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Overexpression of an H^+-PPase Gene from *Thellungiella halophila* in Cotton Enhances Salt Tolerance and Improves Growth and Photosynthetic Performance

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Salinity is one of the major environmental factors limiting plant growth and productivity. An H^+-PPase gene, *TsVP* from *Thellungiella halophila*, was transferred into cotton (*Gossypium hirsutum*) in sense and antisense orientations under control of the cauliflower mosaic virus (CaMV) 35S promoter. Southern and Northern blotting analysis showed that the sense or antisense *TsVP* were integrated into the cotton genome and expressed. Transgenic plants overexpressing the vacuolar H^+-PPase were much more resistant to 150 and 250 mM NaCl than the isogenic wild-type plants. In contrast, the plants from the antisense line (L–2), with lower H^+-PPase activity, were more sensitive to salinity than the wild-type plants. Overexpressing *TsVP* in cotton improved shoot and root growth and photosynthetic performance. These transgenic plants accumulated more Na^+, K^+, Ca^{2+}, Cl^- and soluble sugars in their root and leaf tissues under salinity conditions compared with the wild-type plants. The lower membrane ion leakage and malondialdehyde (MDA) level in these transgenic plants suggest that overexpression of H^+-PPase causes the accumulation of Na^+ and Cl^- in vacuoles instead of in the cytoplasm, thus reducing their toxic effects. On the other hand, the increased accumulation of ions and sugars decreases the solute potential in cells, and facilitates water uptake under salinity, which is an important mechanism for the increased salt tolerance in *TsVP*-overexpressing cotton.

Keywords: Cotton (*Gossypium hirsutum*) — H^+-PPase — Photosynthesis — Salt tolerance.

Abbreviations: BSA, bovine serum albumin; CaMV, cauliflower mosaic virus; CTAB, cetyltrimethylammonium bromide; DTT, ditiothreitol; MDA, malondialdehyde; PFD, photon flux density; PMSF, phenylmethylsulfonyl fluoride; PVP, polyvinylpyrrolidone; RT–PCR, reverse transcription–PCR; TsVP, an H^+-PPase gene from *Thellungiella halophila*; VPP, cotton endogenous H^+-PPase gene; WT, wild type.

Introduction

Salinity is one of the major environmental factors limiting plant growth and productivity. It is estimated that about 20% of the world's cultivated land and nearly half of all irrigated land are affected by salinity (Rhoades and Loveday 1990). Furthermore, the problem of soil salinization is getting more serious, mainly because of unsuitable irrigation practices. Thus, it is imperative to improve the salt tolerance of several major crops, by traditional plant breeding as well as biotechnological approaches.

Salinity interferes with plant growth as it leads to physiological drought, resulting in osmotic stress and ion toxicity (Zhu 2002). Excessive Na^+ in the cytoplasm not only causes ion imbalance and affects critical biochemical processes (Maathuis and Amtmann 1999), but also increases plasma membrane injury, causes malondialdehyde (MDA) accumulation and impairs photosynthetic capacity (Dionisio-Sese and Tobita 1998, Kawasaki et al. 2001). Plants have evolved many mechanisms in response to salt stress, including osmotic adjustment and reducing excessive Na^+ in the cytoplasm. Na^+ extrusion from the cytosol and compartmentation within the vacuole are two main ways to reduce excessive Na^+ in the cytoplasm. The compartmentation of Na^+ into the vacuole, through vacuolar Na^+/H^+ antiporters, provides an efficient mechanism to avert the deleterious effects of Na^+ in the cytosol and maintains an osmotic potential by using Na^+ (and chloride) accumulated in the vacuole to drive water uptake into cells (Apse et al. 1999).

The driving force for Na^+/H^+ antiport is provided by two types of electrogenic, proton-translocating pumps, one hydrolyzing ATP (V-ATPase) and the other hydrolyzing PPi (PPase). In addition, the proton-motive force generated by the vacuolar ATPase (V-ATPase) and vacuolar pyrophosphatase (V-PPase) can also drive the Ca^{2+}/H^+ antiporter, as well as transporters of organic acids, sugars and other compounds to maintain cell turgor

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(Lincoln 1992). In principle, overexpressing either of the vacuolar proton pumps should increase the sequestration of ions in the vacuole by increasing the availability of protons. Overexpression of the vacuolar Na^+/H^+ antiporter has resulted in enhanced plant salt tolerance (Apse et al. 1999, Fukuda et al. 2004, Wu et al. 2004). Gaxiola et al. (2001) overexpressed AVP1 in *Arabidopsis thaliana*, and the transgenic plants displayed enhanced salt and drought tolerance that was correlated with the increased ion content of the plants. Recently, work from the same laboratory demonstrated that AVP1 played an important role in root development through facilitating auxin fluxes, and that transgenic tomatoes expressing AVP1 were more resistant to soil water deficits than controls due to more robust root systems (Li et al. 2005, Park et al. 2005). These results suggest that the enhanced vacuolar H^+ pumping in the transgenic plants provides more energy for vacuolar sodium accumulation, and this is an effective approach to improve crop salt and drought tolerance.

Cotton, one of the most important fiber and oil crops in the world, is classified as a salt-tolerant crop. However, a number of studies have demonstrated that there are obviously varietal differences in response to soil salinity, and its growth and yield are severely inhibited in higher salinity soil (Ashraf 2002). Therefore, it is of agricultural importance to understand and improve the salt tolerance of cotton. Many previous studies suggested that salt-tolerant cotton varieties may have better protection against reactive oxygen species by increasing the activity of antioxidant enzymes under salt stress (Gossett et al. 1994, Rajguru et al. 1999, Meloni et al. 2003). Wu et al. (2004) isolated a cotton gene, *GhNHX1*, encoding a tonoplast Na^+/H^+ antiporter. Their results indicated that the expression of *GhNHX1* in cotton seedlings was induced by salt stress, and the varietal differences in response to soil salinity were consistent with the expression levels of *GhNHX1*. Furthermore, both a transformed yeast Na^+/H^+ antiporter mutant expressing *GhNHX1* and transgenic tobacco plants overexpressing *GhNHX1* showed higher salt tolerance than the yeast mutant and the wild-type (WT) plants, respectively, demonstrating the important role of *GhNHX1* in cotton salt tolerance. Recently, transgenic cotton plants expressing an *Arabidopsis* vacuolar Na^+/H^+ antiporter gene were created and these plants generated more biomass and produced more fibers compared with the WT when grown in the presence of 200 mM NaCl, which was probably due to better photosynthetic performance and higher nitrogen assimilation rates (He et al. 2005). The two studies above suggest that the vacuolar Na^+/H^+ antiporter plays an important role in cotton salt tolerance, and overexpression of the vacuolar H^+-PPase gene to provide a stronger driving force for Na^+/H^+ antiport may also be an effective way to enhance cotton salt tolerance.

We have previously cloned an H^+-PPase gene, *TsVP* from *Thellungiella halophila*. Both a transformed yeast mutant *ena1* expressing *TsVP* and transgenic tobacco plants overexpressing *TsVP* showed higher salt tolerance than the yeast mutants and the WT plants, respectively (Gao et al. 2006). In this study, we overexpressed the *T. halophila TsVP* in cotton to test whether cotton's performance could be improved under salinity stress conditions. Furthermore, to understand better the function of the foreign gene in cotton salt tolerance, we also investigated the performance of transgenic cotton expressing the antisense *TsVP*.

Results

Genetic transformation and molecular characterization of the transgenic plants

Injured shoot apexes of cotton (cultivar 'Luyuan890') were transformed by *Agrobacterium tumefaciens* harboring a plasmid with the *TsVP* gene (as described in Materials and Methods), either in the sense or antisense orientation, under control of the cauliflower mosaic virus (CaMV) 35S promoter. After 3 d of co-culture, the cotton plantlets were transferred to vermiculite and selected by spraying the herbicide sulfometuron (2.5 mg l^{-1}) twice at 24 h intervals at the stage of 2–3 leaves, and about 20–30% of the plantlets survived. The surviving plantlets were then identified by PCR; nine independent *TsVP* sense transformants and seven antisense transformants were identified. The stable integration and transmission of the *TsVP* sense or antisense gene in the genome of T_2 plants were confirmed by Southern blot analysis (Fig. 1A). To examine the expression of the *TsVP* sense or antisense gene in T_2 transgenic plants, total RNA isolated from leaves of WT and transgenic plants was subjected to Northern blot analysis. mRNA transcripts were detected at different levels in all transgenic lines, which confirmed the expression of the *TsVP* sense or antisense gene in the T_2 transgenic plants (Fig. 1B).

