African Journal of Biotechnology Vol. 9(36), pp. 5908-5913, 6 September, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.037
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

OsAPX4 gene response to several environmental stresses in rice (*Oryza sativa L.)*

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Accepted 28 July, 2010

Expression of the gene, OsAPX4, coding for ascorbate peroxidase in leaves and roots of rice were induced by abiotic stresses, such as NaCl, $NaHCO_3$ and Na_2CO_3, polyethylene glycol (PEG) 6000, H_2O_2, $CuCl_2$. Yeast (*Saccharomyces cerevisiae*) over-expressing ascorbate peroxidase exhibited greater tolerance to NaCl and $NaHCO_3$ and transgenic *Arabidopsis* over-expressing OsAPX4 had a greater salt tolerance than wild-type plants in 1/2 Murashige and Skoog (MS) medium with 150, 200 mM NaCl and 5, 7.5 mM $NaHCO_3$. These results suggest that OsAPX4 plays an important role in multiple environmental stresses.

Key words: *Arabidopsis, Oryza sativa*, thaliana, carbonic anhydrase ascorbate peroxides, gene expression stress.

INTRODUCTION

Plants frequently encounter stresses such as salinity, drought, heavy metal, radiation, low temperature, microbial infection among others. Oxidative stress are common secondary stress occurring after many kind of biotic or abiotic stresses which can change the plant internal redox environment and subsequently disturb its growth processes, metabolism and existence. They adversely affect plant growth, development or productivity. In Northern China, the salinity stress, especially the sodium carbonates ($NaHCO_3$, Na_2CO_3) exist in very large rate. This has seriously limited the agriculture and livestock husbandry (Zhang and Liu, 2003). In order to improve plant tolerance to salinity, it is necessary to uncover the molecular mechanisms that allow plants adapts to Salinity.

Ascorbate peroxidase (APX :EC1.11.1.11) acting as an Important reactive oxygen species (ROS) scavenging enzymes plays an important role in making balance of the ROS in cells. APX scavenges hydrogen peroxide (H_2O_2)and converts it to H_2O and O_2 and serve together as a frontline enzymatic antioxidant defenses from yeast to complex organisms like humans and plants (Asada, 1992; Low and Merida, 1996; Asada, 1997, Lamb and Dixon, 1997; Willekens et al., 1997; Foyer and Noctor, 2000; Alscher et al., 2002; Scandalios, 2002; Agrawal et al., 2003). APX isozymes occur in several cell compartments and occur in many plants, including *Arabidopsis*, spinach, pumpkin, tobacco, soybean, potato and rice (Jespersen et al., 1997; Caldwell et al., 1998; Ishikawa et al., 1998). In rice, there are 8 kinds of APX genes confirmed by Southern blot hybridizations (Teixeira et al., 2006). The distinct APX resulted in different biochemical properties of the isoforms such as molecular mass, substrate specificity, ideal pH and stability in the absence of ascorbate (Ishikawa et al., 1998). The transcripts of both *Oryza sativa* ascorbate peroxidase gene 1 (OsAPX1) and OsAPX2 increased under abiotic stress, but OsAPX2 inductions were more potent and rapid than OsAPX1 (Agrawal et al., 2003). Escobar et al. (2003) found that tobacco herbing *Arabidopsis thaliana* APX3 can enhance its tolerance to stress. But up till now, research on OsAPX4 function in stress tolerance is poor.

In this study, the gene expression of OsAPX4 (Gen Bank Accession No. XM507324) were examined under several stresses from salts (NaCl, $NaHCO_3$ and Na_2CO_3),

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Abbreviations: APX, Ascorbate peroxidase; **ROS,** reactive oxygen species; **OsAPX,** *Oryza sativa* ascorbate peroxidase gene; **PEG,** polyethylene glycol; **MS,** Murashige and Skoog; **PCR,** polymerase chain reaction; **DIG,** digoxigenin; **WT,** wild-type.

H_2O_2, polyethylene glycol (PEG) 6000 and metal ions ($CdCl_2$, $ZnCl_2$ and $CuCl_2$) using northern blot analysis. To further investigate the gene's function, salt tolerance of transgenic yeast and *Arabidopsis* over-expressing the OsAPX4 gene were also analyzed.

