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ORIGINAL ARTICLE

Physiological mechanisms underlying OsNAC5-dependent tolerance of rice plants to abiotic stress

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Abstract To understand the functions of transcription factor *OsNAC5* in response to abiotic stress, we generated transgenic rice plants with knockdown *OsNAC5* by RNA-interfered (RNAi) and overexpressing *OsNAC5*, and investigated the effects of cold, drought and salt stress on wild-type (WT), RNAi and overexpression rice lines. Our results demonstrated that RNAi lines became less tolerant to these stresses than WT plants, while overexpression of *OsNAC5* in *Arabidopsis* and rice enhanced tolerance to these stresses. The mechanisms underlying the changes in tolerance of the transgenic rice plants to abiotic stresses were explored by measuring free proline (Pro) and soluble sugar contents in WT and transgenic plants. Accumulation of Pro and soluble sugars was positively correlated with *OsNAC5* expression levels. The less accumulation of Pro in RNAi lines may be accounted for by inhibition of Pro synthesis and transport at transcriptional levels. In addition, knockdown and overexpression of *OsNAC5* enhanced and reduced accumulation of malondialdehyde and H_2O_2, suggesting that knockdown of *OsNAC5* renders RNAi plants more susceptible to oxidative damage. The RNAi lines displayed higher Na^+/K^+ ratio due to greater accumulation of Na^+ ions than WT under salt stress conditions, and expression of genes encoding tonoplast Na^+/H^+ antiporter was lower in RNAi lines than in WT under both control and salt-stressed conditions. Seed germination of RNAi and overexpression plants was more and less inhibited by salt and mannitol than that of WT, respectively. Seed germination of overexpression and RNAi plants was more and less sensitive than that of WT to ABA. These findings highlight the important role of *OsNAC5* played in the tolerance of rice plants to abiotic stress by regulating downstream targets associated with accumulation of compatible solutes, Na^+ ions, H_2O_2 and malondialdehyde.

Keywords Abiotic stress · Abscisic acid · *Arabidopsis* · *Oryza OsNAC5* · Seed germination

Abbreviations

ABA	Abscisic acid
CUC	Cup-shaped cotyledon
GUS	β-Glucuronidase
MDA	Malondialdehyde
MeJA	Methyl jasmonic acid
MS	Murashige and Skoog
NAC	NAM, ATAF and CUC
NAM	No apical meristem
NHX	Na^+/H^+ exchanger
P5CS	Δ^1-Pyrroline-5-carboxylate synthetase
PEG	Polyethylene glycol
Pro	Proline
SNAC	Stress-responsive NAC
TPP	Trehalose-6-phosphate phosphatase

Introduction

Being sessile organisms, plants are frequently exposed to adverse environment such as low temperature, drought and

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high salinity. Plants have evolved sophisticated mechanisms to cope with a variety of abiotic stress. Upon exposure of plants to stressed environment, numerous genes are induced, leading to changes in molecular, cellular and physiological processes that protect plants from stress-induced damages (Thomashow 1999; Shinozaki et al. 2003). As important regulatory proteins, transcription factors, which regulate the expression of many functional genes, play significant roles in response and adaptation of plants to abiotic stress (Nakashima et al. 2009). There have been reports demonstrating that overexpressing transcription factors such as CBF (Liu et al. 1998), OSISAP1 (Mukhopadhyay et al. 2004) and SNAC1 (Hu et al. 2006) confer enhanced tolerance to abiotic stress.

Proteins of the NAC family are a class of plant-specific transcription factors, which contain a highly conserved N-terminal domain known as the NAC domain. The NAC domain, first identified in the NAM, ATAF1 and CUC1/CUC2 proteins (Souer et al. 1996; Aida et al. 1997), may form a helix-turn-helix structure that can bind to promoters of target genes. It has been predicted 151 and 117 NAC genes in rice and *Arabidopsis* genomes, respectively (Nuruzzaman et al. 2010). However, only a limited number of NAC proteins have been characterized so far. For instance, NAM has been shown to control pattern formation of embryos and flowers (Souer et al. 1996). SND1 is involved in formation of secondary walls (Zhong et al. 2006). Xie et al. (2000) reported that AtNAC1 promotes the development of lateral roots via the auxin signal pathway. There is increasing evidence demonstrating that NAC proteins not only participate in plant development, but also play significant roles in the response of plants to biotic and abiotic stress. For instance, *StNAC* from tomato, *AtAF1-2* from *Arabidopsis* and nine NAC-domain genes from Brassicanapus were found to be induced by pathogen infection and mechanical wounding (Collinge and Boller 2001; Hegedus et al. 2003). Overexpression of three *NAC* genes, *ANAC019*, *ANAC055* and *ANAC072*, in *Arabidopsis* conferred tolerance to drought stress (Fujita et al. 2004; Tran et al. 2004). *AtNAC2* can regulate salt tolerance via ethylene and auxin signaling (He et al. 2005). In rice, a stress-responsive *NAC* gene *SNAC1* was identified, and overexpression of *SNAC1* led to enhanced tolerance to drought by modulating stomatal closure (Hu et al. 2006). Another *NAC* gene, *OsNAC6/SNAC2*, is induced by abiotic stress and jasmonic acid treatment, and overexpression of this gene results in enhanced tolerance of rice to cold, drought and salt stress (Ohnishi et al. 2005; Nakashima et al. 2007; Hu et al. 2008).

Recent reports indicate that the NAC domain not only interacts with proteins, but also with other or self NAC domain to form dimers (Olsen et al. 2004). For example, the GEMINIVIRUS REP A-BINDING protein (GRAB) interacts with a geminivirus protein to impair viral replication (Xie et al. 1999). Ren et al. (2000) reported that the function of *HRT* gene requires interaction between a NAC protein (TIP) and viral capsid protein to confer resistance to turnip crinkle virus. Hegedus et al. (2003) revealed that BnNAC14 interacts with other BnNAC proteins by yeast two-hybrid screening. It was reported that OsNAC5 can form homodimers as well as heterodimers with other NAC proteins (Takasaki et al. 2010). These results suggest that NAC domain has complex functions and not only binds to DNA, but also interacts with proteins.

A recent study revealed that OsNAC5 is involved in the accumulation of Fe and Zn in developing rice seeds (Sperotto et al. 2009). Takasaki et al. (2010) reported that OsNAC5 is involved in response and adaptation to salt and drought stress by comparing *OsNAC5*-overexressing rice plants with wild-type plants. The authors also demonstrated that the expression of *OsNAC5* is responsive to cold stress, but they did not evaluate the role of OsNAC5 played in tolerance to cold stress. Moreover, the authors reported that *OsNAC5*-overexpressing rice plants displayed enhanced tolerance to salt and drought stress (Takasaki et al. 2010). However, they did not study the physiological mechanisms underlying the enhanced tolerance to salt and drought stress. In the present study, we investigated the roles of OsNAC5 in tolerance to cold, salt and drought stress by generating transgenic rice plants with overexpressing *OsNAC5* and suppression of *OsNAC5* by RNA interference. More importantly, we demonstrated that *OsNAC5*-dependent increase in tolerance to abiotic stress may be accounted for by its regulation of synthesis of soluble sugars, Pro, antioxidant system and Na^+ accumulation.

Materials and methods

Plant growth and stress treatments

Rice (*Oryza sativa* L. ssp. japonica cv Zhonghua 10) seedlings were grown in the greenhouse at 27/23°C 14 h light/10 h dark. The rice seeds were supplied by the China National Rice Research Institute (Hangzhou, Zhejiang, China). For measurement of *OsNAC5* transcript level, chemical treatment was conducted following those described by Xiang et al. (2007). Briefly, 2-week-old seedlings of the wild type (WT) were exposed to incubation solution containing 0.2 mM salicylic acid (SA), 0.1 mM abscisic acid (ABA), 0.1 mM jasmonic acid (JA), 0.1 mM naphthaleneacetic acid (NAA), 0.1 mM gibberellic acid (GA3), 0.1 mM brassinolide (BR) and 0.1 mM ethylene synthesis precursor aminocyclopropane carboxylic acid (ACC) and leaves were sampled after 2 and 5 h, respectively. All chemicals were obtained from Sigma-Aldrich. For the

treatment of salt and drought stress, seedlings were exposed to solutions containing 200 mM NaCl or 15% PEG 6000 and leaves were sampled after 5 and 10 h, respectively. For cold stress, seedlings were transferred to a growth chamber at 4°C and sampled after varying periods (0, 0.5, 1, 2, 5, 10, 24, 36 and 72 h). A similar treatment regime was also used to study selected gene expression of WT and transgenic rice plants.

Plasmid construction and plant transformation

The full-length cDNA of OsNAC5 was amplified from rice plants with the primers *OsNAC5*-FL-F (5′-CGCGGATCC ATGGAGTGCGGTGGTGCGCTG-3′; *Bam*HI site underlined) and *OsNAC5*-FL-R (5′-CGGGGTACCTTAGAAC GGCTTCTGCAGGTAC-3′; *Kpn*I site underlined). The sequencing confirmed that PCR fragment was directionally cloned into the *Bam*HI-*Kpn*I sites of a pSN1301 or pUN1301 vector to create the pSN1301-*OsNAC5* or pUN1301-*OsNAC5* construct. To construct the RNAi vector, the 344-bp coding sequence was amplified with the primers *OsNAC5*-Ri-F (5′-GGGGTACCACTAGTTCTAC GACCAGGAGCCCG-3′; *Kpn*I and *Spe*I sites underlined) and *OsNAC5*-Ri-R (5′-CGGGATCCGAGCTCCGGCTTC TGCAGGTACG-3′; *Bam*HI and *Sac*I sites underlined), digested by *Kpn*I and *Bam*HI and then *Spe*I and *Sac*I, and ligated to the pTCK303 vector.

