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

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ABSTRACT

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.

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], *OsbZIP72* [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. *NAC*s 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]. *NAC*s 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 *NAC*s 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]. *SNAC1* 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

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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 reference genes for calculating relative transcript levels were *UBQ5* (GenBank Accession No. AK061988) and *eEF-1α* (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. For salt tolerant assay, three-leaf stage WT and transgenic seedlings were incubated in solution containing 100 mM NaCl for 13 days(the salt solution was refreshed every two days), then all the plants were transferred into solution without NaCl 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 pGBKT7 to construct pGBKT7-ONAC045, pGBKT7-ONAC045ΔC, and pGBKT7-ONAC045ΔN, respectively (for primers, see Supplementary Table S1). pGBKT7 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 ß-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 Biolistic 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

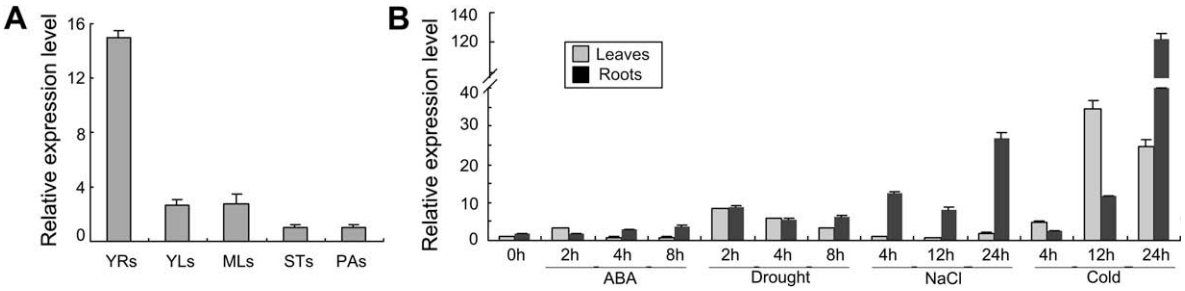

Fig. 1. Expression pattern of *ONAC045* in Guangluai 4 was detected by real-time RT-PCR. The experiments were repeated twice, and similar tendency was observed. The expression levels shown here were according to one measurement. Error bars are standard deviations of three technical repeats. (A) The organ specific expression of *ONA045* in young roots (YRs), young leaves (YLs), mature leaves (MLs), stems (STs), and panicles (PAs). (B) Expression of *ONAC045* in response to ABA (100 μM), drought, NaCl (200 mM), and cold (8–10 °C) treatments in young leaves and young roots.

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 pGBKT7-ONAC045 and pGBKT7-ONAC045ΔN could grow on SD/Trp-/His-/Ade- medium and showed β-galactosidase activity while those containing pGBKT7 and pGBKT7-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.

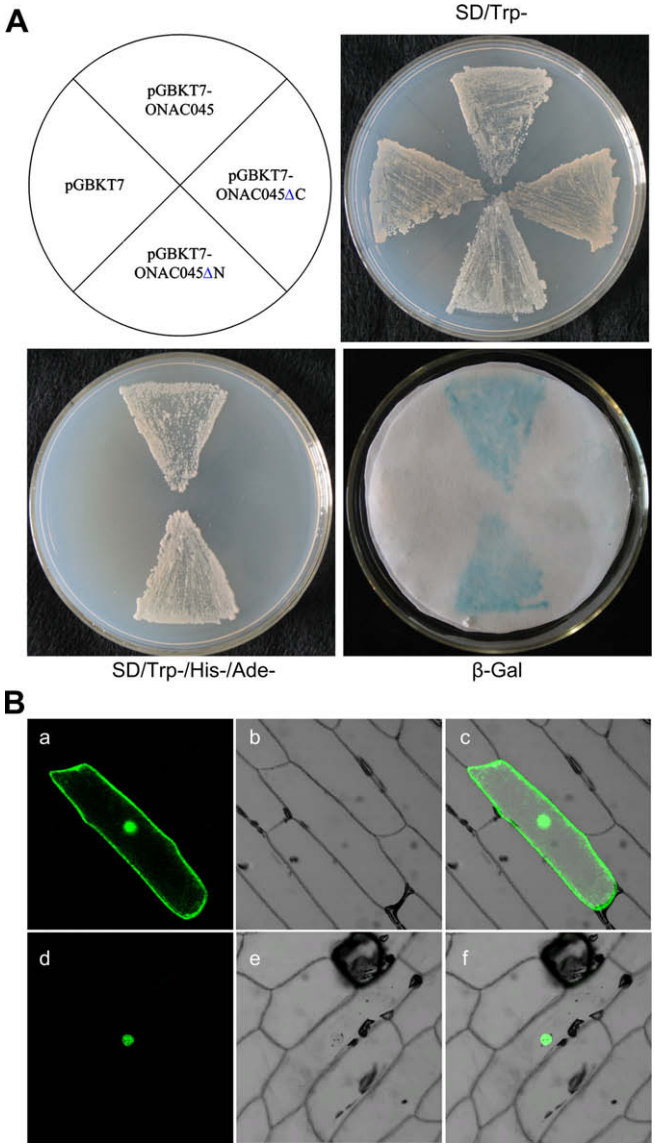

Fig. 2. Transcriptional activation assay and nuclear localization assay of ONAC045. (A) Fusion proteins of pGBKT7-ONAC045, pGBKT7-ONAC045ΔC, and pGBKT7-ONAC045ΔN and pGBKT7 were expressed in yeast strain AH109. The transformant carrying pGBKT7 vector was used as a negative control. The transformants were incubated on SD/Trp- and SD/Trp-/His-/Ade- to examine their growth and tested for β-galactosidase activity. (B) Nuclear localization of OANC045. p35S:GFP (as a control) and p35S:GFP-ONAC045 were transiently expressed in onion epidermal cells. The photographs were taken in the dark field for green fluorescence (a and d), under bright light for the morphology of the cell (b and e) and in combination (c and f), respectively for p35:GFP control plasmid (a–c) and p35S:OsDREB1F-GFP plasmid (d–f).

