

# Characterization of transcription factor gene *SNAC2* conferring cold and salt tolerance in rice

Honghong Hu · Jun You · Yujie Fang · Xiaoyi Zhu ·  
Zhuyun Qi · Lizhong Xiong

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**Abstract** Plants respond to adverse environment by initiating a series of signaling processes including activation of transcription factors that can regulate expression of arrays of genes for stress response and adaptation. NAC (NAM, ATAF, and CUC) is a plant specific transcription factor family with diverse roles in development and stress regulation. In this report, a stress-responsive NAC gene (*SNAC2*) isolated from upland rice IRA109 (*Oryza sativa* L. ssp *japonica*) was characterized for its role in stress tolerance. *SNAC2* was proven to have transactivation and DNA-binding activities in yeast and the *SNAC2*-GFP fusion protein was localized in the rice nuclei. Northern blot and *SNAC2* promoter activity analyses suggest that *SNAC2* gene was induced by drought, salinity, cold, wounding, and abscisic acid (ABA) treatment. The *SNAC2* gene was over-expressed in *japonica* rice Zhonghua 11 to test the effect on improving stress tolerance. More than 50% of the transgenic plants remained vigorous when all WT plants died after severe cold stress (4–8°C for 5 days). The transgenic plants had higher cell membrane stability than wild type during the cold stress. The transgenic rice had significantly higher germination and growth rate than WT under high salinity conditions. Over-expression of *SNAC2* can also improve the tolerance to PEG treatment. In addition, the *SNAC2*-overexpressing plants showed significantly increased sensitivity to ABA. DNA chip

profiling analysis of transgenic plants revealed many up-regulated genes related to stress response and adaptation such as peroxidase, ornithine aminotransferase, heavy metal-associated protein, sodium/hydrogen exchanger, heat shock protein, GDSL-like lipase, and phenylalanine ammonia lyase. Interestingly, none of the up-regulated genes in the *SNAC2*-overexpressing plants matched the genes up-regulated in the transgenic plants over-expressing other stress responsive NAC genes reported previously. These data suggest *SNAC2* is a novel stress responsive NAC transcription factor that possesses potential utility in improving stress tolerance of rice.

**Keywords** *Oryza* · Transcription factor · NAC · Abiotic stress · Expression regulation

## Abbreviations

ABA	Abscisic acid
CUC	Cup-shaped cotyledon
GFP	Green fluorescence protein
NAM	No apical meristem
SNAC	Stress-responsive NAC
PCR	Polymerase chain reaction
RT	Reverse transcription
RWC	Relative water content
NAC	NAM, ATAF, and CUC
NACRS	NAC recognition site

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H. Hu · J. You · Y. Fang · X. Zhu · Z. Qi · L. Xiong (✉)  
National Center of Plant Gene Research (Wuhan), National Key  
Laboratory of Crop Genetic Improvement, Huazhong  
Agricultural University, Wuhan 430070, China  
e-mail: lizhongx@mail.hzau.edu.cn

## Introduction

Plant growth and productivity are frequently threatened by environmental stresses such as drought, salinity or low temperature. To cope with these stresses, plants have

developed arrays of physiological and biochemical strategies to adapt to the adverse conditions (Ingram and Bartels 1996; Pastori and Foyer 2002). Such cascading events were controlled by a battery of genes through intricate regulations. In these regulating processes, the expressions of stress-related genes are largely governed by specific transcription factors. Among these transcription factors, members of AP2/EREBP, bZIP, zinc finger, and MYB families have been well characterized for their regulatory roles in the plant stress or defense responses (Zhu 2002; Seki et al. 2003; Shinozaki et al. 2003). It is generally accepted that activation of a specific transcription factor can often result in expression of many functional genes related to stresses. Many efforts have been reported to improve stress resistance of plants by over-expressing stress-responsive transcription factor genes such as *DREB/CBF* (Liu et al. 1998), *ABF2* (Kim et al. 2004), *OSISAPI* (Mukhopadhyay et al. 2004) and etc.

NAC transcription factors comprise a large gene family. Protein of this family contains a highly conserved N-terminal DNA-binding domain and a diversified C-terminal domain. NAC was derived from the names of the first three described proteins containing the DNA-binding domain, namely NAM (no apical meristem), ATAF1-2 and CUC2 (cup-shaped cotyledon) (Souer et al. 1996; Aida et al. 1997). NAC proteins appear to be widespread in plants but no homolog has been identified thus far in other eukaryotes (Riechmann et al. 2000). Although more than 100 members of this family have been suggested in both rice and *Arabidopsis* genomes (Xiong et al. 2005), only a few of them have been characterized and the reported NAC transcription factors have diverse functions. The early reported NAC genes have been implicated in various aspects of plant development. For examples, *NAM* from *petunia* (Souer et al. 1996) and *CUC1-2* (Aida et al. 1997) from *Arabidopsis* are expressed in the primordial and meristem boundaries and playing important roles in controlling the formation of boundary cell and their mutants failed to develop shoot apical meristem. *NAP* (Sablowski and Meyerowitz 1998) from *Arabidopsis* acts as a target gene of AP3/PI to function in the transition between growth by cell division and cell expansion in stamens and petals. *AtNAC1* mediates auxin signaling to promote lateral root development (Xie et al. 2000). More recently, NAC proteins were found to be involved in plant responses to pathogen and environmental stimuli. Genes in the *ATAF* subfamily, including *StNAC* from tomato and *ATAF1-2* from *Arabidopsis*, were induced by pathogen attack and wounding (Collinge and Boller 2001). In *Brassica napus*, nine members of the NAC (*BnNAC*) were identified for their differential expression after feeding with flea beetle and treatment of cold temperature (Hegedus et al. 2003). In *Arabidopsis*, Tran et al. (2004) and Fujita et al. (2004) reported that three NAC genes *ANAC019*, *ANAC055* and

*ANAC072* were induced by drought, salinity, and/or low temperature, and the transgenic *Arabidopsis* plants over-expressing these genes showed improved stress tolerance compared to the wild type. Furthermore, Proteins of these genes can bind to the promoter sequences with CATGTG motif (Tran et al. 2004). Another stress-related *Arabidopsis* NAC gene is *AtNAC2* that can be induced by high salinity, abscisic acid (ABA), aminocyclopropane carboxylic acid (ACC), and naphthalene acetic acid (NAA), and it has been predicted to be a downstream gene in the ethylene and auxin signal pathways (He et al. 2005). Over-expression of *AtNAC2* resulted in alteration of lateral root development and enhanced salt tolerance (He et al. 2005). A NAC gene *NAM-B1* conferring nutrient remobilization from leaves to developing grains was reported in ancestral wild wheat (Uauy et al. 2006), which further exemplified the functional diversity of NAC gene family.

Recently, a stress-responsive NAC gene *SNAC1* was also characterized in rice (Hu et al. 2006). This gene can be specifically induced in the guard cells of rice under drought stress conditions. Over-expression of this gene in rice resulted in significantly increased stomata closure and drought resistance in the drought-stressed field conditions while the photosynthesis rate and yield of transgenic plants was not affected under normal growth conditions. The *SNAC1*-overexpressing rice plants also showed improved salt tolerance, further emphasizing the usefulness of this gene in stress tolerance improvement (Hu et al. 2006). Another stress-responsive NAC gene *OsNAC6*, which is a member of *ATAF* subfamily (Kikuchi et al. 2000; Ooka et al. 2003), has been reported for its induction by abiotic stresses and jasmonic acid treatment (Ohnishi et al. 2005), and over-expression of this gene in rice resulted in enhanced tolerance to dehydration stresses (Nakashima et al. 2007).

