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Overexpression of a Wheat Aquaporin Gene, *TaAQP8*, Enhances Salt Stress Tolerance in Transgenic Tobacco

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Aquaporin (AQP) proteins have been shown to transport water and other small molecules through biological membranes, which is crucial for plants to combat salt stress. However, the precise role of *AQP* genes in salt stress response is not completely understood in plants. In this study, a *PIP1* subgroup *AQP* gene, designated *TaAQP8*, was cloned and characterized from wheat. Transient expression of TaAQP8–green fluorescent protein (GFP) fusion protein revealed its localization in the plasma membrane. TaAQP8 exhibited water channel activity in *Xenopus laevis* oocytes. *TaAQP8* transcript was induced by NaCl, ethylene and H_2O_2. Further investigation showed that up-regulation of *TaAQP8* under salt stress involves ethylene and H_2O_2 signaling, with ethylene causing a positive effect and H_2O_2 acting as a negative factor. Overexpression of *TaAQP8* in tobacco increased root elongation compared with controls under salt stress. The roots of transgenic plants also retained a high K^+/Na^+ ratio and Ca^{2+} content, but reduced H_2O_2 accumulation by an enhancement of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities under salt stress. Further investigation showed that whole seedlings from transgenic lines displayed higher SOD, CAT and POD activities, increased *NtSOD* and *NtCAT* transcript levels, and decreased H_2O_2 accumulation and membrane injury under salt stress. Taken together, our results demonstrate that *TaAQP8* confers salt stress tolerance not only by retaining high a K^+/Na^+ ratio and Ca^{2+} content, but also by reducing H_2O_2 accumulation and membrane damage by enhancing the antioxidant system.

Keywords: Antioxidant enzyme • Aquaporin • Reactive oxygen species • Salt stress tolerance • Wheat.

Abbreviations: AQP, aquaporin; CaMV, *Cauliflower mosaic virus*; CAT, catalase; DMTU, dimethyl thiourea; GFP, green fluorescent protein; IL, ion leakage; 1-MCP, 1-methylcyclopropene; MDA, malonaldehyde; MeJA, methyl jasmonate; MS, Murashige and Skoog; POD, peroxidase; QPCR, real-time quantitative PCR; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; SA, salicylic acid; SOD, superoxide dismutase; VC, vector control; WT, wild type.

The nucleotide sequences reported in this paper has been submitted to GenBank under the accession number HQ650110 (*TaAQP8*).

Introduction

Soil salinity is one of the most severe abiotic stress factors, which limit growth of most plant species causing significant losses in crop yield. The effects of stress include damage to major plant metabolic processes, such as photosynthesis, growth, energy and lipid metabolism, and protein synthesis (Ruiz-Lozano et al. 2012). To cope with the negative effects of salt stress, plants have evolved biochemical and molecular mechanisms such as regulation of water/osmotic homeostasis, ion balance and damage prevention (Munns 1993, Chen and Polle 2010, Ruiz-Lozano et al. 2012). These also include the regulation of aquaporins (AQPs), transport or compartmentalization of Na^+ and/or K^+, and the antioxidant system.

AQPs are integral membrane proteins that increase the permeability of membranes to water, as well as other small molecules such as CO_2, glycerol and boron (Uehlein et al. 2003, Kaldenhoff and Fischer 2006, Sade et al. 2010), and mediate seed germination, cell elongation, stomatal movement, phloem loading and unloading, reproductive growth and stress responses in plants (Eisenbarth and Weig 2005, Gao et al. 2010). *AQP* was first isolated from *Arabidopsis thaliana* (Maurel et al. 1993), and since then a number of *AQP* genes have been identified including 35 from *A. thaliana* (Johanson et al. 2001), 36 from *Zea mays* (Chaumont et al. 2001), 37 from *Solanum lycopersicum* (Sade et al. 2009) and 33 from *Oryza sativa* (Sakurai et al. 2005), and some of them have been

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characterized. Compared with other species, little is known about the *AQP* genes in wheat because of the unavailability of its complete genome sequence and the allohexaploid nature of its genome.

A large number of studies have shown that environmental stresses such as salt, drought and cold can induce up-regulation of *AQP* genes, and transgenic approaches established that overexpression of some *AQP* genes could improve a plant's tolerance to abiotic stresses (Guo et al. 2006, Yu et al. 2006, Cui et al. 2008, Mahdieh et al. 2008, Peng et al. 2008, Gao et al. 2010, Sade et al. 2010). AQPs confer salt stress tolerance mainly by regulating water uptake and its distribution to plant tissues (Ruiz-Lozano et al. 2012). In Arabidopsis, overexpression of *NIP* aquaporin from wheat increased tolerance to salt stress when compared with untransformed plants. In addition, transformed plants had better root growth as well as higher accumulation of Ca^{2+} and K^+ and lower levels of Na^+ under salinity (Gao et al. 2010). Therefore, there is a need to investigate whether the *PIP* subfamily of *AQP* genes regulate the distribution of these cations under salt stress.

The activity of AQPs is directly regulated by phosphorylation, which is also affected by a number of stimuli including abiotic stresses (Johansson et al. 2000, North and Nobel 2000, Aroca et al. 2005, Horie et al. 2011), phytohormones (Zhao et al. 2008) and H_2O_2 (Aroca et al. 2005). Exogenous application of ABA, a mediator of water stress response, enhanced root hydraulic conductivity in sunflower and maize (Quintero et al. 1999, Hose et al. 2000). In addition, it has been shown that ethylene increases plasma membrane permeability by permitting more water to cross the cells (Woltering 1990). An increase in water transport in hypoxic aspen seedlings exposed to ethylene was attributed to enhanced aquaporin activity, presumably due to the phosphorylation of aquaporins by ethylene (Kamaluddin and Zwiazek 2002). A number of studies have shown that H_2O_2 could change the phosphorylation state of AQPs, thereby altering AQP structure which leads to water channel closure. In addition, H_2O_2 also enhances the internalization of AQPs, resulting in the down-regulation of water transport in plants (Aroca et al. 2005, Boursiac et al. 2008a, Boursiac et al. 2008b, Ehlert et al. 2009, Heinen et al. 2009). This is in contrast to the ability of AQP isoforms to facilitate H_2O_2 transport across the tonoplast and plasma membrane (Bienert et al. 2006, Dynowski et al. 2008). Investigation of AQP function in chilling-tolerant and chilling-sensitive maize genotypes showed higher AQP activities in the chilling-tolerant genotype than in the chilling-sensitive genotype due to less damage to membranes by oxidation (Aroca et al. 2005). However, the role of AQPs in enhancing the antioxidant system that reduces reactive oxygen species (ROS) accumulation and membrane damage under salt stress is unclear.

As an international staple crop, wheat production is constrained by multienvironmental stresses such as drought, salinity and extreme temperature. Therefore, for the genetic improvement of stress resistance in wheat, an understanding of the molecular mechanisms of abiotic stress responses is necessary. Although *AQP* genes respond to salt stress, their exact role in salt stress tolerance is not completely understood. In the present study, we characterized a wheat *AQP*, *TaAQP8*, which imparts salt stress tolerance to transgenic tobacco not only by increasing the K^+/Na^+ ratio and Ca^{2+} content but also by reducing H_2O_2 accumulation and membrane damage by enhancing the expression and activities of antioxidant enzymes.

Results

TaAQP8 encodes a PIP1 subgroup of AQP in wheat

The full-length cDNA of *TaAQP8* (GenBank accession No. HQ650110) was amplified by rapid amplification of cDNA ends (RACE) using mRNA isolated from the leaves of wheat seedlings. *TaAQP8* cDNA is comprised of 909 bp with an 867 bp open reading frame, and the deduced TaAQP8 protein contains 288 amino acid residues with a predicted molecular mass of 30.67 kDa. Blastx analysis revealed that *TaAQP8* had 99% sequence identity with *HvPIP1-1* from *Hordeum vulgare*, 97% with *TaAQP1* from *Triticum aestivum* and 93% with *ZmPIP1-1* from *Z. mays*. The predicted TaAQP8 protein had six putative transmembrane helices, a highly conserved amino acid sequence 'HINPAVTFG' and two 'NPA' motifs (**Supplementary Fig. S1**). Moreover, the conserved sequence (R/K)DYX(E/D)PP(P/R)X_{3-4}(E/D)XXELXXWSF(Y/W)R present in all PIP members was also observed in TaAQP8. Based on the amino acid sequence alignment, a phylogenetic tree was constructed for plant AQPs (**Supplementary Fig. S2**). The sequences of the AQP family from wheat, Arabidopsis and rice were chosen from GenBank. On an evolutionary time scale, TaAQP8 was very close to the PIP1 subfamily, suggesting that the *TaAQP8* isolated in this study may be a member of the wheat PIP1 subfamily.