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 homozygous 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 a 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

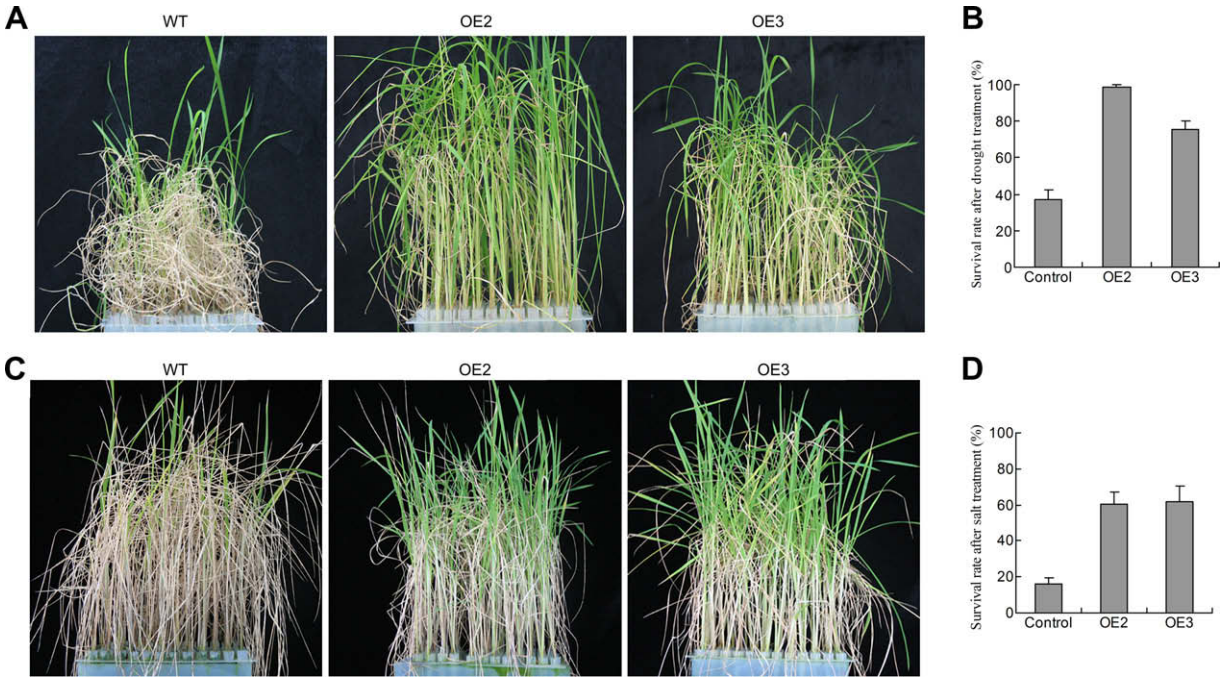

Fig. 3. Drought tolerance assays of ONAC045-overexpressing transgenic rice. (A) Performance of WT control and *ONAC045*-overexpression lines OE2 and OE3 after 9.5 h drought stress and 10 days recovery. (B) Survival rates of the WT control and transgenic rice lines. Error bars are standard deviations based on three replicates ($n > 40$). (C) Performance of WT control and *ONAC045*-overexpression line OE2 and OE3 after 13 days salt stress and 10 days recovery. (D) Survival rates of the WT control and transgenic rice lines. Error bars are standard deviations based on three replicates ($n > 40$).

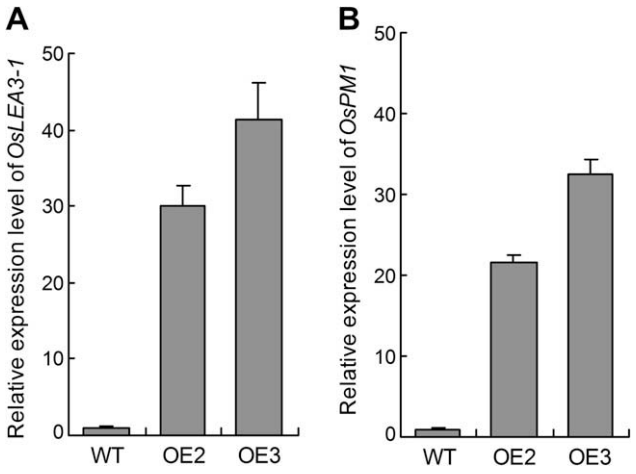

Fig. 4. Expression analysis of two stress-responsive genes *OsLEA3-1* (A) and *OsPM1* (B) in the transgenic lines OE2 and OE3. WT rice was used as control. Error bars are standard deviations of three technical repeats.

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 *OANC045* 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

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

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.163.

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