For *Arabidopsis* transformation, the pSN1301-*OsNAC5* construct was introduced into *Arabidopsis* wild-type plants (Col-0) using floral dip transformation with *Agrobacterium* strain GV3101 (Zhang et al. 2006). For rice transformation, rice seeds were sterilized and cultured on MS plus (4 mg/L 2,4-dichlorophenoxyacetic acid, Sigma) medium for 4 weeks in the dark at 28°C to induce embryogenic callus, and the pSN1301-*OsNAC5* or pTCK303 -*OsNAC5* construct were introduced into the callus by *A. tumefaciens* strain EHA105-mediated transformation, as described by Yang et al. (2004). The positively transformed calli were screened in 1/2 MS medium containing 50 mg/L hygromycin (Sigma), differentiating on differentiation medium (0.5 mg/L naphthylacetic acid and 3 mg/L 6-benzyladenine, Sigma). The positive calli that generated roots and shoots were transferred to 1/2 MS medium to develop into T0 seedlings. The T0 generation was confirmed by GUS stain and transplanted into soil and grown in a greenhouse.

Determination of stress tolerance

To determinate seed germination, 60 seeds of T2 generation from different transgenic lines and WT were sterilized and spread on half-strength MS medium containing ABA, NaCl or mannitol. Seeds were regarded as germinated when the shoot length exceeded half of the seed length. For growth assay, transgenic and WT seeds were germinated on 1/2 MS medium for 3 days, then transferred to 1/2 MS medium containing NaCl for 10 days and used for growth measurements. For survival experiments, 4-week-old seedlings of transgenic and WT rice plants were exposed to cold, salt and drought stress by transferring to 4°C for 6 days, irrigating with 200 mM NaCl for 14 days and withholding water for 15 days, respectively. After the above treatments, plants were transferred to normal conditions and grown for another 7 days. Seedlings that failed to grow were taken as dead (Xiang et al. 2008). For determination of the effect of freezing on transgenic Arabidopsis plants, 4-week-old seedlings were cold acclimated at 4°C for 3 days, transferred to −7°C for 9 h, thawed at 4°C overnight and then transferred to normal conditions. Survival of the seedlings was scored visually (plants with green leaves) after 5 days. All the stress tolerance experiments were repeated at least three times, and data were calculated from the results of three independent experiments.

Assay of electrolyte leakage

To determine electrolyte leakage, leaves from six plants of 4-week-old seedlings treated at 4°C for 6 days were placed in 10 mL tubes containing 6 mL of deionized water. After shaking with a speed of 200 rpm at 25°C for 2 h, electrical conductivity was first determined (C1). Thereafter, the samples were autoclaved at 121°C for 20 min, and total conductivity was determined again (C2) after cooling to room temperature. Relative ion leakage was expressed as percentage of total conductivity after heating to 121°C, i.e., relative ion leakage % = C1/C2 × 100.

Determination of H_2O_2 and MDA contents

Hydrogen peroxide was measured as described previously (Alexieva et al. 2001) with some modification. Briefly, 1-g leaf sample was grounded with 0.1% trichloroacetic acid (TCA) and centrifuged at 10,000*g* for 20 min at 4°C. The supernatant was used and hydrogen peroxide measured spectrophotometrically. The reaction mixture consisted of 1 mL of the extracted supernatant, 1 mL of potassium phosphate buffer and 2 mL of 1 M KI. The reaction was developed for 1 h in darkness and absorbance measured at 390 nm.

For the measurement of MDA, the leaves were weighed and homogenized in 5 mL of 10% TCA solution. The homogenate was centrifuged, and the supernatant was added to 0.6% thiobarbituric acid in 10% TCA. The mixture was incubated in boiling water for 15 min, and the reaction was stopped in an ice bath. Then, the samples were centrifuged and the absorbance of the supernatant was

measured at 450, 532 and 600 nm. MDA contents (nmol g^{-1} fresh weight) were calculated by the following formula: $[6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}]$/fresh weight (Zhang et al. 2009).

Determination of proline and soluble sugar contents

The content of Pro in leaves of rice was determined as described by Bates et al. (1973). Briefly, leaves were harvested, weighed and extracted in 3% sulfosalicylic acid. An aliquot of each extract (2 mL) was incubated with 2 mL of ninhydrin reagent [2.5% (w/v) ninhydrin, 60% (v/v) glacial acetic acid and 40% 6 M phosphoric acid] and 2 mL of glacial acetic acid at 100°C for 40 min, and the reaction was terminated in an ice bath. Toluene (4 mL) was added, followed by vortex and incubation at 23°C for 24 h. The absorbance was measured at 520 nm.

Total soluble sugar content was measured as described previously (Bailey 1958). Leaves of rice were weighed and then extracted by 80% ethanol at 80°C for 30 min with occasional agitation. The superior liquid was filtered and the volume was adjusted to 10 mL with 80% ethanol. The extract was used for measuring sugar content; 1 mL of extract was incubated with 5 mL of anthrone reagent at 95°C for 15 min, and then the reaction was terminated in an ice bath. The absorbance was measured at 620 nm.

Determination of Na^+ and K^+ concentration

Two-week-old seedlings of transgenic and wild-type plants were grown in 1/2 MS solution containing 100 mM NaCl for 5 days, washed with ultrapure water for five times, fixed at 105°C for 10 min and baked at 80°C for 3 days to constant weight. As much as 50 mg of dry material was weighed and placed in a quartz beaker, and 5 mL of nitric acid and 2 mL of hydrogen peroxide were added for digestion. The digested fluid volume was finalized to 100 mL and ion content was measured by ICP-AES.

RNA isolation and quantitative PCR

Total RNAs were extracted from rice seedlings with Trizol reagent (Invitrogen) and treated with RNase free DNase I (Promega). The total RNAs were reverse-transcribed into first-strand cDNA in a 20-μL volume with M-MLV reverse transcriptase (Promega). The samples were diluted to 100 μL with water and 5-μL of each sample (~8 ng RNA equivalent) was amplified using SYBR GreenER™ qPCR SuperMix Universal (Invitrogen) in a 25 μL reaction, containing 5 μL of diluted cDNA, 12.5 μL SYBR GreenER™ qPCR SuperMix Universal, 0.5 μL Rox Reference Dye, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer and 5 μL of water. The thermal cycle used was as follows: 95°C for 3 min; 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The corresponding specific primers were used as follows: for *J033099M14* (5′-CTCAAATCAAGGCGTCAACTAAGA-3′ and 5′-TTTGTCAATATATACGTGGCATATACCA-3′), for *J033031H21* (5′-CGCCCCTCCCCGTATCT-3′ and 5′-AGGAATGCGGCAACAAGTG-3′), for *03g44230* (5′-AGGGACGATGGAGTTCTAAAGCT-3′ and 5′-GGGATTCCAAAGGCAAAAAGA-3′), for *07g01090* (5′-GAGGAGGCTACCTGACTGTCAAC-3′ and 5′-GCTCATGAAGTCGCCAAGGA-3′), for *OsTPP1* (5′-GGAGTTCCTCAATTTCTTGGTG-3′ and 5′-CGCCTCGGAAACTACAGTTATT-3′), for *OsTPP2* (5′-AGGATGCATTCAAGGTTCTGA-3′ and 5′-CAAGATGCCAGTTTCTTCAGG-3′), for *OsNHX1* (5′-ACACGACCTCCGACTAC-3′ and 5′-TCATTGACCCAGCGATT-3′), for *OsNHX2* (5′-CGATGGATGAACGAGTC-3′ and 5′-TGAAGTTGCGGAAAAAT-3′). Rice *Actin1* gene (Accession No. ×16280) was used as internal control with primers 5′-ACCACAGGTATTGTGTTGGACTC-3′ and 5′-AGAGCATATCCTTCATAGATGGG-3′. The relative expression levels were determined as described by Livak and Schmittgen (2001).

Statistics

All data were analyzed by analysis of variance using SAS statistics program. Statistical differences were referred to as significant when $P < 0.05$ or $P < 0.01$.

Results

Expression patterns of *OsNAC5*

The *OsNAC5* was isolated from the salt-stress DNA microarray data. To validate the microarray results, we investigated the response of *OsNAC5* to various abiotic stresses at transcriptional levels. As shown in Fig. 1a, transcripts of *OsNAC5* were found to be accumulated after 30 min of exposure to low temperature (4°C), and the increase was dependent on the cold duration up to at least for 72 h (Fig. 1a). The *OsNAC5* transcripts remained relatively constant at control conditions when determined at the time points used for cold treatment (data not shown), thus discounting the possibility that changes in OsNAC5 transcripts under cold treatment may result from circadian effect. In addition to cold stress, the expression of *OsNAC5* was up-regulated by treatments with PEG and salt stress (Fig. 1b). The expression of *OsNAC5* was up-regulated in response to several plant hormones examined (Fig. 1c). Among the hormones, ABA, ethylene synthesis precursor ACC, MeJA, IAA and BR induced a rapid up-regulation of *OsNAC5* (Fig. 1c). Furthermore, expression of *OsNAC5*

