Regulation of Abiotic Stress Response Through *NtROS2a*-mediated Demethylation in Tobacco

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ABSTRACT DNA methylation is a common and rapid evolving epigenetic mechanism among higher eukaryotic organisms including cytosine residues in CG and CNG sequences. *NtROS2a* gene isolated from tobacco plants (*Nicotiana tabacum* cv Xanthi nc) contains DNA glycosylase domain with significant similarities to *Arabidopsis* ROS1 and DME at the C-terminus. Purified *NtROS2a* protein gene is 5,879 bp long and is designated as *Nicotiana tabacum* Repressor Of Silencing (*NtROS*: accession numbers; AB281588). RT-PCR analysis initially revealed the adaptive response of the lines overexpressing the gene in tobacco to various abiotic stresses. This was validated when phenotypic evaluation to cold, salt, simulated drought and osmotic stress tolerance was performed. Reduction in leaf wilting rate against high salinity treatment is remarkable in transgenic plants which showed at least 50% less wilting than the WT. Moreover, about 70% wilt rate difference was noted between the transgenic and WT in H₂O₂ treatment. This study showed that *NtROS2a* gene encoding DNA glycosylase which caused demethylation could regulate adaptive response of tobacco to various abiotic stress conditions.

Keywords DNA methylation, Demethylation, Repressor of silencing, *Nicotiana tabacum*, Transgenic plant

INTRODUCTION

Epigenetics is a change in gene expression through chromatin modifications without base sequence alteration (Law and Jacobsen 2010), which facilitates recruitment of the RNA polymerase II transcriptional machinery to the promoter. This frequently occurs during somatic cell differentiation, in which the clonal expansion of a single cell leads to a diversity of cell types (Zhang *et al.* 2006). Such a cellular inheritance is common during ontogeny but is usually erased before gametes are produced (Reik and Walter 2001). In eukaryotes, DNA methylation has important roles in the establishment of developmental programs in fungi, plants, and animals. DNA methylation of mammalian and plant is restricted to symmetrical CpG sequences, but plants also have significant levels of

cytosine methylation in the symmetric context CpNpG (where N is any nucleotide), CpNpN (N is A, T or C) and even in a symmetric contexts (Bird 2002). DNA methylation is associated with numerous functions, depending on the model organism and the environment component. In the experiment involving mice, DNA methylation has been shown to be essential for proper embryonic development (Li *et al.* 1992). Similarly, a loss of DNA methylation caused developmental defects in *Xenopus* embryos (Stancheva and Meehan 2000). In parallel with plants, reduced levels of DNA methylation were shown to be responsible for a large number of developmental abnormalities (Finnegan *et al.* 1996; Ronemus *et al.* 1996; Cao and Jacobsen 2002; Kankel *et al.* 2003). The triple mutant that lacks DRM1, DRM2, and CMT3, which has greatly reduced non-CG methylation (Cao and Jacobsen 2002) and the mutant that

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lacks MET1, in which both CG and, to a lesser extent, non-CG methylation are decreased. Superficial similarities in some of the developmental defects have been interpreted to reflect a conserved function of DNA methylation. However, the molecular consequences of genomic DNA methylation have been found to be surprisingly diverse and broad (Cao and Jacobsen 2002; Kankel *et al.* 2003). However, unlike methylation, studies on de-methylation are still limited. In contrast to the well-studied genetics, biology and biochemistry of cytosine DNA methyltransferases, the enzymatic basis of active demethylation have remained elusive (Morales-Ruiz *et al.* 2006). Direct removal of the methyl group from 5-meC residues has been reported, but this mechanism, which involves a thermodynamically unfavorable breakage of the carbon-carbon bond, has been questioned and could not be independently reproduced (Agius *et al.* 2006). Recent work in plants has added genetic evidence for a role of DNA glycosylases in an active demethylation pathway. In Arabidopsis, recent genetic and biochemical studies demonstrated that two bifunctional DNA glycosylase/lyases, ROS1 and Demeter, function as DNA demethylase (Gehring *et al.* 2006; Agius *et al.* 2006; Morales-Ruiz *et al.* 2006). ROS1 can specifically recognize methylated DNA substrate. Its glycosylase activity removes the 5-methylcytosine base, and its lyase activity nicks the DNA backbone at the abasic site by α , β elimination mechanism (Agius *et al.* 2006; Morales-Ruiz *et al.* 2006). Methylation status dynamically changes depending upon cell type, developmental stages and even environmental conditions (Demeulemeester *et al.* 1999; Steward *et al.* 2002; Chakrabarty *et al.* 2003; Wada *et al.* 2004). First, because DNA methylation in nucleosomes induces alteration of gene expression by changing chromatin structures; vast demethylation may serve as a common switch for many genes that are simultaneously controlled upon environmental cues. Second, because artificial demethylation induces heritable changes in plant phenotype (Sano *et al.* 1990); altered DNA methylation may result in epigenetic inheritance, in which gene expression is modified without changing the nucleotide sequence. To investigate the function of *NtROS2a* gene encoding DNA glycosylase in various abiotic stress conditions, we developed transgenic tobacco plants using overexpression and RNAi technique and subjected them to