TaAQP8 locates in the plasma membrane

To determine the cellular localization of TaAQP8, the *TaAQP8* gene was cloned into the pCAMBIA1304-GFP (green fluorescent protein) vector downstream of the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter and upstream of the *GFP* gene to create the TaAQP8–GFP fusion construct. The TaAQP8–GFP fusion protein and pm-rk (a plasma membrane marker) were then co-expressed in onion epidermal cells. The results showed that green fluorescence and red pm-rk were both confined to the plasma membrane (**Fig. 1**), indicating that TaAQP8 protein was localized in the plasma membrane of cells.

Expression of *TaAQP8* in *Xenopus laevis* oocytes enhances water permeability of cells

To determine whether TaAQP8 is a functional AQP, water channel activity of the protein was assayed in *X. laevis* oocytes. Two days after cRNA or water injection, the rate of change in

cell volume (**Fig. 2A**) and the osmotic water permeability coefficient (Pf) (**Fig. 2B**) were calculated in the presence of osmotic solution. Swelling in *TaAQP8*-expressing oocytes and controls is displayed in **Supplementary Video S1**. The rate of change in cell volume was higher in *TaAQP8*-expressing oocytes than in water-injected oocytes after 15 min of osmotic treatment. Oocytes expressing *TaAQP8* yielded a 1.64-fold higher Pf than water-injected oocytes, suggesting that TaAQP8 is a functional AQP with low water channel activity.

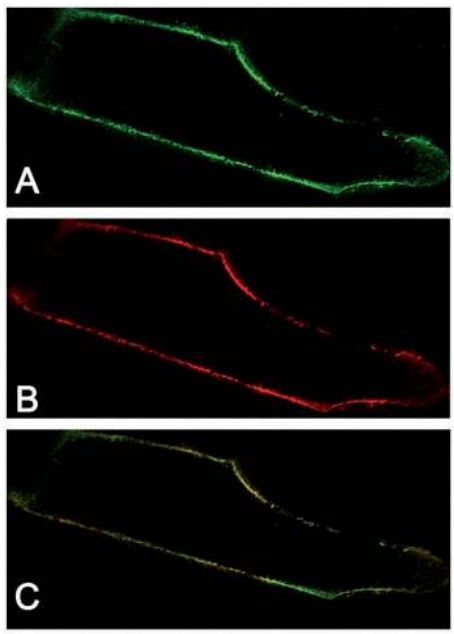

Fig. 1 Subcellular localization of TaAQP8 protein. Onion epidermal cells transiently co-transformed with TaAQP8::GFP and pm-rk (a plasma membrane marker). (A) Fluorescence image of an epidermal cell expressing the p35S-TaAQP8::GFP fusion protein. (B) Fluorescence image of an epidermal cell expressing pm-rk. (C) Merged fluorescence image of an epidermal cell expressing the p35S-TaAQP8::GFP fusion protein and pm-rk marker. Two biological experiments were performed, which produced similar results.

TaAQP8 is ubiquitously expressed in wheat tissues

AQP genes play a significant role in various plant tissues when exposed to unfavorable environmental conditions (Li et al. 2009). To determine the expression patterns of *TaAQP8* in different wheat tissues, quantitative real-time PCR (QPCR) was carried out with mRNAs isolated from different tissues as templates (**Supplementary Fig. S3**). The results showed that *TaAQP8* was expressed in all tissues examined, including root, stem, leaf, stamen, pistil and lemma, with high expression levels in root, stem and leaf.

TaAQP8 is up-regulated in response to NaCl, ethylene and H_2O_2 treatments

To investigate the response of *TaAQP8* to salt stress, *TaAQP8* transcript levels were determined after NaCl treatment. The results showed that expression of *TaAQP8* was induced by NaCl and reached the highest level (5.3-fold) after 2 h, followed by a decrease (**Fig. 3**). Because salt stress induces accumulation of various signal molecules, the effects of ABA, ethylene, methyl jasmonate (MeJA), salicylic acid (SA), gibberellin and H_2O_2 on *TaAQP8* transcription were also examined. The results showed that the *TaAQP8* expression increased (2.0-fold) at 2 h and peaked at 6 h (19.5-fold) with ethylene treatment and was induced to the highest level at 2 h (5.4-fold) with H_2O_2 treatment (**Fig. 3**). *TaAQP8* transcript levels were induced marginally by gibberellin (**Supplementary Fig. S4**) while ABA inhibited the expression of *TaAQP8* at 12 and 24 h

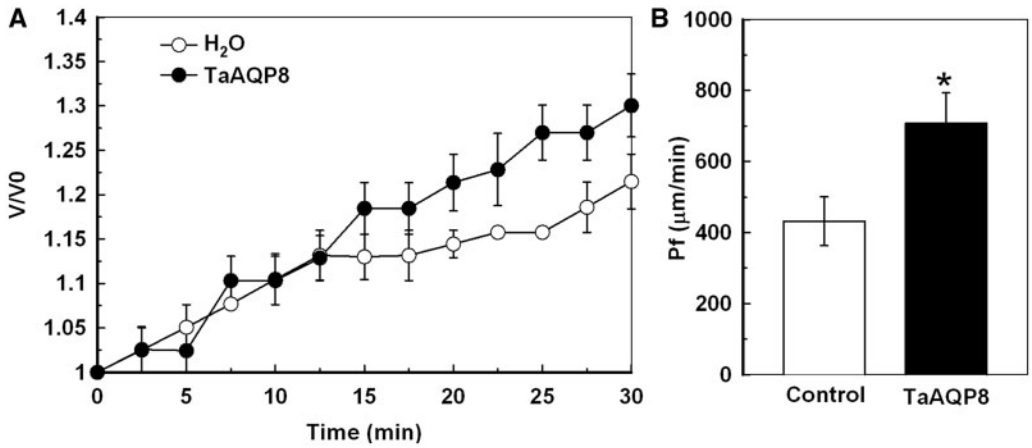

Fig. 2 Water channel activity test of TaAQP8. (A) The swelling rates of *Xenopus laevis* oocytes injected with cRNA encoding TaAQP8, or water (as negative control). The rate of oocyte swelling upon immersion in hypo-osmotic medium is plotted as V/V_0 vs. time, where V is the volume at a given time point and V_0 is the initial volume. (B) Osmotic water permeability coefficient (Pf) of oocytes injected with cRNA encoding TaAQP8, or water. The Pf values were calculated from the rate of oocyte swelling. Vertical bars indicate ± SE of three replicates on one sample (each replicate contains three oocytes). When no bar is shown, the deviation is smaller than the symbol. Asterisks indicate a significant difference between the control and *TaAQP8*-expressing oocytes ($^*P < 0.05$; $^{**}P < 0.01$). Three biological experiments were performed, which produced similar results.