In this study, a stress-responsive NAC transcription factor gene termed *SNAC2* was isolated from upland rice IRAT109 and the expression profile of *SNAC2* under normal growth and various stress treatments was investigated by checking the expression of reporter genes under the control of *SNAC2* promoter in rice. The transgenic rice over-expressing *SNAC2* showed significantly improved tolerance to cold as well as to salinity and dehydration stresses. Genomic expression profile of the *SNAC2*-overexpressing rice was investigated to help elucidate the molecular basis of enhanced cold tolerance conferred by this gene.

## Materials and methods

### Constructs and genetic transformation

The full length cDNA of *SNAC2* was amplified from total RNA of IRAT109 by RT-PCR with a 5'-terminal

*KpnI*-adapted primer (5'-TTGGTACC GACGATCACCG GAGAAGG-3') and a 3'-terminal *BamHI*-adapted primer (5'-AAGGATCCCTCGCCTGAGTCAAAGTTCA-3'). The product was sub-cloned into the vector pCAMBIA-1301 (accession number AF234297) digested with *KpnI* and *BamHI* under the control of maize promoter *Ubiquitin1*. For GUS activity analysis of the *SNAC2* promoter, the promoter fragment was isolated with *BamHI*-adapted primer (5'-AAGGATCCTCGAGAAGGATTGGTACAAG TT-3') and *EcoRI*-adapted primer (5'-TTGAATTC TAG TGTTGGGAAGAGGAGAGG-3') from total genomic DNA of IRAT109 and then ligated into the vector pCAMBIA-1391Z. For determination of sub-cellular localization of *SNAC2* protein, the coding region of *SNAC2* was fused to green fluorescence protein (GFP) reporter gene in the vector p1381-EGFP (Wu et al. 2003; Hu et al. 2006) under the control of its native promoter. All the constructs were transformed into rice *Zhonghua11* (*O. sativa* L. ssp *japonica*) according to the rice genetic transformation method (Hiei et al. 1994).

#### Plant growth, stresses treatment and measurement

For expression level analysis, seeds of upland rice IRAT109 were germinated and grown on sandy soil (with paddy soil mixed with two thirds of river sand) in greenhouse (28°C and a 14-h-light [ $>3000$  lux]/10-h-dark cycle). At the four-leaf stage, the seedlings were treated with different stresses. Drought stress was applied by withholding water and leaves were sampled according to the drought stressed phenotype (the degree of leaf-rolling). For high salinity treatment, sodium chloride (NaCl) solution was added with a final concentration of about 200 mM. Abscisic acid (10 mM stock solution in ethanol) was diluted to 100  $\mu$ M with surfactant (0.02% Tween-20) water and sprayed onto the leaves of seedlings. For cold stress, the vigorous seedlings were transferred to a growth chamber (with a temperature set at 4°C but it varied 3–8°C, and a 12-h-light [ $>3000$  lux]/12-h-dark cycle) in the morning and kept in the chamber for up to 48 h for sampling (as indicated in the Fig. 1).

T<sub>2</sub> or T<sub>3</sub> seeds of transgenic rice lines and WT control were sterilized and germinated in dark at 26°C on two kinds of medium: MS and MS plus 150 mM NaCl. The germination rate was recorded. To test the response of young seedlings to stress conditions, seeds were germinated on MS medium plus 100 mg/l hygromycin for 4 days and then transferred to MS medium plus 150 mM NaCl, 15% PEG6000, or 3  $\mu$ M ABA and normal MS medium as controls. The plant height and root length of the seedlings were measured after 7, 14, and 20 days of growing in ABA, PEG, and salinity medium respectively.

For cold stress tolerance testing, seeds were germinated on MS medium containing 100 mg/l hygromycin for 7 days and the seedlings were transferred into soil. Half of each pot was used for planting transgenic plants while the other half for wild type plants with same vigor as transgenic plants when transplanted. The seedlings were grown for another three weeks before applying the stress treatment. For cold stress, the seedlings were transferred to a growth chamber at 4°C with 16 h light /8 h dark for 5 days and then back to normal growth conditions for recovery. Cell membrane stability was evaluated by relative conductance (R1/R2) of cell membrane under cold stress. The top full expanded leaves were sampled and washed by double sterilized water (ddH<sub>2</sub>O) for several times. The sampling was consistent in replicated experiments. The conductance of leaf segment surging in 20 ml ddH<sub>2</sub>O for 6 h (R1) (before boiling) and after in boiling water for 15 min (R2) were measured. All stress tolerance testing experiments were repeated three times.

#### Northern and Southern blot analysis and GFP imaging

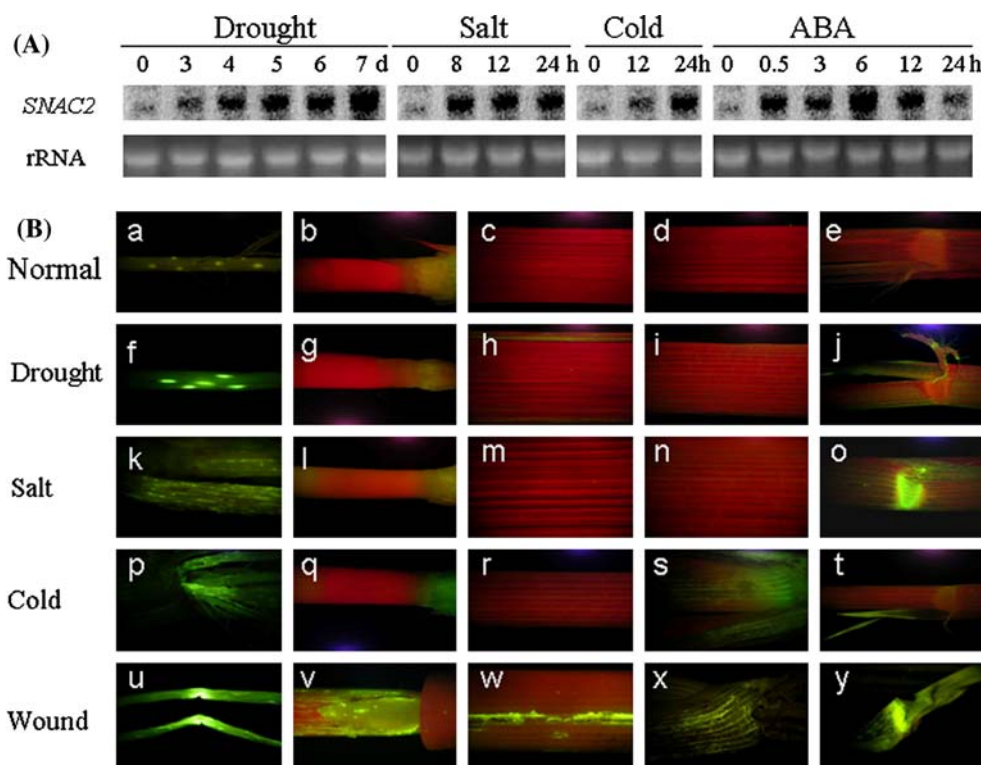
Total RNA was isolated from leaves according to the instruction of Trizol reagent (Invitrogen, Carlsbad, CA). Fifteen micrograms of total RNA of each sample were separated on 1.2% agarose gel containing 6% formaldehyde in 3-(*N*-morpholino)-propanesulfonic acid buffer (pH 7.0) and transferred to Nylon membrane. Total genomic DNA was extracted from transgenic plants by CTAB method, 3  $\mu$ g DNA were digested by *EcoRI* and separated on 0.8% agarose gel in TAE buffer and transferred to nylon membrane. Hybridization was performed with a <sup>32</sup>P-labeled DNA probe according to a published protocol (Church and Gilbert 1984), and the membranes were washed with 2 $\times$  SSC containing 0.1% SDS at room temperature (22–24°C) for 10 min once and with 2  $\times$  SSC containing 0.1% SDS at 65°C for 5 min. The membranes were dried and visualized directly with a phosphorimager (Fujifilm, Japan).

