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Overexpression of *OsNAC6* transcription factor from Indonesia rice cultivar enhances drought and salt tolerance

Agus Rachmat[1,2*], Satya Nugroho[1], Dewi Sukma[2], Hajrial Aswidinnoor[2] and Sudarsono[3]

[1]Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong, Bogor, West Java, Indonesia
[2]Plant Breeding and Biotechnology Program, IPB Graduate School, Bogor Agricultural University (IPB), Darmaga Campus, Bogor 16680, West Java, Indonesia
[3]PMB Lab, Department of Agronomy and Horticulture, Faculty of Agriculture, Bogor Agricultural University (IPB), Darmaga Campus, Bogor 16680, West Java, Indonesia

Abstract

Drought is a major constrain in crop production that reduce growth and cause yield loss of up to 70%. Transcription factor plays a major role in cellular regulation and physical changes of plants as a response to stress. A number of transcription factors, such as CBF/DREB, NAC, zinc finger protein are regulators during stress. The *Oryza sativa* NAC6 (*OsNAC6*) gene is one of the transcription factor in rice that can regulate gene expression during stress conditions. Thus, pCambia 1305 harboring *OsNAC6* chimaeric gene with CaMV 35S promoter was introduced into rice zygotic embryo using *Agrobacterium tumefaciens* mediated transformation to regenerate transgenic rice overexpressing the transgene. As many as 39 putative transgenic lines in which 21 lines possitively harbored *hpt* gene have been regenerated. The positive identification of *hpt* in the regenerated transgenic rice indirectly indicated integration of the targeted *OsNAC6* since both transgenes were part of the same T-DNA. Further analysis indicated the presence of 1-3 copies of transgene integration in the genome. The expression of *OsNAC6* transgene in the transgenic rice line#C.73, C.83 and C.91 were higher than wild type non-transgenic one. Further analysis indicated those three transgenic lines carrying *OsNAC6* transgene exhibited higher tolerance against drought and salinity stresses. Moreover, three known stress-associated regulatory genes (AP2, Zincfinger protein and MYB) were up-regulated in those three transgenic lines. These findings demonstrated that *OsNAC6* might be a candidate of stress-responsive NAC regulatory gene that can be used to develop either drought or salt tolerant tolerant transgenic plants.

Key words: *Agrobacterium tumefaciens*, Drought stress, *Oryza sativa* cv. Ciherang, Salt stress, Transgenic rice

Introduction

Rice (*Oryza sativa* L.), a major staple crop to Indonesian and to other developing coutries in the world. Dehydration stress is a major constrain in rice production in certain areas or seasons. Such dehydration stress may be initiated by either drought or high salinity. Therefore, availability of dehydration stress tolerance variety is highly desirable. Dehydration stress tolerance is a character controlled by multi-genes, making the development of drought tolerance transgenic crops a challenging task. One need to understand the physiology and molecular processes associated with dehydration stress responses to be able to develop crop with higher tolerance to dehydration stress.

A number of transcription factors (TFs) are associated with dehydration stress responses in plants. The TFs control expression of a number of target genes and may associate with a number of pathways. Therefore, modifying complex metabolic pathways in plants can be done by transforming a single TF (Hussain et al. 2011). Interaction among transcription factors with the cis-acting elements of the target genes regulate the expression of the target gene and eventually direct a number of cellular activities (Xiong et al., 2005).

The NAC is a drought inducible gene, initially studied in *Arabidopsis*. Improved stress tolerance in *Arabidopsis* could be achieved by over-expressing the *Arabidopsis thaliana* (*At*) NAC genes (ANAC019, ANAC055, and ANAC072). In these

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*Corresponding Author

Agus Rachmat
Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong, Bogor, West Java, Indonesia