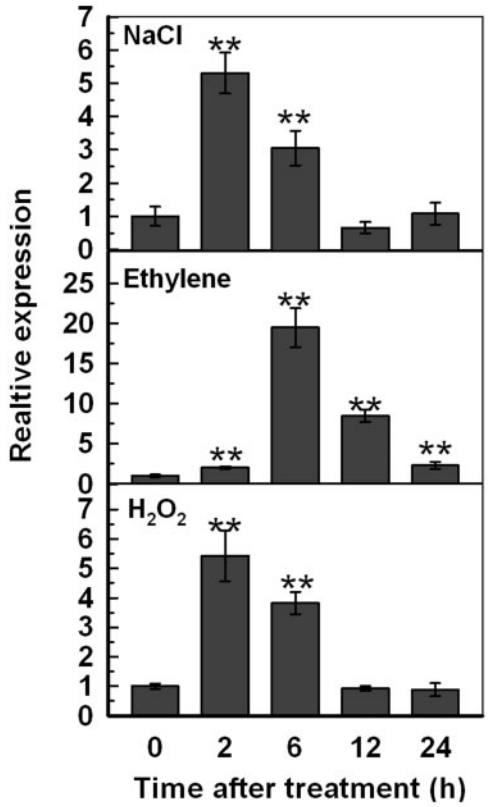

Fig. 3 Expression patterns of *TaAQP8* under NaCl, ethylene and H_2O_2 treatments in wheat by QPCR analysis. Ten-day-old wheat seedlings were treated with 200 mM NaCl, 100 μM ethylene and 10 mM H_2O_2. Wheat leaves were sampled at different time points to extract RNA for QPCR analysis. The *y*-axis represents the relative fold difference in mRNA level, which is calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001) with *TaActin* as internal control. The mRNA fold difference is relative to that of distilled water-treated samples used as a calibrator. Vertical bars indicate ± SE of four replicates on one sample. Asterisks indicate a significant difference between the control and treated samples ($^*P < 0.05$; $^{**}P < 0.01$). Three biological experiments were performed, which produced similar results.

(**Supplementary Fig. S4**). MeJA and SA had no effect on the expression of *TaAQP8* (**Supplementary Fig. S4**). These results suggest that robust expression of *TaAQP8* was induced by NaCl, ethylene and H_2O_2.

Induction of *TaAQP8* by salt stress involves ethylene and H_2O_2

To explore whether up-regulation of *TaAQP8* under salt stress involves ethylene and H_2O_2 signaling, 1-methylcyclopropene (1-MCP) was chosen as the inhibitor of ethylene signaling transduction, and dimethyl thiourea (DMTU) was chosen as the scavenger of H_2O_2 (Desikan et al. 2006, Zhang et al. 2006). For this experiment, wheat seedlings were pre-treated with 1-MCP and DMTU under NaCl treatment. The results clearly showed the induction of *TaAQP8* at 2 h (5.8-fold) and 6 h (2.6-fold) during treatment with NaCl (**Fig. 4A**) which is consistent with the results in **Fig. 3**. Moreover, the up-regulation of *TaAQP8* during NaCl treatment was inhibited by 1-MCP at 2 h (2.4-fold) and 6 h (1.3-fold) but was increased by DMTU at 2 h (21.2-fold) and 6 h (12.0-fold) (**Fig. 4A**). These results imply that the up-regulation of *TaAQP8* under salt stress involves ethylene and H_2O_2 signaling, with ethylene causing a positive effect and H_2O_2 acting as a negative factor.

H_2O_2 plays an important role in mediating signal transduction and is proposed to function downstream of ethylene (Desikan et al. 2006). Therefore, we determined whether the up-regulation of *TaAQP8* expression induced by ethylene involves H_2O_2 signaling. For this, wheat seedlings were pre-treated with DMTU for 2 and 6 h to block the production of H_2O_2 completely (Jiang and Zhang 2002) and then exposed to ethylene for 2 and 6 h. The results showed that *TaAQP8* was induced by ethylene at 2 h (3.0-fold) and 6 h (17.1-fold) (**Fig. 4B**), which is consistent with the results in **Fig. 3**. In addition, pre-treatment with the H_2O_2 scavenger had no effect on the up-regulation of *TaAQP8* in ethylene-treated wheat seedlings (**Fig. 4B**). These results suggest that ethylene-induced up-regulation of *TaAQP8* did not involve H_2O_2 signaling.

Generation of transgenic tobacco overexpressing *TaAQP8*

To investigate further the role of *TaAQP8* in salt stress, transgenic tobacco plants overexpressing *TaAQP8* under the control of the CaMV 35S promoter were generated. A total of 14 transgenic lines (T_1) were confirmed by hygromycin resistance analysis and PCR using primers specific to *TaAQP8* and *GFP* (data not shown). Among these lines, OE4, OE7 and OE11 segregated at a rate of 3 : 1 for hygromycin resistance. Moreover, all three transgenic T_2 line seedlings survived on Murashige and Skoog (MS) medium containing 40 mg l^{-1} hygromycin. Transgenic plants overexpressing the vector control (VC) were also subjected to similar analysis. *TaAQP8* expression in the three T_2 lines was investigated by reverse transcription–PCR (RT–PCR) analysis (**Supplementary Fig. S5**). The result showed that *TaAQP8* mRNA was detected in all three transgenic lines but not in the wild type (WT) and VC, with OE7 and OE11 showing a higher expression levels than OE4.

Overexpression of *TaAQP8* enhances salt tolerance in transgenic tobacco

For salt tolerance analysis, seeds from WT, VC and transgenic lines were germinated on MS medium or MS medium supplemented with 100 or 200 mM NaCl for 8 d to detect the germination rate. Higher germination rates were observed in transgenic lines than in the WT and VC under NaCl treatment (**Fig. 5**). Further, seedlings from WT, VC and transgenic lines were cultured in MS medium for 1 week and then transplanted to fresh MS medium or MS medium supplied with 100 or 200 mM NaCl for 1 week to observe root length. The result suggested that suppression of root growth was lower in transgenic lines than in WT and VC under 100 or 200 mM NaCl

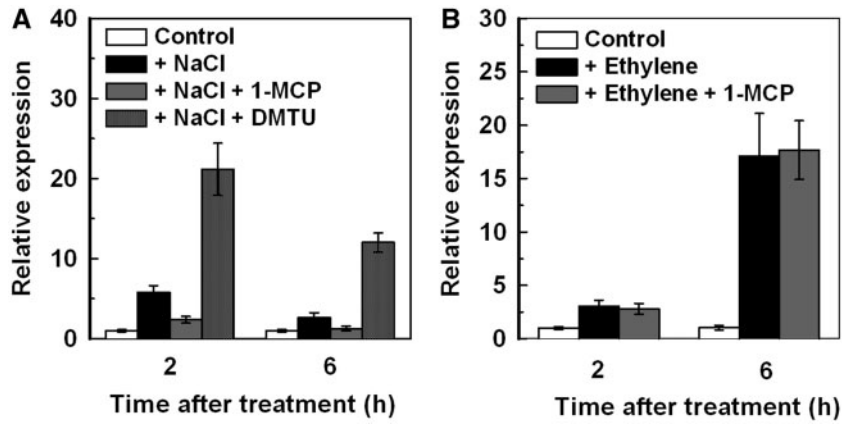

Fig. 4 Effects of inhibitors of signaling molecules on the *TaAQP8* transcripts under NaCl and ethylene treatments. (A) Effects of pre-treatment with inhibitors of ethylene and H_2O_2 on the expression of *TaAQP8* in the leaves of wheat seedlings exposed to NaCl. The plants were pre-treated with distilled water, 300 p.p.b. of 1-MCP and 5 mM DMTU for 2 and 6 h, respectively, and then exposed to 200 mM NaCl for 2 and 6 h respectively. (B) Effects of pre-treatment with inhibitors of H_2O_2 on the expression of *TaAQP8* in the leaves of wheat seedlings exposed to ethylene. The plants were pre-treated with distilled water and 5 mM DMTU for 2 and 6 h, respectively, and then exposed to 100 μM ethylene for 2 and 6 h, respectively. The plants treated with distilled water for 2 or 6 h were used as control. The *y*-axis represents the relative fold difference in mRNA level calculated using the $2^{-\Delta\Delta Ct}$ formula with *TaActin* as internal control. The mRNA fold difference was relative to that of distilled water-treated samples used as a calibrator. Vertical bars indicate ± SE of four replicates on one sample. Three biological experiments were performed, which produced similar results.