MATERIALS AND METHODS

Plant materials and treatments

Rice (*O. sativa L.*, cv. Nipponbare) was grown in a growth chamber with a 12 h illumination period at 26°C. Seedlings, 10-days-old, were treated with 20 mM NaHCO3, 20 mM Na_2CO_3, 100 mM NaCl. 10% H_2O_2 or 10% (w/v) PEG 6000, 100 mM $CdCl_2$,100 mM $ZnCl_2$,100 mM $CuCl_2$ for 24 h . The leaves and roots of seedlings were harvested for Northern blot analysis separately.

A. thaliana (ecotype: Columbia) seeds were planted in1/2 MS agar plate. About 8 to 12 days old, seedlings were transferred to soil and grown to maturity. Plants were watered twice a week with 1/8 MS (Murashige and Skoog, 1962). Temperature in the growth room was 23 ± 2°C. Light provided by cool-white fluorescent bulbs was 60 to 90 $\mu Em^{-2}sec^{-1}$ for seedlings (14 h light/8 h dark).

Construction of expression vector and transformation of *Arabidopsis*

The complete coding region of the rice *(O. sativa* L.) peroxisomal ascorbate peroxidase gene (OsAPX4, GenBank Accession No.XM507324) amplified by polymerase chain reaction (PCR) with sense primer, 5'-GGATCCATGGCCGCCCCG-3' (BamHI site underlined), and antisense primer, 5'-GAGCTCTTACTTGCTCTT CTTAGAAGCCT-3', (SacI site underlined) was fused into the pMD-18-T vector (TaKaRa Japan) to construct the PMD-OsAPX4 plasmid. The PMD-OsAPX4 plasmid DNA was digested with BamHI/SacI and the digested PMD-OsAPX4 cDNA was ligated into the pBI121 binary vector (Clontech) digested with BamH and Sac I to construct the plasmid pBI121- OsAPX4. The plasmid containing the chimeric cauliflower mosaic virus 35S promoter (CaMV35): OsAPX4 was introduced into *Arabidopsis* (*A.* thaliana ecotype: Columbia) by *Agrobacterium tumefaciens*-mediated transformation using the vacuum infiltration method (Bechtold and Pelletier, 1998). The *Arabidopsis* seeds collected were screened in MS medium supplemented with 40 mg kanamycin l^{-1}. Expression of the introduced gene in different lines was identified by PCR and Northern blot.

For construction of the $pYES_2$-OsAPX4, the PMD-OsAPX4 plasmid DNA was digested with BamHI and Sphl. The fragment was ligated into the BamHI and Sphl sites in pYES2 (Clontech) to construct the plasmid pYES2- OsAPX4. Then, the plasmid $pYES_2$-OsAPX4 was induced into InVSc1 (Invitrogen). For Northern blot analysis, plant total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Plant total RNA (10 µg) was separated on a 1.2% denaturing formaldehyde agarose gel and blotted onto a nylon membrane. RNA blot analyses were performed using a digoxigenin (DIG)-labelled OsAPX4 cDNA probe. Hybridization signals were detected with CDP-StarTM (Amersham Pharmacia).

Salinity stress tolerance assay

To assess the relative salinity tolerance, seedlings of the T3 generation over-expressing OsAPX4 were used. The transgenic and wild-type seeds were planted in 1/2 MS solid medium supplemented with three kind of salts (5 and 7.5 mM $NaHCO_3$, 150 and 200 mM NaCl), respectively.

Expression of PMD-OsAPX4 in yeast (*Saccharomyces cerevisiae*) cells under several stresses

The pYES2 vector (clontech) driven by the GAL1 promoter was introduced for inducible expression of recombinant proteins in yeast. High level expression of the fusion protein was induced by galactose, repressed by glucose and maintained by raffinose. Both plasmid pYES2- OsAPX4 and pYES2 empty vector were transformed into competent yeast strain INVSc1 (clontech) using the electric impulse method. Yeast cells culture method was performed as described by Dali et al. (2006). Briefly, solid medium was obtained by adding 1% (w/v) agar to the YPGDR medium (rich medium + 1.94% galactose, 0.06% glucose and 1% raffinose). To test salt tolerance, cells were first grown in YPGDR medium to a density of about 10^7 cells per ml and then diluted into 10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} in the same medium and cells containing pYES2 empty vector were used as a control. 0.5, 0.75, 1 mol/L NaCl or 25, 50, 75 mM $NaHCO_3$ mM were added to media for stress treatment, respectively.