The GFP fluorescence in different tissues was observed directly by fluorescence microscopy. To determine the sub-cellular localization of *SNAC2*, a slice of transgenic callus was stained with propidium iodide (10  $\mu$ g/ml) as reported (Ito and Shinozaki 2002) and the GFP fluorescence was observed by confocal microscopy (TCS SP2, Leica).

#### Biochemical assay in yeast

For transactivation assay, the open reading frame of *SNAC2* generated by PCR was fused in frame to the yeast GAL4 DNA binding domain in the vector pDEST32 by recombination reactions (Invitrogen). The fused gene was

**Fig. 1** Expression pattern of *SNAC2* in *japonica* rice IRAT109. **(a)** RNA gel blot analysis of expression of the *SNAC2* in IRAT109 seedlings under drought, salt (200 mM NaCl), cold (4°C) and ABA treatment (100 μM). **(b)** Expression pattern of *GFP* driven by the *SNAC2* promoter in transgenic seedlings under different stresses including drought (no water-supply for 7 d), salt (200 mM NaCl for 12 h), cold (4°C for 12 h), wounding (tissues were slightly crushed by forceps). Root: a, f, k, p, u; Stem and internodes: b, g, i, q, v; Leaf: c, h, m, r, w; Sheath: d, i, n, s, x; Ligule: e, j, o, t, y



expressed in yeast strain MV203 (Invitrogen). The transformed yeast strain was plated on SD/Leu<sup>-</sup> medium and cultured for three days, and then colony-lift filter assay ( $\beta$ -gal assay) was performed as described by the manufacture (Invitrogen). For yeast one-hybrid assay, the open reading frame of *SNAC2* was fused to the GAL4 activation domain in the vector pGADT7-Rec2 (Clontech, Palo Alto, CA) and co-transformed with the reporter vector pHIS2-*cis* (containing triple tandem repeats of NACRS from the *OsERD1* promoter (Hu et al. 2006) into yeast strain Y187 and colony-lift filter assay was followed to verify the DNA-protein interactions (Yeast Protocols Handbook, Clontech).

#### DNA chip analysis

Affymetrix DNA chip representing all putative genes of the rice genome was used for genes expression pattern of transgenic rice. For DNA chip analysis, leaf samples of two independent transgenic lines and wild type plants (4-leaf stage) under normal growth and low temperature conditions (4–8°C, 12 h light/12 h dark for two days) were used for DNA chip analysis. Three independent biological samples were used for each material/treatment. Chip hybridization and data processing were carried out with Affymetrix custom service (CapitoBio, Beijing, China) by following the standard protocol for Affymetrix DNA chip. The up- or down-regulated genes from DNA chip analysis

were confirmed by real time PCR analysis by using rice *Actin* gene as an internal control for calibration of relative expression.

#### Results

##### Isolation and sequence analysis of *SNAC2*

Toward identification of drought-responsive NAC genes in rice, we conducted a comparative expression profiling analysis of an upland rice variety IRAT109 (*Oryza sativa* L. ssp. *japonica*) and a low land rice variety Zhenshan 97 (*O. sativa* L. ssp. *indica*) with various stress treatments (drought, salt, and cold) using a custom Affymetrix DNA chip (CapitalBio, Beijing, China) (unpublished data). Based on the expression files, several putative NAC genes including the previously reported *SNAC1* gene (Hu et al. 2006) showing strong induction by stresses were identified. In this study, one of the stress-responsive NAC genes, designated *SNAC2*, was chosen for further functional analysis. The full length cDNA of this gene was isolated from the upland rice IRAT109 by RT-PCR. The cDNA sequence (1529 bp in length) shows 98% of sequence identity to the predicted cDNA of gene model LOC\_Os01g66120.1 in the rice annotation database (<http://www.tigr.org/tdb/e2k1/osa1/>) and encodes a peptide of 303 amino acids. Sequence alignment analysis suggested that

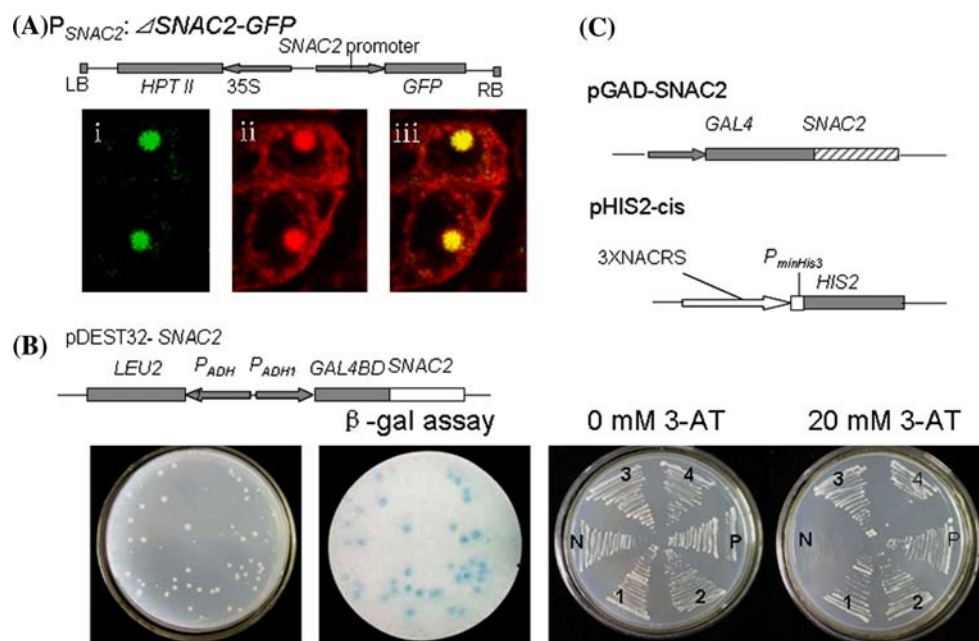
the SNAC2 contained a conserved DNA-binding domain of 163 amino acids in the N-terminus and a variable domain in the C-terminus. A putative nuclear localization signal (NLS) was predicted in the first 144 amino acids (Supplementary Fig. 1).