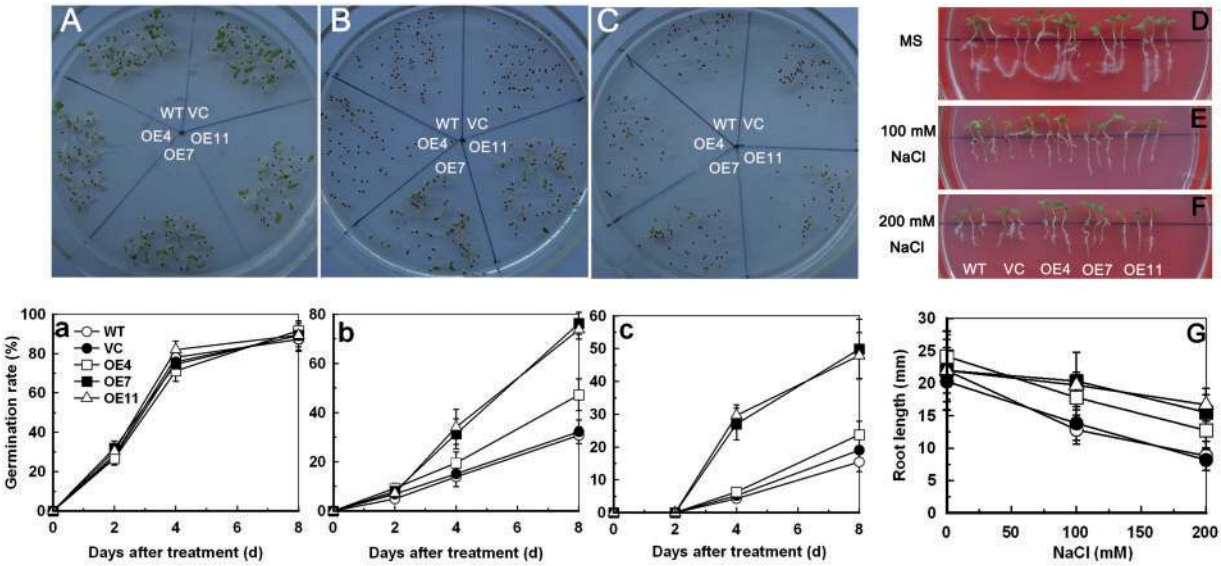

Fig. 5 Salt tolerance analysis of *TaAQP8*-overexpressing plants during the early seedling development stage. A total of 200 surface-sterilized seeds of each transgenic line, WT or VC were germinated on MS medium containing 0 (A, a), 100 (B, b) or 200 mM (C, c) NaCl for 8 d, and the germination rate was calculated. (A–C) Photographs of the first 8 d after germination on medium. The WT, VC and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the seedlings were transplanted to fresh MS medium or MS medium supplied with 100 or 200 mM NaCl for 1 week. The photographs were taken (D–F) and the root length was calculated (G). Vertical bars indicate ± SD calculated from four replicates (each replicate contains three seedlings). Three biological experiments were performed, which produced similar results.

treatment (**Fig. 5**). Little difference was observed between transgenic plants and the two controls grown on MS medium. In a second experiment, 3-week-old seedlings from transgenic and WT tobacco plants grown in pots were exposed to 300 mM NaCl stress for 40 d. While 13.1% of the WT plants survived, all three transgenic lines had higher rates of survival, with 37.2% for OE4, 32.1% for OE7 and 44.5% for OE11 (**Fig. 6A**, **B**). Because the adult tobacco plants (6 weeks old) had some tolerance to salt stress, the high concentration of NaCl was chosen to treat tobacco plants for an extended

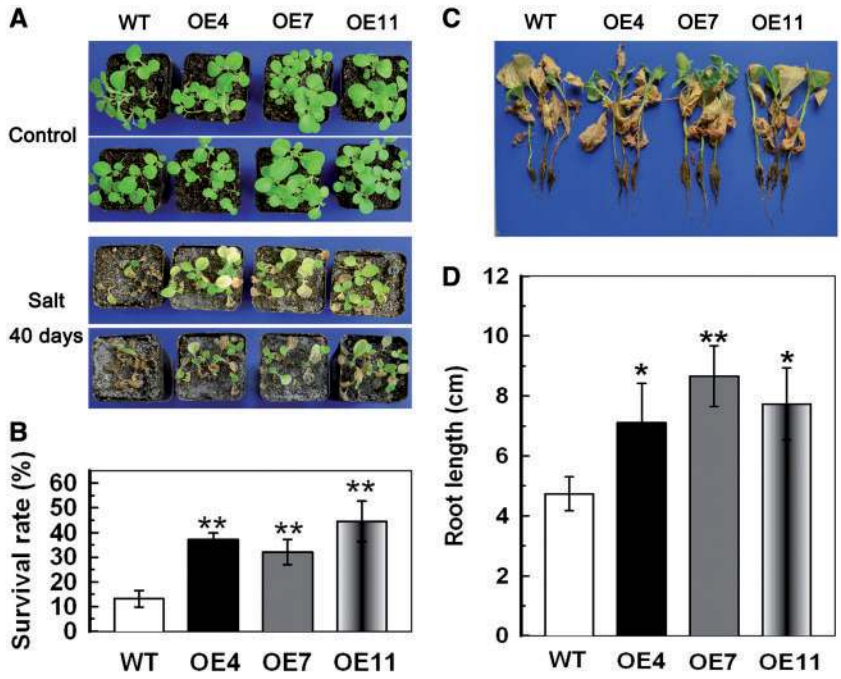

Fig. 6 Analysis of the enhanced salt tolerance in transgenic lines. The WT and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the plants were transplanted to containers filled with a mixture of soil and sand (3 : 1) where they were regularly watered for 2 or 5 weeks. Thirty seedlings of each line or the WT of 3-week-old tobacco plants with a consistent growth state were subjected to 300 mM NaCl stress for 40 d, the photographs were taken (A) and the survival rate was calculated (B). Six-week-old tobacco plants with a consistent growth state were subjected to 300 mM NaCl stress for 80 d, the photographs were taken (C) and root length was calculated (D). Data are means ± SD calculated from three replicates. Asterisks indicate a significant difference between the WT and the three transgenic lines (*$P < 0.05$; **$P < 0.01$). Three biological experiments were performed, which produced similar results.

time. When these tobacco plants were subjected to 300 mM NaCl stress for 80 d, the leaf phenotype in transgenic lines was similar to that of the WT. However, root length in transgenic lines (OE4 7.1, OE7 8.7 and OE11 7.7) was longer than in the WT (4.7) (**Fig. 6C, D**). These results suggest that overexpression of *TaAQP8* increased acclimatization of transgenic tobacco plants to salt stress during seed germination and root elongation.

Overexpression of *TaAQP8* affects Na^+, K^+ and Ca^{2+} distribution in different tissues of transgenic plants under salt stress

The observed differences in the leaf and root phenotypes of transgenic plants compared with the WT under salt stress prompted us to look at physiological differences. To determine the effect of *TaAQP8* overexpression on the accumulation of Na^+, K^+ and Ca^{2+}, the contents of these cations in leaves, roots and stems of WT and transgenic plants were examined under salt stress and normal conditions. Without salt stress, no difference was detected between the transgenic lines and the WT in Na^+, K^+ and Ca^{2+} contents (**Fig. 7**). However, transgenic plants under salt stress had elevated Na^+ and K^+ in roots, and reduced Na^+ and increased K^+ in stems compared with the WT (**Fig. 7**), suggesting that the overexpressed *TaAQP8* played a role in regulating cation contents. Further analysis indicated that the roots and stems of transgenic lines maintained a higher K^+/Na^+ ratio than the WT during salt treatment (**Fig. 7**). In addition, overexpression of *TaAQP8* decreased the Ca^{2+} content in leaves and improved Ca^{2+} in roots and stems under salt stress. These results suggest that the distribution of Na^+, K^+ and Ca^{2+} displayed different patterns in different tissues of transgenic lines, with roots and stems maintaining a higher K^+/Na^+ ratio and Ca^{2+} content and leaves exhibiting a lower Ca^{2+} content than WT.