cold, salt and heat stress.

MATERIALS AND METHODS

Gene isolation and cloning

Total RNA was isolated from tobacco samples by acid guanidium thiocyanate-phenol -chloroform (AGPC) method (Chomczynski and Sacchi 1987) with a slight modification, and used for cDNA library construction with the ZapII vector (Stratagene, La Jolla, CA, USA). In brief, cDNAs were ligated to the vector at the *EcoRI* and *XhoI* sites, and after transformation, the library was screened with ³²P-labeled probes. Positive clones were rescued in the pBluescript SK-phagemid vector by *in vivo* excision, and amplified in *Escherichia coli* DH5 α . The nucleotide sequences were determined by the dideoxynucleotide chain termination method (PRISM BigDye Terminator, ABI, Sunnyvale, CA, USA), and analyzed with appropriate computer software (GeneWorks, National Center for Biotechnology Information, and PSORT server). Genomic sequence was obtained by PCR using genomic DNA as the template and appropriate primer sets designed after the cDNA sequence. Resulting fragment was 10,243 bp, including a 1918 bp upstream region from the transcription initiation site (accession number AB267678).

Ti-plasmid vector construction for plant transformation

To produce transgenic plants carrying *NtROS2a* gene, the *NtROS2a* cDNA was ligated into the pBI121 and pKANNIBAL vector and electroplated into *Escherichia coli* strain XL10-Gold (Stratagene) resulting in 1.5×10^6 transformants. The construct was confirmed by PCR amplification using the 35s promoter (F-5' TGAGAC-TTTTCAACAAAGGGTA), and *NtROS2a* (R-3' GGG-AACTGATGAGCGGTTTG) primers. The construct was electroporated into *Agrobacterium tumefaciens* strain LBA4404 (Koncz and Schell 1986) and 2.6×10^6 cfu/ml inoculum was obtained 48 hours after shake-incubation of the bacteria in 25 ml LB medium at 240 rpm speed at 28°C and stored in 50% glycerol at -80°C until use.

Generation of *NtROS2a* transgenic tobacco

Seeds of wild type tobacco were surface sterilized by washing with 70% ethanol for 1 min, 3% sodium hypochlorite for 10 min, and rinsed four times with sterile distilled water. The seeds were then allowed to germinate in glass containers with 25 cm³ of MS-based medium (Murashige and Skoog 1962). For transformation experiments, tobacco was grown on MS medium in a Magenta culture box (Sigma, USA) in a culture room at 28°C under an 18 h (day)/6 h (night) photoperiod. Sterile leaf disks were co-cultivated with *Agrobacterium* and planted onto a medium without antibiotics. After incubation at 28°C for two days, the leaf discs were transferred to MS medium supplemented with 6-benzylaminopurine (BA) 1 mg/L and 1-naphthalene acetic acid (NAA) 0.1 mg/L for callus and shoot induction. For root induction, MS medium containing hygromycin 50 mg/L was used. The media for callus and shoot, and root induction contained 8 g/L of agar, 50 mg/L hygromycin and the cultures were maintained at 28°C under light condition. After root induction, putative transgenic plants were transferred immediately to MS medium without plant hormones.