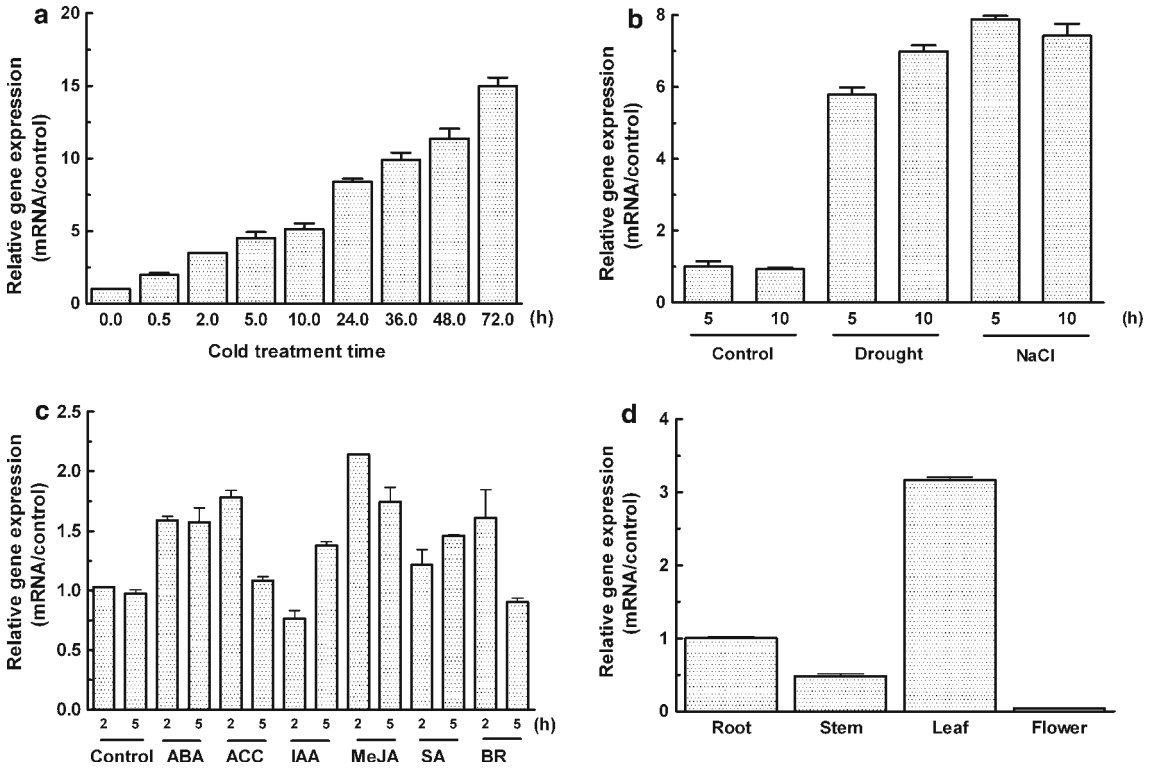

Fig. 1 Quantitative polymerase chain reaction (PCR) analysis of *OsNAC5* expression in response to cold treatment at 4°C for varying periods (**a**), salt stress (200 mM NaCl) for 5 and 10 h and PEG-induced drought stress (15% PEG6000) for 5 and 10 h (**b**). Response of *OsNAC5* expression to plant hormones (ABA, ACC, IAA, MeJA, SA and BR) (**c**). Expression patterns of *OsNAC5* in different organs (**d**). Data are means ± SE of three biological replicates

was found in leaves, roots, stems and flowers with the expression being highest in leaves and lowest in flowers (Fig. 1d).

Generation of transgenic plants with altered expression of *OsNAC5*

To understand the function of *OsNAC5*, we first generated transgenic Arabidopsis by constructing an overexpression construct (pSN1301-*OsNAC5*) containing the *OsNAC5* full-length ORF fused to a GUS reporter gene under the control of cauliflower mosaic virus 35S promoter. *Agrobacterium* strain GV3101 containing this construct was used to transform wild-type *A. thaliana* (Col-0), and several transgenic lines were obtained and confirmed by genome PCR (data not shown). In addition, we also generated transgenic rice by constructing an RNA interference (RNAi) construct (pTCK303) with part of the coding sequence fragment to be knocked down *OsNAC5* expression, as well as an overexpression construct (pUN1301) with the *OsNAC5* full-length ORF fused to a GUS reporter gene driven by a maize ubiquitin promoter. Both constructs were introduced into rice calli via *Agrobacterium*-mediated transformation, and several transgenic lines were obtained. The expression levels of knockdown and *OsNAC5*-overexpressing lines were examined by real-time quantitative PCR (q-PCR). The expression of *OsNAC5* in the knockdown lines (Ri4, Ri5, Ri6 and Ri10) was reduced to approximately 30% of that in WT, while *OsNAC5* transcript in the overexpression lines was more than twofold higher than in WT (Fig. 2). Among them, two knockdown lines (Ri5, Ri6) and one overexpression line (OE1) were chosen based on their expression levels of *OsNAC5* and

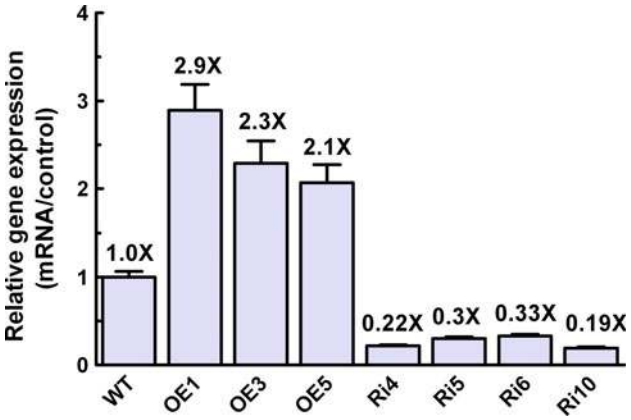

Fig. 2 Quantitative PCR analysis of *OsNAC5* expression in wild-type (WT), overexpression (OE1, OE3, OE5) and RNAi (Ri4, Ri5, Ri6 and Ri10) plants. Data are means ± SE with three biological replicates

seed availability to study the physiological function of OsNAC5 using T2 generations of those transgenic as well as wild-type rice plants. A recent study has reported that that overexpressing OsNAC5 confers tolerance of rice plants to salinity and drought (Takasaki et al. 2010). We had data showing that overexpressing lines of OE1, OE3 and OE5 displayed comparable phenotypes under both control and stressed conditions (data not shown). Therefore, the observed changes in tolerance of the OE1 line are unlikely to result from the effects of insertion position in the rice genome.

Overexpression of *OsNAC5* confers tolerance to low temperature

Seedlings of both overexpressing and knockdown lines showed comparable phenotype to WT plants under non-stressed, control conditions (Fig. 3c). Given that expression of *OsNAC5* was rapidly up-regulated by cold, we compared the tolerance of *Arabidopsis* overexpression of *OsNAC5* to cold with that of WT *Arabidopsis*. After being frozen at −7°C for 9 h, wild-type *Arabidopsis* plants exhibited symptoms of flagged and whitened leaves. In contrast, no such stressed symptoms were observed in the transgenic *Arabidopsis* lines overexpressing *OsNAC5* (OE1, OE5 and OE6) following the identical freezing treatments (Fig. 3a). In addition, the transgenic *Arabidopsis* had a much higher survival rate than wild-type plants after 7 days of recovery following the freezing regimes of −7°C for 9 h. For instance, the survival rates for WT and three overexpressing *Arabidopsis* lines (OE1, OE5 and OE6) were found to be 45.0, 70.0, 84.4 and 89.4%, respectively (Fig. 3b). This result suggests that overexpressing *OsNAC5* confers more tolerance to freezing stress in Arabidopsis.

To further characterize the function of *OsNAC5*, we studied the effect of chilling stress on two knockdown and one overexpression rice lines. Exposure of WT and transgenic rice plants to 4°C for 6 days and recovering for 7 days at 22°C led to withered leaves in WT plants (Fig. 3c). In contrast, leaves of the two knockdown lines (Ri5 and Ri6) became yellow and severely withered following the same chilling and recovery treatments as used for WT (Fig. 3c). Accordingly, the two knockdown lines also exhibited lower survival rate than WT plants following 7 days of recovery at 22°C after chilling stress at 4°C for 6 days (Fig. 3d). We also measured the effect of chilling stress on the relative electrolyte leakage, which is an indicator for the damage caused by abiotic stress (Verslues et al. 2006), using WT and transgenic rice seedlings of knockdown and overexpressing *OsNAC5* lines. Under non-stressed control conditions, all the plants examined (WT, OE1, Ri5 and Ri6) showed a low electrolyte leakage (Fig. 3e). A marked increase in the relative electrolyte leakage was observed for all the plants upon exposure to the chilling stress, and the increase in the two knockdown lines was higher than that in WT, while OE1 plants exhibited a lower relative electrolyte leakage than WT (Fig. 3e). These results suggest that the expression level of *OsNAC5* in rice plants is positively correlated with tolerance to chilling stress such that knockdown of *OsNAC5* makes rice plants less tolerant to chilling stress compared to WT.

Alterations of *OsNAC5* expression affect the sensitivity of seed germination to abiotic stress and ABA.

Germination of wild-type, *an OsNAC5-overexpressing and RNAi rice* seed was comparable in the absence of NaCl, mannitol and ABA, since all these seeds were fully germinated after 5 days of incubation. Seed germination was significantly inhibited by NaCl in both WT and transgenic lines, and the inhibition was positively dependent on NaCl concentration in the external medium (Fig. 4a, d). More importantly, the inhibitory effect of NaCl on seed germination was greater for the two knockdown lines than for WT. On the other hand, germination of seeds overexpressing *OsNAC5* was less inhibited by NaCl than WT seeds (Fig. 4d). A similar inhibition of germination was observed for Ri5 and Ri6 seeds compared to WT seeds due to osmotic stress, resulting from the addition of mannitol to the incubation medium (Fig. 4b, d). These findings show that knockdown of *OsNAC5* renders seed germination more sensitive to salt and osmotic stresses.

Given that the expression of *OsNAC5* was closely associated with tolerance to cold stress (Fig. 4) and the expression of *OsNAC5* was induced by ABA (Fig. 1c), we thus examined the sensitivity of germination of WT and transgenic seeds to ABA. In contrast to salt and osmotic stress, seed germination of RNAi lines was less inhibited by ABA than that of WT, while seed germination of overexpressing lines was more suppressed by ABA than that of WT (Fig. 4c, d). For example, germination of WT seeds was reduced to 73% when 6 μM ABA was present in the incubation medium, while the same concentration of ABA reduced germination rate of seeds for OE1, Ri5 and Ri6 line to 33, 93 and 90%, respectively (Fig. 4e). Moreover, *OsNAC5*-overexpressing plants were more sensitive to ABA at seedling growth stage (data not shown). These results suggest that ABA is likely to be involved in mediating *OsNAC5*-dependent increase in tolerance to abiotic stress.