Email: guess_btk@yahoo.com

NAC gene expressing transgenic *Arabidosis*, there were a number of altered expression of genes induced by drought, salinity, and low-temperature stress (Tran et al., 2004). The function of NAC gene in drought tolerance response was further supported by Hu et al. (2006). In their study, transgenic rice overexpressing SNAC1 were more tolerance to drought stress during flowering in the field and yielded more seeds than the control non-transgenic one (Hu et al. 2006). Hu et al. (2006) also demonstrated that the function of SNAC1 gene is also associated with plant responses to cold and salt stresses (Hu et al., 2006). The NAC6/SNAC2 and OsNAC10 genes originated from *Oryza sativa* (Os) have also been reported as transcription factors regulating the expression of genes associated with stress responses (Nakashima et al., 2007; Jeong et al., 2010). These two genes belong to the ATAF gene sub-family (Kikuchi et al., 2000; Ooka et al., 2003). Expression of the AtNAC2 gene from *Arabidopsis* was induced by both salt stress and by abscisic acid. The function of AtNAC2 is associated with tolerance to salt stress and with root development (He et al., 2005).

Regeneration of transgenic rice has been proposed as an alternative route for developing drought tolerant rice genotype. Drought tolerance is a character regulated by many genes. Shinozaki and Yamaguchi (2007) have identified the two gene groups associated with response of plant to drought. In group one includes genes that protect plant cells under dehydration stress, such as genes encoding osmotin, detoxification enzymes, cell recovery and structural adaptation proteins. The second group includes one dealing with those that regulate signal transduction and dehydration stress inducible gene, such as one coding for protein kinases and TFs (Shinozaki and Yamaguchi, 2007).

The TF proteins have an important function in regulating gene expression and physical changes of plants as a response to stresses. A number of the known TFs include CBF/DREB, NAM, ATAF, CUC, zinc finger protein regulators during stress (Huang et al., 2009). Overexpression of three *NAC* genes from *Arabidopsis thaliana, such as ANAC019*, *ANAC055* and *ANAC072* in transgenic plant has increased stress tolerance and changed gene expression induced by drought, salinity and low temperature (Tran et al., 2004). Therefore, these three genes might actually play a major role in stress tolerance responses. Either *OsNAC6* or *OsNAC10* gene was also reported to encode protein during drought stress (Jeong et al., 2010; Nakashima et al., 2007). The expression of stress-induced *SNAC-A* domain carrying gene such as *RD26*, Arabidopsis *ATAF*, and rice *SNAC* (*SNAC1, OsNAC6* and *OsNAC5*) were also reported as associated with increasing drought and salinity tolerance (Nakashima et al., 2012).

Rice (*Oryza sativa*) NAC6 gene (*OsNAC6*) has been isolated from drought tolerance upland rice cv. Batutegi and the appropiate transgene is constructed. Overexpression of *OsNAC6* was done by introducing the transgene into Indica rice cv. Ciherang, the most widely planted rice variety in Indonesia and regenerating transgenic rice lines. This research was conducted to evaluate drought and salt stress responses of the regenerated transgenic Indica rice cv. Ciherang overexpressing *OsNAC6*.

Materials and Methods

Rice cultivars

Seedlings of upland rice (*Oryza sativa* L.) cv. Batutegi and lowland rice cv. Ciherang were obtained from Muara Experimental Station, Indonesian Center for Rice Research (ICRR), Bogor, West Java, Indonesia. Rice cv. Batutegi is more tolerant to drought stress than Ciherang. However, Ciherang is more commonly planted rice variety by farmers in Indonesia.

Transgene construct and transformation

Plasmid pCAMBIA 1305 was obtained from CAMBIA-Australia. The complete cDNA of *OsNAC6* was generated by PCR amplification using gene specific primer pairs developed based on NAC cDNA sequences available in the GenBank DNA Database (Accession No. B028185.1). The total cDNA was generated from total mRNA isolated from upland rice (*Oryza sativa*) cv. Batutegi and it was used as template. The resulted PCR amplified product was sequenced to determine its identity and the correctly identified full length *OsNAC6* cDNA fragment was fused into appropiate site of pCAMBIA 1305 overexpression vector. The transgene construct of pCNAC6 (Figure 1) was introduced using *Agrobacterium*-mediated transformation method into rice cv. Ciherang and a number of rice transgenic lines were regenerated. Hygromycin phosphotransferase gene (*hpt*) in the overexpression vector allows transformed cells expressing the transgenes to be selected out of the non-transformed cell populations using selective medium supplemented with hygromycin. As part of this rice transformation system, a selective and toxic hygromycin that interferes with the cellular metabolism of non-transformed cells is applied to population of putatively transformed ones.