Genomic DNA isolation

Total DNA of transgenic tobacco was extracted from a ground freeze-dried leaf tissue (200 mg) using the cetyltrimethyl ammonium bromide (CTAB) method. After adding the CTAB buffer, the samples were incubated in a 55°C water bath for 15 min. After incubation, the extract mixture was spun at 1200 rpm for 5 min and the supernatant were transferred into clean tubes and added with 250 µl of chloroform: isoamyl alcohol (25:1). The mixture was mixed gently by inverting for 5 min and was centrifuge at 13,000 rpm for 3 min at room temperature. The aqueous phase were collected into a clean tube added with 0.08 vol. of cold 7.5 M ammonium acetate and 0.8 vol. of cold isopropanol (-20°C). The mixture was kept in the freezer for 30 min, and the DNA was collected by centrifugation at 13,000 rpm for 10 min. The pellet was washed with 500 µl 70% ethanol, air-dried and resuspended in 50-100 µl of ddH₂O.

PCR analysis

The presence of transgene in putative transgenic plants was detected by PCR method with 35S promoter F-5' (5'-TGAGACTTTTCAACAAAGGGTA-3') and *NtROS2a* gene R-(5'-GGGAAGTGTGAGCGGTTTG-3') primer pair. The assay was performed in a total 50 µl volume containing 100 pg DNA, 10 pmol each of primers, 2.5mM dNTPs, 10X PCR buffer, and 0.5 U *Taq*-polymerase (Takara, Japan). The PCR reaction was carried out by denaturing the template at 94°C for 5 min followed by 35 cycles of amplification (1 min at 94°C, 1 min at 50°C and 2 min at 72°C) and by extension at 72°C for 5 min.

Gene expression analysis under the abiotic stresses

Tobacco Seeds (*Nicotiana tabacum* cv Xanthine) were grown under controlled conditions (28°C day / 25°C night temperatures, 14 h light / 10 h darkness, 75% relative humidity) in MS medium in a Magenta culture box (Sigma, USA). After three weeks of germination, seedlings were exposed to high-salinity (250 mM), cold (4°C), H₂O₂ (3%), and 100 µM ABA stresses. The plant tissues were harvested after 0, 30 min, 6h, and 8h of stress treatment, immediately frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Real-time PCR was conducted using RT pre-mix (TOYOBO Co., Japan) in a Bio-RAD I Cycler IQ5 machine. The threshold cycle (Ct) values of the triplicate PCRs were averaged and the relative quantification of the expression levels was performed using the comparative Ct method (Livak *et al.* 2001). The fold change in total RNA (target gene) relative to the reference gene (actin gene) was determined by the following formula: fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{actin gene}})_{\text{transgenic plants}} - (Ct_{\text{target gene}} - Ct_{\text{actin gene}})_{\text{wild-type plants}}$. The following primers were used for the analysis, *NtROS2a*: (5'-CACTT-TTGAAGGGTTGGACA-3') and R (5'-CGTATGCT-GTTGCATGAGGAA-3'), and Actin F: (5'-CGCCCGT-CATCGTGC GGTTTC-3') and R: (5'-TCCGCGGGGTCC-ATGG TCTG-3'). RT-PCR analysis of vegetative tissue (leaf, stem, and root) was conducted using 50 ng of total RNA treated with DNase I. The amplification program

consisted of an initial step at 42°C for 60 min, 94°C for 5 min, 40 cycles (94°C for 15 sec, 55°C for 30 sec, 72°C for 30 sec), and a final step at 72°C for 10 min, and the amplified fragments were loaded on 1.2% agarose gel.

Germination assays under salt and drought stresses

All seeds in the following assays were surface sterilized and kept at 4°C for 2 d to break dormancy before subjecting them to any of the experimental treatments. For the germination assay, the tobacco seeds were plated in one-half MS medium (0.6% agar) added separately with PEG6000 or NaCl, and the number of germinated seeds was counted every day. For root elongation assay, the germinated seeds were first plated upright in MS plates until the roots were about 0.5 cm, then the seedlings were transferred into the same medium supplemented with PEG6000 or NaCl, in the growth chamber. The root lengths were recorded before and after the treatments.