OsNAC5 and salt and drought stress tolerance

To test whether *OsNAC5* plays a role in tolerance of rice to salt and drought stress, we compared the tolerance of

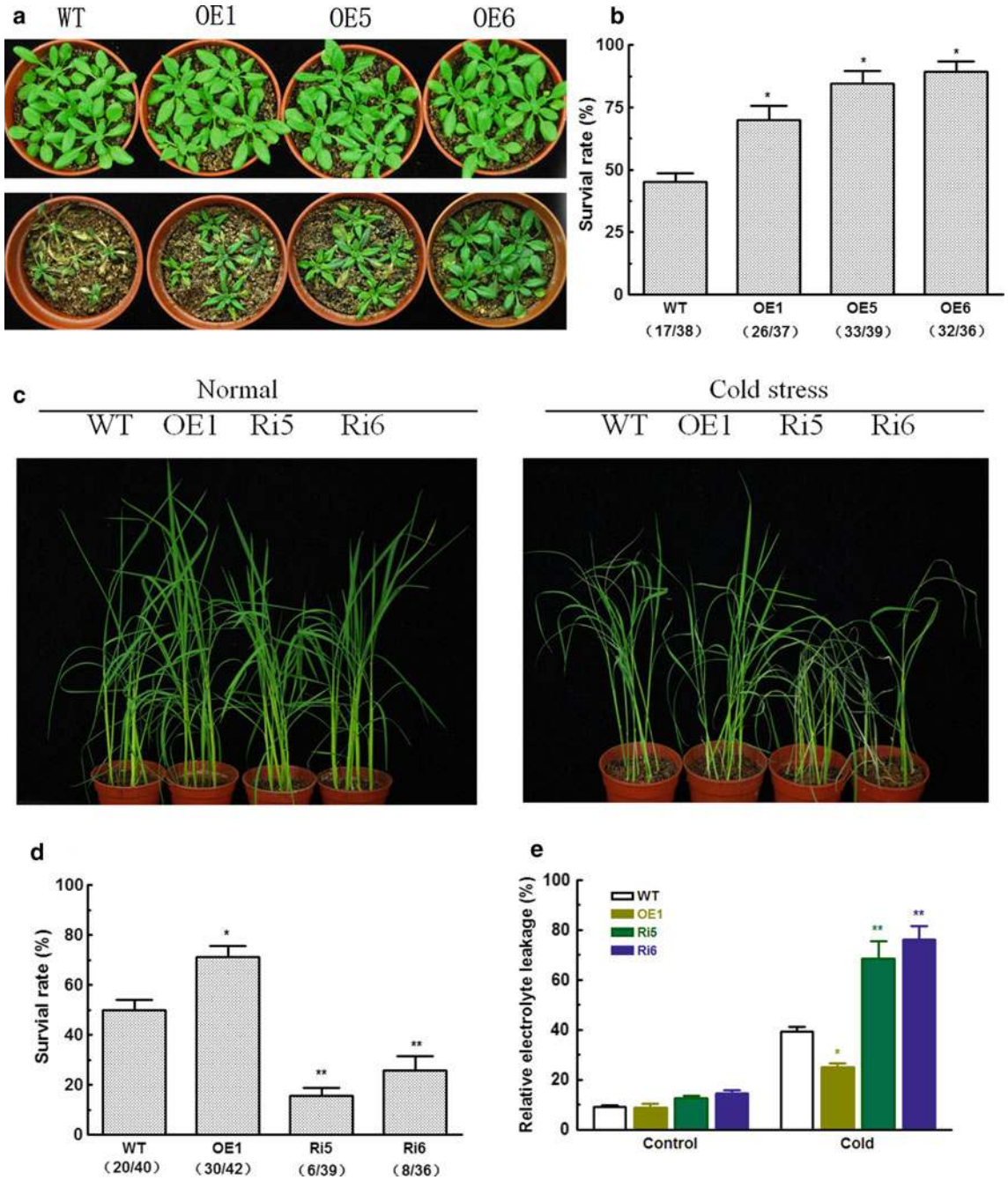

Fig. 3 Transgenic Arabidopsis and rice plants with overexpression of *OsNAC5* and underexpression of *OsNAC5* differed in their tolerance to cold stress. Photographs showing Arabidopsis WT (Col-0) and transgenic lines (OE1, OE5 and OE6) with overexpression of *OsNAC5* before and after exposure to −7°C for 9 h (**a**). Survival rates of WT and transgenic Arabidopsis after recovery from the freezing regime (−7°C for 9 h) for 7 days (**b**). Photographs of WT, OE1, Ri5 and Ri6 rice seedlings before and after cold treatment at 4°C for 6 days (**c**). Survival rate of WT and transgenic rice after recovery at 22°C for 7 days following treatment at 4°C for 6 days (**d**). Relative electrolyte leakage in WT, OE1, Ri5 and Ri6 leaves after cold stress (4°C, 6 days; **e**). Data are means ± SE with four replicates. *Asterisks* represent statistically significant differences between WT and transgenic plants (OE1, Ri5 and Ri6). * $P < 0.05$, ** $P < 0.01$

transgenic rice to salt and drought stress with that of WT plants. As shown in Fig. 5a, no difference in shoot length among WT, OE1, Ri5 and Ri6 seedlings was observed when grown in the control medium. Addition of NaCl to the incubation medium inhibited shoot growth for both WT and transgenic rice plants, and the inhibitory effect increased with increase in NaCl concentration. However, shoots of the two knockdown lines (Ri5 and Ri6) were

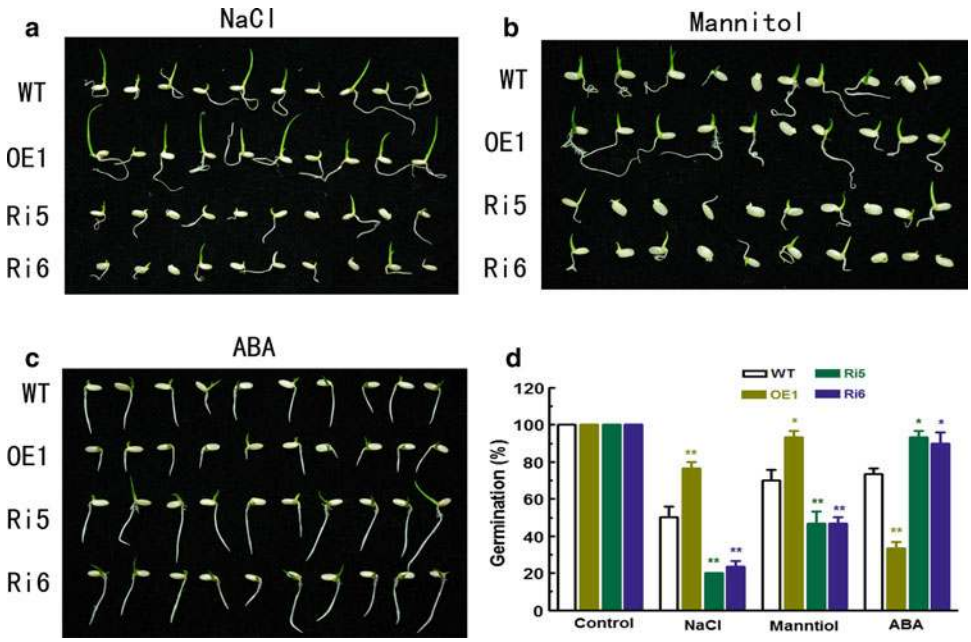

Fig. 4 Effect of salt stress (**a**), mannitol (**b**) and ABA (**c**) on germination of WT, OE1, Ri5 and Ri6 rice seeds. Seeds of WT, OE1, Ri5 and Ri6 rice were germinated in control solution (CK), and 100 mM NaCl, 200 mM mannitol and 6 μM ABA for 5 days. Germination rate for WT, OE1, Ri5 and Ri6 rice seeds after incubation in control medium and in the presence of 100 mM NaCl, 200 mM mannitol and 6 μM ABA for 7 days (**d**). Data are means ± SE of three replicates with each replicate containing 30 seeds. *Asterisks* represent statistically significant differences between WT and transgenic plants (OE1, Ri5 and Ri6) under conditions of different treatments. * $P < 0.05$, ** $P < 0.01$

significantly shorter than that of WT, while the overexpression line showed longer shoots than WT when NaCl was present in the medium (Fig. 5a). Similar to the effect of NaCl on shoot length, a greater reduction in fresh weight of the two knockdown lines than that of WT was observed when challenged by NaCl (Fig. 5b). In addition, a lower survival rate for the two knockdown lines than for WT was found following irrigation with water containing 200 mM NaCl for 14 days and then recovery for 7 days in the medium without NaCl (Fig. 5c, d). In contrast, there was a higher survival rate for the overexpression line (OE1) than for WT after treatment with 200 mM NaCl for 14 days (Fig. 5c, d). The higher survival rate of OE1 plants than that of WT, Ri5 and Ri6 under salt conditions further supports the improved tolerance of *OsNAC5*-overexpressing plants to salt stress.

To examine whether *OsNAC5* is involved in the tolerance of rice to drought stress, we studied the effect of drought stress on the survival rate of WT and transgenic rice plants by withholding water for 15 days and then allowing them to recover for 7 days by watering them daily (Fig. 5e). The survival rate for RNAi lines was lower than that for WT, while the overexpression line displayed higher survival rates than WT and the RNAi lines (Fig. 5f). These results indicate that overexpressing *OsNAC5* also enhances tolerance of rice seedlings to drought stress.

Proline, soluble sugars, MDA and H_2O_2 contents in transgenic plants

To explore the physiological mechanism responsible for the changes in the tolerance of transgenic rice plants with altered expression levels of *OsNAC5* to low temperature, salt and drought stress, we monitored changes in Pro and soluble sugar contents in WT and transgenic rice in response to varying stresses. Under non-stressed, control conditions, no significant difference in Pro content was detected between WT and the RNAi plants, while Pro content in the overexpression OE1 plants was significantly higher than in WT and RNAi plants (Fig. 6a). There were no significant differences in soluble sugars among WT, RNAi and overexpression plants under control conditions (Fig. 6c). Upon exposure to cold, drought and salt stress, there were marked increases in Pro and soluble sugars contents in both WT and transgenic rice plants (Fig. 6a, c). However, the increases were much less in the two *OsNAC*5-knockdown lines than in WT, while the overexpression line exhibited greater increases in Pro and soluble sugars than WT and the RNAi lines in response to cold, drought and salt stress (Fig. 6a, c).