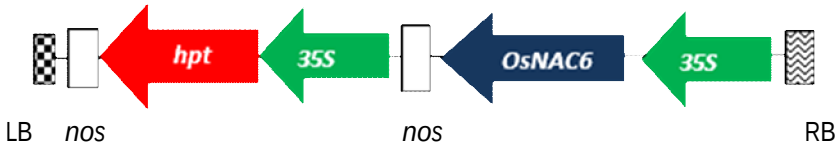

Figure 1. Structure of pCNAC6 construct, carrying both *OsNAC6* and *hpt* transgenes in its T-DNA. This construct was used for rice cv. Ciherang genetic transformation mediated by *Agrobacterium*. *35S*: 35S CaMV promoter; *OsNAC6*: *OsNAC6* coding sequence; *hpt*: hygromycin-phosphotransferase coding sequence; *nos*: nopaline synthase terminator; LB and RB: left and right border of the T-DNA structure.

Inoculation and co-cultivation

Agrobacterium tumefaciens was cultured on AB medium (Chilton et al., 1974) containing hygromycin (50 mg l^{-1}) and kanamycin (50 mg l^{-1}) for 3 days at 28 C. The bacteria were harvested by centrifugation and the bacterial pellets were resuspended in AA-inf medium at 3x109 cfu ml^{-1}, containing 0.1 mM acetosyringone (co-cultivation medium). The immature rice embryos were used as explants and cultured scutellum-side up on the co-cultivation medium. The *Agrobacterium* suspension (5 µl) was dropped on top of the rice immature embryos and they were co-cultivated at 25°C in the dark for 7 days. Effectiveness of two different co-cultivation media were evaluated, suc as N6-As and NB-As. The N6-As consisted of N6 salts and vitamins (Chu, 1978), vitamin assay casamino acids (0.5 g l^{-1}), L-proline (0.5 g l^{-1}), sucrose (20 g l^{-1}), D-glucose (10 g l^{-1}), 2,4-D (2 mg l^{-1}), NAA (1 mg l^{-1}), BA (1 mg l^{-1}), acetosyringone (0.1 mM), and agarose Type I (5.5 g l^{-1}), pH 5.2. The NB-As consisted of N6 major salts, B5 minor salts and vitamins (Gamborg et al., 1968), sucrose (20 g l^{-1}), D-glucose (10 g l^{-1}), 2,4-D (2 mg l^{-1}), NAA (1 mg l^{-1}), BA (1 mg l^{-1}), acetosyringone (0.1 mM), and agarose Type I (5.5 g l^{-1}), pH 5.2 (Hiei and Komari, 2006).

Selection and regeneration of transgenic rice

Elongated rice shoots were removed using scalpel from co-cultivated rice immature embryos.. In all of the next steps, unless otherwise stated, the co-cultivated embryos were maintained at 30°C under 24 hours illumination (ca.35 lmol $m^{-2}s^{-1}$). The co-cultivated embryos were grown for 5 days on NBM medium consisted of N6 major salts, B5 minor salts and vitamins, vitamin assay casamino acids (0.5 g l^{-1}), L-proline (0.5 g l^{-1}), L-glutamine (0.3 g l^{-1}), D-maltose (20 g l^{-1}), D-mannitol (36 g l^{-1}), 2,4-D (2 mg l^{-1}), NAA (1 mg l^{-1}), BA (0.2 mg l^{-1}), Gelrite (5 g l^{-1}), pH 5.8 supplemented with cefotaxime (250 mg l^{-1}) and carbenicillin (100 mg l^{-1}). Subsequently, they were sub-cultured onto the NBM primary selection medium containing hygromycin (30 mg 1^{-1}) and cefotaxime (250 mg l^{-1}) for primary selection and maintained for 3 weeks. Hygromycin-resistant callus lines developed from the rice scutella were isolated and cultured onto NBM primary selection medium for another 10 days and ones clearly hygromycin resistant were transferred onto NBPR medium consisted of N6 major salts, B5 minor salts and vitamins, vitamin assay casamino acids (0.5 g l^{-1}), L-proline (0.5 g l^{-1}), L-glutamine (0.3 g l^{-1}), D-maltose (30 g l^{-1}), 2,4-D (2 mg l^{-1}), NAA (1 mg l^{-1}), BA (1 mg l^{-1}), Gelrite (7 g l^{-1}), pH 5.8, supplemented with hygromycin (40 mg l^{-1}) and cefotaxime (250 mg l^{-1}) for 10 days. Proliferated calli with green spots were isolated and cultured on an RNM regeneration medium consisted of N6 major salts, B5 minor salts and vitamins, vitamin assay casamino acids (0.3 g l^{-1}), L-proline (0.3 g l^{-1}), L-glutamine (0.3 g l^{-1}), D-maltose (30 g l^{-1}), NAA (1 mg l^{-1}), BA (3 mg l^{-1}), agarose type I (4 g l^{-1}), pH 5.8, supplemented with hygromycin (30 mg l^{-1}) and cefotaxime (250 mg l^{-1}) as described by Hiei and Komari (2006).