Leaf discs assay and chlorophyll contents in transgenic plants

Tobacco seeds (*Nicotiana tabacum* cv Xanthi nc) were grown under controlled conditions having 28°C day / 25°C night temperatures, 14 h (light) / 10 h (darkness) in MS medium in a Magenta culture box (Sigma, USA), and 75% relative humidity. Three weeks after germination, healthy and fully expanded leaves of wild-type and transgenic plants were detached. Leaf discs of about 1 cm in diameter were cut and floated in 1/2 MS medium containing separately either 250 mM NaCl or 3% H₂O₂ for 6 d. The treatment was performed in continuous white light at 25°C. To examine the chlorophyll contents, the leaf discs obtained from transgenic and WT plants were floated in 1/2 MS liquid medium containing 250 mM NaCl, 3% H₂O₂ without sucrose (MSH) for 7 d under light at 25°C. Measurement of chlorophyll fluorescence was performed according to Aono *et al.* (1993).

RESULTS

Characterization of *NtROS2a* genes

Two cDNA fragments were initially isolated from

tobacco leaves RNA by reverse transcriptase-PCR using a set of degenerate primers which were designed based on the conserved DNA glycosylase domain being known as plant 5-methylcytosine DNA glycosylase proteins (<http://www.chromdb.org>). One of the two fragments cloned, designated as *Nicotiana tabacum* Repressor Of Silencing 2a (*NtROS2a*), (Fig. 1A) (accession numbers AB281588 for *NtROS2a*) is 5879bp long and is highly associated to XP_002267310 (*Vitis vinifera*) and ABC61677 (*A. thaliana*). This clone encoded a protein of 1,673 amino acids with predicted molecular mass of 186 kDa. A pair-wise comparison of their deduced amino acid sequences with ABC61677 (*A. thaliana*) showed the similarity of *NtROS2a* at 32.2%. However, the similarity at the glycosylase domain with ABC61677 (*A. thaliana*) was 94.3% for *NtROS2a* (amino acid positions, 1,291-1,459). A pair-wise comparison of *NtROS2a* indicated the similarity of *NtROS2b* to be 99.6%. Notably, only five out of 1,673 total amino acids (0.4%) differed between *NtROS2a* and *NtROS2b*, suggesting them to be isoforms. Clustering starts in the root node with the training and evaluation of 17 DNA glycosylase classifiers (Fig. 1B). The species representing the DNA glycosylase group are divided into two groups under the same parent roots. *NtROS2a* gene is grouped to other roots compared with genes related to demethylation. This result indicates that *NtROS2a* is a unique gene related to demethylation. GFP fluorescence in BY-2 cell suspensions was examined after transformation. About 70% of round-shaped calli that were obtained after the *Agrobacterium*-mediated transformation of BY-2 cells displayed GFP fluorescence intensity sufficient for reliable evaluation of its homogeneity (data not shown). Subcellular localization of *NtROS2a* gene was analyzed by *Agrobacterium*-mediated transient expression of a *NtROS2a*-GFP fusion gene in onion epidermal cells (Fig. 2). The cells transformed with *NtROS2a*-GFP displayed fluorescence specifically in the nucleus, suggesting that *NtROS2a* is a nucleus-localized protein.

Gene expression under various stresses

In plants, *NtROS2a* play a direct regulatory role in response to multiple signal stimulation. To clarify the potential function of *NtROS2a* in response to different

Fig. 1. (A); Alignment of conserved m⁵C DNA glycosylase domain of *NtROS2a*, AB281588; *NtROS2b*, AB281589 (*Nicotian tabaccum*); XP_002267310 (*Vitis vinifera*); ABC61677 (*A. thaliana*) Common amino acids among for proteins are indicated by *closed box*, and *grey boxes* indicate conservative changes. Conserved lysine and aspartic acid residues in the glycosylase/lyase active site (InterPRO search, IPRO11257) are indicated by *closed arrowheads*, and four cysteine residues predicted to function in DNA binding are indicated by *closed circles*. (B); Unrooted phylogenetic tree. Amino acid sequences were aligned using the ClustalW program. Genebank accession numbers are *NtROS1*, AB281587; *NtROS2a*, AB281588; *NtROS2b*, AB281589; *NtROS3*, AB281590 (*N. tabacum*); XP_002267310 (*Vitis vinifera*); *Nth1*, CAC16135; *AtDME*, Q8LK56; *AtDML2*, Q9SR66; *AtDML3*, Q49498; *DME*, NP_196076; *DEMETER*, ABC61677 (*A. thaliana*); *MutY*, Q9UIF7 *Ogg1*, O15527; *A2*, CAI21775 (*Homo sapiens*); *Nth*, P20625 (*Escherichia coli*); *A2*, BAD25304 (*Oryza sativa japonica* Group); *GB-M1*, CAD26190 (*Encephalitozoon cuniculi* GB-M1 Eukaryota; Fungi; Microsporidia; Unikaryonidae; Encephalitozoon).