The changes in Pro accumulation under abiotic stress due to alteration of *OsNAC5* expression in the RNAi and overexpression lines prompt us to investigate the effect of the abiotic stress on Pro synthesis and transport at the transcriptional level. The response of transcripts for the putative Δ^1-pyrroline-5-carboxylate synthetase (*J033099M14*) and two putative Pro transporters (*03g44230* and *07g01090*) in WT and transgenic plants to cold, drought and salt stress was monitored by real-time qPCR. Transcripts of Pro synthetase (*J033099M14*) were greater in OE1 than in WT plants under conditions of cold, drought and salt stress with the highest transcript level occurring in response to *cold* stress (Fig. 6b), while they were less in Ri5 plants under conditions of varying stress. In contrast, transcripts of Pro synthetase

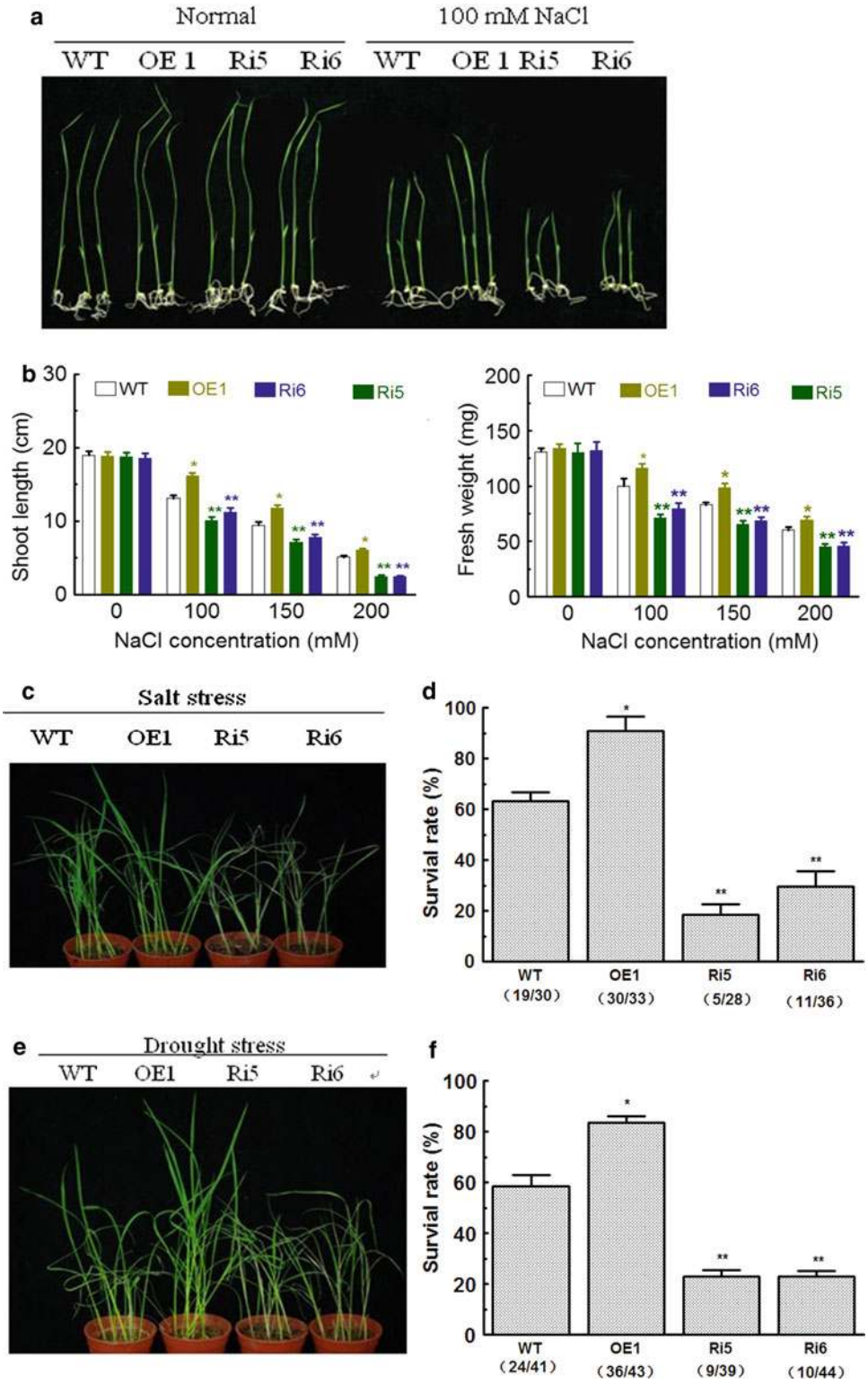

Fig. 5 Phenotypes of wild-type (WT), *OsNAC5*-overexpressing and RNAi seedlings grown in normal and solutions containing 100 mM NaCl (**a**). Effect of various NaCl concentrations applied for 10 days on shoot length and fresh weight of WT, OE1, Ri5 and Ri6 rice seedlings (**b**). Data are means ± SE of 15 seedlings for each treatment with three replicates. Effect of 200 mM NaCl (**c**, **d**) and drought (**e**, **f**) on survival rates of WT, OE1, Ri5 and Ri6 rice seedlings treated with NaCl for 14 days and withholding of water for 15 days. The survival rate data are means ± SE of 8–12 seedlings for each treatment with four replicates. *Asterisks* represent statistically significant differences between WT and transgenic plants (OE1, Ri5 and Ri6) in response to drought and salt stress. * $P < 0.05$, ** $P < 0.01$

(*J033099M14*) were less in Ri5 plants than in WT plants under the stress conditions (Fig. 6b). There were higher transcript levels of Pro transporter (*07g01090*) in OE1 plants than WT under cold and drought, while these transcripts in Ri5 plants were less than in WT under conditions of cold and drought (Fig. 6b). In contrast to cold and drought stress, transcript of Pro transporter (03g44230) was higher and lower in overexpression and RNAi lines than WT plants under salt stress, respectively (Fig. 6b). The transcripts of Pro transporter (07g0101090) were insensitive to salt stress,

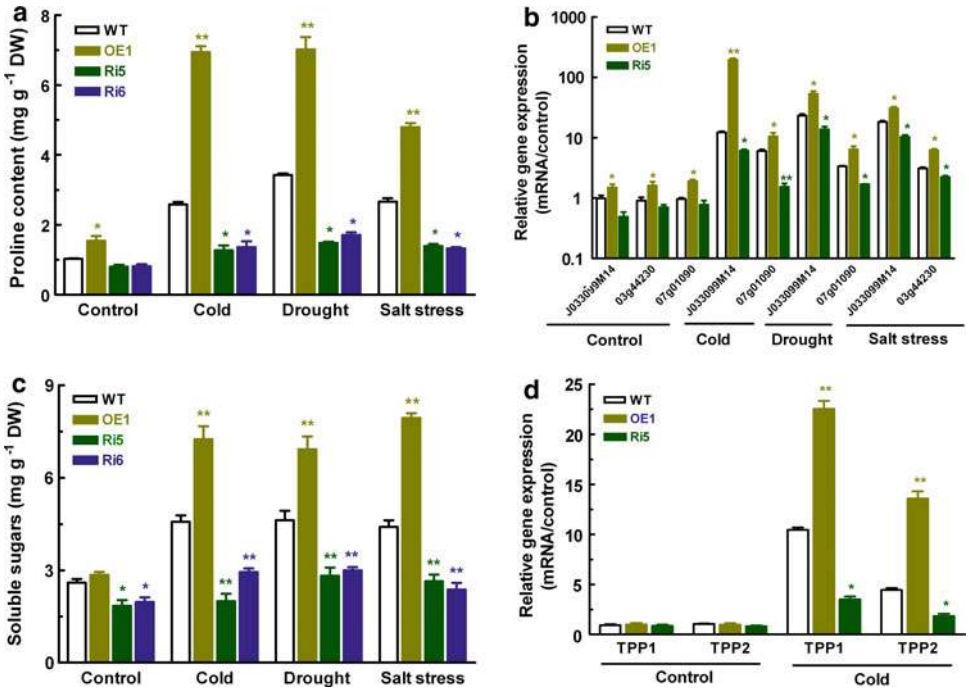

Fig. 6 Effect of cold (4°C for 24 h), drought (withholding water for 6 days) and NaCl (200 mM for 6 days) on Pro contents of WT, OE1, Ri5 and Ri6 rice seedlings (**a**). Expression of genes encoding Pro synthetase (*J033099M14*) and Pro transporters (*03g44230, 07g01090*) (**b**) and on soluble sugar contents of WT, OE1, Ri5 and Ri6 rice seedlings (**c**). Expression of *OsTPP1* and *OsTPP2* of WT, OE1 and Ri5 rice seedlings in response to cold stress (4°C for 24 h; **d**). The control samples grown in 1/2 MS solution were collected at identical time points as samples treated with cold, drought and salt stress. Data are means ± SE with three replicates. *Asterisks* represent statistically significant differences between WT and transgenic plants (OE1, Ri5 and Ri6) in response to cold, drought and salt stress. * $P < 0.05$, ** $P < 0.01$

while the transcripts of Pro transporter (03g44230) were not responsive to cold and drought (data not shown).