Overexpression of *TaAQP8* increases antioxidant enzyme activities and decreases H_2O_2 content in roots under salt stress

Because Na^+, K^+ and Ca^{2+} play important roles in cell metabolism, protein biosynthesis and enzyme activation, activities of enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidase (POD; EC 1.11.1.7) were measured in the leaves and roots from potted plants under normal conditions and salt treatment. After 30 and 60 d of salt stress, leaves from transgenic lines showed higher SOD activities and H_2O_2 contents than the WT, but exhibited no difference in CAT and POD activities compared with WT leaves (**Fig. 8A–D**). However, roots from transgenic lines displayed higher SOD, CAT and POD activities and lower H_2O_2 contents than WT roots (**Fig. 8E–H**). These results indicated that overexpression of *TaAQP8* leads to scavenging of excess H_2O_2 by

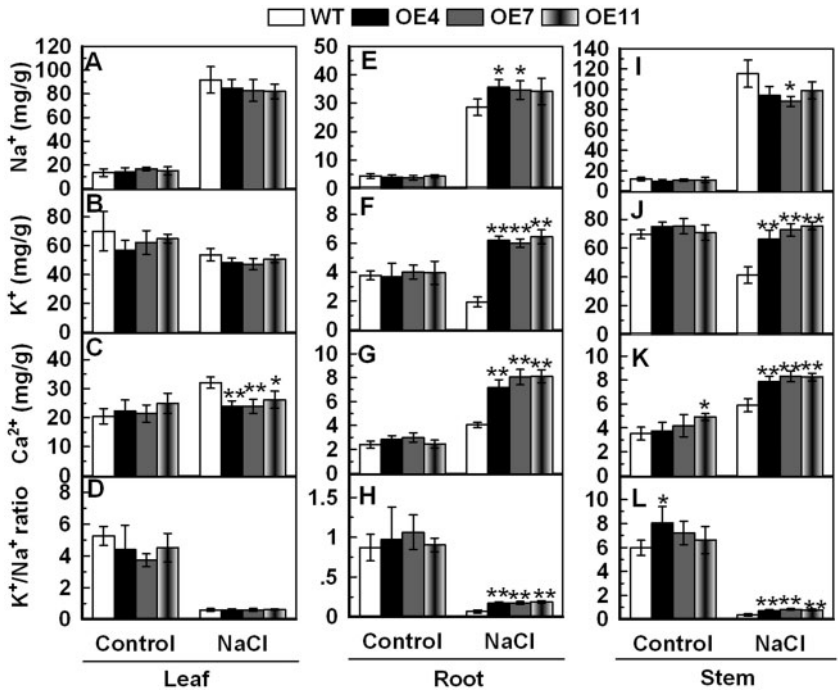

Fig. 7 Ion content in transgenic lines and the WT under salt stress. The WT and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the plants were transplanted to containers filled with a mixture of soil and sand (3 : 1) where they were regularly watered for 5 weeks. Six-week-old tobacco plants were subjected to 300 mM NaCl stress for 60 d. Leaves, roots and stems were collected from transgenic lines and the WT to detect the content of Na^+, K^+, Ca^{2+} and the K^+/Na^+ ratio. Data are means ± SD calculated from four replicates. Asterisks indicate a significant difference between the WT and the three transgenic lines (*$P < 0.05$; **$P < 0.01$). Three biological experiments were performed, which produced similar results.

activating antioxidant enzymes in the roots of transgenic plants under salt stress.

Overexpression of *TaAQP8* increases antioxidant enzyme expression and activities under salt stress

To evaluate the expression and activities of antioxidant enzymes, whole seedlings from transgenic lines and the WT were used. In normal growth conditions, SOD, CAT and POD activities displayed no difference between transgenic lines and the WT (**Fig. 9**). After 7 d of salt stress, however, transgenic lines had significantly higher SOD, CAT and POD activities than the WT (**Fig. 9A–C**). The expression of *NtSOD* and *NtCAT* was also higher in transgenic lines than in the WT (**Fig. 9D**, **E**), with no difference in the expression of *NtPOX* between transgenic lines and the WT (**Fig. 9F**). These results suggested that overexpression of *TaAQP8* enhances SOD, CAT and POD activities under salt stress, and the up-regulation of *NtSOD* and *NtCAT* could contribute to this increase.

Overexpression of *TaAQP8* decreases H_2O_2 accumulation, ion leakage (IL) and malonaldehyde (MDA) under salt stress

Antioxidant enzymes play significant roles in ROS scavenging and thereby lower membrane lipid peroxidation. To estimate H_2O_2 accumulation, IL and MDA contents, seedlings from transgenic lines and the WT were grown on MS medium for 7 d and transferred to fresh MS medium or MS medium supplemented with 200 mM NaCl for 7 d. In normal growth conditions, H_2O_2 accumulation, IL and MDA contents were similar between transgenic lines and the WT (**Fig. 10**). However, under salt stress, transgenic lines showed lower accumulation of H_2O_2 than the WT (**Fig. 10A**). IL, an important indicator of membrane injury, was significantly higher in WT than in transgenic plants under salt stress, suggesting that transgenic plants suffered from less membrane damage than the WT (**Fig. 10B**). MDA is the product of lipid peroxidation caused by ROS and is in general used to evaluate ROS-mediated injuries in plants. MDA measurements displayed a pattern similar to H_2O_2 and IL, with lower levels in transgenic lines relative to the WT under salt stress (**Fig. 10C**). These physiological indices demonstrated that overexpression of *TaAQP8* reduces H_2O_2 accumulation and membrane lipid peroxidation under salt stress.

Discussion

When sodium salts accumulate in the soil, plants take in more Na^+ and less K^+. Since Na^+ is toxic to cell metabolism, its uptake and distribution determine the salt sensitivity of plants (Ruiz-Lozano et al. 2012). However, plants have evolved several biochemical and molecular mechanisms to cope with

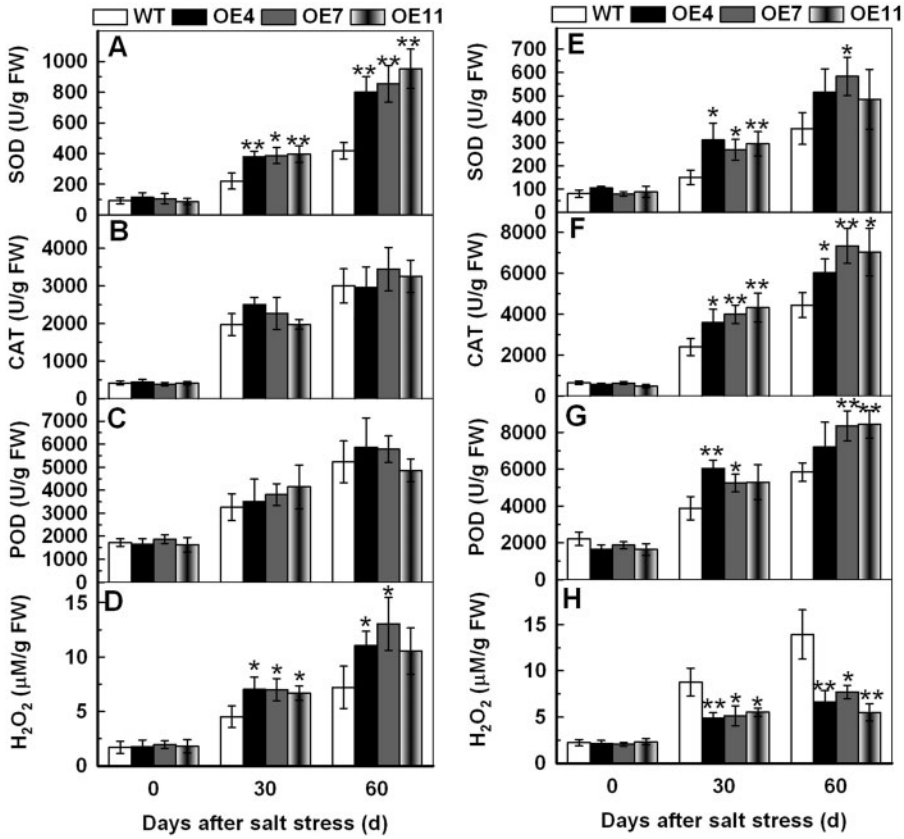

Fig. 8 Analysis of the activities of SOD, CAT and POD, and H_2O_2 content in leaves (A–D) and roots (E–H) of transgenic lines and the WT. The WT and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the plants were transplanted to containers filled with a mixture of soil and sand (3 : 1) where they were regularly watered for 5 weeks. Six-week-old tobacco plants were subjected to 300 mM NaCl stress for 60 d. Tobacco leaves and roots were sampled from WT and transgenic lines with 30 or 60 d salt stress treatment to detect activities of SOD, CAT and POD, and H_2O_2 content. Data are means ± SD calculated from four replicates. Asterisks indicate a significant difference between the WT and the three transgenic lines (*$P < 0.05$; **$P < 0.01$). Four biological experiments were performed, which produced similar results.