RESULTS AND DISCUSSION

Expression of OsAPX4 in rice under several stresses

To understand the function of OsAPX4 gene in rice, expression of OsAPX4 in leaf and root under normal or several stresses was analyzed at the transcript level. OsAPX4gene expression was examined under several stresses from salts (NaCl, $NaHCO_3$ or Na_2CO3) and10% PEG 6000, H_2O_2 and three kind of metals ($CdCl_2$, $ZnCl_2$, $CuCl_2$). In leaves and roots, levels of OsAPX4 transcripts were increased by treatment with stress, especially for H_2O_2 (Figure 1). For three metals, both in leaves and roots, levels of OsAPX4 transcripts were decreased by treatment with $CdCl_2$ and $ZnCl_2$ stress, but increased by treatment with $CuCl_2$ (Figure 2). This indicated that OsAPX4 has different response relationship with different metals.

To confirm the OsAPX4 responses to H_2O_2, we analyzed the OsAPX4 expression under stresses under different H_2O_2 concentration from 2 to 15% (Figure 3). The results that the transcriptional level of OsAPX4 increased with the concentration of H_2O_2 indicated that the OsAPX4 play a key role in protecting cells against oxidative stress. To further confirm OsAPX4 responds to salt stresses, we analyzed the OsAPX4 expression under stresses from $NaHCO_3$, NaCl (Figure 4) in time course. The levels of OsAPX4 transcript both in leaves and roots increased when exposed to 100 mM NaCl with time course. For treatment with 100 mM NaCl and 20 mM $NaHCO_3$, the transcript levels in leaves decreased first and then were induced strongly, especially in 48 h. But up to 72 h, the level decreased again under 20 mM NaHCO3. This could be due to the concentration of $NaHCO_3$ being too strong or the treatment time too long to induce plant apoptosis. The transcript levels in roots increased continually under 100 mM NaCl and decreased first, but then increased continuously under 20 mM $NaHCO_3$ and up to the highest level in 72 h for both treatment. These results suggest that the expression of

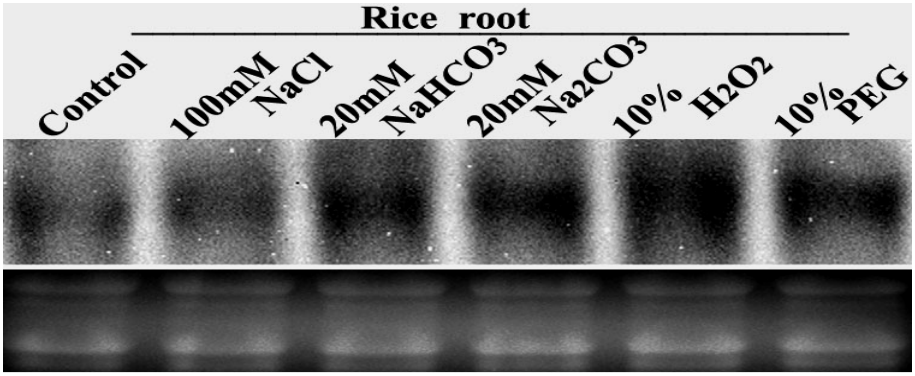

Figure 1. Northern blot analysis of OsAPX4gene in rice under several stresses. Total RNA was isolated from roots of rice treated under stresses from 100 mM NaCl, 20 mM $NaHCo_3$, 10% H_2O_2 and 10% PEG for 24 h and hybridized with DIG-labelled cDNA probe for OsAPX4. Equal loading of RNA samples into each was confirmed by ethidium-bromide staining of rRNAs.