Drought and salt tolerance assay

The rice seeds derived from selected transgenic rice cv. Cihearng - lines#C.72, C.83 and C.91 were germinated on Yoshida medium (Yoshida et al., 1973) containing hygromicin (100 mg/l) at room temperature for 7 days. For drought and salt tolerance assay, hygromycin resistance-transgenic rice seedlings growing in the selective medium were grown in Yoshida medium containing either

100 µM abscisic acid (ABA), 20% polyethylene glycol (PEG) or 200 mM sodium chloride (NaCl) for 14 days in a culture room at room temperature. Wild type (WT) rice cv. Ciherang of the same stage used as control were parallelly grown under the similar conditions. After 14 days, the length of the seedling root and leaf were recorded.

Quantitative real-time RT-PCR (qRT-PCR)

For the gene expression analysis, seedling leaves of WT and transgenic rice were harvested. The leaf samples were collected at 0 and 24 hours after stress treatments. Total RNA was extracted from collected rice leaves using RNA isolation kit (the Trizol reagent of Invitrogen). After DNase-treatment, cDNA was synthesized from total RNA aided by M-MLV reverse transcriptase (Invitrogen). Real-time RT-PCR was performed using SYBR Premix with the Eco Ilumina real time PCR System. The PCR amplification conditions were as follow: one cycle of 5 minutes denaturation at 95°C, followed by 40 cycles of amplification steps of 5 seconds denaturation at 95°C; 30 seconds primer annealing at 60°C, and 30 seconds primer elongation at 70°C; and one cycle of final extension at 70°C for 5 minutes. Relative transcript levels was determined by using actin rice gene expression as internal reference.

Table 1. Primer sequences used for quantitative real time RT-PCR of the respective genes.

Primer name	Sequences
OsNAC6 _F	5' gccggcgttcccggacctggcggcg 3'
OsNAC6 _R	5' ccgccgccgccgaggccgccgaggc 3'
AP2_F	5' ttaccgcggtgtcaggcaacggaca 3'
AP2_R	5' tgtgcagccagagttggcatctgtg 3'
Zincfinger_F	5' ccatcggcgttcggcgactcggtgt 3'
Zincfinger_R	5' ggtggtggcagatcccgacgacgaa 3'
MYB_F	5' tgccgaggcaggccggccttctccg 3'
MYB_R	5' cacacattcttgatctcgttgtccg 3'

Results and Discussion

Drought, salt, cold, and biotic stresses are major constrains in rice production. Developing stress tolerance rice cultivars has been a major research areas in a number of institutions. One of the route undertaken to develop stress tolerant rice cultivars are regenerating transgenic lines expressing a number of stress induced genes (AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger or WRKY) (Zhang et al., 2004; Umezawa et al., 2006).

As many as 39 plantlets were regenerated after *Agrobacterium* – mediated transformation to introduce OsNAC6 transgene into rice genome. PCR analysis was also conducted using specific primer for *hpt* gene. Based on the PCR results, twenty one of the regenerated plantlets were positively harbored *hpt* transgene (Figure 2), indirectly indicating that *OsNAC6* has also been integrated in the genome of the regenerated plantlets. The resulting PCR (+) for the *hpt* gene is an indirect indicator of integration of the other gene that was in one T-DNA structure into the putative transgenic rice genome (Zaidi et al., 2006).