the negative effects of salinity. A number of genes that play an important role in the maintenance of ionic homeostasis by regulating transport or compartmentalization of Na^+ and/or K^+ have been characterized. These include the Na^+/H^+ antiporter *SOS1*, the Na^+ influx transporter family *HKT* and the tonoplast Na^+/H^+ antiporter family *NHX* (Munns 2005, Ruiz-Lozano et al. 2012). Although >35 *AQP* genes have been identified to date in the wheat genome, the functions of only a small number of them, such as *TaNIP*, *TdPIP1;1* and *TdPIP2;1*, are known (Forrest and Bhave 2008, Gao et al. 2010, Ayadi et al. 2011). The precise role of AQPs in osmotic and salt stress tolerance is not completely understood. In the present study, a *PIP1* subfamily gene, designated as *TaAQP8*, was cloned and characterized from wheat.

TaAQP8 plays a positive role during salt stress

Several studies have demonstrated the importance of *AQP* genes in salt stress tolerance (Guo et al. 2006, Gao et al. 2010, Sade et al. 2010, Ayadi et al. 2011). *TaAQP8* transcript was rapidly induced after 2 h treatment with NaCl, suggesting that it was induced by salt (Figs. 3, 4). Therefore, to understand further the function of *TaAQP8* under salt stress, we generated transgenic tobacco plants overexpressing *TaAQP8* under the control of the constitutive CaMV 35S promoter. As shown in Figs. 5 and 6, 1-, 3- and 6-week-old tobacco plants overexpressing *TaAQP8* displayed improved tolerance against salt stress when compared with the WT. These results are consistent with previous studies on *AQP* genes conferring salt stress tolerance in transgenic plants (Jang et al. 2007, Gao et al. 2010, Sade et al. 2010, Ayadi et al. 2011).

Enhanced salt stress tolerance is related to Na^+, K^+ and Ca^{2+} distribution in different tissues of transgenic plants

The role of AQP in regulating Na^+, K^+ and Ca^{2+} distribution under salt stress was reported in a recent study (Gao et al. 2010). *TaNIP*-overexpressing Arabidopsis accumulated higher K^+ and Ca^{2+} and lower Na^+ levels than WT plants under salt stress (Gao et al. 2010). In the present study, leaves of transgenic plants failed to maintain a higher K^+/Na^+ ratio and Ca^{2+}

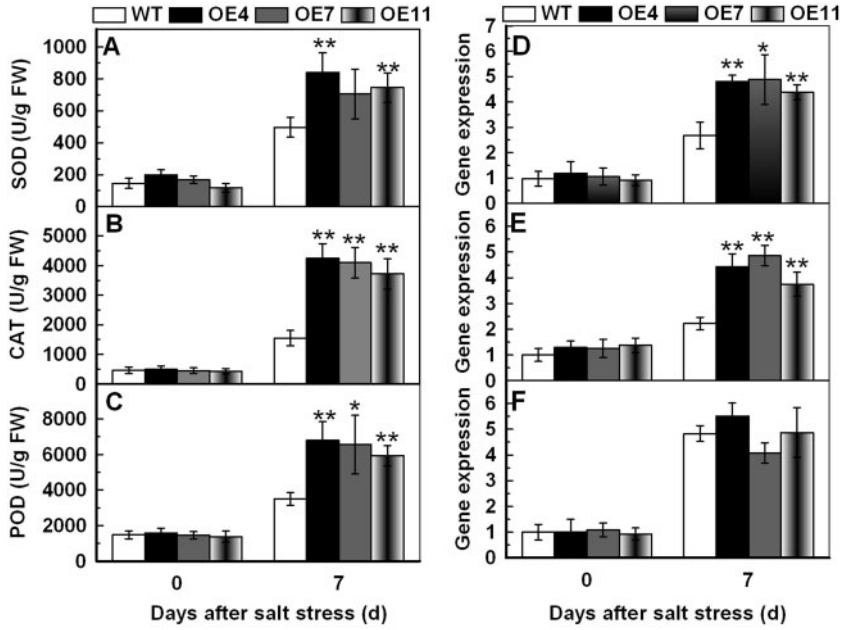

Fig. 9 Analysis of the activities and expression of SOD, CAT and POD in WT and transgenic lines. The WT and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the seedlings were transplanted to MS medium without NaCl or MS medium supplied with 200 mM NaCl for a week. The whole seedlings were used to detect activities and expression of SOD, CAT and POD. (A) SOD activity; (B) CAT activity; (C) POD activity; (D) NtSOD expression; (E) NtCAT expression; (F) NtPOX expression. Data are means ± SD calculated from four replicates. Asterisks indicate a significant difference between the WT and the three transgenic lines (*$P < 0.05$; **$P < 0.01$). Four biological experiments were performed, which produced similar results.

content compared with the WT (**Fig. 7**), which explains the lack of difference in leaf phenotype between transgenic and WT plants under salt stress. In contrast, overexpression of *TaAQP8* resulted in a higher Ca^{2+} content and K^+/Na^+ ratio in roots and stems of transgenic lines when compared with the WT under salt stress (**Fig. 7**). In recent years, a high cytosolic K^+/Na^+ ratio has become an accepted marker of salinity tolerance (Ruiz-Lozano et al. 2012). A higher K^+/Na^+ ratio in roots and stems of transgenic lines under salt stress indicates the ability of *TaAQP8*-overexpressing plants to tolerate high salt concentrations.

Ca^{2+} is known to enhance the activity of AQPs (Cabañero et al. 2006) and also to play a crucial role in the salt tolerance signaling pathway (Sheen 1996). Higher Ca^{2+} accumulation in roots and stems of transgenic lines under salt stress implied that the Ca^{2+}-mediated signaling pathway may work more efficiently in transgenic plants under salt stress. The control by Ca^{2+} or environmental factors of the opening and closing of AQPs has been reported (Steudle and Henzler 1995, Azad et al. 2004). In addition, the effects of Ca^{2+} seem to involve root water channel activity under high salinity conditions (Carvajal et al. 2000, Cabañero et al. 2004, Cabañero et al. 2006). In Arabidopsis, Ca^{2+} triggers a signaling cascade to activate Na^+/H^+ antiporters (Liu and Zhu 1998, Quintero et al. 2002). It has also been shown that Ca^{2+} is essential for the maintenance of membrane integrity and K^+/Na^+ selectivity, which ameliorates Na^+ toxicity in a variety of plant species (Cramer et al. 1985, Chen and Poole 2010).

Because *AQP* genes mediate and regulate rapid transmembrane water flow during growth and development, *TaAQP8*-overexpressing plants may be more efficient in regulating water transport across membranes under stress conditions. It is speculated that the physiological effects are beneficial for plants in maintaining the protein machinery that regulates nutrient uptake and distribution.

The antioxidant mechanism is involved in *TaAQP8* conferring salt stress tolerance

Na^+ is toxic to cell metabolism and has a deleterious effect on some enzymes. High Na^+ levels also reduce photosynthesis and lead to ROS accumulation (Mahajan and Tuteja 2005). In contrast, K^+ is a cofactor for many enzymes and is involved in protein biosynthesis and participates in the binding of tRNA to ribosomes (Blaha et al. 2000). Thus, a higher K^+/Na^+ ratio may be beneficial to enhance the activities of enzymes and maintain cellular ROS homeostasis. In addition, Ca^{2+}, an important second messenger in stress signaling, stimulates the catalytic activity of antioxidant enzymes, thereby decreasing H_2O_2 levels (Knight and Knight 2001, Yang and Poovaiah 2002, Hu et al. 2007, Xu 2010, Shoresh et al. 2011). An important consequence of salinity stress is the generation of excess ROS which leads to cell toxicity, membrane dysfunction and cell death (Gouiaa et al. 2012). There is a constant need for efficient mechanisms to avoid oxidative damage to cells, and antioxidant enzymes play an important role in the defense of plants against ROS (Ruiz-Lozano et al. 2012).