Trehalose can protect proteins and cellular membranes from inactivation caused by a variety of abiotic stress (Elbein et al. 2003). Trehalose-6-phosphate phosphatase (TPP) is an enzyme catalyzing the final step of trehalose biosynthesis (Goddijn and van Dun 1999). There is evidence demonstrating that overexpression of *TPP* confers tolerance to cold stress by accumulating trehalose (Garg et al. 2002; Jang et al. 2003; Pramanik and Imai 2005; Ge et al. 2008). We found that expression of *OsTPP1* and *OsTPP2* was suppressed and enhanced in the RNAi (Ri5) and *OsNAC5*-overexpressing (OE1) lines in response to cold stress, respectively (Fig. 6d).

Plants suffering from cold stress often exhibit symptoms of oxidative stress as evidenced by accumulation of reactive oxygen species such as H_2O_2 and malondialdehyde (MDA) (Chinta et al. 2001; Verslues et al. 2007). To test whether the difference in tolerance to cold stress between WT and transgenic rice was related to oxidative stress, effect of cold, drought and salt stress on the contents of H_2O_2 and MDA in WT, RNAi and overexpressing lines was investigated. Both MDA and H_2O_2 contents in the two RNAi lines were higher than in WT plants under non-stressed, control conditions, while overexpression line had lower MDA and H_2O_2 contents than WT under control conditions (Fig. 7). After treatment with cold, drought and salt stress, there was a marked increase in MDA contents in the RNAi lines (Fig. 7a). In contrast, MDA contents in the overexpression line remained relatively unchanged, while WT plants exhibited a moderate increase in MDA contents in response to the treatments (Fig. 7a). A similar change in H_2O_2 content in WT, RNAi and overexpressing lines was observed in response to cold, drought and salt stress, i.e., RNAi lines (Ri5 and Ri6) showed greatest accumulation of H_2O_2, while the overexpressing line (OE1) exhibited the lowest increase in H_2O_2 content in response to cold, drought and salt stress (Fig. 7b). These results are indicative that the expression level of *OsNAC5* in rice is positively correlated with the capacity to counteract oxidative stress evoked by cold, drought and salt stress.

Na^+ content of transgenic plants under salt stress

To test whether the *OsNAC5*-dependent increase in tolerance to salt stress results from the alleviation of the toxic effect of Na^+ ions, we also determined the effect of salt stress on Na^+ and K^+ concentration in leaves of WT, OE1, Ri5 and Ri6 plants. No difference in both Na^+ and K^+ concentrations in shoots of WT, OE1, Ri5 and Ri6 plants

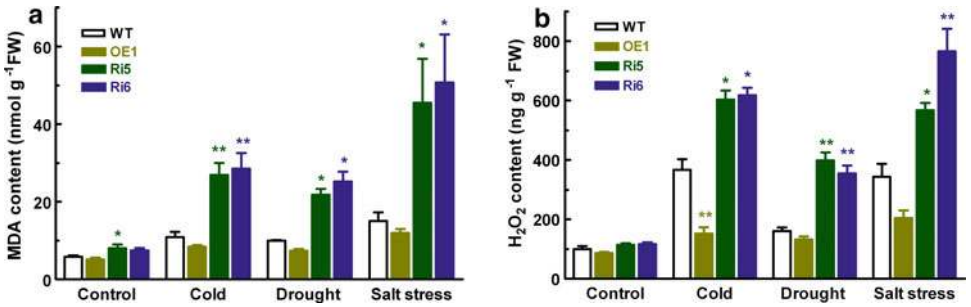

Fig. 7 Effect of cold (4°C, 6 days), drought (withholding water, 10 days) and salt stress (200 mM NaCl, 10 days) on the contents of MDA (**a**) and H_2O_2 (**b**) in WT, OE1, Ri5 and Ri6 rice plants. Data are means ± SE with three replicates. *Asterisks* represent statistically significant differences between WT and transgenic plants (OE1, Ri5 and Ri6) in response to cold, drought and salt stress. * $P < 0.05$, ** $P < 0.01$

was found under control conditions (Fig. 8a, b). There were marked increases in Na^+ concentration in the three types of plants when treated with 100 mM NaCl, and the salt stress-induced increase in Na^+ concentration was highest in Ri5 and Ri6 plants, while Na^+ concentration in OE1 plants was lower than that in WT plants (Fig. 8a). In contrast to Na^+, salt stress equally inhibited K^+ accumulation in all the plants examined (Fig. 8b). Accordingly, salt stress led to an increase in Na^+/K^+ ratio in all the plants with the OE1 and Ri5 displaying the lowest and highest Na^+/K^+ ratio, respectively (Fig. 8c).

The effects of salt stress on the expression of *OsNHX1* and *OsNHX2* in WT and transgenic rice plants were examined at transcriptional levels by real-time qPCR. As shown in Fig. 8d, transcripts of *OsNHX1* and *OsNHX2* in Ri5 plants were less than in WT, and they were higher in the *OsNAC5*-overexpression line than in WT under control conditions. Exposure of WT, Ri5 and OE1 plants to NaCl led to increases in *OsNHX1* and *OsNHX2* transcripts, leading to lower expression of *OsNHX1* and *OsNHX2* in Ri5 plants than in WT, while it was higher in OE1 plants than in WT under salt stress (Fig. 8d). These results imply that OsNAC5 may regulate the salt tolerance by compartmentalizing Na^+ into the vacuoles, minimizing the toxic effect of cytosolic Na^+.

Discussion

NAC is a plant-specific protein family with 151 and 117 members in rice and Arabidopsis genomes, respectively, and the known NAC is divided into five groups according to phylogenetic relationship (Fang et al. 2008; Nuruzzaman et al. 2010). Among the known NAC proteins, the third subgroup that includes *SNAC1, OsNAC6/SNAC2* and *OsNAC5* has been identified to be responsive to abiotic

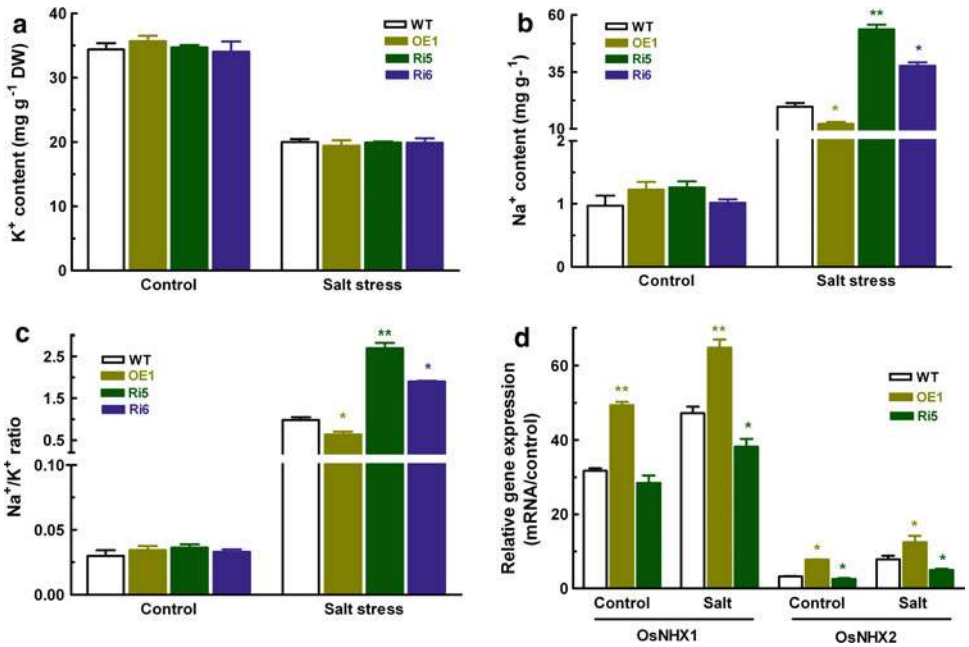

Fig. 8 Effect of 100 mM NaCl on K^+ content (**a**), Na^+ content (**b**), Na^+/K^+ ratio (**c**) and expression of *OsNHX1* and *OsNHX2* (**d**) in leaves of WT, OE1, Ri5 and Ri6 rice plants. The 2-week-old rice seedlings were treated with 100 mM NaCl for 5 days and K^+ and Na^+ contents were determined. Effect of NaCl on expression of *OsNHX1* and *OsNHX2* was determined by exposing WT, OE1, Ri5 and Ri6 rice seedlings to 100 mM NaCl for 24 h. Data are means ± SE with three replicates. *Asterisks* represent statistically significant differences between WT and transgenic plants (OE1, Ri5 and Ri6) in response to salt stress. * $P < 0.05$, ** $P < 0.01$

stress (Hu et al. 2006, 2008; Takasaki et al. 2010). Previous studies have shown that the expression of *OsNAC5* was induced by a number of abiotic stresses such as drought, cold and salt stress (Takasaki et al. 2010). In addition to abiotic stress, expression of *OsNAC5* was also responsive to ABA (Sperotto et al. 2009; Takasaki et al. 2010) and MeJA (Takasaki et al. 2010). In the present study, we confirmed that the expression of *OsNAC5* was up-regulated by cold, drought and salt stress, as well as ABA and MeJA. Our results also revealed that, in addition to ABA and MeJA, other plant hormones such as ethylene, IAA, SA and BR could also induce rapid up-regulation of *OsNAC5* (Fig. 1).

Although it has been reported that overexpression of *OsNAC5* in rice confers tolerance to salt and drought stress (Takasaki et al. 2010), the physiological mechanisms underlying the enhanced tolerance remain to be elucidated. In the present study, we generated transgenic rice plants with knockdown of *OsNAC5* by RNAi technique, and conducted detailed physiological studies using the RNAi lines. Our results demonstrated that knockdown of *NAC5* rendered the transgenic rice plants less tolerant to cold, drought and salt stress, while overexpression of *OsNAC5* in Arabidopsis conferred greater tolerance of Arabidopsis to cold stress. The involvement of OsNAC5 in tolerance of rice plants to abiotic stress was confirmed by the observations that overexpression of *OsNAC5* made the rice more tolerant to cold, drought and salt stress than WT. We further explored the physiological basis responsible for the changes in tolerance of *OsNAC5*-overexpressing and RNAi rice plants to abiotic stress.