The results of Southern blot analysis (Table 2 and Figure 3) indicate that the evaluated putative first generation (T1) of transgenic rice integrated a range of 1-3 copies of *hpt* transgenes. The duplicate Southern blot analysis resulted in the same estimate of transgene integration number. The presence of different number of integrated *hpt* transgenes indicate that the evaluated putative transgenic lines have originated from independent transgenic regeneration events. Since the *hpt* transgene was in the same T-DNA structure as the *OsNAC6* transgene; therefore, the presence of *hpt* could be used as indirect evidence for the presence of *OsNAC6*. In such case, it could be assumed that the *OsNAC6* transgene might have also been integrated in the putative rice transgenic line in a range of 1-3 copies.

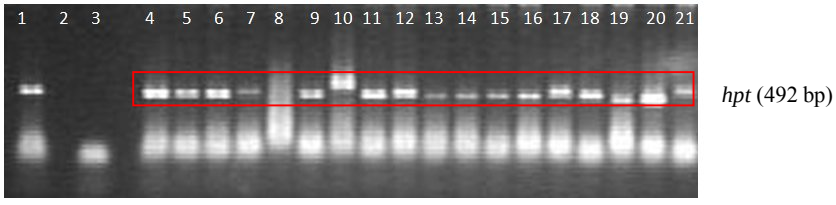

Figure 2. PCR amplification using *hpt* specific primer pairs. Lane 1: positive control, lane 2: negative control (only water, without template cDNA), lane 3: negative control (rice cv. Ciherang), and lane 4-21: samples of putative transgenic line.

Table 2. Estimation of number of *hpt* transgene integration in the genome of the first generation (T1) putative transgenic rice cv. Ciherang as indicated in Figure 3.

No	Genotype	Lane no.	Interpreted copy no.
1	NB.15	4,5	-
2	NB.41	6,7	-
3	NB.62	8,9	-
4	C.72	10,11	1
5	C.83	12	1
6	C.91	13	2
7	C.22	14	3

Note : NB.15, NB.41, and NB.62 were T1 individuals without transgene and C.72, C.83, C.91, C.22 were ones carrying transgenes.

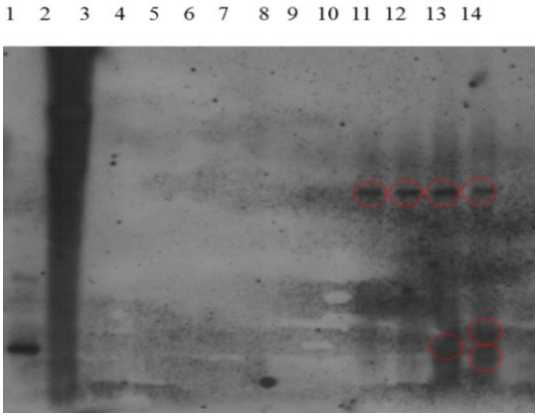

Figure 3. Southern blot analysis of the T1 generation samples of putative rice cv. Ciherang transgenic lines. Column: (1) control + *hpt* fragmen*;* (2) control + plasmid 1305, (3) control – rice cv. Bengawan, (4-5) NB.15, (6-7) NB.41, and (8-10) NB62 – the T1 segregated lines showed negative results in Southern blot analysis, while (11) C.72, (12) C.83, (13) C.91, and (14) C.22 – the T1 segregated lines showed possitive results of Southern blot, respectively.

Putative T1 transgenic lines C.72 and C.83 integrated 1 copy of the inserted hpt transgene, while genotype C.91 integrated 2 copies (Table 2 and Figure 3). The above southern blot results also indicated that there were some putative T1 transgenic lines showed negative result. Those genotype showed positive result when analysed for PCR using hpt gene specific primers. Such cases may indicate that the analyzed samples might have low qualities and or quantities of DNA for southern analysis. However, the quality and the quantity of the DNA were high enough for PCR analysis.