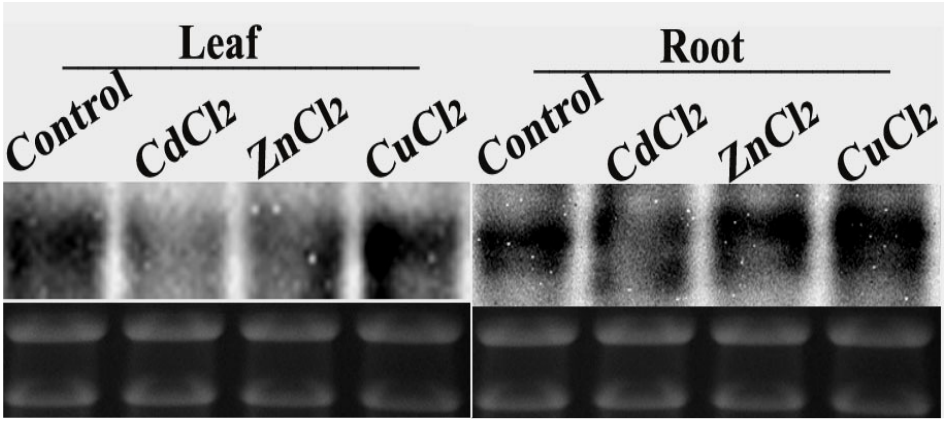

Figure 2. Northern blot analysis of OsAPX4 gene in rice under three kind of metal stresses. Total RNA was isolated from leaves and roots of rice treated under stresses from 100mM $CdCl_2$, 100 mM $ZnCl_2$, 100 mM $CuCl_2$ for 24 h and hybridized with DIG-labelled cDNA probe for OsAPX4. Equal loading of RNA samples into each was confirmed by ethidium-bromide staining of rRNAs.

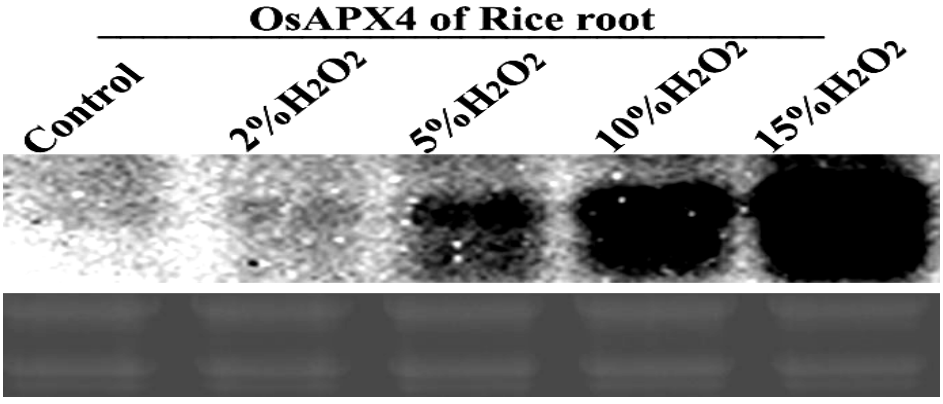

Figure 3. Northern blot analysis of OsAPX4 gene in rice under different concentrations of H_2O_2 stresses. Total RNA was isolated from roots of rice treated with 2, 5, 10 and 15% H_2O_2, respectively, and hybridized with DIG-labelled cDNA probe for OsAPX4. Equal loading of RNA samples into each was confirmed by ethidium-bromide staining of rRNAs.