Overexpression of TsVP enhanced cotton salt tolerance

In preliminary experiments, T_2 plants from *TsVP* transgenic homozygous lines (sense or antisense) and the WT plants were used for salt tolerance assays. There were six sense lines and two antisense lines that showed obviously different salt tolerance compared with the WT. Subsequently, seedlings at the stage of five leaves from the six sense lines and two antisense lines were subjected to salt stress by adding 150 or 250 mM NaCl to the nutrient solution. After 21 d of salt treatment, plants were harvested and dried. Under non-stressed conditions the dry shoot masses of the *TsVP* sense transgenic plants were from 7 to 47% more than those of WT plants, and this difference in the dry root masses was from 19 to 62% (Table 1). When treated with 150 mM NaCl, the dry shoot masses and root masses of

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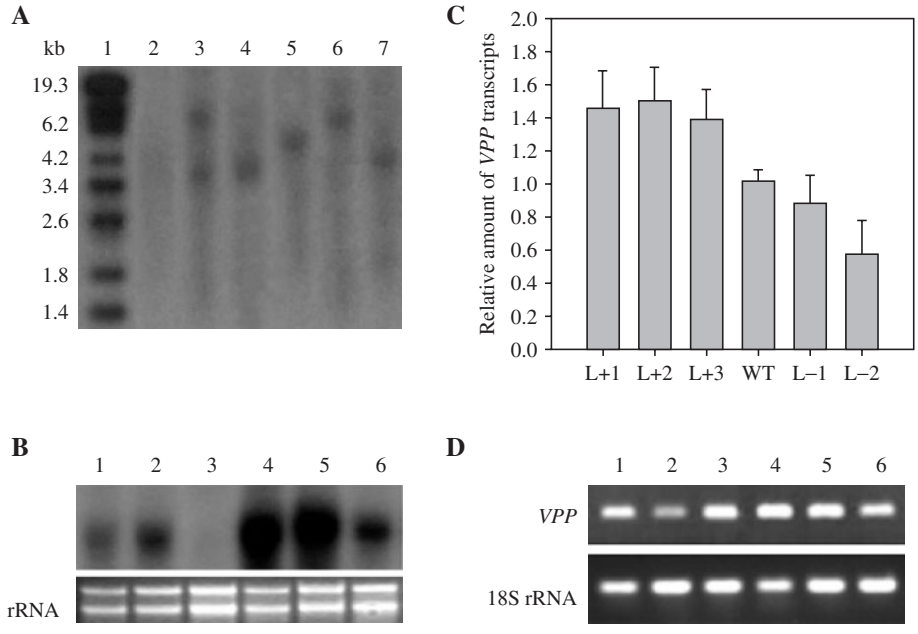

Fig. 1 Molecular characterization of the transgenic cotton plants. (A) Southern blot analysis of genomic DNA probed with full-length *TsVP* cDNA: lane 1, λ-*Eco*T14 molecular weight marker; lane 2, WT; lanes 3 and 4, antisense lines L–1 and L–2; lanes 5–7, sense lines L+1, L+2 and L+3. (B) Northern blot: lanes 1 and 2, antisense lines L–1 and L–2; lane 3, WT; lanes 4–6, sense lines L+1, L+2 and L+3. Total RNA was extracted from young leaves of WT and transgenic plants and probed with full-length cDNA of *TsVP*. Ethidium bromide-stained rRNA bands are shown as a loading control. (C) The relative expression levels of the cotton endogenous *VPP* gene by real-time quantitative RT–PCR. The relative gene expression levels in WT and transgenic lines were calculated with the 18S rRNA gene as an internal control. The expression level of the WT was arbitrarily set to 1.0. Values are means of five replicates ± SD. (D) Products from real-time RT–PCR analyzed in 2% agarose gels: lanes 1 and 2, antisense lines L–1 and L–2; lane 3, WT; lanes 4–6, sense lines L+1, L+2 and L+3. The transcription level of the 18S rRNA gene was used as a reference.

the sense transgenic plants were 30–53% and 56–89% more than those of the WT, respectively (Table 1). When treated with 250 mM NaCl, the dry shoot masses and root masses of the sense plants were 27–56% and 50–87% more than those of the WT, respectively, and the root/shoot ratios of all sense lines were significantly higher than those of the WT (Table 1). There was no significant difference in biomass between the antisense transgenic lines and the WT under non-salt stressed and salt conditions with 150 mM NaCl (Table 1). However, the shoot and root biomasses in the antisense line L–2 were 33 and 38% lower than those of the WT under 250 mM NaCl, respectively (Table 1). Three sense lines (L + 1, L + 2 and L + 3) and two antisense lines (L–1 and L–2) were selected from these lines for the analysis of salt tolerance and photosynthetic performance.

Repression of the cotton endogenous H^+-PPase gene in the antisense transgenic lines

The relative expression levels of the cotton endogenous *VPP* gene encoding H^+-PPase were analyzed with real-time reverse transcription–PCR (RT–PCR). The *VPP* transcripts were detected in all lines examined (Fig. 1D). To validate the repression of the endogenous *VPP* in cotton plants by the antisense gene construct, the relative transcript levels in WT and transgenic lines were calculated according to the data of real-time RT–PCR. The expression level of the WT was arbitrarily set to 1.0. As shown in Fig. 1C, the relative amounts of *VPP* transcripts in the two antisense lines were about 88 and 57% of that of the WT, respectively.

V-PPase and V-ATPase activity

To confirm the function of *TsVP*, the hydrolytic activity of H^+-PPase of plants from *TsVP* sense lines (L + 1, L + 2 and L + 3), a *TsVP* antisense line (L–2) and the WT was determined under non-stressed and salt stress conditions. Seedlings grown in Hoagland solution, in 2 liter plastic boxes, were subjected to salt treatment with 150 or 250 mM NaCl for 21 d, then the roots were collected for membrane vesicle isolation and enzyme assays. The purity of the tonoplast vesicles was between 52 and 66%, as determined by the hydrolytic activity of V-ATPase in the presence or absence of the specific V-ATPase inhibitor 50 mM NO_3^-. As shown in Fig. 2A, under normal conditions, the PPase activity in *TsVP* sense plants was 22–75% higher than that in the WT, while that in *TsVP* antisense line L–2 was about 24% lower than that in the WT.

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Table 1 The biomasses (dry weights) and root/shoot ratios of cotton plants after salt treatment with 150 and 250 mM NaCl for 21 d

Genotype	NaCl concentration (mM)	Shoot biomass (g)	Root biomass (g)	Total biomass (g)	Root/shoot ratio
WT	0	1.98 ± 0.20	0.26 ± 0.07	2.24 ± 0.26	0.13 ± 0.03
	150	0.70 ± 0.14	0.09 ± 0.02	0.79 ± 0.16	0.13 ± 0.02
	250	0.55 ± 0.07	0.08 ± 0.01	0.64 ± 0.08	0.15 ± 0.01
L + 1	0	2.73 ± 0.47*	0.42 ± 0.06**	3.15 ± 0.48*	0.16 ± 0.03
	150	1.07 ± 0.13**	0.17 ± 0.03*	1.24 ± 0.16*	0.16 ± 0.02*
	250	0.86 ± 0.16*	0.15 ± 0.03**	1.01 ± 0.19**	0.17 ± 0.02*
L + 2	0	2.92 ± 0.47*	0.42 ± 0.09*	3.34 ± 0.55*	0.14 ± 0.02
	150	0.98 ± 0.14*	0.15 ± 0.02**	1.13 ± 0.16*	0.15 ± 0.01
	250	0.76 ± 0.16*	0.13 ± 0.02*	0.90 ± 0.18*	0.18 ± 0.02*
L + 3	0	2.11 ± 0.17	0.31 ± 0.05	2.42 ± 0.20	0.14 ± 0.02
	150	1.06 ± 0.17*	0.17 ± 0.02**	1.23 ± 0.19*	0.16 ± 0.01*
	250	0.78 ± 0.15*	0.14 ± 0.03*	0.92 ± 0.18*	0.17 ± 0.02*
L + 4	0	2.78 ± 0.45*	0.39 ± 0.09*	3.17 ± 0.53*	0.14 ± 0.01
	150	1.02 ± 0.12*	0.16 ± 0.03*	1.18 ± 0.15**	0.15 ± 0.01
	250	0.80 ± 0.15*	0.13 ± 0.02*	0.93 ± 0.17*	0.17 ± 0.02*
L + 5	0	2.22 ± 0.17	0.32 ± 0.05	2.53 ± 0.21	0.14 ± 0.02
	150	0.91 ± 0.13*	0.14 ± 0.02*	1.05 ± 0.14*	0.15 ± 0.01
	250	0.71 ± 0.14	0.12 ± 0.02*	0.83 ± 0.16*	0.17 ± 0.02*
L + 6	0	2.54 ± 0.43*	0.38 ± 0.05*	2.92 ± 0.47*	0.15 ± 0.02
	150	1.01 ± 0.16*	0.16 ± 0.03**	1.17 ± 0.18*	0.16 ± 0.02*
	250	0.70 ± 0.14	0.12 ± 0.03*	0.83 ± 0.16*	0.18 ± 0.02*
L–1	0	2.30 ± 0.47	0.32 ± 0.06	2.62 ± 0.52	0.14 ± 0.02
	150	0.81 ± 0.16	0.12 ± 0.04	0.93 ± 0.19	0.15 ± 0.02
	250	0.65 ± 0.14	0.10 ± 0.02	0.75 ± 0.15	0.16 ± 0.01
L–2	0	1.74 ± 0.22	0.23 ± 0.06	1.97 ± 0.27	0.13 ± 0.02
	150	0.72 ± 0.19	0.10 ± 0.02	0.82 ± 0.21	0.14 ± 0.03
	250	0.37 ± 0.09*	0.05 ± 0.01*	0.42 ± 0.11*	0.14 ± 0.02

Cotton seedlings grown in Hoagland solution were subjected to salt stress by adding 150 or 250 mM NaCl to the nutrient solution at the five-leaf stage. After 21 d of salt treatment, plants were harvested for biomass determination after drying for 72 h in an oven at 70°C. Values are the means of three independent experiments ± SD. Asterisks indicate a significant difference from the WT under the same salt conditions at $^{*}P < 0.05$ or $^{**}P < 0.01$ by *t*-test.