Expression pattern of *OsNAC6* in rice seedling stage was analyzed using quantitative real time RT-PCR. Results of the real time RT-PCR indicated the expression level of *OsNAC6* was generally higher in young transgenic leaves than that of non-transgenic rice cv. Ciherang (Figure 4). There was no significant increased in *OsNAC6* expression in ABA treated transgenic rice cv. Ciherang at 0 and 24 hour after treatment. However, slight increase in *OsNAC6* expression was observed at 24 hour after treatment of ABA in WT rice cv. Ciherang (Figure 4.A.). Both NaCL (200 mM) and PEG (20%) treatment induced slight increased in *OsNAC6* expression in both WT and transgenik rice cv. Ciherang (Figure 4.B and 4.C), indicating that the expression of *OsNAC6* in rice is affected more by either NaCL or PEG treatment than by ABA.

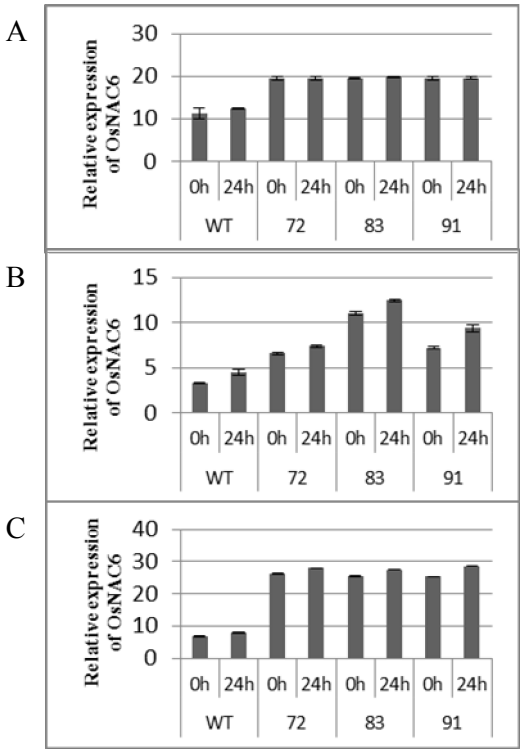

Figure 4. Expression pattern of *OsNAC6* in young leaf of transgenics rice cv. Ciherang were detected by quantitative real-time RT-PCR. The experiments were repeated twice. Error bars are standard deviations of two technical repeats. Wild type (WT) rice cv. Ciherang was used as non-transgenic control. Expression of *OsNAC6* in young rice leaves in response to (A) ABA (100 uM), (B) NaCl (200 mM), and (C) PEG (20%) treatments.

ABA is the most important phyto-hormones involved in growth and development of plants and in a number of stress adaptation (Schroeder et al., 2001; Shinozaki and Yamaguchi-Shinozaki, 2000; Verslues et al., 2006). High cellular ABA levels leads to synthesis of storage proteins in seeds, promote seed desiccation tolerance, dormancy (Finkelstein et al., 2002, 2008) and inhibit seed germination. ABA is also reported to control lateral root formation and seedling growth (Xiong et al., 2006). It also associated with the reduction of water transpiration through promotion of stomatal pore closure (Hetherington, 2001; Kim et al., 2010). Moreover, ABA controls the expression of a number of stress-responsive genes. Based on those, ABA is aptly called as a stress hormone (Hoth et al., 2002; Nemhauser et al., 2006; Seki et al., 2002).

Although the *OsNAC6* is a stress induced regulatory gene, in this study, transgenic rice cv. Ciherang overexpressing *OsNAC6* transgene did not response to the ABA at 24 hours after treatment. Increased in expression of *OsNAC6* was not observed at 24 hours after ABA treatment among evaluated transgenic rice cv. Ciherang. Such failure in observing an increased in *OsNAC6* expression in the ABA treated transgenic rice cv. Ciherang might be due to the use of 35S CaMV promoter in the *OsNAC6* transgene construct (Figure 1). The 35S promoter is a constitutive-strong promoter, therefore, slight increase in native *OsNAC6* gene epression because of ABA treatment may not be observable in the transgenic line. This was supported by the data shown in Figure 4.A. ABA treatment to the WT rice cv. Ciherang could only induce a slight increase in *OsNAC6* native gene expression.