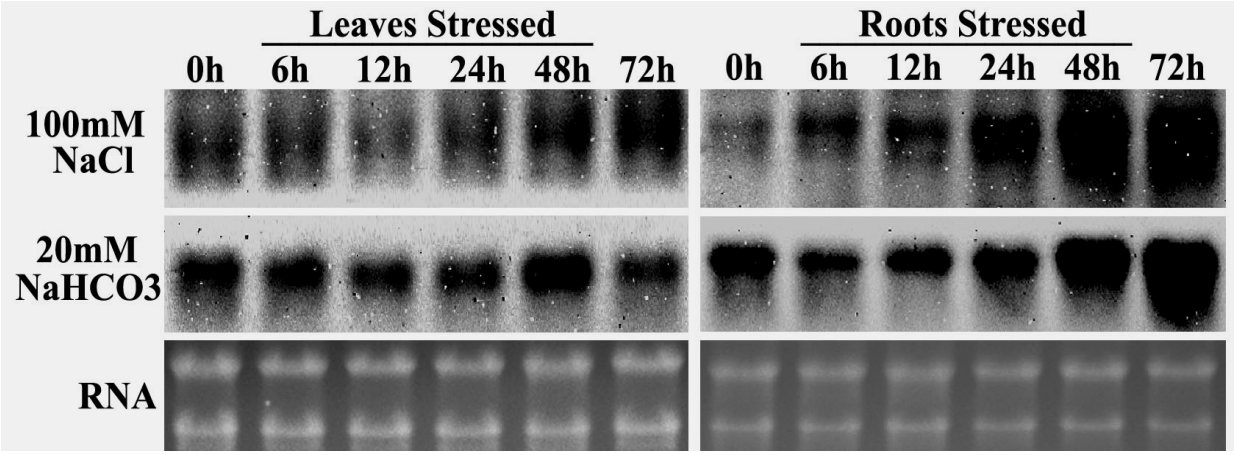

Figure 4. Northern blot analysis of OsAPX4 gene in rice under salt stresses. Total RNA was isolated from leaves and roots of rice treated under stresses from 100 mM NaCl, 20 mM $NaHCO_3$ for the times indicated and hybridized with DIG-labelled cDNA probe for OsAPX4. Equal loading of RNA samples into each was confirmed by ethidium-bromide staining of rRNAs.

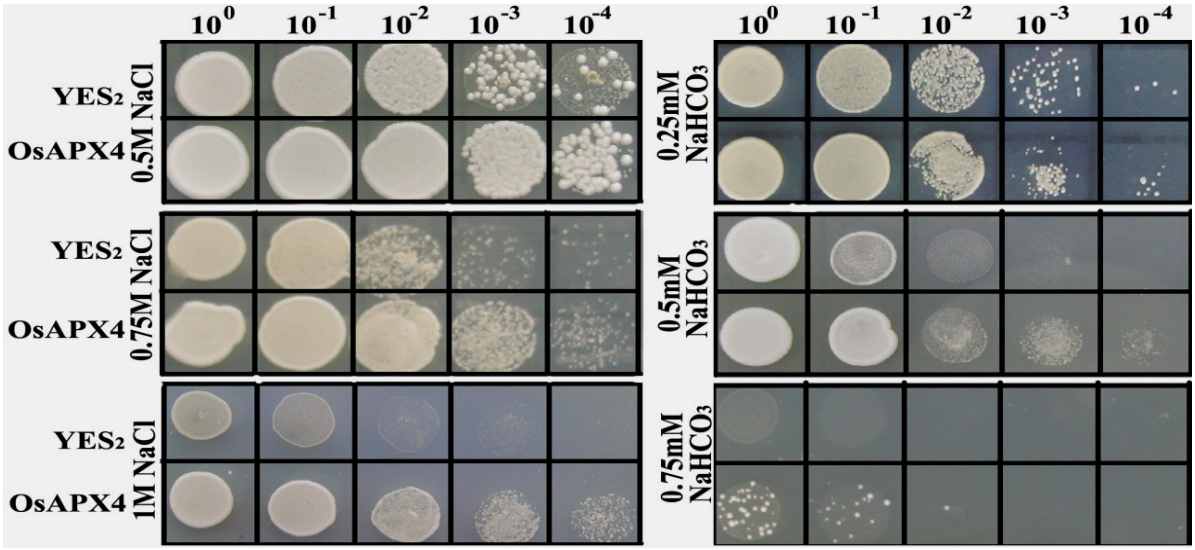

Figure 5. Growth in the presence or in the absence of different concentrations of NaCl (500, 750 or 1000 mM) or $NaHCO_3$ (25, 50, 75 mM) of yeast expressing OsAPX4 constructions or transformed by the empty plasmid pYES2. Yeast were grown in YPGDR agar medium to mid-log phase and incubated for 36 h at 30°C.

OsAPX4 was related to environmental stresses.

Expression of OsAPX4 in yeast cells under several stresses

To determine whether OsAPX4 protected cells against environmental stress, we used yeast (*S. cerevisiae*) as a model because yeast is a single-cell eukaryote whose metabolic pathways are similar to those in higher plants. We inserted the OsAPX4 gene into the pYES2 vector (clontech) driven by the GAL1 promoter, which is induced by galactose and repressed by glucose. The growth of cells over-expressing OsAPX4 was better than the growth of the control under NaCl and $NaHCO_3$ stress (Figure 5). These results suggest that over-expression of OsAPX4 in yeast increased the cells' tolerance to sodium stresses.