The hydrolytic activity of H^+-PPase in all plants increased under 150 mM NaCl treatment, but it declined under 250 mM NaCl treatment. However, the differences in H^+-PPase activity between transgenic plants and the WT still existed, with that in sense plants 22–62 and 44–94% higher than that in the WT under 150 and 250 mM NaCl treatment, respectively. However, H^+-PPase activity in L–2 plants was 31% lower than that in the WT under 250 mM NaCl treatment. In contrast, the hydrolytic activity of the V-ATPase in all plants was induced by both 150 and 250 mM NaCl treatments, and there was no significant difference between transgenic lines and the WT (Fig. 2B).

Growth of WT and transgenic cotton plants under salt stress

Overexpression of the *TsVP* gene in cotton promoted plant growth, as shown in Fig. 3. Under non-stressed conditions, the *TsVP*-overexpressing lines exhibited greater development of the shoot and root system compared with the WT (Fig. 3A, D). Under salt stress conditions, the phenotypic differences between WT and the sense transgenic plants became more apparent (Fig. 3B, C, E, F). Growth of plants was severely inhibited by both of the salt treatments, and this inhibitory effect increased with increasing NaCl concentration, but growth of *TsVP*-overexpressing plants was less inhibited compared with the WT (Fig. 3B, C, E, F).

To validate further the observation from the liquid culture assays, we selected two sense lines (L + 1 and L + 2) and one antisense line (L–2) to examine resistance to salinity under natural weather conditions in May to July in Jinan. Transgenic and WT plants were grown together in large plastic containers with vermiculite. In this assay, the more robust root system in the sense lines was also observed under both MS solution alone and 150 mM NaCl treatment (Fig. 4).

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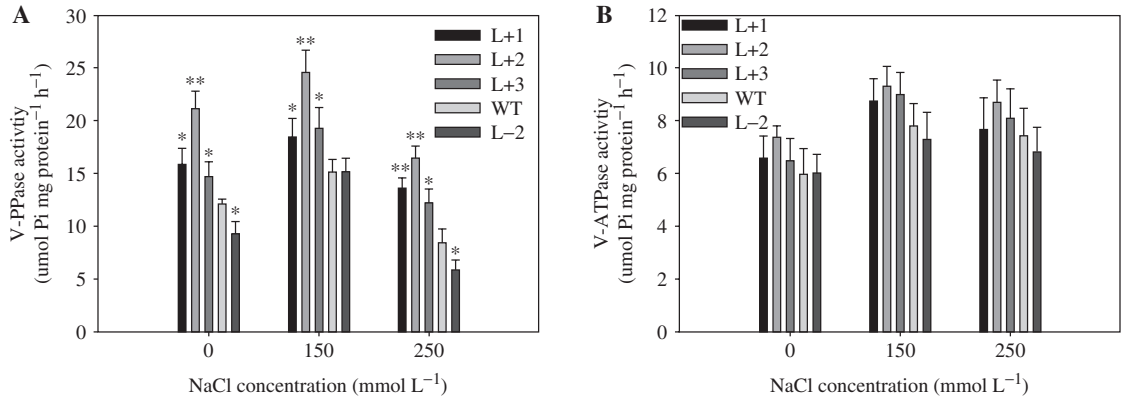

Fig. 2 V-PPase activity (A) and V-ATPase activity (B) in transgenic and WT plants under non-stressed and salt stress conditions. Plants from *TsVP* sense lines (L+1, L+2 and L+3), *TsVP* antisense line (L–2) and the WT were grown in Hoagland solution and then subjected to 21 d of salt treatment in 150 or 250 mM NaCl. Roots were then collected for membrane vesicle isolation and enzyme assays. Values are the means of three independent experiments ± SD. Asterisks indicate a significant difference from the WT under the same salt-treated conditions at $^{*}P<0.05$ or $^{**}P<0.01$ by *t*-test.

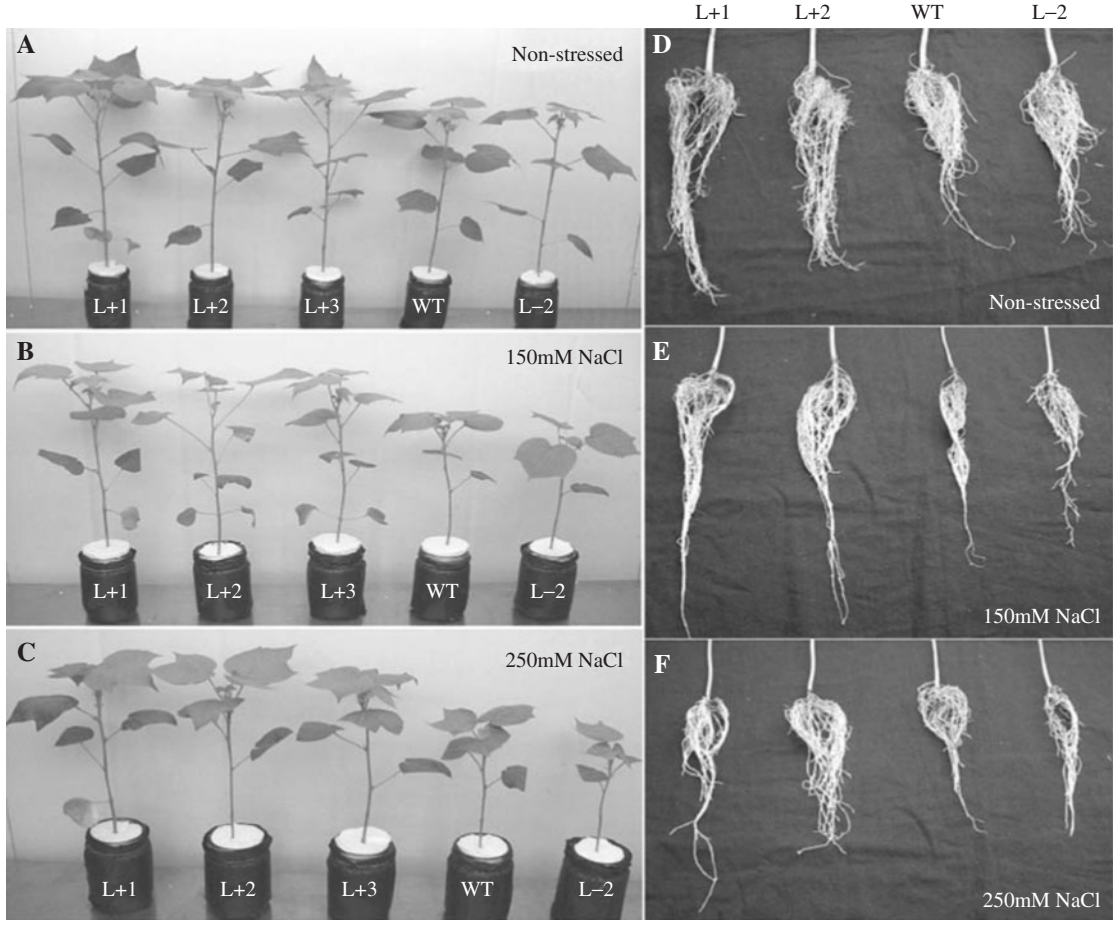

Fig. 3 Phenotypes of shoots (A, B and C) and roots (D, E and F) of WT and transgenic cotton plants after treatment with 150 and 250 mM NaCl for 21 d. Cotton seedlings grown in Hoagland solution were subjected to salt stress by adding 150 or 250 mM NaCl to the nutrient solution at the stage of five leaves.

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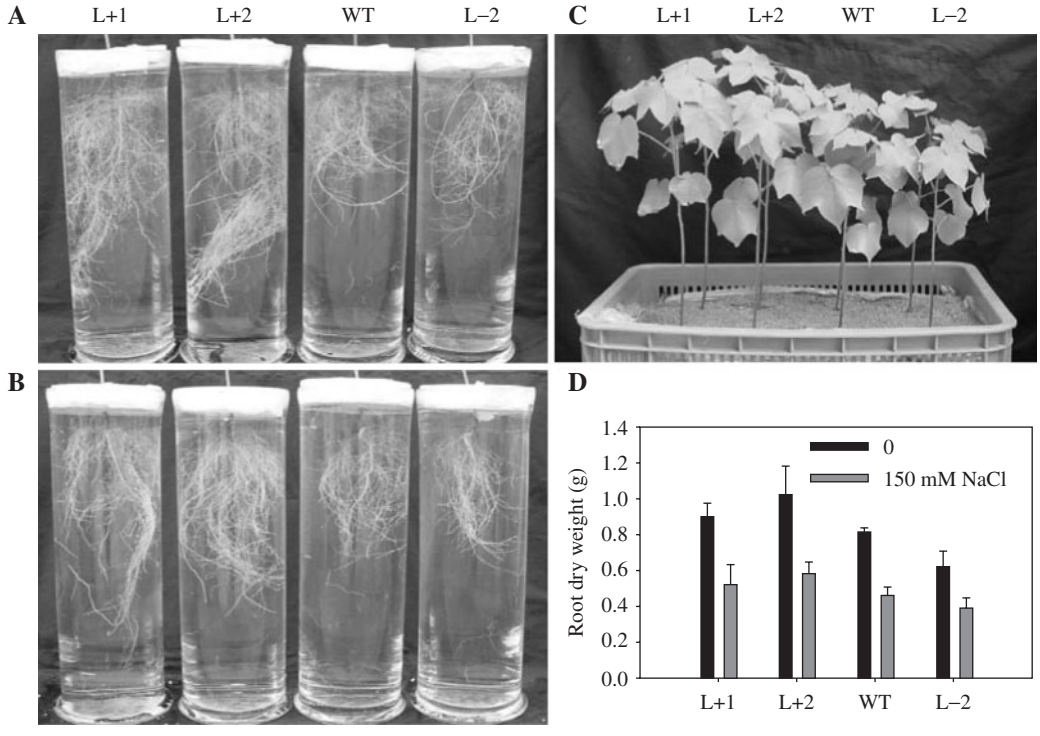

Fig. 4 Phenotypes of roots and shoots and the root biomass of WT and transgenic lines L+1, L+2 and L–2 grown in vermiculite with MS solution. (A) Roots of non-stressed cotton plants; (B) roots of salt-stressed plants; (C) non-stressed plants; (D) root dry weight. Beginning at the fifth week after germination, plants in one container were exposed to salt stress, for 21 d, by supplying NaCl to the nutrient solution with a final concentration of 150 mM. Plants in the other container act as non-stressed controls.