The *OsNAC6* gene is a regulatory gene for many other stress-responsive TFs, such as AP2, Zincfinger protein, and MYB, respectively. In this experiment, expression level of those genes were evaluated in either the WT or the transgenic rice cv. Ciherang over expressing *OsNAC6* transgene. Result of the evaluation indicated that the expression levels of the AP2, Zincfinger protein and MYB, were up-regulated in transgenic lines (Figure 5). The AP2, Zincfinger protein and MYB are regulatory genes for many plant stress responses. The AP2/EREBP is the largest plant TF family and they play an important role for regulating responses for abiotic or biotic stresses and for a number of plant developments (Ohto et al., 2009).

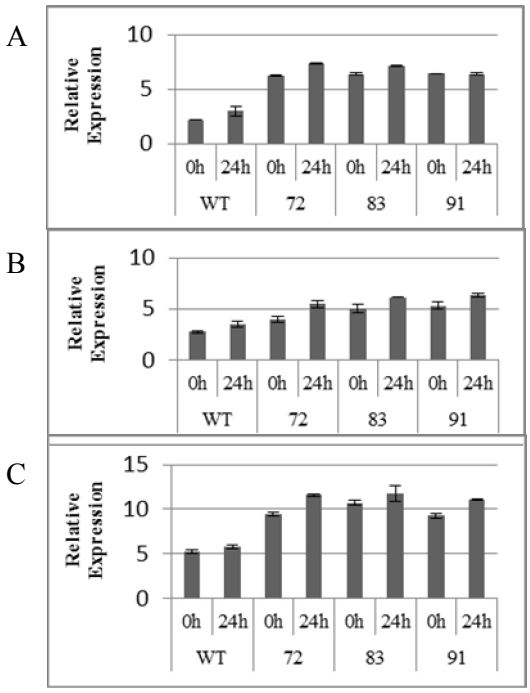

Figure. 5. Expression analysis of three stress-responsive genes in the transgenic rice cv. Ciherang line#C.72, C.83 and C.91. Wild type (WT) rice cv. Ciherang was used as non-transgenic control. Error bars are standard deviations of two technical repeats. (A) AP2, (B) Zincfinger protein, and (C) MYB are stress-responsive genes, respectively.

A number of NAC protein are also involved in plant responses to stress (Delessert et al., 2005). In this study, we again demonstrated the function of a novel rice NAC gene - *OsNAC6* associated with stress responses. Analysis of *OsNAC* expression in the regenerated rice transgenic showed that the evaluated *OsNAC6* transgene was associated with both tolerance to drought and salinity stresses in seedlings of rice young leaves and roots (Figure 6).

The effect of *OsNAC6* overexpression in transgenic rice grown under PEG and salt stress was also investigated. As shown in Figure 6.A. and 6.B., transgenic rice cv. Ciherang line#C.72, C.83 and C.91 showed better growth, longer roots and leaves length than those of WT. Such data indicated that constitutive expression of *OsNAC6* in transgenic rice cv. Ciherang increased rice tolerance to salt and drought stresses. Such data also indicated that constitutive expression of *OsNAC6* might be used to improve drought and salt tolerance in rice.

In this experiment, level of drought tolerant for the evaluated non-transgenic and transgenic rice cv. Ciherang was calculated based on the recovery rate

of rice seedlings after drought treatment. The drought stress arrangement was applied to 14 days old rice seedlings as follow: no watering for up to 7 days followed by normal watering for 7 days of recovery periods in the greenhouse. At the end of recovery period, recovery rate of the evaluated rice lines was recorded.

The three transgenic rice cv. Ciherang line#C.72, C.83 and C.91 showed higher survival rate than the wild type one (Figure 7), indicating that constitutive expression of *OsNAC6* increased the tolerance of the rice transgenic lines against the stresses. The recovery rate of the WT rice cv. Ciherang and the transgenic rice cv. Ciherang - lines #C.72, C.83 and C.91 were 33.3%, 65.5%, 74.4% and 67.8% (Figure 7).