Salt stress tolerance of transgenic *Arabidopsis* seedlings harboring OsAPX4

Transgenic plants harboring OsAPX4 were screened on 1/2 MS agar medium containing 40 mg/ml kanamycin and the presence of the transgene in T3 generation were

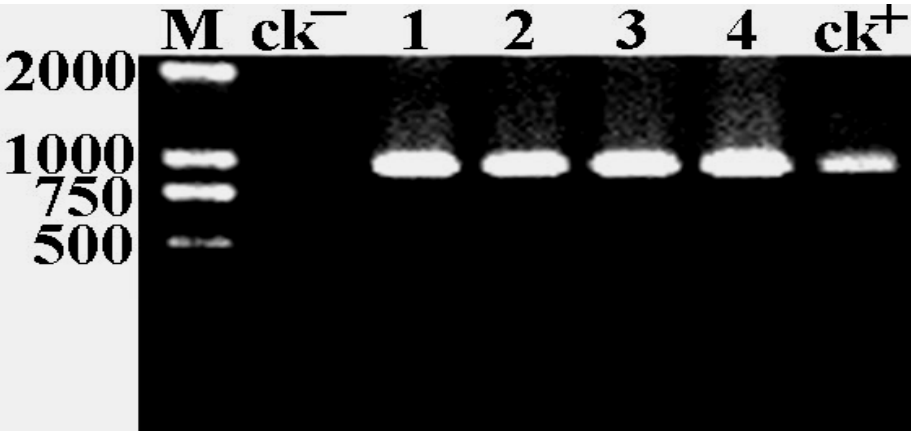

Figure 6. PCR results of TRX m1-type gene from transformed *Arabidopsis thaliana* plants; M: 2000 bp DNA marker, CK: negative control; 1 - 4: transformed plants; CK^+: positive control.

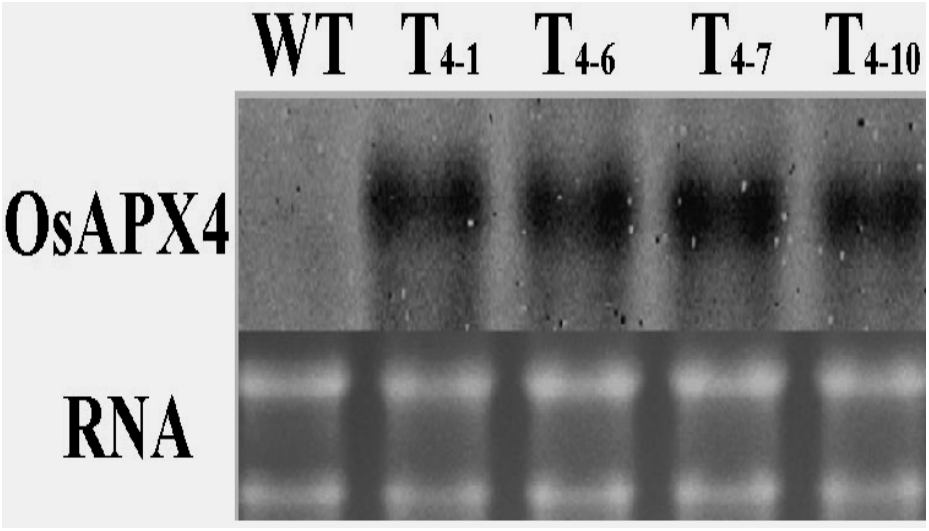

Figure 7. Northern blot analysis of OsAPX4 expression in wild-type and transformed *Arabidopsis*. Total RNA(10 µg) extracted from 4-week-old seedlings of T4 generation plants was separated on a 1.2% formaldehyde agarose gel, transferred to a nylon membrane and hybridized with a DIG-labelled OsAPX4 cDNA probe. WT is wild type plants. T4-1, T4-6, T4-7 and T4-10 are transgenic lines over-expressing the OsAPX4 gene driven by the 35S promoter