#### Expression profile of SNAC2 under different stresses

To confirm the DNA chip data for SNAC2, the expression level of SNAC2 in the upland rice IRAT109 under different stresses was checked by RNA gel blot analysis (Fig. 1a). Transcript of SNAC2 was induced in the seedling leaves at early stage of drought stress (with relative water content in leaves about 90%, a time point at which no obvious stressed symptom of plants was observed) and the enhanced transcript level maintained throughout the development of drought stress. Increased transcript level of this gene was also detected in the leaves of seedlings irrigated with 200 mM NaCl for 8 h. When the seedlings were exposed to 4°C, the SNAC2 transcript was induced within 1 h and peaked at 8 h. The transcript of SNAC2 gene was also induced by ABA treatment and peaked at 6 h after treatment.

To further elucidate the expression pattern of SNAC2, the green fluorescence protein (GFP) reporter gene under the control of the SNAC2 promoter was transformed into rice variety Zhonghua 11. The expression pattern of the reporter gene was analyzed in different rice seedling tissues under different stresses and normal growth conditions (Fig. 1b). Under normal growth condition, weak GFP expression was observed only in roots and internodes. Under drought stress, strong induction of GFP expression was observed in roots and ligules. A similar induction pattern as under drought stress was observed under salt stress. Under low temperature and wounding conditions, strong induction of GFP was observed in roots, stems, ligules, and sheaths.

The tempo-spatial expression pattern of SNAC2 was also determined by histochemical  $\beta$ -glucuronidase (GUS) staining of transgenic plants harboring a SNAC2 promoter::GUS reporter construct. The results also suggested that the GUS reporter gene was expressed mainly in roots, internodes, ligules, spikelets, and callus under normal conditions (Supplementary Fig. 2). GUS activity assay also supported that the SNAC2 promoter had strong induction activity under dehydration stress conditions (Supplementary Fig. 2).



**Fig. 2** SNAC2 features a transcription factor. (a) Construct of  $P_{SNAC2}::\Delta SNAC2::GFP$  (The sequence encoding the first 144 amino acids of SNAC2 was under the control of SNAC2 promoter) was transformed into rice and GFP signal was checked in calli cells with confocal microscopy. i: fluorescent image of GFP; ii: fluorescent image stained with propidium iodide; iii: merged image. (b) Transactivation assay of SNAC2 in yeast. Full SNAC2 protein was fused to the GAL4 binding domain (GAL4 BD) and transformed into

yeast strain MV203, and  $\beta$ -gal assay was performed to identify the transactivation activity (*LacZ* expression). (c) The pGAD-SNAC2 plasmid and the reporter construct pHIS-cis (Hu et al. 2006) were co-transformed into yeast strain Y187. The positive transformants were determined by growth performance on SD/Leu<sup>-</sup>/Trp<sup>-</sup>/His<sup>-</sup> plates with or without 3-AT. N: Negative control (p53HIS2 + pGAD-SNAC2); P: positive control (p53HIS2 + pGAD-Rec2-53); 1–4: four different colonies containing pGAD-SNAC2 and pHIS-cis

## SNAC2 features a transcriptional activator

Sequence analysis revealed a putative nuclear localization signal (NLS) in the first 144 amino acids of N-terminus (Supplementary Fig. 1). To confirm this prediction, SNAC2-GFP fusion construct was used for in vivo protein targeting in rice. The sequence encoding the first 144 amino acids of SNAC2 was fused to GFP and the construct was transformed into rice Zhonghua 11. Slices of transgenic callus were made for GFP observation under confocal microscopy. GFP signal was detected only in the nucleus (Fig. 2a), suggesting that SNAC2 is a nuclear protein and the first 144 amino acids containing the putative NLS can mediate the nuclear targeting of the protein.

To validate that the SNAC2 functions as a transcriptional activator, the open reading frame of SNAC2 was fused to the GAL4 DNA binding domain (GAL4-DB) in the vector pDEST32 and the construct was transformed into yeast strain MV203. Colony-lift filter assay showed that the reporter gene *LacZ* was expressed only in the yeast cell transformed with the GAL-DB-SNAC2 construct (Fig. 2b), indicating that the SNAC2 had transcriptional activity in yeast cell.

Our previous work showed that SNAC1 can bind to the NAC recognition site (NACRS)-like sequence in the promoter of gene *OsERD1* (Hu et al. 2006). To verify whether SNAC2 has the same DNA-binding activity, the full length of SNAC2 was fused to the GAL4 activation domain of vector pGAD-RecT7 and the fused construct was co-transformed with pHIS-*cis* construct containing NACRS-like sequence (Hu et al. 2006) into yeast strain Y187. The result showed that only the co-transformants of pGAD-SNAC2 and pHIS-*cis* could grow on the SD/Leu-/Trp-/His-medium with 30 mM 3-AT (Fig. 2c), suggesting that SNAC2 could bind to the NACRS-like sequence to activate the *HIS* reporter gene expression in yeast.

## Improved cold tolerance of SNAC2-overexpressing plants

To test the effect of SNAC2 on improving stress resistance, an over-expression construct with SNAC2 gene under the control of maize *Ubiquitin* promoter was transformed into rice Zhonghua11. The expression level and copy number of T-DNA were analyzed by northern and Southern blot analyses (Supplementary Fig. 3). Three independent over-expression lines (U7 and U14 each with one copy of transgene and U12 with two copies of transgene) were chosen for stress tolerance testing. Under normal conditions, there was no significant difference in plant morphology (such as root depth and volume, plant height, and numbers of tillers and spikelets) and yield (data not

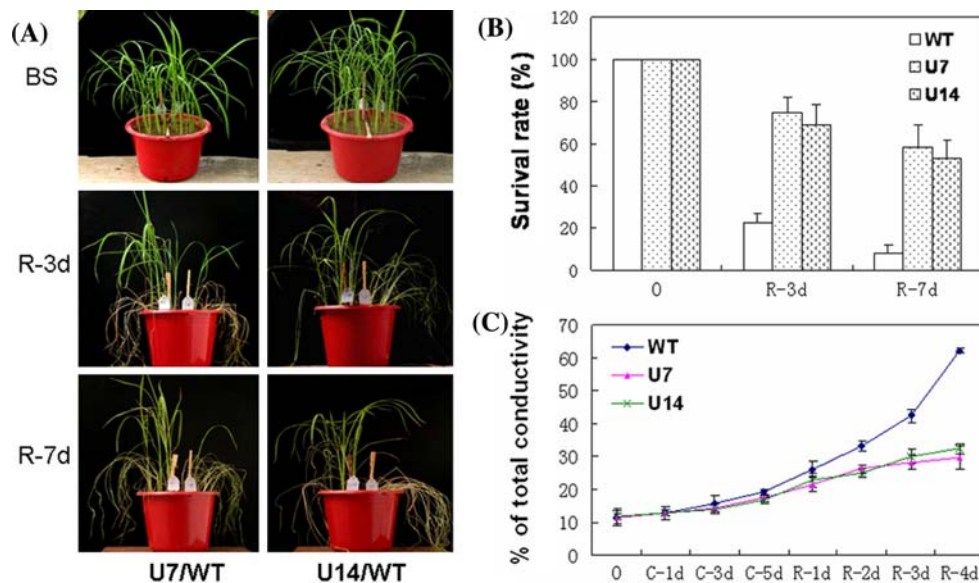
shown) between the transgenic lines and wide type (WT). This result suggested that over-expressing SNAC2 had no detrimental effects on the growth and development of rice.