Changes of chlorophyll content in cotton leaves during salt treatment

Seedlings grown in Hoagland solution were subjected to salt stress by adding 150 or 250 mM NaCl to the nutrient solution at the stage of five leaves. Chlorophyll content was determined before addition of salt and after 1, 7, 14 and 21 d of salt stress. As shown in Fig. 5, before salt stress, the chlorophyll content in the sense lines L + 1 and L + 2 was slightly higher than in the WT, and that in L–2 was lower than in the WT, but these differences were not significant. During salt stress, the chlorophyll content in all plants tested increased in the first week after salt stress, and then decreased remarkably in the following 2 weeks, but it was significantly higher in *TsVP*-overexpressing lines than in the WT. Chlorophyll content in the antisense transgenic lines showed no significant difference from the WT after salt stress. Compared with plants subjected to 150 mM NaCl, plants subjected to 250 mM NaCl displayed a greater variation in chlorophyll content (Fig. 5).

Effect of salt stress on photosynthesis and chlorophyll fluorescence of cotton seedlings

In order to examine possible reasons for the higher biomass of *TsVP*-overexpressing lines, the photosynthetic performance of plants under non-stressed and salt stress conditions was investigated. Under non-salt stressed conditions, the *TsVP*-overexpressing cotton plants displayed higher photosynthesis rate and stomatal conductance than WT plants, to various extents. However, the photosynthesis rate of the antisense transgenic line L–2 was significantly lower than that of the WT (Fig. 6A, B). The net photosynthetic rate and stomatal conductance of both WT and transgenic plants were significantly inhibited with increasing salinity. However, plants from the sense transgenic lines showed much less inhibition by salinity than the WT plants, while the antisense transgenic lines showed much more inhibition than the WT plants. After 21 d salt treatment in 150 mM NaCl, the net photosynthetic rate and stomatal conductance in the sense transgenic lines were 11–19 and 10–18% higher than those of the WT, respectively, while those of the antisense transgenic lines were 36–52 and 27–33% lower than those of the WT, respectively. Under 250 mM NaCl treatment, these differences in the net photosynthetic rate and stomatal conductance between transgenic lines and the WT were still observed (Fig. 6A, B).

Regarding the intercellular CO_2 concentration, the transgenic lines showed no difference from the WT under non-salt stressed conditions, except L + 2 for which the

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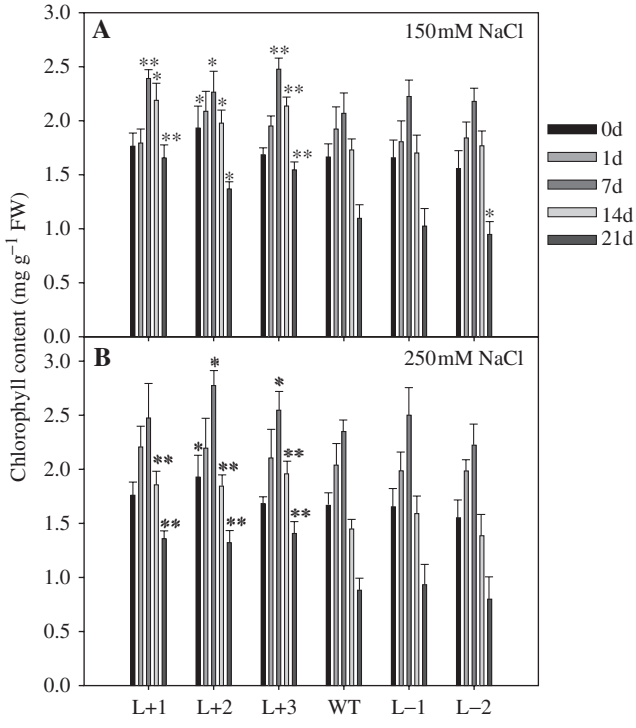

Fig. 5 Changes in chlorophyll content of cotton leaves during salt treatment with 150 mM NaCl (A) and 250 mM NaCl (B). Seedlings grown in Hoagland solution were subjected to salt stress at five-leaf stage. Chlorophyll content was determined before addition of salt and after 1, 7, 14 and 21 d of salt stress. Values are the means of three independent experiments ± SD. Asterisks indicate a significant difference from the WT under the same salt treatment conditions at $^{*}P<0.05$ or $^{**}P<0.01$ by *t*-test.

concentration was significantly higher than that of the WT, and L–2 for which the concentration was significantly lower than that of the WT. The intercellular CO_2 concentrations in all transgenic lines and the WT decreased when plants were exposed to 150 mM NaCl for 21 d, but those of the sense transgenic lines were higher than those of the WT (Fig. 6C). Under 250 mM NaCl treatment, the intercellular CO_2 concentrations in transgenic lines L + 1, L + 2, L + 3 and L–1 decreased compared with those of non-stressed plants, but those of WT and L–2 increased (Fig. 6C).

To investigate further the factors limiting photosynthesis under salt stress, the potential maximum quantum yield of PSII photochemistry estimated using the F_v/F_m ratio in dark-acclimated leaves was measured. The F_v/F_m ratio is the most frequently used parameter to indicate injury to the PSII complexes due to stress factors, including salinity. Compared with that under normal condition, no significant changes in the F_v/F_m ratio were observed in either transgenic or WT plants under 150 mM NaCl for 21 d (Fig. 6D). However, the F_v/F_m ratio decreased significantly in all lines (except transgenic line L + 2) under 250 mM NaCl for 21 d, though that of the sense transgenic lines was higher than that of the WT (Fig. 6D). It appears that 150 mM NaCl has no significant effect on the reaction center of PSII, whereas 250 mM NaCl depresses the reaction center of PSII significantly in cotton plants acclimated to long-term salt treatment.

Data for stomatal conductance, intercellular CO_2 concentration and the F_v/F_m ratio indicated that the reduction in supply CO_2, mainly due to the decrease in stomatal conductance, was the dominant limiting factor for photosynthesis under 150 mM NaCl, whereas the decrease in photosynthesis rate under 250 mM NaCl was due to both the decrease in stomatal conductance and the depressed PSII activity.

Leaf cell membrane ion leakage and MDA content under salt stress

Excessive Na^+ in the cytoplasm usually increases membrane injury and causes MDA accumulation (Dionisio-Sese and Tobita 1998). To study the effects of salt stress on cotton seedlings further, leaf cell membrane ion leakage and MDA content were determined in non-stressed plants and plants salt stressed for 21 d. As shown in Fig. 7A and B, there were no significant differences in ion leakage and MDA content between the WT and all transgenic lines under normal conditions. Ion leakage and MDA content increased with increased salt concentrations (Fig. 7). However, significant differences appeared in the ion leakage and MDA content between *TsVP* sense lines and the WT under increased salt conditions. Leaf cell ion leakage of *TsVP* sense lines was 4–9 and 5–10% less than that of the WT under 150 and 250 mM NaCl treatments, respectively (Fig. 7A). The amounts of MDA in the sense transgenic lines were 7–10 and 9–15% less than that of the WT under the two salt treatments, respectively (Fig. 7B). Under increased salt conditions, there were no significant differences in ion leakage and MDA content between the antisense transgenic lines and the WT (Fig. 7). These results indicated that overexpressing *TsVP* in cotton could alleviate the cell membrane injury caused by salt stress.

