A, all transformants grew well on SD/Trp- medium. However, only transformants containing pGBK7-ONAC045 and pGBK7-ONAC045ΔN could grow on SD/Trp-/His-/Ade- medium and showed β-galactosidase activity while those containing pGBK7 and pGBK7-ONAC045ΔC could not. These results indicated that ONAC045 functioned as a transcriptional activator and the activation domain was located in the C-terminal region.

To determine the subcellular localization of ONAC045, p35S-GFP-ONAC045 and p35S-GFP were transiently expressed in onion epidermal cells. As shown in Fig. 2B, the onion cells transformed with p35S-GFP vector displayed fluorescence throughout the whole cells (Fig. 2B, a–c). In contrast, fluorescence in the onion cell transformed with p35S-GFP-ONAC045 was detected exclusively in the nucleus (Fig. 2B, d–f), indicating that ONAC045 encoded a nuclear localized protein.

**Overexpression of ONAC045 in transgenic rice improved drought and salt tolerance**

In order to characterize the *in vivo* function of ONAC045, transgenic rice plants overexpressing this gene were generated. The T2 generations of two homoyzogous transgenic lines, overexpression line 2 (OE2) and overexpression line 3 (OE3), were used for stress tolerance assay (for overexpression level, see Supplementary Fig. S1).

We tested the effect of ONAC045 overexpression on drought tolerance. As shown in Fig. 3A and B, more than 90% of OE2 and more than 70% of OE3 remained vigorous respectively after recovery, while only about 35% of wide type survived, suggesting that overexpression of ONAC045 could improve drought tolerance in transgenic rice (*t* test, *P* < 0.01).

The effect of ONAC045 overexpression on salt tolerance was also investigated. As shown in Fig. 3C and D, the survival rates of OE2 and OE3 were more than 60%, significantly higher than that of WT plants (16%), suggesting that overexpression of ONAC045 could improve salt tolerance in transgenic rice (*t* test, *P* < 0.01).

**Overexpression of ONAC045 induced expression of two stress-responsive genes**

To better understand the mechanisms of drought and salt tolerance conferred by overexpressing ONAC045, we investigated the expression of several known drought and salt induced genes in transgenic rice plants. As shown in Fig 4, the expression levels of two late embryogenesis abundant (LEA) gene (OsLEA3-1) [27], GenBank Accession No. Z68090), and a homologue gene of wheat plasma membrane protein (WPM-1) [28] (termed as OsPM1 here, GenBank Accession No. NM_001061933) were strongly induced in transgenic rice compared with that in wild type rice under normal growth condition.

**Discussion**

Rice is one of the most important crops in the world. The growth and productivity of rice are often threatened by environmental factors, such as drought, salt, cold, and biotic stresses. Many efforts have been undertaken to generate stress tolerant rice by manipulating the expression of stress-responsive genes [29,30].

Some members of NAC family have been shown to be involved in plant stress responses [15–19]. In this study, we functionally characterized a novel rice stress-responsive NAC gene ONAC045. Expression analysis showed that ONAC045 was highly induced by drought, salt, cold, and ABA in leaves and roots (Fig. 1B). Interestingly, the expression pattern was different between leaves and roots. For example, after salt treatment, the induced expression was much higher in roots than that in leaves at all three examined time points (Fig. 1B), suggesting that the expression of ONAC045 was differently regulated in leaves and roots. A previous study showed that ONAC045 was not induced under drought treatment
in leaves [11], which was different with our results here (Fig. 1B). This might be explained by the use of different rice cultivars (indica rice Minghui63 was previous used) and/or somewhat different conditions of drought treatment between the two studies. Promoter sequence analysis of ONAC045 showed that there were several core sequences of MYB and MYC binding sites, including 5'–CNGTTR and 5'–CANNTG [11], which may at least partially explain the stress-responsive expression. Like SNAC1 [20] and OsNAC6/SNAC2 [21,22], ONAC045 was also localized in the nucleus (Fig. 2B) and had transcriptional activation, suggesting that it functioned as a transcriptional activator (Fig. 2A). Furthermore, the C-terminal part may be responsible for the transcriptional activation (Fig. 2A).

Recently, 140 NAC or NAC-like genes were identified in rice, and they were divided into five subgroups according to phylogenetic relationship [11]. All of the known stress-related NAC genes were grouped into family III, including the two well characterized stress-responsive NAC genes, SNAC1, and OsNAC6/SNAC2. Different from these two genes, ONAC045 was grouped into family I, which comprises all the published development-related NAC genes [11]. We did not find any obvious developmental differences between the transgenic plants and the wide type plants under normal growth condition. However, we still cannot rule out the possibility that ONAC045 plays a role in rice development.

Transgenic rice overexpressing ONAC045 showed significantly increased tolerance to drought and salt at the seedling stage (Fig. 3). Previous studies showed that transgenic plants overexpressing some stress-responsive genes, such as OsNAC6/SNAC2 [21,22], OsDREB1A, OsDREB1B, AtDREB1A, and AtDREB1B [31], led to growth retardation under normal condition, which may finally cause significant reduction of potential yield. We did not observe similar phenotype in transgenic rice overexpressing ONAC045 under the greenhouse condition. It will be interesting to investigate whether overexpressing ONAC045 can enhance the stress tolerance under field conditions.

We observed that the expression levels of two stress-responsive genes OsLEA3-1 and OsPM1, were upregulated in transgenic lines (Fig. 4). LEA proteins are involved in many stress responses of plants. OsLEA3-1 belongs to group 3 LEA family, and expression of this gene is induced by ABA, drought, and salt, but not by cold [27]. Overexpressing OsLEA3-1 in rice showed significantly increased drought tolerance under the field condition. OsPM1 is a homologue gene of wheat WPM1 which may be closely associated with the ABA-induced freezing tolerance in wheat cultured cells [28]. OsPM1 is also induced by ABA, drought, salt, and cold (data not shown). The two target genes were both strongly induced by ABA, implying that ONAC045 might be involved in ABA signaling pathway. Expressions of OsLEA3-1 and OsPM1 were not affected.

![Fig. 3. Drought tolerance assays of ONAC045-overexpressing transgenic rice.](image)

![Fig. 4. Expression analysis of two stress-responsive genes OsLEA3-1 (A) and OsPM1 (B) in the transgenic lines OE2 and OE3. WT rice was used as control. Error bars are standard deviations of three technical repeats.](image)
in either SNAC1 or SNAC2 transgenic rice according to previous microarray analysis [20,22], which is consistent with the result that no genes regulated by SNAC1 were also found to be regulated by SNAC2 [22]. This indicates that these different NAC genes have non-redundant functions even though they are all involved in stress responses.

Overall, we characterized a stress-responsive NAC gene ONAC045. Overexpression of this gene could significantly enhance drought and salt tolerance in rice, making it a potential candidate for engineering stress tolerant rice.

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Appendix A. Supplementary data


References