Strong induction of SNAC2 by cold stress prompted us to test the tolerance of transgenic rice plants to low temperature. The transgenic seeds of T<sub>2</sub> generation were germinated on MS medium containing 100 mg/l hygromycin (for selecting positive transgenic plants) and the young seedlings were transplanted in soil along with WT seedlings with same age and vigor. The healthy plants at four-leaf stage were shifted to a growth chamber with low temperature (ranging 4–8°C) and illumination of 16 h-light/8 h-dark for 5 days. During the cold treatment, there was no visual phenotypic difference between transgenic plants and WT. During the recovery period (cold-stressed plants were transferred to normal growth conditions), however, the transgenic plants performed significantly better than WT in terms of stay-green of leaves and survival rate (Fig. 3a). After seven days of recovery, more than 50% of the transgenic plants remained vigorous while almost all WT plants died (Fig. 3b). Similar result (improved tolerance to low temperature stress) was obtained by using homozygous T<sub>3</sub> transgenic plants (not shown).

We further measured the cell membrane penetrability and chlorophyll content, two well recognized parameters reflecting the damage by cold stress. During the cold stress, cell membrane penetrability measured by the relative penetrability (ratio of the conductance of leaves before and after boiling, see Methods) was gradually increased but there was no significant difference between transgenic and WT plants. During the recovery period, however, transgenic plants had significantly lower cell membrane penetrability than WT (Fig. 3c). These results suggested that over-expressing SNAC2 in rice could significantly improve cold tolerance.

## Improved tolerance of SNAC2-overexpressing plants to salinity and PEG treatments

To test salinity tolerance, germinated positive transgenic (selected by germinating the seeds on MS medium containing 100 mg/l hygromycin) and WT seeds (with 2–3 mm shoots) were transplanted on MS medium containing 150 mM NaCl and the normal MS medium without NaCl as control. In the salinity medium, transgenic seedlings grew faster and their shoots were significantly longer than WT after growing for 20 days (Fig. 4a). However, there was no difference in root length or root numbers between transgenic and WT seedlings grown in the salinity medium. Nevertheless, no difference in growth performance was observed between transgenic and WT seedlings



**Fig. 3** *SNAC2*-overexpressing transgenic rice plants showed improved cold tolerance. **(a)** Growth performance of *SNAC2*-overexpressing transgenic and wild type seedling of four-leaf stage in the same barrel (left, transgenic plants; right, wild type) before and after stress (4–8°C and 16 h light/8 h dark cycle for 5 days). BS, before stress; R-3d, recovery for 3 days after stress; R-7d, recovery for 7 days after stress. The experiment was repeated three times. **(b)**

Survival rate after stress. **(c)** Relative penetrability (R1/R2) of leaves during the cold stress (4–8°C, 16 h light/8 h dark). R1, conductance of the water solution with leaves dipped in for 6 h; R2, conductance of water solution with leaves in boiled for 15 min. C-d, days after cold stress; R-d, days after recovery. WT: wild type Zhonghua 11. Values are the mean ± SD (n = 10)

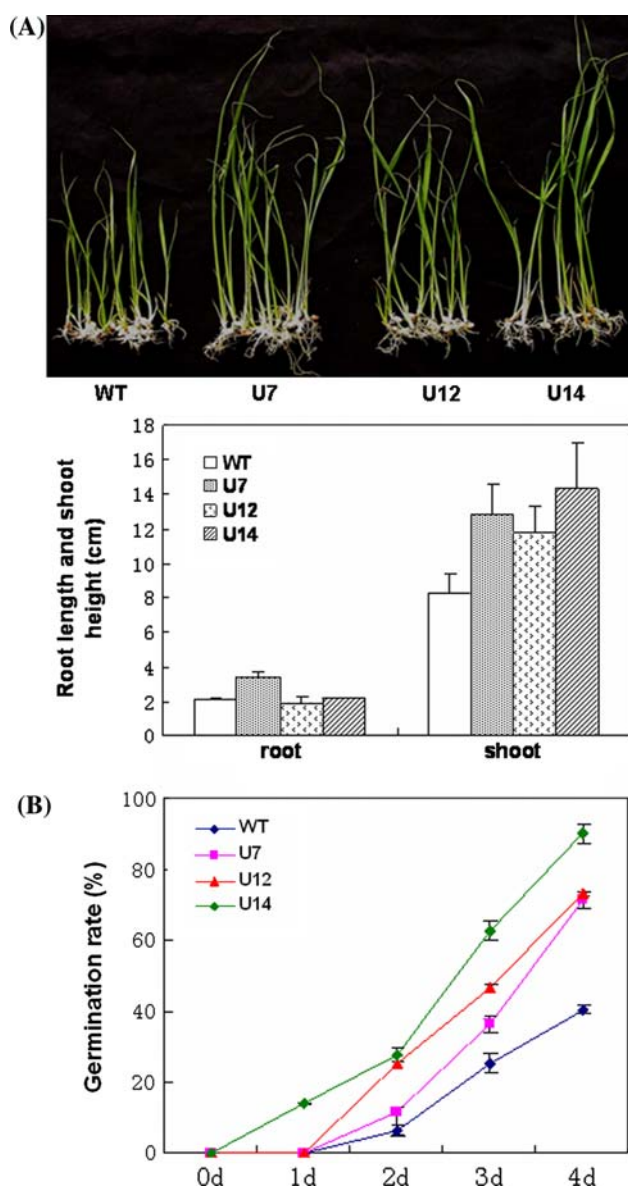
in the normal MS medium (data not shown). We also evaluated germination ability of transgenic lines harboring *SNAC2* under salt-stress condition. After 4 days of germination on the medium containing 150 mM NaCl, only 40% of WT seeds were poorly germinated, whereas more than 70% of transgenic seeds germinated very well (Fig. 4b). In the MS medium, however, more than 90% of both transgenic and WT seeds germinated very well and there was no significant difference in germination rate, suggesting that over-expression of *SNAC2* does not affect seed germination under normal conditions. The significantly higher germination rate of transgenic seeds than that of WT under salinity conditions further supported the improved salt tolerance of *SNAC2*-overexpressing plants.

The growth performance of transgenic seedlings on MS medium containing 15% PEG6000 (to mimic dehydration stress) was measured to evaluate the effectiveness of this gene on improving dehydration resistance. The positive transgenic and WT shoots were transferred to two kinds of MS mediums, one with 15% of PEG6000 and the other without PEG6000 as control. In the control, there was no significant difference in plant height and root length between transgenic and WT seedlings after growing for 2 weeks (data not shown). In the medium with 15% of PEG6000, the plant height of WT was significantly lower (only about half of the transgenic plant height) than that of transgenic plants (Fig. 5). However, no significant

difference of relative yield, a parameter for evaluation of drought resistance of rice at reproductive stage by Yue et al (2006), was detected between *SNAC2*-overexpression lines and wild type under the drought-stressed field conditions (data not shown).

#### Enhanced ABA sensitivity of *SNAC2*-overexpressing plants

Induction of the *SNAC2* gene by ABA (Fig. 1) and the improved stress tolerance of *SNAC2*-overexpressing plants gave us a hint that the ABA sensitivity of transgenic plants might be altered. The transgenic seeds germinated on the medium containing hygromycin were transferred to MS medium containing 3 μM ABA and normal MS medium. In the ABA-containing medium, the growth of *SNAC2*-overexpressing seedlings was significantly inhibited compared to WT in terms of plant height (Fig. 6a). The root growth of *SNAC2*-overexpressing seedlings was also severely inhibited (nearly no root growth) compared to WT in the ABA-containing medium. In the medium without ABA, however, there was no significant difference in either root or shoot length between transgenic and WT seedlings (Fig. 6b). These results suggested that over-expression of *SNAC2* in plants resulted in increased sensitivity to ABA.