Fig. 2. Cellular localization. Onion epidermal cell layers were bombarded with gold particles coated 35SGFP alone or 35SNtROS2a-GFP, and observed by epifluorescence for GFP. Bright field mages are merged to show positions of nucleus indicated by *arrowheads*.

stimuli, Expression pattern of *NtROS2a* gene was investigated under various abiotic stresses (Fig. 3). The *NtROS2a* gene was induced expression after ABA, cold, salt and H₂O₂ treatments. *NtROS2a* mRNA was accumulated at 6 hr. after stresses treatment except for cold. At 8 hr. after stresses treatment, the expression level was declined back to the levels at 6 hr. treatment. However, there was no

significant accumulation of *NtROS2a* mRNA under cold stress. The lowest expression was observed in plants under normal conditions (data not shown).

Vector construction and transgenic plants

To generate transgenic plants, *NtROS2a* cDNA was cloned into the binary expression vector pBI121 under the

Fig. 3. Expression of *NtROS2a* in response to abiotic stress in tobacco. The real-time qRT-PCR analysis of *NtROS2a* in tobacco seedlings subjected to abiotic stresses including cold (4 °C), salt (250 mM NaCl), and ABA (100 μM), H₂O₂ (3%) stress. Total RNA was purified from the tobacco seedlings at Three-leaf stage after various treatments and used in real-time qRT-PCR assays.

Fig. 4. Ti-plasmid vectors for overexpression and silencing *NtROS2a* gene and transgenic plants. (A); Ti-plasmid vector for overexpression of *NtROS2a* gene. (B); Ti-plasmid vector for gene silencing (RNAi) of *NtROS2a* gene. (C); Transformation with *Agrobacterium* on MS medium with selectable kanamycin, (a); Survival response of explants in the selection medium after 4 weeks of co-cultivation (b); Appearance of shoot buds from the cut edges of the surviving green explants and development of small multiple shoots (c); Differentiation and elongation of green resistant shoots under selection after 8 weeks of culture (d); Acclimation of transgenic plants.

control of the 35S promoter (Fig. 4A). RNAi vector was designed for directional insertion of PCR products on either side of the PDK intron (Fig. 4B). The *NtROS2a* gene fragment was amplified through PCR, appended with the restriction sites and cloned sequentially on either side of the intron to become the two arms of the hairpin. The tobacco leaf discs infected with *Agrobacterium* on MS medium with selectable kanamycin showed callus formation. The callus formation initiated from only the putative transformed leaf discs and the untransformed leaf discs began to yellow after 3-5 days. During the following 5-10 days, the leaf discs turned brown and did not initiate callus formation. The transformed leaflets developed into bright green calli and then the shoots emerged with 3 - 5 leaflets in 25-30 days (Fig. 4C-a, b). The emerging shoots with 3-5 leaflets showed root formation in 20-25 days (Fig. 4C-c). The roots continued to grow in the putative transgenic plants, but the root growth in untransformed plants did not develop (data not shown). All transgenic plants showed normal phenotype. The T₁ putative transgenic plants were transferred from the 1/2 MS medium to a selectable kanamycin supplemented MS medium. The plantlets remained green and developed elongated roots as they were resistant to kanamycin, while the untransformed plants became yellow while the roots ceased to elongate because of their sensitivity to the antibiotic (Fig. 4C-d). PCR analysis of transgenic lines in T₂ generation was confirmed integration to tobacco genome both *NtROS2a* and *nptII* gene.