Ion contents in plant tissues

To investigate whether overexpressing *TsVP* increases the concentration and sequestration of ions into the vacuole, we measured the amounts of Na^+, K^+, Ca^{2+} and Cl^- in the leaves and roots of WT and transgenic plants under different NaCl concentrations (0, 150 and 250 mM). As shown in Fig. 8, the Na^+ and Cl^- concentrations increased and the K^+ and Ca^{2+} concentrations decreased with increasing NaCl concentrations in both roots and leaves. In roots, the Na^+ content was 30–66% higher in the sense transgenic lines than in the WT, whereas the Na^+ content in the antisense line L–2 was about 24% lower than in the WT. In leaves, the Na^+ content was 32–76% higher in the sense transgenic lines than

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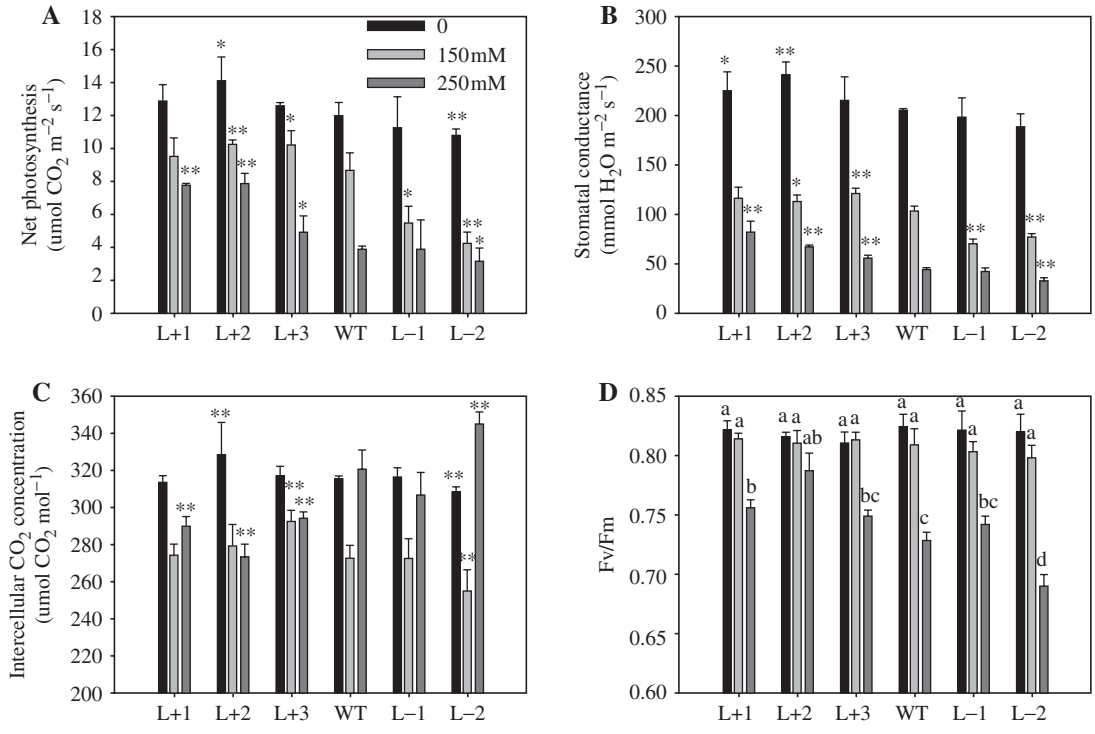

Fig. 6 Net photosynthesis (A), stomatal conductance (B), intercellular CO_2 concentration (C) and F_v/F_m (D) of WT and transgenic plants under non-salt stressed and salt conditions. Values are the means ± SD of six plants per line. Asterisks indicate a significant difference from the WT under the same salt-treated conditions at $^*P<0.05$ or $^{**}P<0.01$ by *t*-test. In (D), values followed by the same letter (a, b, c and d) are not significantly different from each other.

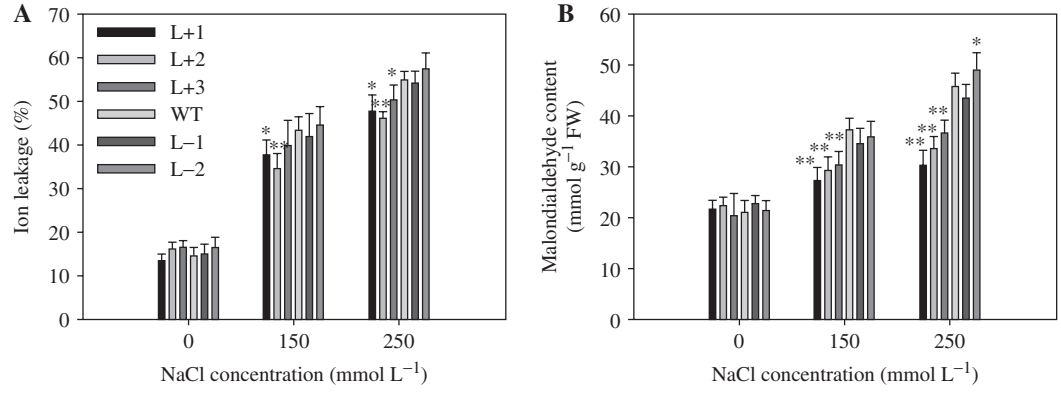

Fig. 7 Changes in membrane ion leakage (A) and MDA content (B) in leaves of WT and transgenic plants grown in Hoagland solution and subjected to 21 d of salt stress from the five-leaf stage. Values are the means of three independent experiments ± SD. Asterisks indicate a significant difference from the WT under the same salt-treated conditions at $^*P<0.05$ or $^{**}P<0.01$ by *t*-test.

in the WT, while that in L–2 was about 17–25% lower than in the WT (Fig. 8A). For Cl^-, the sense transgenic lines was also higher than the WT, and the difference was significant in roots of L+1 and L+2 and leaves of L+2 (Fig. 8D). The K^+ or Ca^{2+} content in the sense transgenic lines was slightly higher than that in the WT with different salt treatments, but the difference was not significant (Fig. 8B, C).

The TsVP-overexpressing plants accumulated more soluble sugars and maintained lower solute potential than wild-type plants

To investigate the mechanism of the improvement of salt tolerance in the *TsVP*-overexpressing cotton plants, we measured the solute potential (Ψs, refers to the concentration of osmotically active particles dissolved in water) from the

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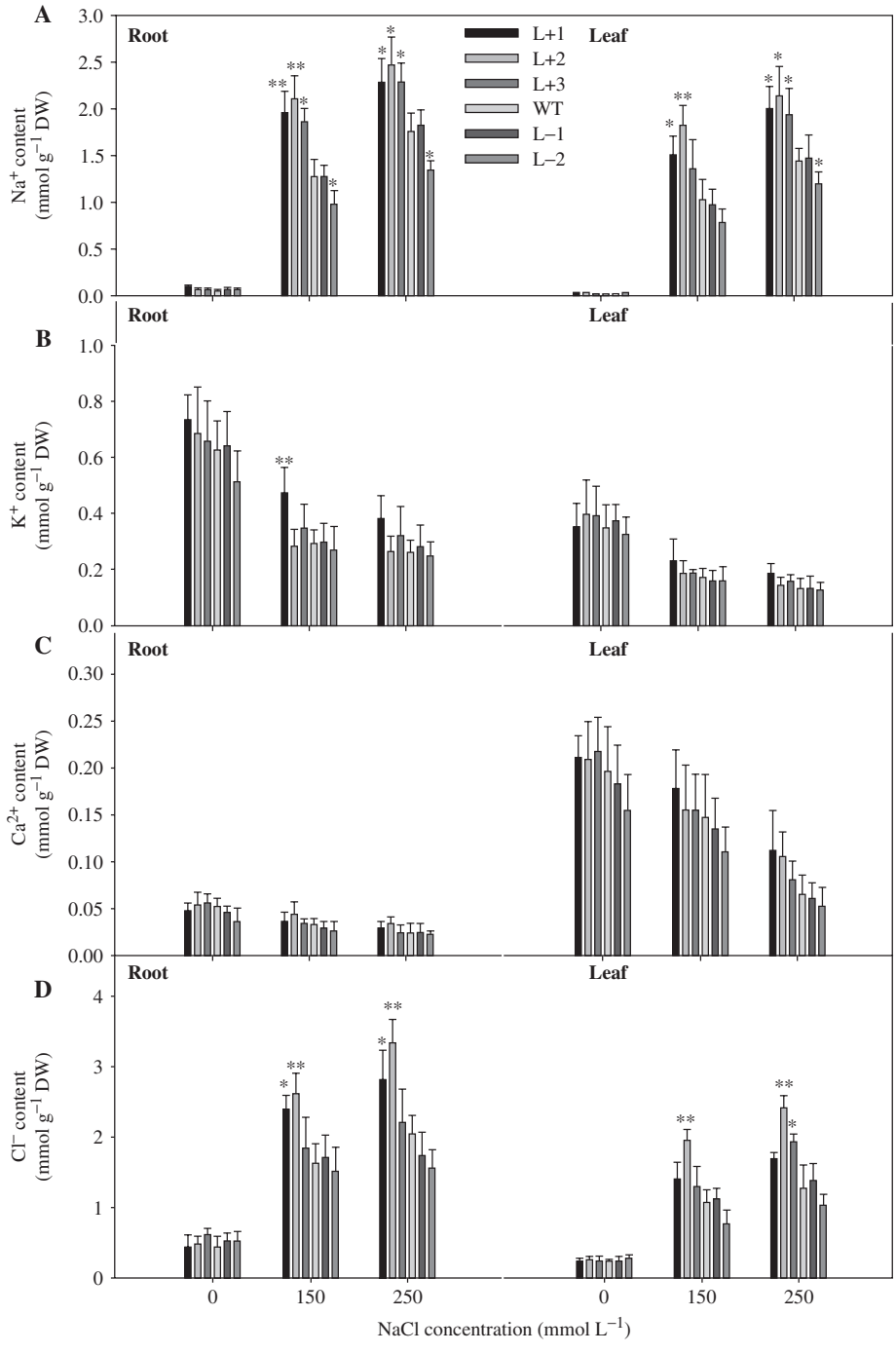