**Fig. 4** *SNAC2*-overexpressing transgenic rice showed improved salinity tolerance. **(a)** Root length and plant height of transgenic and control rice on MS medium with 150 mM NaCl for 14 d were measured. CK: wild type Zhonghua 11. Values are the mean  $\pm$  SD ( $n = 10$ ). **(b)** Germination rate of transgenic and control lines on MS medium with 150 mM NaCl was recorded every day. The experiment was repeated three times

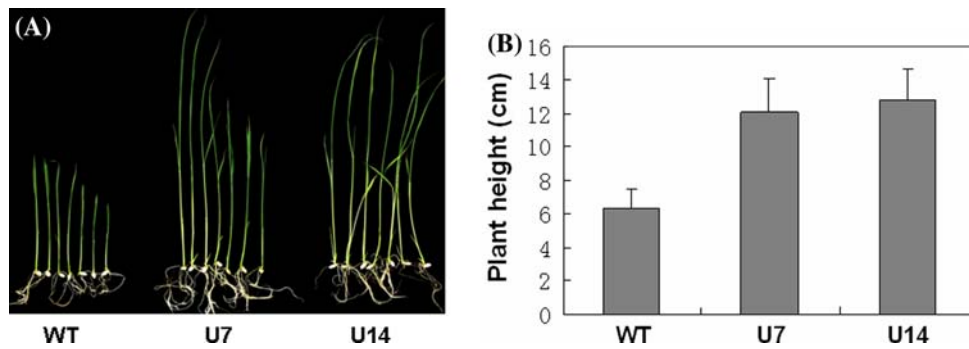
#### Genomic expression profile of *SNAC2*-overexpressing rice

To help elucidate the possible mechanisms of the enhanced stress tolerance, we checked genomic expression profiles of *SNAC2*-overexpressing rice plants under both normal and cold stress conditions using an Affymetric DNA chip. At a threshold of 2.5-fold ( $P < 0.001$ ) change of expression level, 36 and 9 genes were up-regulated and down-regulated, respectively, in both the independent transgenic lines

compared to WT under both normal and cold stress conditions (Table 1). We randomly picked up 16 genes that were up- or down-regulated in the transgenic plants with different folds based on DNA chip data to confirm by real time PCR analysis. The results suggested all the 15 genes detected showed similar up- or down-regulation patterns as in DNA chip analysis (Table 1). Based on the annotations of these genes in TIGR database, more than half of the up- and down-regulated genes encode proteins with diverse functions including transcription regulation (7 transcription factors involved), growth regulation (such as cell division-related AAA-type ATPase, cyclin-related protein, calcium binding EGF protein), carbohydrate and amino acid metabolism (such as UDP-glucose 4-epimerase, isocitrate lyase, lysine decarboxylase, and lysine ketoglutarate reductase), protein turnover (such as carboxyl-terminal peptidase), and many stress protection-related proteins such as peroxidase, ornithine aminotransferase (involved in proline synthesis), heavy metal-associated protein, sodium/hydrogen exchanger, heat shock protein, GDSL-like lipase, BURP domain containing protein, and phenylalanine ammonia lyase (Table 1). Nevertheless, about one third of the up- or down-regulated genes were annotated with unknown function. We checked the stress responsiveness of the up- or down-regulated genes based on the microarray profiling data of drought and salt stresses in rice Minghui 63 (Zhou et al. 2007) and cold stress in rice Zhonghua 11 (this study) and found that 27 genes were responsive to drought, salt, and/or cold stresses (Table 1). We also analyzed the sequence of 1 kb upstream the predicted start codon of each up- or down-regulated gene to identify putative NAC recognition sites (NACRS) and core DNA-binding sequences as identified in *Arabidopsis* (Tran et al. 2004). Among the 45 genes, 26 genes contain both NACRS and core DNA-binding sequences in their promoter regions, while almost all the other genes contain either NACRS or core DNA-binding sequences (Table 1), suggesting that some of these genes might be the transcriptional targets of *SNAC2*.

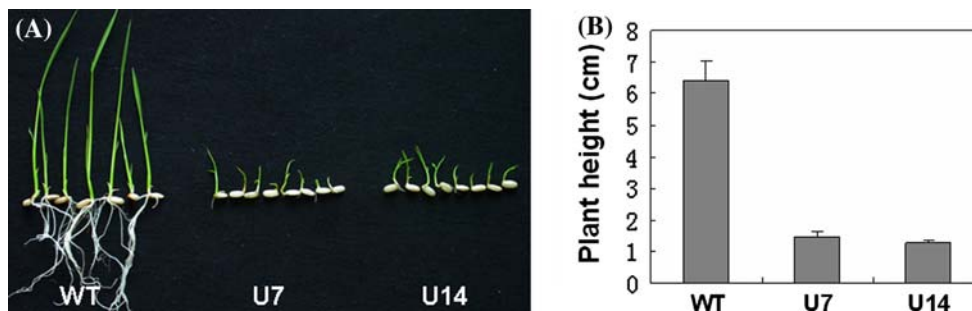
#### Discussion

To date, a number of transcription factor genes from different families such as DREB (Liu et al. 1998; Kasuga et al. 1999; Yamaguchi-Shinozaki and Shinozaki 2001; Haake et al. 2002), MYB (Abe et al. 1997), bZIP (Uno et al. 2000), zinc finger (Mukhopadhyay et al. 2004) and NAC (Fujita et al. 2004; Tran et al. 2004) has been reported as having effect on improving stress tolerance. However, the evidence of improved stress resistance for most of these genes is from model species such as *Arabidopsis*. Rice is one of the most important crops



**Fig. 5** *SNAC2*-overexpressing transgenic plants confer improved tolerance to PEG treatment. Seeds were germinated on MS medium containing 100 mg/l hygromycin for 4 days and then transferred to

MS medium containing 15% PEG6000. Plant height was measured after the seedlings growing for 14 days. Values are the mean  $\pm$  SD ( $n = 10$ )



**Fig. 6** *SNAC2*-overexpressing transgenic plants were highly sensitive to ABA. Seeds germinated on MS medium containing 100 mg/l hygromycin were transferred to the MS medium with 3  $\mu$ M ABA (a)

or normal MS medium (b) and the growth performance was investigated at 7 d after transplanting. Values are the mean of plant height ( $n = 10$  for error bar) for each line

worldwide. In our previous study, over-expression of a stress-responsive gene *SNAC1* in rice can significantly improve drought and salt resistance (Hu et al. 2006). In this study, another stress inducible gene *SNAC2* was over-expressed in rice and the transgenic rice plants showed significantly improved tolerance to cold and salt stresses as well as PEG treatment. Our results further support that some of the stress-responsive NAC genes such as *SNAC1* and *SNAC2* are playing important roles in the regulation of stress responses in rice.