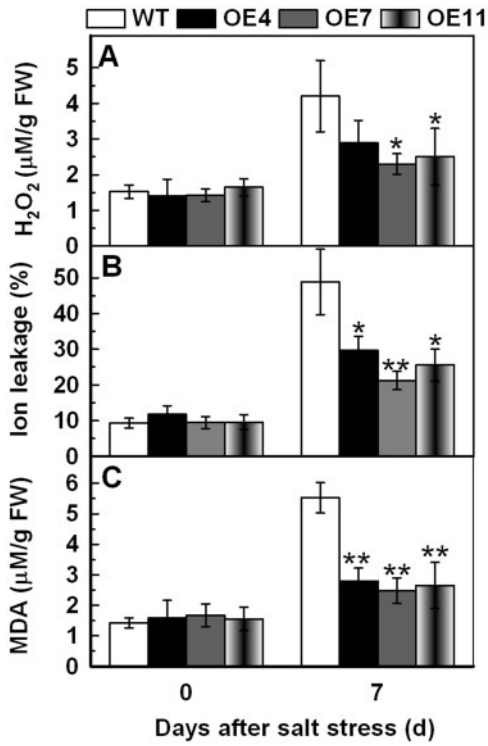

Fig. 10 Analysis of H_2O_2 accumulation (A), IL (B) and the content of MDA (C) in transgenic lines and the WT. The WT and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the seedlings were transplanted to fresh MS medium or MS medium with 200 mM NaCl for 1 week. The whole seedlings were used to detect H_2O_2 accumulation, IL and the content of MDA. Vertical bars indicate ± SD calculated from four replicates. Asterisks indicate a significant difference between the WT and the three transgenic lines (*$P < 0.05$; **$P < 0.01$). Four biological experiments were performed, which produced similar results.

Therefore, we were interested in observing the activities of antioxidant enzymes, ROS accumulation and lipid peroxidation in transgenic lines and the WT. Our results showed that leaves of transgenic plants had a higher H_2O_2 content and SOD activity than those of the WT (**Fig. 8A**, **D**). However, overexpression of *TaAQP8* improved SOD, CAT and POD activities and decreased H_2O_2 accumulation in roots compared with the WT (**Fig. 8E–H**). Moreover, transgenic seedlings also displayed higher expression and activities of antioxidant enzymes, and lower H_2O_2, MDA and IL contents relative to the WT (**Figs. 9**, **10**). ROS have been shown to alter AQP structure, which leads to channel closure through a direct oxidative mechanism (Kourie 1998), or to induce internalization of PIPs and reduce hydraulic conductivity through cell signaling mechanisms (Boursiac et al. 2008a, Boursiac et al. 2008b). An H_2O_2-mediated decrease in water transport under abiotic stresses was also identified in previous studies (Lee et al. 2004, Aroca et al. 2005, Boursiac et al. 2008a). *BjPIP1*-overexpressing plants showed higher activities of antioxidant enzymes and lower levels of IL and MDA than the WT under Cd stress (Zhang et al. 2008), implying that the antioxidant system may be involved in the ability of *AQP* genes to confer abiotic stress tolerance in plants. Here, we have provided physiological evidence that *TaAQP8* confers salt stress tolerance by enhancing expression and activities of antioxidant enzymes that scavenge ROS and reduce membrane injury.

In conclusion, an *AQP* gene from the *PIP1* subgroup, designated as *TaAQP8*, was cloned and characterized from wheat. *TaAQP8* plays a role in root elongation during salt stress not only by retaining a higher K^+/Na^+ ratio and Ca^{2+} content but also by enhancing the antioxidant system. The role of *TaAQP8* in conferring salt stress tolerance is presumed. When transgenic tobacco plants are subjected to salt stress, the function of *TaAQP8* is effectively induced by ion toxicity or signaling molecules such as ethylene and H_2O_2. *TaAQP8*-overexpressing plants may be more efficient in regulating water transport across membranes under salt conditions, consistent with the primary function of AQPs which is to enhance water permeability. This physiological effect is beneficial for plants in maintaining the protein machinery which regulates nutrient uptake and distribution as well as the elevated antioxidant system. In future work, emphasis would be on deciphering the mechanism of *AQP* regulation of ions and activation of the antioxidant system under salt stress.

Materials and Methods

Plant materials and treatments

Wheat (*T. aestivum* L. cv. Chinese Spring) seeds were surface-sterilized with 75% (v/v) ethanol for 2 min and 1% (v/v) bichloride for 10 min, and then washed with sterile water five times. The sterilized seeds germinated on sterile water and grew in MS liquid medium under a 16 h light/8 h dark cycle at 25°C. For NaCl treatment, the 10-day-old seedlings were transferred into Petri dishes containing 200 mM NaCl solution and the plants were incubated under light for different times. For treatments with signaling molecules, the 10-day-old seedlings were sprayed with 100 μM ABA, 100 μM ethylene, 100 μM MeJA, 5 μM gibberellin, 2 mM SA or 10 mM H_2O_2, and the plants were incubated under light for different times. For treatment with inhibitors, the plants were pre-treated with 300 p.p.b. 1-MCP and 5 mM DMTU for 2 and 6 h, respectively, and then exposed to 200 mM NaCl for 2 and 6 h, respectively. The plants were pre-treated with 5 mM DMTU for 2 and 6 h respectively, and then exposed to 100 μM ethylene for 2 and 6 h, respectively. In order to get reliable results, for all of the above treatments, the wheat seedlings with consistent growth were subjected to various treatments and the untreated wheat seedlings were used as control for each series of treatments. For the organ differential expression assays, roots, stems and leaves were cut from sterile seedlings, and pistils, stamens and lemma were obtained from wheat plants in the field. The samples from the treated or control plants were subsequently frozen in liquid nitrogen and stored at −80°C for extraction of total RNA and QPCR assay.

Cloning and bioinformatics analysis of the *TaAQP8* gene

The wheat expressed sequence tags (ESTs) are available on the DFCI wheat gene index database (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=wheat), from which an EST sequence (CJ711427) belonging to the major intrinsic protein (MIP) family was acquired. Sequence analysis by ORF Finder showed that the 3′ end was missing. Employing RACE, the 3′ end of the gene from wheat was amplified with the SMART RACE cDNA amplification kit (Clontech) using the primer P1 (**Supplementary Table S1**), and the cDNA obtained from leaves of wheat seedlings treated with 200 mM NaCl, 20% PEG6000 and cold (4°C) for 2 h as template. The amplified products of the 3′ cDNA ends were inserted into the pMD18-T vector (TAKARA). The nucleotide sequence of the inserted cDNA fragment was determined on an ABI PRISM310 Genetic Analyzer (Perkin Elmer Applied Biosystems) using the BigDye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The full-length cDNA sequence was identified with the help of DNAMAN software and was amplified by RT–PCR with primer P2 (**Supplementary Table S1**), and wheat poly(A)$^+$ mRNA as template. The amplified product was inserted into the pMD18-T vector (TAKARA). The nucleotide sequence was determined on an ABI PRISM310 Genetic Analyzer (Perkin Elmer) using the BigDye Termination Cycle Sequencing Ready Reaction Kit (Perkin Elmer). The sequence was analyzed by BLAST (http://ncbi.nlm.nih.gov/blast).

QPCR analysis

The expression of *TaAQP8* in different wheat organs and leaves after different treatments was analyzed by QPCR using the fluorescent intercalating dye SYBRGreen (ToYoBo) in a detection system (MJ Research Opticon2). Each 200 ng of poly(A)$^+$ mRNA was converted into cDNA using AMV Reverse Transcriptase (Promega) at 42°C in a final volume of 20 μl, which subsequently served as the template in QPCR. The primers (P3–P8; **Supplementary Table S1**) used in the QPCR excluded the highly conserved protein domain and had high efficiency and specificity, which was detected by Opticon monitor2 QPCR software and agarose gel electrophoresis. The resulting PCR products obtained by all the primers were subjected to sequencing to confirm the specificity. In all of the experiments, appropriate negative controls containing no template RNA were subjected to the same procedure to exclude or detect any possible contamination. Before proceeding with the actual experiments, a series of template and primer dilutions were tested to determine the optimal template and primer concentration for appropriate amplification of the target during the experiments. The amplification efficiencies for the internal control and the target genes were determined with the range from 0.9 to 1.1. Each sample was amplified in four independent replicates, and the data were analyzed using Opticon monitor2 QPCR software. *TaActin* or *NtUbiquitin* was used as the internal control for wheat and tobacco, respectively, which served as a benchmark to which other genes examined were normalized. The mRNA fold difference was relative to that of untreated samples used as calibrator. The relative expression level of genes was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen 2001).