Fig. 8 Content of ions in roots and leaves of WT and transgenic plants at different concentrations of NaCl. (A) Na^+ content; (B) K^+ content; (C) Ca^{2+} content; and (D) Cl^- content. WT and transgenic plants grown in Hoagland solution were subjected to 21 d of salt stress by adding 150 or 250 mM NaCl to the nutrient solution at the five-leaf stage. Values are the means of three independent experiments ± SD. Asterisks indicate a significant difference from the WT under the same salt-treated conditions at $^*P<0.05$ or $^{**}P<0.01$ by *t*-test.

leaves of the wild-type, *TsVP* sense and antisense lines. As shown in Fig. 9A, under normal conditions, values of Ψs for *TsVP* sense lines were lower (–0.95 to –1.01 MPa) than the values for the WT (–0.91 MPa), while values of Ψs for the antisense line L–2 (–0.85 MPa) were higher than for the WT. The solute potential of all lines decreased with increasing salinity concentration (Fig. 9A). However, the decline in Ψs was greater in *TsVP* sense lines than in WT and *TsVP* antisense lines. The values of Ψs for *TsVP* sense lines decreased from –1.16 to –1.21 and from –1.38 to –1.52 MPa under

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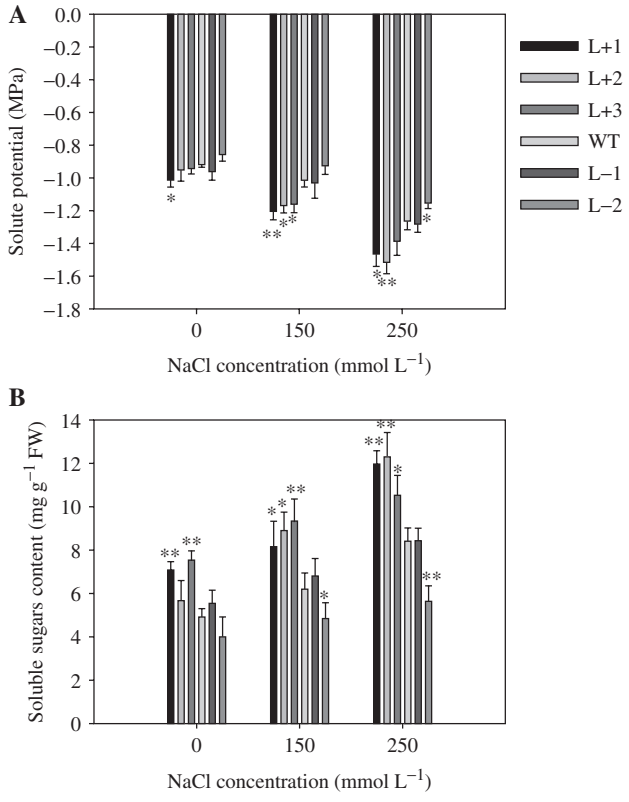

Fig. 9 Solute potential (A) and total soluble sugars content (B) in seedlings under non-salt stressed aconditions and following 21 d of salt stress from the five-leaf stage. Values are the means of three independent experiments ± SD. Asterisks indicate a significant difference from the WT under the same salt-treated conditions at $^{*}P<0.05$ or $^{**}P<0.01$ by *t*-test.

150 and 250 mM NaCl treatments, respectively, whereas the values for WT decreased to –1.01 and –1.26 MPa, respectively. The value for the antisense line L–2 (–1.15 MPa) was significantly higher than that of the WT (–1.26 MPa) under salt treatment with 250 mM NaCl (Fig. 9A).

To investigate further factors other than inorganic ions leading to the lower solute potential in *TsVP*-overexpressing plants, we measured the total soluble sugars in the same leaves used for Ψs measurement. As expected, *TsVP*-overexpressing plants accumulated more soluble sugars than WT and *TsVP* antisense plants under both non-salt-stressed and salt-stressed conditions (Fig. 9B). These results indicated that the sense *TsVP* plants retained more solutes, including cations and sugars, in cells. This would enhance the capability for water uptake at low soil solution water potential, which can occur due to salinization.

Discussion

Salinity is one of the major hazards, usually confined to arid or semi-arid regions of the world, which constitute a primary limit on crop productivity (Ashraf 1994). Although cotton is classified as a salt-tolerant crop, with a salinity threshold level of 7.7 dSm^{-1}, its sensitivity varies greatly among genotypes, and its growth and seed yield are severely reduced at high salinity levels (Ashraf 2002). Therefore, there is an urgent need to focus efforts on developing salt-tolerant varieties of cotton. In recent years, research in plant biology has led to the identification of genes that might be useful in crop improvement, and, among the genes identified, those encoding vacuolar H^+-PPase appear to hold great promise in improving agricultural productivity under salt and drought conditions (Gaxiola et al. 2001, Park et al. 2005, Gao et al. 2006). In the present study, we transferred an H^+-PPase gene, *TsVP* from *T. halophila*, in sense or antisense orientation into cotton. It was shown that overexpression of *TsVP* in cotton resulted in improved salt tolerance. Plants from all the three sense *TsVP* transgenic lines maintained better shoot and root growth, higher photosynthesis capacity, lower percentage of ion leakage and less lipid membrane peroxidation, and retained more solutes than the WT under increased NaCl concentrations. In contrast, plants from the antisense line (L–2), with lower H^+-PPase activity, were more sensitive to salinity than the WT.

In this study, the overexpression of *TsVP* in cotton enhanced shoot and root growth under both normal and increased salt conditions (Figs. 3, 4). The measurement of shoot and root dry weight (Table 1) was in agreement with visual observations. Furthermore, the root/shoot ratio in the sense transgenic cotton was significantly higher than that in the WT under 250 mM NaCl (Table 1). These results are consistent with previous work reported by Li et al. (2005). They reported that overexpression of *AVP1* increased the number and size of rosette leaves and enhanced root growth in *Arabidopsis*, and deduced that AVP1 appeared to function in both shoot and root development. They ascribed these phenotypes to the enhanced auxin (indole acetic acid) polar transport by increased AVP1 activity. Thus, the enhanced shoot and root growth may also result from the enhanced capacity for auxin polar transport in *TsVP*-overexpressing cotton. A more robust root system can facilitate water uptake, especially when plants are exposed to low soil water conditions (such as drought and salinity). Thus, the greater development of the root system in *TsVP*-overexpressing cotton plants may provide the morphological and/or physiological basis for enhanced performance under salinity conditions, resulting in less severe symptoms than those observed in the WT.

The decline in productivity observed in many plant species subjected to excess salinity is often associated with a reduction in photosynthetic capacity. Salt may affect growth indirectly by decreasing the rate of photosynthesis

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(Meloni et al. 2003). In this study, the photosynthetic rate and stomatal conductance decreased dramatically with increasing salinity concentrations (Fig. 6A, B), which is consistent with previous studies in cotton (Brugnoli and Lauteri 1991, Plaut and Federman 1991, Meloni et al. 2003). However, higher rates of photosynthesis and stomatal conductance were observed in the sense transgenic plants than in the WT under both non-salt-stressed and salinity conditions (Fig. 6A, B). Salt stress decreases photosynthesis through stomatal and non-stomatal (direct effect on the photosynthetic apparatus) factors. Stomatal factors are generally more significant at medium salinities and non-stomatal limitations are more relevant at high salinity (Everard et al. 1994). In this study, parallel decreases in stomatal conductance and intercellular CO_2 concentration under treatment with 150 mM NaCl were observed (Fig. 6B, C), which indicated that the reduction in supply of CO_2, mainly due to the decrease of stomatal conductance, was the dominant limiting factor for photosynthesis. Under the 250 mM NaCl treatment, the stomatal conductance decreased more than under 150 mM NaCl, while the intercellular CO_2 concentration in each line (except L + 2) was higher than under 150 mM NaCl (Fig. 6B, C). These results suggested that the photosynthetic apparatus may be adversely affected by 250 mM NaCl stress, which is consistent with the significant decline in the F_v/F_m ratio (Fig. 6D), the most frequently used parameter for determining injury to the PSII complexes by stress factors, including salinity. Thus, the decrease in the photosynthesis rate was due to both stomatal closure and the depressed PSII activity under 250 mM NaCl.

Under non-salt-stressed conditions, the higher photosynthetic rate in the sense lines may be due to the slightly higher chlorophyll content than in the WT, which was supported by results from the antisense line L–2, with lower chlorophyll content and photosynthetic rate (Figs. 5, 6A). It is interesting to note that the chlorophyll content in all plants tested increased in the first week after salt stress, and then decreased remarkably in the following 2 weeks (Fig. 5). Plaut and Federman (1991) also reported that a rise in chlorophyll concentration occurred in salinity- and water-stress-acclimated plants. This may be an adaptation to the salt stress in salinity-acclimated plants, which may be responsible for enhanced photosynthetic electron transport (Plaut and Federman 1991). In fact, the rate of photosynthesis also increased in the first week during salt stress (data not shown).