Plants have evolved various mechanisms to adapt to abiotic stress such as cold, drought and salinity. Accumulation of compatible solutes, including free Pro (Liu and Zhu 1997; Armengaud et al. 2004; Xiang et al. 2007) and soluble sugars (Gilmour et al. 2000; Garg et al. 2002; Gupta and Kaur 2005), is a common phenomenon in response to abiotic stress. The accumulated Pro and soluble sugars facilitate osmoregulation and/or act as a molecular chaperone to stabilize the structure of proteins and play a role in the regulation of antioxidant systems (Hare et al. 1999; Székely et al. 2008). In the present study, we found that the RNAi plants accumulated much less Pro and soluble sugars than WT plants under cold, drought and salt stress (Fig. 6a, c), and that they exhibited reduced tolerance to these stresses (Figs. 3, 5). In contrast, *OsNAC5*-overexpressing rice plants had greater amounts of Pro and soluble sugar contents (Fig. 6a, c) and displayed enhanced tolerance to cold, drought and salt stress (Figs. 3, 5). These findings provide direct evidence in support of the argument that enhanced tolerance of *OsNAC5*-overexpressing plants is related to greater accumulation of Pro and soluble sugars. Our results also revealed that the transcripts of Pro synthetase (*P5CS*) and Pro transporter in *OsNAC5*-overexpressing plants were higher than those in WT plants, while these transcripts in RNAi plants were much less than in WT plants (Fig. 6b). In addition, the alteration of Pro accumulation in transgenic rice may also result from Pro degradation. For example, cold acclimation-induced Pro accumulation in Arabidopsis is related to increases in transcript of P5CS1 and decrease in transcript of ProDH, a gene responsible for Pro degradation (Zhao et al. 2009). Although we cannot exclude the possible involvement of ProDH in the changes in Pro accumulation, our results suggest that the up-regulation of *OsNAC5* facilitates Pro synthesis and transport, leading to greater accumulation of Pro in *OsNAC5*-overexpressing plants. Several reports demonstrate that overexpression of transcriptional factors enhances tolerance to abiotic stress due to Pro accumulation resulting from up-regulation of Pro synthetase and transport genes (Kishor et al. 1995; Liu and Zhu 1997; Xiang et al. 2008).

Trehalose, the key enzyme catalyzing the final step of trehalose biosynthesis (Goddijn and van Dun 1999), is a disaccharide sugar that is widely found in fungi, insects and higher plants (Strom and Kaasen 1993; Elbein et al. 2003; Shima et al. 2007). Trehalose can protect proteins and cellular membranes from inactivation induced by a variety of stress (Elbein et al. 2003). It has been reported that the expression of *OsTPPs* is induced by chilling stress (Su et al. 2010). There is evidence that overexpression of *TPP* enhances the accumulation of trehalose and tolerance to cold (Garg et al. 2002; Jang et al. 2003; Pramanik et al. 2005; Ge et al. 2008). Our findings that the expression of *OsPP1* and *OsPP2* are positively dependent on expression levels of *OsNAC5* imply that overexpression of *OsNAC5* may also result in greater accumulation of trehalose due to up-regulation of genes encoding TPP. In addition to increase in accumulation of trehalose, it has also been shown that overexpression of *OsTPP1* triggers expression of several abiotic stress-induced genes, conferring tolerance of rice to cold and salt stress without alteration of trehalose contents (Ge et al. 2008). Regardless of the nature underlying the enhanced tolerance of *OsTPP1*-overexpressing plants, our findings indicate that OsTPP is likely to be a downstream target of OsNAC5 in response to abiotic stresses.

Abiotic stress such as drought, cold and salinity often cause accumulation of ROS and induce lipid peroxidation, leading to oxidative stress (Xiong et al. 2002). In the present study, we found that *OsNAC5*-overexpressing rice plants generated less amounts of H_2O_2 and MDA than wild-type plants under conditions of drought, cold and salt stress (Fig. 7). In contrast, *OsNAC5*-underexpression lines (Ri5, Ri6) exhibited greater accumulation of H_2O_2 and MDA than both wild-type and overexpression plants when

challenged by cold, drought and salt stress (Fig. 7). MDA is a decomposition product of polyunsaturated fatty acids, and has been widely used as a parameter for lipid peroxidation (Mittler 2002). These findings suggest that expression of *OsNAC5* can suppress oxidative stress associated with cold, drought and salt stress, thus making the *OsNAC5*-overexpressing plants suffer less from oxidative damage than wild-type plants.

Exposure of plants to solution containing NaCl would cause osmotic stress, oxidative stress and sodium toxicity. The greater accumulation of soluble sugars and Pro and less accumulation of H_2O_2 and MDA in *OsNAC5*-overexpressing plants than in wild-type plants under salt stress suggests that *OsNAC5* may confer a higher tolerance to osmotic and oxidative stress to plants. To unravel the mechanism underpinning the enhanced tolerance of *OsNAC5*-overexpressing plants to salt stress, we also measured Na^+ contents in leaves of both wild-type and transgenic rice plants in the absence and presence of 100 mM NaCl in the growth medium. One finding is that overexpression of *OsNAC5* reduced Na^+ contents in shoots; while knockdown of *OsNAC5* stimulated Na^+ accumulation in comparison with wild-type plants under salt stress (Fig. 8a). In contrast to Na^+, K^+ contents in rice shoots were independent of *OsNAC5* expression, such that there was no difference in K^+ contents among *OsNAC5* overexpression, knockdown and wild-type plants in the absence and presence of NaCl in the growth medium(Fig. 8b). Accordingly, RNAi plants exhibited a higher Na^+/K^+ ratio than WT, while a lower Na^+/K^+ ratio in *OsNAC5*-overexpressing plants than in WT plants was observed (Fig. 8c). The lower Na^+/K^+ ratio would be beneficial for plants to tolerate salt stress as observed in the *OsNAC5*-overexpressing plants. Under salt stress, Na^+ can enter the cytosol via several pathways and can become toxic to cytosolic enzymes at high concentrations. Sequestration of Na^+ into vacuoles mediated by Na^+/H^+ antiporters in the tonoplast is an important mechanism to minimize toxic effect and maintain cytosolic pH under salt stress (Orlowski and Grinstein 1997; Apse et al. 1999). In addition, the sequestration reduces Na^+ concentration in the cytoplasm and contributes to water uptake from saline solutions by osmotic adjustment (Zhu 2003). Therefore, compartmentalization of Na^+ into vacuoles is an essential strategy for salt tolerance in plants. There have been numerous reports showing that overexpression of *NHX* enhances tolerance to salt stress. For instance, Fukuda et al. (2004) demonstrated that overexpression of *OsNHX1* confers tolerance of rice to salt stress. In the present study, we found that the expression levels of *OsNHX1* and *OsNHX2*, which encode tonoplast Na^+/H^+ antiporters, were positively correlated with the expression levels of *OsNHX1* and *OsNHX2* under both control and salt-stressed conditions (Fig. 8d). The greater expression of *OsNHX1* and *OsNHX2* in *OsNAC5*-overexpressing plants would allow more Na^+ ions to be stored in the vacuoles, thus conferring plants more tolerance to salt stress.

In addition to abiotic stress, expression of *OsNAC5* was also sensitive to several phytohormones (Fig. 1d). Previous studies have shown that the expression of *OsNAC5* is induced by ABA (Sperotto et al. 2009; Takasaki et al. 2010) and MeJA (Takasaki et al. 2010). Our finings confirmed these results. ABA plays a critical role in stress signaling pathway (Choi et al. 2000; Kang et al. 2002; Kim et al. 2004; Zou et al. 2008; Xiang et al. 2008). We investigated the response of germination of *OsNAC5*-overexpressing and *OsNAC5*-underexpressing rice seeds to exogenous ABA. We found that overexpressing *OsNAC5* rendered seed germination more sensitive to ABA than of wild-type, while germination of RNAi seeds was less inhibited by ABA (Fig. 4c, d). Given that over- and knockdown expression of *OsNAC5* made seed germination more and less tolerant to NaCl and mannitol (Fig. 4), and abiotic stress such as cold, drought and salt stress often induced increase in ABA synthesis (Nambara and Marion-Poll 2005), it remains to be established whether ABA plays a regulatory role in the function of *OsNAC5* in response to abiotic stress.

Tran et al. (2004) identified the complete NAC recognition sequences, which include CATGT and CACG and with the latter one being the core sequence. Takasaki et al. (2010) determined that OsNAC5 can specially bind to the NAC recognition sequence of the OsLEA3 promoter (−56 to −85 bp, containing the complementary sequence of CACG). We analyzed the promoters of downstream genes and found that all of the genes examined in the present study contained the NAC core motif, CACG, in their 1.5-kb promoter regions. Further analyses revealed that there were 15 and 33 CACG motifs in *OsNHX1* and *OsNHX2* promoters, respectively, with four CACG motifs being identified in the region of −17 to −104 in *OsNHX1* promoter. Therefore, it is likely that OsNAC5 can bind to the NAC recognition sequence in the *OsNHX1* and *OsNHX2* promoters to enhance the expression of *OsNHX1* and *OsNHX2*. Promoters of sugar-related genes, *OsTPP1* and *OsTPP2*, contained one CACG motif in −140 to −150 and two CACG motifs in −70 to −110 regions, respectively. Similarly, promoters of *J033099M14* and *03g44230* had one CACG motif in −270 to −280 and two CACG in −200 to −220 regions, respectively. In contrast, only two CACG motifs were found in the promoter of *07g01090* and they were far from the transcriptional start site (1 kb). These findings suggest that *OsTPP1*, *OsTPP2*, *J033099M14* and *03g44230*, but not *07g01090*, may be direct targets of OsNAC5.