*SNAC2*, featuring a transcription activator as proven by yeast one-hybrid assay, may have an ability to activate downstream target genes in rice. The whole genome expression profile analysis revealed that 36 and 9 genes were up- and down-regulated (2.5 folds or higher) respectively in the *SNAC2*-overexpressing transgenic plants, and most of these genes are also responsive to stresses including drought, salt and cold. Although such profiling data is preliminary for revealing the molecular and physiological basis of the improved stress resistance of the transgenic rice, some of these up- and down-regulated genes can be helpful clues for further investigation of the molecular basis of the improved stress resistance. Actually, homologous genes of many up-regulated genes have been documented with functions in the responses or adaptations

to abiotic stresses in plants. The products of these genes include peroxidase (Murgia et al. 2004), ornithine aminotransferase (Roosens et al. 1998), lysine ketoglutarate reductase (Galili et al. 2001), heavy metal-associated protein (Barth et al. 2004), sodium/hydrogen exchanger (Shi et al. 2003), heat shock protein (Sun et al. 2001), GDSL-like lipase (Naranjo et al. 2006), BURP domain (RD22) containing protein (Goh et al. 2003), and phenylalanine ammonia lyase (Vincent et al. 2005), ATP-dependent helicase (Vashisht et al. 2005), Tat pathway signal sequence family protein (Ochsner et al. 2002). Plant growth is often suppressed under stress conditions. We noticed that quite a few genes related to plant growth such as putative RADIALIS, cell division AAA ATPase, and calcium binding EGF domain containing protein were down-regulated in the transgenic plants, although the relevance of these down-regulated genes to stress adaptation needs further experimental evidence. We also noticed that a gene encoding carboxyl-terminal peptidase was dramatically suppressed in the transgenic plants, which might suggest that protein degradation or turnover was affected in the transgenic plants.

Putative core DNA-binding sequence and NAC recognition site identified in the promoter of *AtERD1* gene (Fujita et al. 2004; Tran et al. 2004) can be also found in

**Table 1** Genes up- and down-regulated in the *SNAC2*-overexpressed rice plants identified by DNA chip analysis

Gene or locus ID	Description of putative gene function	Normal growth <sup>a</sup>		Cold stress <sup>a</sup>		NACRS/ CDBS <sup>b</sup>	Stress response <sup>c</sup>
		U7/WT	U14/WT	U7/WT	U14/WT		
GI:150775	Hygromycin B phosphotransferase (marker gene)	188.8/n.d.	599.2/n.d.	126.8/n.d.	342.8/n.d.		
LOC_Os03g43100	Expressed protein	39.5/21.4	49.6/17.5	19.5/11.3	25.4/14.7	1/2	
LOC_Os06g21369	Hypothetical protein	18.5/6.2	8.6/3.7	12.9/3.4	4.6/2.1	2/3	
LOC_Os01g35330	Expressed protein	17.6/n.d.	18.3/n.d.	20.0/n.d.	27.9/n.d.	3/2	
LOC_Os11g17954	Transposon protein, Pong sub-class	9.3/4.9	8.9/5.1	31.6/7.2	28.7/6.4	0/6	
LOC_Os02g41630	Phenylalanine ammonia lyase	9.0/n.d.	8.9/n.d.	2.7/n.d.	17.3/n.d.	0/3	
LOC_Os11g08210	No apical meristem (NAM) family protein	8.5/3.5	8.7/4.4	2.6/2.0	9.6/3.3	1/9	D
LOC_Os03g19600	Retrotransposon protein, Ty3-gypsy subclass	7.3/n.d.	5.0/n.d.	2.8/n.d.	3.7/n.d.	0/6	
LOC_Os05g41940	Transposon protein, En/Spm sub-class	6.9/n.d.	7.2/n.d.	4.1/n.d.	6.3/n.d.	5/1	
LOC_Os04g27190	Metal binding domain containing protein	6.5/2.3	7.4/3.2	4.4/2.1	5.7/2.0	0/1	C
LOC_Os01g66120	NAM family protein	5.9/16.7	5.3/22.1	4.9/18.2	5.5/15.1	1/8	D, S, C
LOC_Os03g44150	Putative ornithine aminotransferase	5.4/4.3	5.8/4.4	2.8/1.8	7.3/2.6	2/9	D, S, C
LOC_Os02g54254	Putative lysine ketoglutarate reductase	4.2/3.1	5.5/3.6d	4.0/2.1	8.5/2.8	2/0	
LOC_Os08g04210	Putative 33 kDa secretory protein	4.2/n.d.	6.3/n.d.	16.3/n.d.	17.1/n.d.	2/0	
LOC_Os11g04400	Scarecrow-like transcription factor	3.9/n.d.	5.1/n.d.	6.6/n.d.	4.8/n.d.	1/3	S
LOC_Os09g17560	O-methyltransferase family protein	3.8/4.1	4.9/3.7	2.9/2.5	2.6/1.8	0/2	S
LOC_Os01g35330	Expressed protein	3.7/2.3	3.8/2.1	4.0/1.8	5.3/2.3	3/2	C
LOC_Os09g21180	Homeobox domain-containing protein	3.7/3.2	3.6/2.9	2.6/1.7	5.0/2.4	1/1	
LOC_Os02g41650	Phenylalanine ammonia lyase	3.6/2.5	2.9/1.6	2.8/1.9	7.5/2.5	1/7	S
LOC_Os03g25340	Peroxidase family protein	3.6/2.2	7.3/2.8	2.6/1.7	4.0/2.2	0/2	C
LOC_Os05g50390	Expressed protein	3.5/n.d.	3.5/n.d.	5.8/n.d.	6.3/n.d.	3/2	
LOC_Os05g11910	GDSL-like lipase/ Acylhydrolase family	3.4/n.d.	3.5/n.d.	5.6/n.d.	9.9/n.d.	6/4	D, S, C
LOC_Os01g45640	Tat pathway signal sequence family protein	3.4/n.d.	3.9/n.d.	3.1/n.d.	3.8/n.d.	1/6	D
LOC_Os01g51210	Putative lysine decarboxylase	3.4/n.d.	4.6/n.d.	3.6/n.d.	3.8/n.d.	0/4	C
LOC_Os07g34520	Putative isocitrate lyase	3.3/n.d.	4.4/n.d.	6.3/n.d.	9.3/n.d.	1/1	S, C
LOC_Os03g41390	Zinc finger, C2H2 type family	3.2/n.d.	2.7/n.d.	2.6/n.d.	5.7/n.d.	3/1	C
LOC_Os08g32540	Cyclin, N-terminal domain containing protein	3.2/n.d.	5.3/n.d.	5.7/n.d.	5.9/n.d.	0/1	
LOC_Os07g47100	Sodium/hydrogen exchanger 3 family protein	3.1/2.5	3.9/2.7	4.2/2.4	8.9/2.2	2/7	D, S
LOC_Os09g35800	UDP-glucose 4-epimerase	3.1/n.d.	4.9/n.d.	2.6/n.d.	4.3/n.d.	0/13	D, S
LOC_Os01g74490	Heavy metal-associated protein	3.0/n.d.	4.4/n.d.	3.9/n.d.	11.2/n.d.	2/1	S, C
LOC_Os10g42220	3-hydroxyisobutyryl-coenzyme A hydrolase	3.0/n.d.	3.2/n.d.	2.8/n.d.	5.3/n.d.	1/1	
LOC_Os10g26940	BURP domain containing protein	2.9/n.d.	3.6/n.d.	3.5/n.d.	5.3/n.d.	1/0	D, S, C