In principle, enhanced expression of the vacuolar proton pumps can increase vacuolar solute accumulation by increasing the availability of protons. The sequestration of ions such as sodium in the vacuole could confer salt tolerance. Gaxiola et al. (2001) have reported that the overexpression of the vacuolar H^+-PPase gene AVP1 in transgenic *Arabidopsis* plants resulted in drought and salt tolerance, and ascribed these properties to the increased accumulation of solutes. In this work, the cotton plants overexpressing *TsVP* showed a tendency to accumulate more Na^+, Cl^- and soluble sugars under salinity condition than WT plants (Figs. 8, 9B). The increased accumulation of Na^+ is likely to be due to the activity of the vacuolar secondary transporters, such as Na^+/H^+ antiporters, in the presence of increased proton supply. The sequestration of Na^+ in the vacuole may prevent Na^+ toxicity and facilitate cellular K^+ uptake. The increased accumulation of Cl^- may influx to the vacuole, mediated by Cl^- channels and a Cl^-/H^+ antiport in the tonoplast (White and Broadley 2001) as a compensatory transport to maintain electroneutrality (Gaxiola et al. 1998).

Excessive Na^+ in the cytoplasm usually increases membrane injury and causes MDA accumulation (Dionisio-Sese and Tobita 1998). To study further the effects of excessive Na^+ on cotton plants, cell membrane ion leakage and MDA content were measured in leaf tissues. The percentage of ion leakage and the MDA content were less in the sense transgenic plant than in the WT under salt stress conditions (Fig. 7). The correlation in our results between higher Na^+ and less damage in the sense transgenic leaf tissues supports the hypothesis that the overexpression of *TsVP* in cotton can enhance the accumulation of Na^+ in vacuoles.

Besides avoiding Na^+ and Cl^- toxicity, the compartmentation of Na^+ and Cl^- into the vacuole also maintains an osmotic potential to drive water uptake into cells (Apse et al. 1999). Plaut and Federman (1991) indicated that the accumulation of Na^+ and Cl^- in the salinity-acclimated plants fully accounted for the decrease in leaf osmotic potential, whereas the organic solutes contributed only 8–10% to this osmotic adjustment. In the present study, the solute potential (Ψs) of all cotton lines decreased with increasing salinity concentration. However, Ψs in the sense lines decreased much more than that in the WT and the antisense lines under salinity conditions (Fig. 9A). Na^+ and Cl^- increased significantly with increased salinity, and the concentration of Na^+ and Cl^- in the *TsVP*-overexpressing lines was higher than in the WT (Fig. 8A, D). Thus, the lower solute potential in *TsVP*-overexpressing plants may be due mainly to the greater accumulation of Na^+ and Cl^-, although K^+, Ca^{2+} and other solutes such as soluble sugars are also involved. The net increase in the concentration of solutes in the cell must lead to an increase in the uptake of water so that the sense transgenic plants can maintain turgor under low soil solution water potential conditions, which can occur due to salinization. In addition, maintenance of greater leaf turgor can lead to the maintenance of greater photosynthetic capacity and growth in plants exposed to low soil water potential (Gupta and Berkowitz 1987, Plaut and Federman 1991).

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In this study, to understand better the function of the H^+-PPase gene in transgenic cotton, we created not only *TsVP*-overexpressing plants, but also the so-called *TsVP* antisense plants to explore the effect of antisense *TsVP* on the expression of the cotton endogenous H^+-PPase gene (*VPP*). In agreement with the heterogenous suppressing efficiency of the antisense gene in cotton, we found a partial cDNA sequence of the H^+-PPase gene (*VPP*) in cotton in GenBank (accession No. AF009568), reported by Smart et al. (1998), which showed 81% identity to the full-length cDNA of *TsVP* (GenBank accession No. AY436553). Thus, it is very likely that the antisense *TsVP* can suppress the expression of the endogenous *VPP* gene in cotton. Moreover, the lower amount of *VPP* transcripts and the decreased H^+-PPase activity in the antisense line further validate the hypothesis (Figs. 1C, 2A). However, of the two antisense lines used in the study, only one (L–2) appeared efficient, possibly due to the low transcript level of the transgene in L–1 (Fig. 1B). The higher amount of *VPP* transcripts in the sense lines than in the WT detected by real-time RT–PCR may be due to the low specificity of the primers for *VPP*. It is difficult to design effective, specific primers for the endogenous *VPP* gene because of the high level of identity between the sequences of *VPP* and *TsVP* cDNA. In fact, three pairs of primers for *VPP* were tested in this study, but all the results showed a higher amount of transcripts in the sense lines than in the WT. However, this should not affect the conclusion that the antisense *TsVP* did suppress the endogenous *VPP* gene in the antisense cotton lines.

The results of Gaxiola (2001) and Park (2005) suggested that the genetic manipulation of vacuolar proton-pumping pyrophosphatases in economically important crops could provide an important avenue for crop improvement. Here, we report for the first time that overexpression of an H^+-PPase gene from *T. halophila* can significantly enhance salt tolerance in cotton, one of the most important fiber and oil crops in the world. The transgenic cotton plants maintained improved growth and photosynthetic performance compared with the WT under salinity conditions. We ascribe these properties to the increased accumulation of solutes and the more robust root systems. Recently, He et al. (2005) reported that overexpressing *Arabidopsis* vacuolar Na^+/H^+ antiporter in cotton resulted in increased salt tolerance. Notably, the AtNHX1-expressing plants exhibited improved fiber yield under irrigation in the field. They ascribed this to the decreased water potential, which facilitates the uptake of water during cell elongation. If this is the case, the *TsVP*-overexpressing cotton generated in the present study may also exhibit increased fiber yield, since these plants also maintained decreased water potential (Fig. 9A). Furthermore, the robust root system may also help to take up water. In addition, Smart et al. (1998) reported that in cotton, the peak of PPase activity occurred at about 20 d post-anthesis, a few days after the peak rate of fiber expansion, which may suggest that the PPase plays an important role in the process of secondary wall deposition. Thus, increased PPase activity in cotton may lead to increased fiber strength. This possibility will be explored in the future.

In the study of He et al. (2005), it was suggested that the vacuolar $\Delta\mu H^+$ might become a limiting factor at soil NaCl concentrations >200 mM. Here, our results further demonstrated the important role of the vacuolar proton-pumping pyrophosphatases in cotton salt tolerance. As suggested by He et al., the simultaneous overexpression of both a vacuolar H^+-PPase and a vacuolar Na^+/H^+ antiporter will probably be required to increase salt tolerance further in cotton.

Materials and Methods

Plasmid construction and cotton transformation

The full-length cDNA of *TsVP* was cloned into the *Xba*I site of a pCAMBIA1300-*als* plasmid in sense and antisense orientation under the control of the CaMV 35S promoter. The resultant plasmids contained the sense or antisense *TsVP* gene and a mutant *als* gene, coding for acetolactate synthase, conferring resistance to the herbicide sulfometuron as a selectable marker. Injured shoot apexes of cotton (cultivar 'Luyuan890') were transformed with *Agrobacterium tumefaciens* LBA4404, carrying the above recombinant plasmids, according to the procedure reported by Lv et al. (2004). Transgenic plants were selected by spraying the herbicide sulfometuron (2.5 mg l^{-1}) twice at 24 h intervals on the seedlings. The resistant plantlets were verified by PCR with primers P1 (5′-CAGAACTCGCCGTAAAGACT-3′) and P2 (5′-GCAGAAACCGAAGATAACG-3′) for sense *TsVP* transgenic cotton, and primers P1 and P3 (5′-TAAAGAACC AACCAAATGATGT-3′) for antisense *TsVP* transgenic plants.

Southern and Northern blot analysis

Genomic DNA was isolated from young leaves using the modified cetyltrimethylammonium bromide (CTAB) protocol (Permingeat et al. 1998) with the addition of 2% (w/v) polyvinylpyrrolidone (PVP) 40 in extraction buffer. After *Bam*HI digestion of 20 μg of genomic DNA and electrophoresis on a 0.8% TBE agarose gel, DNA was transferred to a Hybond-N^+ nylon membrane (Roche, Mannheim, Germany).

Total RNA was extracted from young leaves of WT and transgenic plants according to the method of Salzman et al. (1999). For Northern blot analysis, 20 μg of total RNA was separated on 1.2% formaldehyde-containing agarose gels and transferred onto a Hybond-N^+ nylon membrane (Roche).

The full-length cDNA of *TsVP* was labeled with [^{32}P]dCTP by a Random Primer DNA Labeling Kit (TAKARA SHUZO CO. LTD., Kyoto, Japan) and used as the probe for Southern and Northern blotting. The membranes were pre-hybridized and hybridized at 65°C. After stringent washing, radioactive membranes were exposed to X-ray film (Kodak, USA) for 48 h at –70°C.

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Expression analysis of the cotton endogenous VPP gene

Five-leaf stage cotton seedlings were used to analyze the expression of the cotton endogenous *VPP* gene by real-time quantitative RT–PCR. Total RNA was extracted from young leaves of WT and transgenic plants according to the method of Salzman et al. (1999). cDNA synthesis was performed with the RT reagent kit (TAKARA, Dalian, China) according to the manufacturer's protocol. The gene-specific primers were: *VPP*1 (5′GCGTTGGTGTCTTTGGCT-3′) and *VPP*2 (5′-CAACAATA AAGGGTGTGAGCAT-3′) for cotton *VPP*; 18S-P1 (5′-AACCAT AAACGATGCCGACCAG-3′) and 18S-P2 (5′-AGCCTTGCGA CCATACTCCC-3′) for the cotton 18S rRNA gene (GenBank accession No. L24145). The cotton 18S rRNA gene was used as an internal control. Real-time quantitative RT–PCRs were performed on Chromo 4^{TM} (MJ Research, Waltham, MA, USA) with the SYBR® RT-PCR Kit (TAKARA, Dalian, China) in a 10 µl reaction volume, which contained 5 µl of SYBR Green PCR mix, 0.2 µl of each forward and reverse primer, 1 µl of diluted cDNA template and an appropriate amount of sterile ddH_2O. Amplification conditions were: 2 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 58°C and 30 s at 72°C. The relative cotton *VPP* gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with the 18S rRNA gene as an internal control. All experiments were repeated five times.