In summary, we found that *OsNAC5* expression was rapidly induced by abiotic stresses such as salt, drought, cold as well as a number of plant hormones. The involvement of *OsNAC5* in tolerance to cold, drought and salt stress was demonstrated by the fact that overexpression and underexpression of *OsNAC5* conferred greater and less tolerance to these stresses, respectively. We further identified that the reduced tolerance by underexpression of *OsNAC5* may result from their capacity to accumulate less soluble sugars and Pro, and greater accumulation of H_2O_2 and MDA. This argument was supported by the fact that *OsNAC5*-overexpressing rice plants accumulated greater amounts of Pro and soluble sugars, and less amounts of MDA and H_2O_2. These metabolic changes would protect plants from dehydration and oxidative damage under stressed conditions. We also found that knockdown of *OsNAC5* led to greater accumulation of toxic Na^+ in leaves due to suppressed up-regulation of genes encoding tonoplast Na^+/H^+ antiporters. Therefore, taken together, our findings highlight that OsNAC5 is an important regulator involved in modulation of several downstream functional genes in response to abiotic stresses.

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References

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. Plant Cell 9:841–857

Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24:1337–1344

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis thaliana*. Science 285:1256–1258

Armengaud P, Thiery L, Buhot N, Grenier-de March G, Savouré A (2004) Transcriptional regulation of proline biosynthesis in *Medicago truncatula* reveals developmental and environmental specific features. Physiol Plant 120:442–450

Bailey RW (1958) The reaction of pentoses with anthrone. Biochemistry J 68:669–672

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205–207

Chinta S, Lakshmi A, Giridarakumar S (2001) Changes in the antioxidant enzyme efficacy in two high yielding genotypes of mulberry (*Morus alba* L.) under NaCl salinity. Plant Sci 161: 613–619

Choi HI, Hong JH, Ha JO, Kang JY, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem 275: 1723–1730

Collinge M, Boller T (2001) Differential induction of two potato genes, Stprx2 and StNAC, in response to infection by *Phytophthora infestans* and to wounding. Plant Mol Biol 46:521–529

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13:17R–27R

Fang YJ, You J, Xie KB, Xie WB, Xiong LZ (2008) Systematic sequence analysis and identification of tissue-specific or stress-responsive genes of NAC transcription factor family in rice. Mol Genet Genomics 280:547–563

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J 39:863–876

Fukuda A, Nakamura A, Tagiri A, Tanaka H, Miyao A, Hirochika H, Tanaka Y (2004) Function, intracellular localization and the importance in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. Plant Cell Physiol 45:146–159

Garg AK, Kim JK, Owens TG, Ranwala AP, Choi YD, Kochian LV, Wu RJ (2002) Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 99:15898–15903

Ge LF, Chao DY, Shi M, Zhu MZ, Gao JP, Lin HX (2008) Overexpression of the trehalose-6-phosphate phosphatase gene OsTPP1 confers stress tolerance in rice and results in the activation of stress responsive genes. Planta 228:191–201

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol 124:1854–1865

Goddijn OJM, van Dun K (1999) Trehalose metabolism in plants. Trends Plant Sci 4:315–319

Gupta AK, Kaur N (2005) Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. J Biol Sci 30:761–776

Hare PD, Cress WA, van Staden J (1999) Proline synthesis and degradation: a model system for elucidating stress-related signal transduction. J Exp Bot 50:413–434

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J 44:903–916

Hegedus D, Yu M, Baldwin D, Gruber M, Sharpe A, Parkin I, Whitwill S, Lydiate D (2003) Molecular characterization of *Brassica napus* NAC domain transcriptional activators induced in response to biotic and abiotic stress. Plant Mol Biol 53:383–397

Hu HH, Dai MQ, Yao JL, Xiao BZ, Li XH, Zhang QF, Xiong LZ (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proc Natl Acad Sci USA 35:12987–12992

Hu HH, You J, Fang YJ, Zhu Xiaoyi, Qi ZY, Xiong LZ (2008) Characterization of transcription factor gene SNAC2 conferring cold and salt tolerance in rice. Plant Mol Biol 67:169–181

Jang IC, Oh SJ, Seo JS, Choi WB, Song SI, Kim CH, Kim YS, Seo HS, Choi YD, Nahm BH, Kim JK (2003) Expression of a bifunctional fusion of the *Escherichia coli* genes for trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase in transgenic rice plants increases trehalose accumulation and abiotic stress tolerance without stunting growth. Plant Physiol 131:516–524

Kang JY, Choi HI, Im MY, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell 14:343–357

Kim SY, Kang JY, Cho DI, Park JH, Kim SY (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. Plant J 40:75–87

Kishor PBK, Hong ZL, Miao GH, Hu CAA, Verma DPS (1995) Overexpression of Δ-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108:1387–1394

Liu J, Zhu JK (1997) Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of Arabidopsis. Plant Physiol 114:591–596

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell 10:1391–1406

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)). Methods 25:402–408

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Mukhopadhyay A, Vij S, Tyagi AK (2004) Overexpression of a zinc finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. Proc Natl Acad Sci USA 101:6309–6314

Nakashima K, Tran LS, Van Nguyen D, Fujita M, Maruyama K, Todaka D, Ito Y, Hayashi N, Shinozaki K, Yamaguchi-Shinozaki K (2007) Functional analysis of a NAC-type transcription factor OsNAC6 involved in abiotic and biotic stress-responsive gene expression in rice. Plant J 51:617–630

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional regulatory networks in response to abiotic stresses in Arabidopsis and grasses. Plant Physiol 149:88–95

Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56:165–185

Nuruzzaman M, Manimekalai R, Sharoni AM, Satoh K, Kondoh H, Ooka H, Kikuchi S (2010) Genome-wide analysis of NAC transcription factor family in rice. Gene 465:30–44

Ohnishi T, Sugahara S, Yamada T, Kikuchi K, Yoshiba Y, Hirano HY, Tsutsumi N (2005) *OsNAC6*, a member of the *NAC* gene family, is induced by various stresses in rice. Genes Genet Syst 80:135–139

Olsen AN, Ernst HA, Lo LL, Johansson E, Larsen S, Skriver K (2004) Preliminary crystallographic analysis of the NAC domain of ANAC, a member of the plant-specific NAC transcription factor family. Acta Crystallogr D Biol Crystallogr 60:112–115

Orlowski J, Grinstein S (1997) Na^+/H^+ exchangers of mammalian cells. J Biol Chem 272:22373–22376

Pramanik MH, Imai R (2005) Functional identification of a trehalose 6-phosphate phosphatase gene that is involved in transient induction of trehalose biosynthesis during chilling stress in rice. Plant Mol Biol 58:751–762

Ren T, Qu F, Morris TJ (2000) HRT gene function requires interaction between a NAC protein and viral capsid protein to confer resistance to turnip crinkle virus. Plant Cell 12:1917–1925

Shima S, Matsui H, Tahara S, Imai R (2007) Biochemical characterization of rice trehalose-6-phosphate phosphatases supports distinctive functions of these plant enzymes. FEBS J 274:1192–1201

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6:410–417

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996) *The no apical meristem* gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell 85:159–170

Sperotto RA, Ricachenevsky FK, Duarte GL, Boff T, Lopes KL, Sperb ER, Grusak MA, Fett JP (2009) Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. Planta 230:985–1002

Strøm AR, Kaasen I (1993) Trehalose metabolism in *Escherichia coli*: stress protection and stress regulation of gene expression. Mol Microbiol 8:205–210

Székely G, Ábrahám E, Cséplo Á, Rigo G, Zsigmond L, Csiszár J, Ayaydin F, Strizhov N, Jásik J, Schmelzer E, Koncz C, Szabados L (2008) Duplicated *P5CS* genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. Plant J 53:11–28

Takasaki H, Maruyama K, Kidokoro S, Ito Y, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K, Nakashima K (2010) The abiotic stress-responsive NAC-type transcription factor OsNAC5 regulates stress-inducible genes and stress tolerance in rice. Mol Genet Genomics 284:173–183

Thomashow MF (1999) Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 50:571–599

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress. Plant Cell 16:2481–2498

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water. Plant J 45:523–539

Verslues PE, Batelli G, Grillo S, Agius F, Kim YS, Zhu JH, Agarwal M, Katiyar-Agarwal S, Zhu JK (2007) Interaction of SOS_2 with nucleoside diphosphate kinase 2 and catalases reveals a point of connection between salt stress and H_2O_2 signaling in *Arabidopsis thaliana*. Mol Cell Biol 27:7771–7780

Xiang Y, Huang YM, Xiong LZ (2007) Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. Plant Physiol 144:1416–1428

Xiang Y, Tang N, Du H, Ye HY, Xiong LZ (2008) Characterization of OsbZIP23 as a key player of the Basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. Plant Physiol 148:1938–1952

Xie Q, Sanz-Burgos AP, Guo HS, Garcia JA, Gutierrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol 39:647–656

Xie Q, Frugis G, Colgan D, Chua NH (2000) *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 14:3024–3036

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14:S165–S183

Yang YZ, Peng H, Huang HM, Wu JX, Ha SR, Huang DF, Lu TG (2004) Large-scale production of enhancer trapping lines for rice functional genomics. Plant Sci 167:281–288

Zhang XR, Henriques R, Lin SS, Niu QW, Chua NH (2006) *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nat Protoc 1:641–646

Zhang L, Tian LH, Zhao JF, Song Y, Zhang CJ, Guo Y (2009) Identification of an apoplastic protein involved in the initial phase of salt stress response in rice root by two-dimensional electrophoresis. Plant Physiol 149:916–928

Zhao MG, Chen L, Zhang LL, Zhang WH (2009) Nitrate reductase-mediated nitric oxide production is involved in cold acclimation and freezing tolerance in Arabidopsis. Plant Physiol 151:755–767

Zhong RQ, Demura T, Ye ZH (2006) SND1, a NAC domain transcription factor, is a key regulator of secondary wall synthesis in fibers of Arabidopsis. Plant Cell 18:3158–3170

Zhu JK (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441–445

Zou MJ, Guan YC, Ren HB, Zhang F, Chen F (2008) A bZIP transcription factor, OsABI5, is involved in rice fertility and stress tolerance. Plant Mol Biol 66:675–683