**Table 1** continued

Gene or locus ID	Description of putative gene function	Normal growth <sup>a</sup>		Cold stress <sup>a</sup>		NACRS/ CDBS <sup>b</sup>	Stress response <sup>c</sup>
		U7/WT	U14/WT	U7/WT	U14/WT		
LOC_Os01g04370	16.9 kDa class I heat shock protein	2.8/n.d.	5.4/n.d.	4.6/n.d.	6.5/n.d.	0/2	
AK119979	Serine carboxypeptidase S10 family	2.8/n.d.	3.2/n.d.	2.9/n.d.	6.4/n.d.	N/A	
LOC_Os07g27350	Expressed protein	2.7/n.d.	2.5/n.d.	3.0/n.d.	2.7/n.d.	1/6	<u>C</u>
LOC_Os06g20820	Expressed protein	2.6/n.d.	4.0/n.d.	4.1/n.d.	5.1/n.d.	1/0	
LOC_Os04g44240	Cytokinin-O-glucosyltransferase 3, putative	2.5/n.d.	4.6/n.d.	3.1/n.d.	5.2/n.d.	0/1	
LOC_Os06g48210	ATP-dependent helicase DDX41, putative	2.5/n.d.	3.2/n.d.	2.6/n.d.	2.5/n.d.	1/4	D, C
LOC_Os06g16640	Carboxyl-terminal peptidase, putative	-23.4/n.d.	-37.5/n.d.	-15.4/n.d.	-25.4/n.d.	4/2	<u>D</u>
LOC_Os03g62670	Thiopurine S-methyltransferase family protein	-5.4/n.d.	-8.5/n.d.	-4.4/n.d.	-3.5/n.d.	0/4	<u>D</u>
LOC_Os11g03230	GDA1/CD39 family protein	-4.3/-1.8	-4.7/-2.0	-3.2/-1.7	-3.7/-1.9	2/2	
LOC_Os05g50340	RADIALIS, putative	-4.0/n.d.	-7.1/n.d.	-2.3/n.d.	-2.3/n.d.	0/2	<u>D</u>
LOC_Os12g24320	Cell division protein AAA ATPase family	-3.6/n.d.	-3.8/n.d.	-2.2/n.d.	-3.9/n.d.	2/2	D,S, <u>C</u>
LOC_Os04g29680	Calcium binding EGF domain containing protein	-3.3/n.d.	-6.1/n.d.	-2.5/n.d.	-3.2/n.d.	0/0	<u>C</u>
LOC_Os01g01840	Helix-loop-helix DNA-binding protein	-2.1/n.d.	-2.8/n.d.	-2.9/n.d.	-2.0/n.d.	1/1	
LOC_Os07g13770	UDP-glucosyl transferase family protein	-2.1/n.d.	-3.7/n.d.	-2.5/n.d.	-5.0/n.d.	3/7	<u>D</u> , <u>S</u>
LOC_Os09g28210	Helix-loop-helix DNA-binding protein	-2.1/n.d.	-2.5/n.d.	-4.5/n.d.	-11.6/n.d.	0/1	D, S, C

<sup>a</sup> Values represent the changed folds of expression level based on DNA chip and real-time PCR (separated by “/”) analyses with three replications. Negative values indicate down-regulated expression in transgenic plants. n.d.: no data

<sup>b</sup> Numbers of putative NAC recognition sequence (NACRS) and core DNA binding sequence (CDBS) in the 1 kb region upstream of the start codon of each gene

<sup>c</sup> Responsiveness of the gene to drought (**D**) and salt (**S**) stresses is based on our microarray profiling data (Zhou et al. 2007) and responsiveness to cold (**C**) stress is based on the expression profile of rice Zhonghua 11 in this study. A threshold of 2.5-fold ( $P < 0.001$ ) of induction or suppression is used. Underlined letter indicates down-regulation of the gene by the stress

the promoter sequences of many genes up- or down-regulated in the *SNAC2*-overexpression plants, suggesting that some of these genes might be transcriptionally regulated directly by *SNAC2*. We also noticed that a few transcription factors were significantly up-regulated in the transgenic plants. This may partially explain the fact that some genes without the core DNA-binding sequence or NAC recognition site in their promoter sequences were also up- or down-regulated in the transgenic plants.

Both *SNAC2* and *SNAC1* genes encode typical NAC transcription factors closely related to each other based on protein sequence analysis (Ooka et al. 2003) and have similarity in both stress-induced expression (both genes are induced by drought, salt, cold, and ABA treatment) and ABA sensitivity. However, *SNAC2* gene differs from

*SNAC1* in a few aspects. First, the two genes have different stress-induced expression patterns. *SNAC2* gene was strongly induced in roots and internodes and slightly induced in leaves by drought, salt, and cold stress (Fig. 1b). The induced expression in leaves had no cell-type preference (data not shown). In addition, *SNAC2* gene was strongly induced by wounding in all the vegetative tissues investigated (Fig. 1b). The *SNAC1* genes, however, was strongly induced by drought stress and the induced expression is quite specific in guard cells (Hu et al. 2006), but *SNAC1* was not induced by wounding (data not shown). Second, over-expressions of the two genes have different effect on stress resistance. Over-expression of *SNAC1* can significantly improve drought resistance under the field conditions, which may be mainly due to the function of this

gene in regulating stomata closure and water use efficiency (Hu et al. 2006). Over-expression of *SNAC2*, however, showed no significant effect on drought resistance in the field conditions (data not shown) even though the transgenic plant showed improved tolerance to osmotic stress by PEG treatment. In addition, *SNAC2*-overexpression can improve cold tolerance while over-expression of *SNAC1* had no significant effect on improving cold tolerance (not shown) even though *SNAC1* gene is induced by cold. Third, the potential transcriptional target genes of *SNAC1* and *SNAC2* are different. We compared the genes up- or down-regulated in the *SNAC1*- and *SNAC2*-overexpression plants and noticed that there is no overlapping between the two sets of genes up- or down-regulated in the two over-expression plants respectively. Such a sharp difference in the expression-altered genes in *SNAC1*- and *SNAC2*-overexpression plants may partially explain the difference of the two over-expression transgenic plants in stress tolerance. We also compared the genes up- or down-regulated in rice plants over-expressing *SNAC1* and *SNAC2* with the genes up- or down-regulated in *Arabidopsis* plants over-expressing three stress-inducible *AtNAC* genes (Tran et al. 2004). Interestingly, very few of them have matches with similar functional annotations. Notably, quite a few transcription factor genes (including another putative NAC homolog) were up-regulated in *SNAC1*- or *SNAC2*-overexpressing rice plants but no matched transcription factors were found in the list of up-regulated genes in the *AtNAC*-overexpressing *Arabidopsis* plants (Fujita et al. 2004; Tran et al. 2004). This data may suggest that different stress-responsive NAC transcription factors may activate the transcription of different set of target genes, thus conferring diverse functions that jointly lead to stress resistance. A comparison of the flanking sequences of the core DNA binding sites in the putative *SNAC1* and *SNAC2* target genes revealed different conserved flanking sequences of the core binding sites of the target genes between *SNAC1* and *SNAC2* (Supplementary Fig. 4). This may suggest that DNA-binding specificity of different NAC transcription factors may be determined by the sequences flanking the core DNA binding site.

In conclusion, this study has identified a novel NAC transcription factor gene *SNAC2* that can be induced by various abiotic stresses. The significantly enhanced cold and dehydration stress tolerance of the *SNAC2*-overexpressing rice suggest a promising utility of this gene in genetic improvement of stress tolerance in economically important crops such as rice.

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