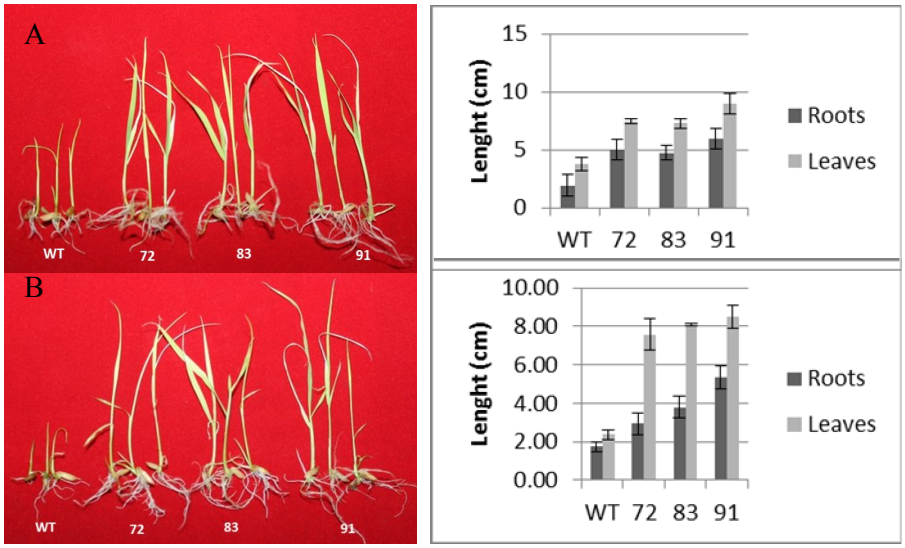

Figure 6. Representative samples of rice seedling growth in response to drought and salinity stress of transgenic rice cv. Ciherang lines#C.72, C.83 and C.91 overexpressing *OsNAC6*. Wild type (WT) rice cv. Ciherang was used as non-transgenic control. Error bars are standard deviations of two technical repeats. The observations were conducted at 14 days after treatment. (A) PEG (20%) and (B) NaCl (200 µM).

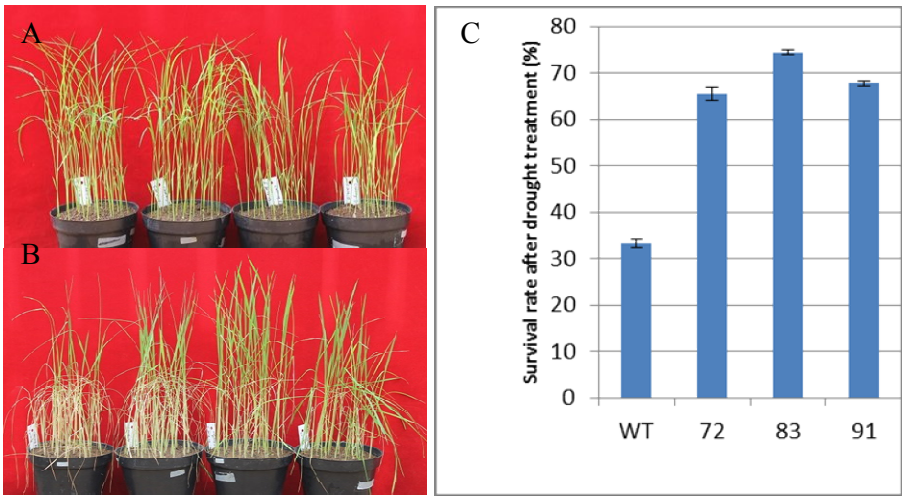

Figure 7. Drought tolerance assays of transgenic rice overexpressing *OsNAC6*. Left to right - performance of wild type (WT) rice cv. Ciherang as non-transgenic control and transgenic rice cv. Ciherang line#C.72, C.83 and C.91. Error bars are standard deviations of two technical repeats. (A) under normal condition and (B) after 7 days of drought stress, followed by 7 days of recovery periods. (C) Survival rates of the WT and the three transgenic rice lines after 7 days of drought stress, followed by 7 days of recovery periods.

Conclusion

Overexpression of *OsNAC6* transgene in rice cv. Ciherang improved the growth of the transgenic lines under PEG induced stress and salinity treatments. Moreover, it also enhances the expression of other transcription factor-related drought and salinity stress responses, such as Zincfinger protein, MYB and AP2. Two times higher expression of *OsNAC6* in transgenic rice cv. Ciherang lines#C.72, C.83 and C.91 resulted in higher recovery rate after drought stress than the wild type one.

Acknowledgments

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