further confirmed by PCR using primers specific for the OsAPX4 cDNA (Figure 6). Over-expression of the gene in the transgenic lines was also confirmed by Northern analysis (Figure7). It shows the accumulation of OsAPX4 transcripts in three independent transgenic lines (T3-1, T3-6, T3-7 and T3-10). To investigate whether the OsAPX4 gene has a role in salinity tolerance, the salt tolerances of the seedlings of the three T4 transgenic lines over-expressing the OsAPX4 gene were compared with the salt tolerance of wild-type (WT) plants. The growth of the three independent transgenic lines (T4-1, T4-6 and T4-7) and WT plants were comparable in the absence of salts (Figure 8). However, in the presence of 5 and 7. 5 mM $NaHCO_3$, 150 mM NaCl or 200 mM NaCl, the growth of WT plants was less than with the growth of the transgenic lines (Figure 8). These results indicate that over-expression of the OsAPX4 gene conferred improved salt tolerance to *Arabidopsis* plants.

In conclusion, expression of OsAPX4 gene in yeast can enhance the resistance to salts (NaCl, $NaHCO_3$). Trans-genic *Arabidopsis* over-expressing the OsAPX4gene also exhibited growth advantage under NaCl and $NaHCO_3$ stress environments. These examinations imply that the OsAPX4 gene plays an important role in the response of

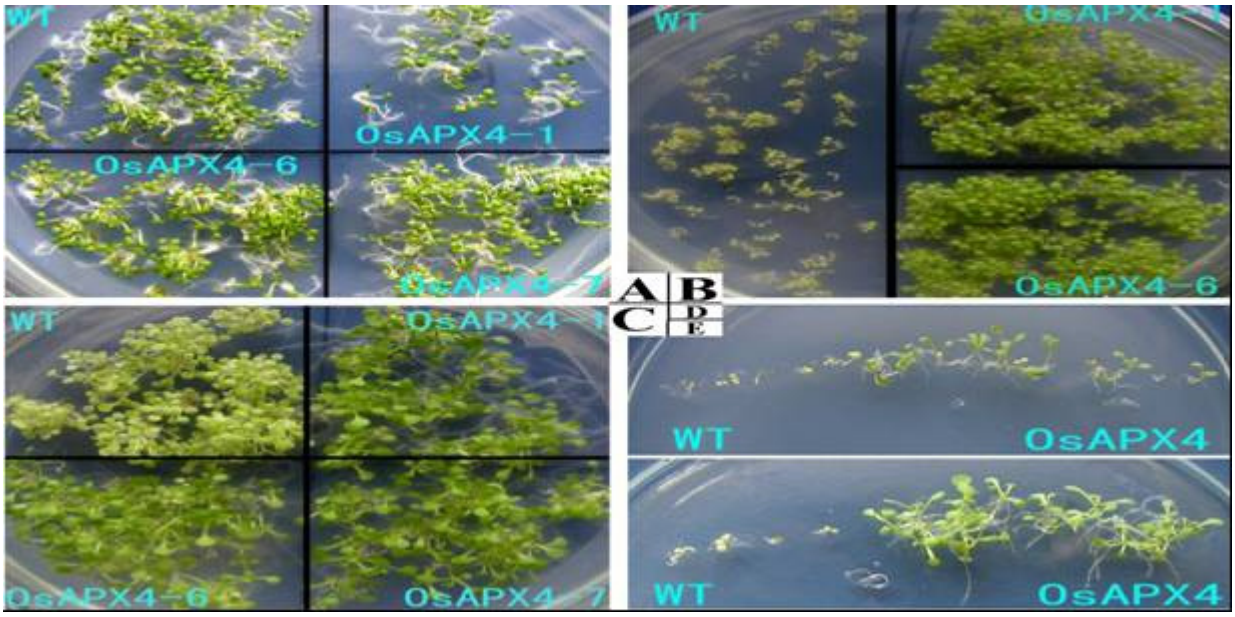

Figure 8. Relative salt tolerance of wild-type and the transgenic plants over-expressing the OsAPX4 gene under salt stress. Wild-type (WT) and transgenic seeds germinated in 1/2 MS solid medium agar plates in the absence or in the presence of NaCl or $NaHCO_3$. A: 1/2 MS, 7 day old; B: 1/2 MS+5 MM NaHCO3, 7 day old; C: 1/2MS + 150MM NaCl, 15 day old; D: 1/2 MS+7.5 MM NaHCO3, 7 day old; E: 1/2 MS+200 MM NaCl , 15 day old.

plants to several environmental stress conditions.

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