Subcellular localization of TaAQP8 protein

The coding sequence of *TaAQP8* containing the *Nco*I/*Spe*I restriction site was amplified using primers (P9; **Supplementary Table S1**) for transient expression in onion epidermal cells. The PCR products were subcloned into *Nco*I/*Spe*I sites of the pCAMBIA1304-GFP expression vector under the control of the CaMV 35S promoter. pCAMBIA1304-TaAQP8-GFP and the pm-rk were transiently expressed in onion epidermal cells using a gene gun (PDS-1000, BIO-RAD), where pm-rk was used as the plasma membrane-localized maker (Nelson et al. 2007). Fluorescence was observed by confocal laser scanning microscopy (LSM700; Carl Zeiss) after incubation at 25°C for 24 h on MS medium.

cRNA synthesis, oocyte preparation, cRNA injection, and osmotic water permeability assay

The cDNAs of *TaAQP8* were subcloned into pCS107 vector using the flanking restriction sites *Bam*HI and *Eco*RI. The cRNA transcripts were synthesized in vitro with an mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion) with *Asc*I-linearized vector. Oocyte preparation, injection and expression were performed as described by Daniels et al. (1996). Stage VI *X. laevis* oocytes were isolated and stored in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.4) containing 10 μg ml^{-1} streptomycin. The follicular cell layer was removed via 2 h of incubation with 2 mg ml^{-1} collagenase in ND96 buffer at 25°C with continuous gentle agitation. The defolliculated oocytes were injected with 50 nl of cRNA (1 mg ml^{-1}) or water used as a negative control, and then the oocytes were incubated at 18°C for 48 h in ND96 solution supplemented with 10 μg ml^{-1} penicillin and 10 μg ml^{-1} streptomycin. The osmotic water permeability coefficient of oocytes was determined as described by Zhang and Verkman (1991). To measure the osmotic water permeability coefficient, oocytes were transferred to 5-fold diluted ND96 solution. Changes in the oocytes volume were monitored at room temperature with a microscope video system by taking digital images at 150 s intervals. Oocytes volumes (Vs) were calculated from the measured area of each oocyte. The osmotic Pf was calculated for the first 30 min using the formula $Pf = V_0\,[d(V/V_0)/dt]/[S_0 \times V_W\,(Osmin - Osmout)]$. V_0 and S_0 are the initial volume and surface area of each individual oocyte, respectively; d(V/V0)/dt is the relative volume increase per unit time; *Vw* is the molar volume of water (18 cm^3 mol^{-1}); and Osmin – Osmout is the osmotic gradient between the inside and outside of the oocyte.

Plant transformation and generation of transgenic plants

The coding sequence of *TaAQP8* containing the *NcoI*/*SpeI* restriction site was amplified by using primers (P9; **Supplementary Table S1**), and was cloned into the pCAMBIA1304 vector as a GFP-fused fragment driven by the CaMV 35S promoter. The transformation vectors pCAMBIA1304-TaAQP8-GFP and pCAMBIA1304 were introduced into the *Agrobacterium tumefaciens* strain LBA4404. The transgenic tobaccos were generated using the *Agrobacterium*-mediated transformation method (Horsch et al. 1985). The seeds from transgenic plants were selected on MS medium containing 40 mg l^{-1} hygromycin. The hygromycin-resistant T_1 seedlings were confirmed by RT–PCR amplification using the primers for the *TaAQP8* (P10; **Supplementary Table S1**) and *GFP* (P11; **Supplementary Table S1**) gene. Three independent transgenic T_2 lines and the T_2 VC seedlings that almost all survived on MS medium containing 40 mg l^{-1} hygromycin were used in the experiments. The expression of *TaAQP8* in the three independent T_2 lines was investigated by semi-quantitative RT–PCR analysis using primers P10 and P5 (**Supplementary Table S1**), with the *NtUbiquitin* gene used as an internal control.

Stress tolerance assays of the control and transgenic plants

For salt stress tolerance assay during early seedling development, the WT, VC and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week, and then the seedlings were transferred to fresh MS medium or MS medium supplied with 100 or 200 mM NaCl for 1 week. The whole seedlings were used to perform physiological experiments, in which the root length, activities and expression of SOD, CAT and POD, the content of H_2O_2 and MDA, and IL were measured. A total of 200 surface-sterilized seeds of each transgenic line or the two controls were sown on MS medium without NaCl or MS medium supplied with 100 or 200 mM NaCl for 8 d to detect the germination rate. For salt stress tolerance assay, the WT and transgenic lines were cultured in MS medium under a 16 h light/8 h dark cycle at 25°C for 1 week and then were transplanted to containers filled with a mixture of soil and sand (3 : 1) where they were regularly watered for 2 or 5 weeks for the salt stress tolerance assay. Three-week-old tobacco plants with a consistent growth state were subjected to 300 mM NaCl for 40 d, which was used to detect the survival rate under NaCl stress. Six-week-old plants with a consistent growth state were subjected to 300 mM NaCl for 80 d and the root length was measured. After 60 d salt stress treatment, the leaves, roots and stems of tobacco plants were collected to detect the content of ions. After 30 or 60 d of salt stress treatment, the leaves and roots were collected from tobacco plants to measure the activities of SOD, CAT and POD, and H_2O_2 content.

Measurement of the content of Na^+, K^+ and Ca^{2+}

For ion content determination, the plant materials were washed with ultrapure water, treated at 105°C for 10 min and baked at 80°C for 48 h. Then 50 mg of the dry material was weighed and dissolved with 6 ml of nitric acid and 2 ml of H_2O_2 (30%) and heated at 180°C for 15 min. The digested samples were diluted to a total volume of 50 ml with ultrapure water and transferred into new tubes before analysis by using an inductively coupled plasma-mass sepectrometer (ICP-MS, ELAN DRC-e).

Measurement of SOD, CAT and POD activities, the content of H_2O_2 and MDA, and IL

The activities of SOD, POD and CAT were spectrophotometrically measured. For extraction of SOD, POD and CAT, about 0.5 g of samples was ground in liquid and homogenized in 5 ml of extraction buffer containing 0.05 M phosphate buffer (pH7.8) and 1% polyvinyl pyrrolidone. The homogenate was centrifuged at 10,000 × *g* for 10 min at 4°C and the resulting supernatant was collected for enzyme activity analysis. SOD and CAT activities were spectrophotometrically measured by using a SOD and CAT Detection Kit (A001 and A007, Jiancheng) according to the manufacturer's instructions. Total POD activity was measured by the change in absorbance at 470 nm due to guaiacol oxidation according to the method described in a previous study (Polle et al. 1994). Samples were collected for H_2O_2 measurements as described in a previous study (Jiang and Zhang 2001). MDA content was determined by the thiobarbituric acid (TBA)-based colorimetric method as described by Heath and Packer (1968). IL was measured based on the method of Jiang and Zhang (2001) with slight modification. The collected samples were cut into strips and incubated in 10 ml of distilled water at room temperature for 12 h. The initial conductivity (C1) was measured with a conductivity meter (DDBJ-350). The samples were then boiled for 30 min to result in complete IL. After cooling down at room temperature, the electrolyte conductivity (C2) was measured. IL was calculated according to the equation: IL (%) = C1/C2 × 100.

Statistical analysis

Statistical analyses were performed using the software in Excel and SPSS. Analysis of variance was used to compare the statistical difference based on Student's *t*-test, at a significant level of $P < 0.05$, $P < 0.01$.

Supplementary data

Supplementary data are available at PCP online.

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