Transcript levels of *NtROS2a* in the transgenic plants

The transcript levels of *NtROS2a* in the transgenic plants

Table 1. Primers sequence used for *NtROS2a* analysis.

Primers	Sequence(5'-3')
NtROS2a-RT F	TGTAAGTCTGAGCGAAGAGC
NtROS2a-RT R	GGAAAGCTAGGTGGTGAAGT
actin-RT F	TACATGTTCAACCACCACTGC
actin-RT R	AAGCTCCTGCTCGTAGTCAA
35s-F	TGAGACTTTTCAACAAAGGGTA
NtROS2a-R	ACAATGGGCTCTGGTGTTC
Kana F	ATGATTGAACAAGATGGATTGCAC
Kana R	TCAGAAGAAGCTCGTCAAGAAGGC
URP250	CCAGTTGCTATAAGAGGCAT
URP251	ATCCAAGGTCCGAGACAACC
URP253	CCCAGCAACTGATCGCACAC
URP255	ATGTGTGCGATCAGTTGCTG

were determined by RT-PCR analysis using gene-specific primer sets (Table 1). Total RNAs were extracted from leaf of 14-d-old seedlings grown under normal growth conditions. *NtROS2a* expression level was higher in all pBI121:: *NtROS2a* transgenic lines than in the non-transgenic (WT) controls (Fig. 5A). By contrast, the pKANNIBAL:: *NtROS2a* transgenic lines showed lower expression level of *NtROS2a* gene than in non-transgenic (Fig. 5B).

Transgenic tobacco enhanced Salt- and H₂O₂-stress tolerance

To examine the effect of abiotic stress in transgenic tobacco plants, gene expression patterns were investigated between the transgenic and non-transgenic plants under salt and H₂O₂ stress (Fig. 6). *NtROS2a* transcript was induced by salt- and H₂O₂ stress. The leaves of T₂ *NtROS2a*

Fig. 5. Quantitative RT-PCR analysis. WT; Wild type tobacco (*Nicotiana tabacum* cv Xanthi nc), (A); pBI121:: *NtROS2a* (OX lines), (B); pKANNIBAL:: *NtROS2a* (RNAi lines). CT values were calculated using actin expression level as a control. Error bars show the standard error of the mean for three replicate measurements.

Fig. 6. NaCl and H₂O₂ sensitivity in transgenic plants. (A) and (B) showed the phenotype of leaf discs from the WT plants, OX lines and RNAi lines after incubation in 250 mM NaCl and 3% H₂O₂ solution for 5 d, and corresponding measurement of chlorophyll contents respectively. Leaf discs floating in water served as the control. The standard deviation is represented by the vertical bar in graph (n=3).

transgenic lines OX 1 and RNAi 13 and non-transgenic WT were incubated at 250 mM NaCl and 3% H₂O₂ for 5 days. In NaCl, most of WT leaves (83%) wilted, whereas only 30.7% seedlings of transgenic line 1 and 24.4% of line 13 wilted (Fig. 6A). In H₂O₂, most leaves of WT (90%) and transgenic line 13 (99%) were wilted, while only 20% seedlings of transgenic line 1 showed the symptom (Fig. 6B).

Effects of abiotic stress on root elongation in transgenic lines

Transgenic lines (OX 1, RNAi 13) were subjected to simulated osmotic stress, and the effects on root elongation were assessed after germination. Germination in OX 1 lines was clearly higher than that in WT and RNAi 13 (Fig. 7A and B). After 9 d of 125 mM NaCl treatment, seed germination in OX 1 line was 40% which is higher than the 15% germination in WT. Similarly, the germination percentage in OX 1 lines was twice higher than that in WT after 2% PEG6000 treatment for 9 d. After 14 d of the same treatment, the roots of OX 1 line plants were about twice longer than those in wild type and RNAi 13. Interestingly, when subjected to higher concentrations of PEG (2%) for the same duration, roots of OX 1 line were not only about

34% longer than those of wild types but also produced more lateral roots. These results indicate that *NtROS2a* increases the adaptation to salt and osmotic stress during seed germination and root elongation in tobacco (Fig. 7C). Also, the change of DNA methylation affects the gene expression and stability. Insertion of *NtROS2a* is expected to cause changes in the genome of recipient plant. Total DNA of transgenic lines was digested with *Msp*I and *Hpa*II, which are unable to cleave the sites m⁵CCGG and Cm⁵CGG, respectively. DNA methylation affects was analyzed by RAPD analysis, but there were no different bands amplified (data not shown). This became part of the band overlap in demethylation, or a very specific band that a fragment is considered.