Plant material and salt treatments

T_2 plants from six sense *TsVP* transgenic homozygous lines (L + 1 to L + 6), two antisense *TsVP* transgenic homozygous lines (L–1 and L–2) and the WT ('Luyuan890') plants were used for salt tolerance assays. The delinted seeds of WT and transgenic plants were germinated in vermiculite at 28°C. At the two-leaf stage, healthy WT and transgenic seedlings were transferred to Hoagland solution (Hoagland and Arnon 1950). Nine seedlings of the WT and each transgenic line were cultured by aeration in a greenhouse at 30/25°C (day/night) with a photon flux density (PFD) of 500 µmol m^{-2} s^{-1}, a relative humidity of 60–70% and a photoperiod of 14/10 h (light/dark). The nutrient solution was changed twice a week and tap water was added daily to replace the water lost by transpiration. At the five-leaf stage, six seedlings of the WT and each transgenic line were exposed to salinity by adding NaCl to the nutrient solution in 50 mM increments every 24 h, until the final concentrations of 150 and 250 mM were reached. The other three seedlings of the WT and each transgenic line continued to be cultured in Hoagland solution as a control. After 21 d of salt treatment, the gas exchange parameters, chlorophyll fluorescence, ion concentrations, soluble sugar content, solute potential, MDA content and electrolyte leakage of leaf cells were measured on plants from three of the sense lines (L + 1, L + 2 and L + 3), the two antisense lines and the WT. Then plants from all transgenic lines and the WT were harvested for biomass determination after drying for 72 h in an oven at 70°C.

To observe the root system better, two plants from transgenic lines L + 1, L + 2 and L–2 and the WT were grown in each of two large plastic containers containing vermiculite for 4 weeks with a constant supply of MS solution every day (Fig. 4). Beginning at the fifth week after germination, plants in one container were exposed to salt stress by supplying NaCl to the nutrient solution, to a final concentration of 150 mM. Plants in the other container acted as non-stressed controls. The experiment was conducted under natural weather conditions in June to July in Jinan, a city in Northern China. The temperature was about 35/20°C (day/night) with a photoperiod of 14/10 h (day/night) and a relative humidity of 40–60%. After 21 d of salt treatment, roots were harvested and the dry weights were measured after drying for 72 h in an oven at 70°C.

Membrane vesicle isolation, V-ATPase and V-PPase assays

Plants from the transgenic lines L + 1, L + 2, L + 3 and L–2, and the WT were grown in Hoagland solution in 2 liter plastic boxes with 16 seedlings in each box. The growth conditions and salt treatment were as described above. After 21 d of salt treatment with 150 or 250 mM NaCl, roots were collected for membrane vesicle isolation and enzyme assays. Three replications were performed.

Tonoplast-enriched membrane vesicles were prepared by sucrose density gradient ultracentrifugation according to de Michelis and Spanswick (1986) with minor modifications. About 20 g of root segments for each sample were rinsed three times with deionized water and then homogenized in 80 ml of ice-cold buffer containing 50 mM HEPES-Tris (pH 7.8), 0.25 M sucrose, 10% (v/v) glycerol, 2 mM EGTA and 0.5% (w/v) bovine serum albumin (BSA), 2% (w/v) PVP40, 5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF) with a homogenizer. The tissue homogenate was set aside at 0°C for 10–15 min to allow foam to settle and then filtered through three layers of gauze. The filtrate was centrifuged at 4,200×*g* at 4°C for 10 min to remove the cell debris, and then the supernatant was centrifuged at 50,228×*g* for 2 h at 4°C in a SORVALL SS-34 rotor. The pellets then were gently resuspended in an appropriate volume of ice-cold buffer containing 5 mM HEPES-Tris (pH 7.4), 2 mM $MgSO_4$, 2 mM DTT and 1 mM PMSF.

To purify vacuolar membrane vesicles (tonoplast), the microsomal suspension was layered over a 10/35% (w/w) discontinuous sucrose gradient in 5 mM HEPES-Tris (pH 7.4), 2 mM DTT and 1 mM PMSF. After centrifugation at 100,000×*g* at 4°C for 2 h, the vesicles at the 10/35% sucrose interface were collected, and diluted in an equal volume of buffer containing 5 mM HEPES-Tris (pH 7.4), 2 mM $MgSO_4$, 2 mM DTT and 1 mM PMSF. Vesicle pellets were collected by centrifugation at 146,000×*g* for 1 h and resuspended in the storage buffer with 5 mM HEPES-Tris (pH 7.4), 2 mM $MgSO_4$ and 2 mM DTT, and were then ready for use.

The hydrolytic activity of V-ATPase and V-PPase was determined by measuring the release of inorganic phosphate (Pi) according to the method of Smart et al. (1998). Inorganic phosphate was determined using the method of Lin and Morale (1977). V-ATPase activity is presented as the difference of the measured values in the absence or presence of 50 mM NO_3^-. V-PPase hydrolytic activity was expressed as the difference in activity measured in the presence or absence of 50 mM KCl (K^+-stimulated PPase activity). PPase activity was calculated as one-half of the rate of Pi liberation because the hydrolysis of 1 mol of PPi yields 2 mol of Pi.

Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

Chlorophyll determination

Leaf samples (about 200 mg) were homogenized with 80% acetone (v/v), and the homogenate was then filtered through filter paper. The total chlorophyll content was determined spectrophotometrically according to Arnon (1949).

Gas exchange and chlorophyll fluorescence measurements

Net photosynthesis, stomatal conductance and intercellular CO_2 content were measured on the second fully expanded leaves from the apex of seedlings using a portable infrared gas

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analyzer-based photosynthesis system (LI-6400, Li-Cor Inc., Lincoln, NE, USA). The photosynthetic PFD was maintained at 800 μmol $m^{-2} s^{-1}$ by an internal 6400-02BLED source. All the measurements were carried out from 09:00 to 10:00 a.m. During collection of the measurements, the air relative humidity was about 70% and the ambient CO_2 concentration about 400 μmol CO_2 mol^{-1}.

Chlorophyll fluorescence was determined in intact plants using a pulse amplitude modulation fluorometer PAM 2000 (Walz, Effeltrich, Germany). Leaves previously selected for the measurement of gas exchange were used for fluorescence measurements. After dark adaptation for 30 min, the potential maximum photochemical efficiency of PSII (F_v/F_m) was measured by application of a 1 s saturation flash.

Measurement of ion leakage and MDA levels

Leaves of salt-stressed and non-stressed plants were used to determine ion leakage and MDA levels. The MDA content was determined according to Peever and Higgins (1989). The absorbance of the supernatant at 450, 532 and 600 nm was determined with a spectrometer. The concentration of MDA was calculated by the following formula:

$$C(\text{mol } 1^{-1}) = 6.45(OD_{532} - OD_{600}) - 0.56OD_{450}$$

The ion leakage from the cellular membranes was determined via conductivity measurements. Ion leakage expressed as a percentage was calculated as described previously (Fan et al. 1997).

Determination of ion concentrations

WT and transgenic plants grown in Hoagland solution were subjected to salt stress at the five-leaf stage. After 21 d of salt stress, root tips (5–7 cm in length) and the third fully expanded leaves from the apex of salt-stressed and non-stressed plants were excised and rinsed three times in deionized water. They were then dried for 72 h at 70°C, and the dry weight measured. The dried leaves and roots were digested with 0.1 M HNO_3, and Na^+, K^+, Ca^{2+} and Cl^- contents were determined by an ion selective electrode method using an ion analyzer (PXSJ-216, Leici, Shanghai, China) according to Rieger and Litvin (1998).

Quantification of solute potential and total soluble sugars

WT and transgenic plants grown in Hoagland solution were subjected to salt stress at the stage of five leaves. After 21 d of salt stress, part of the second fully expanded leaves from the apex of salt-stressed and non-stressed plants was excised and rehydrated in deionized water in Petri dishes at 4°C for 24 h, then blotted dry with filter paper, frozen in liquid nitrogen, and thawed to express sap, which was analyzed with a freezing point micro-osmometer (Fiske, Model 210, Norwood, MA, USA). The solute potential (Ψs) expressed in MPa was calculated according to Gaxiola et al. (2001).

The same leaves used for Ψs detection were used to determine the total soluble sugars. Total soluble sugars of leaves (about 100 mg) were extracted in boiling water for 30 min and determined by anthrone reagent using glucose as the standard (Yemm and Willis 1954).

Statistical analysis

All data were presented as mean ± SD. Comparisons between transgenic plants and WT plants were performed using Student's *t*-test. A *P*-value of <0.05 was considered statistically significant. All statistical analyses were done using SigmaPlot 9.0.

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