DISCUSSION

DNA glycosylases are subdivided into four groups according to their substrate specificity: excision of uracil, uracil-containing mismatches, alkylated bases and oxidized bases (Begum *et al.* 2007). NtROS proteins are structurally related to *Arabidopsis* ROS1 and DME, which belong to a

Fig. 7. Germination assay under salt and osmotic stresses in transgenic lines (A); 125 mM NaCl stresses, (B); 2% PEG stresses. (C); Root elongation under salt and osmotic stresses in transgenic lines. Root growth under 125 mM NaCl and 2% PEG for 2 weeks after germination.

large family of uracil-containing mismatch DNA glycosylases. However, apart from the m⁵C DNA glycosylase domain and regions at the carboxyl-terminus, their similarity was low, suggesting specific function of NtROS proteins. Since NtROS1 and NtROS2a were shown to possess m⁵C removal activity, and to be localized in nucleus, it is conceivable that they are closely associated with DNA and engaged in DNA demethylation. Conserved lysine and aspartic acid residues located in the helix-hairpin-helix domain in DNA glycosylases (Krokan *et al.* 1997) and four cysteine residues adjacent to the DNA glycosylase domain were also shown in NtROS proteins. The latter cluster is considered to play a role in DNA binding. NtROS proteins also possess nuclear localization signal and histone deacetylase interacting domains (<http://smart.embl-heidelberg.de/>) (Fig. 1), suggesting them to localize in nucleus and to interact with DNA or chromatin (Gehring *et al.* 2006). In our studies, subcellular localization of *NtROS2a* gene was localized a nucleus protein (Fig. 2).

Their induction profile upon abiotic stresses is indicative, as DNA glycosylases are considered to deal with oxidative damages, which are commonly caused by reactive oxygen species (Choi and Sano 2007; Begum *et al.* 2007). The *NtROS2a* gene was induced expression after ABA, cold, salt and H₂O₂ treatments. *NtROS2a* mRNA was accumulated at 6 hr. after stresses treatment except for cold. At 8 hr. after stresses treatment, the expression level was declined back to the levels at 6 hr. treatment. However, there was no significant accumulation of *NtROS2a* mRNA under cold stress (Fig. 3). To cope with such damages, a set of genes must be expressed to repair and/or strengthen defense reactions. If these genes are locked by methylation under non-stressed condition, demethylation is absolutely necessary to globally switch them on. It is tempting to speculate that NtROS proteins participate in this task, thereby finely adjusting gene expression through methylation/demethylation pathways. This study showed a close correlation between

demethylation and expression of a tobacco gene encoding a glycerophosphodiesterase-like protein upon exposure to abiotic stresses. This speculation is in accordance with the present finding, showing that *NtROS2a* is distantly related to other members, and localized not only at cell surface (plasma membrane) but also in nucleus, suggesting a novel, so far unknown biological functions. In healthy leaves grown under non-stressed conditions, *NtROD2a* was not expressed, and its coding region was found to be heavily methylated. Upon exposure to abiotic stress, its transcripts were induced and genomic locus was partly demethylated, while upon biotic stress, neither transcripts nor demethylation were induced. In this report, transgenic lines (OX 1, RNAi 13) were subjected to simulated osmotic stress, and the effects on root elongation were assessed after germination. Germination in OX 1 lines was clearly higher than that in WT and RNAi 13 (Fig. 7). After 9 d of 125mM NaCl treatment, seed germination in OX 1 line was 40% which is higher than the 15% germination in WT. This strengthened the close correlation between gene expression and methylation, and shows clear demethylation activity towards m⁵C in DNA and ubiquitously expressed in plant tissues (Aguis et al. 2006). Its involvement in stress response has yet to be confirmed, but the present findings indicate that this possibility warrants attention.

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