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Enhanced Tonoplast H^+-ATPase Activity and Superoxide Dismutase Activity in the Halophyte *Suaeda salsa* Containing High Level of Betacyanin

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Abstract In this study, high-betacyanin *Suaeda salsa* seedlings were developed and used to explore whether the betacyanin accumulation is related to salinity tolerance in *S. salsa*. After 8 days of culture, betacyanin content decreased markedly in both high-betacyanin *S. salsa* seedlings and the control under nonsalt stress, but the decreases were suppressed by NaCl treatments. Betacyanin content in high-betacyanin seedlings was much higher than that in the control throughout the salt treatments. Growth of *S. salsa* plants was significantly promoted by NaCl treatments, and the fresh weight of high-betacyanin seedlings was much higher than that of the control when grown in 400 mmol L^{-1} NaCl. Similar cell sap osmolarity and K^+/Na^+ ratios were observed in high-betacyanin seedlings and the control. No obvious differences in V-ATPase (tonoplast H^+-ATPase) activity, leaf SOD (superoxide dismutase) activity, and total chloroplast SOD (including thylakoid-bound SOD and stroma SOD) activity were detected between high-betacyanin seedlings and the control under nonsalt stress conditions. However, V-ATPase hydrolytic activity increased dramatically in *S. salsa* seedlings when subjected to different levels of NaCl, and the increases in V-ATPase activity in high-betacyanin seedlings were much higher than that in the control. No clear pattern was observed for NaCl-dependent activity changes of P-ATPase (plasma membrane H^+-ATPase) and V-PPase (tonoplast H^+-pyrophosphatase). Similar changes were demonstrated in leaf SOD activity and chloroplast SOD activity under salt stress. Both leaf SOD activity and chloroplast SOD activity were markedly enhanced with the increase of NaCl or with time, especially thylakoid-bound SOD activity. Furthermore, the increases in chloroplast SOD activity and thylakoid-bound SOD activity were much higher in high-betacyanin seedlings than that in the control at different levels of NaCl treatment. The higher V-ATPase activity, chloroplastic SOD activity, and thylakoid-bound SOD activity demonstrated in high-betacyanin seedlings, but lower in the control, suggest that high-betacyanin *S. salsa* seedlings may have higher potential to be energized by the electrochemical gradient for ion uptake into the vacuole and to scavenge $O_2^{-\bullet}$ in situ produced in the chloroplasts, which may lead to higher salt tolerance than the control under salt stress. Thus, betacyanin may be involved in salt tolerance of *S. salsa*.

Keywords *Suaeda salsa* · Betacyanin · V-ATPase · Leaf total SOD activity · Chloroplastic SOD activity

Introduction

Increased salinity affects primary carbon metabolism, plant growth, and development by ion toxicity, nutritional deficiency, water deficits, and oxidative stress (Flowers 2004; Sairam and Tyagi 2004). To maintain growth and productivity, plants must adapt to stress conditions and exercise specific tolerance mechanisms. One mechanism involves removal of Na^+ from the cytoplasm by transporting it into the vacuole via Na^+/H^+ exchangers driven by the electrochemical gradient of protons (H^+) generated by the tonoplast H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (V-PPase) (Niu and others 1995; Qiu and others 2004; Sze and others 1999). Increased salt tolerance also has been observed in transgenic rice carrying the *OsNHX1*

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(*NHX1* from *Oryza sativa*) and *AgNHX1* (*NHX1* from *Atriplex gmelini*) genes (Fukuda and others 2004; Ohta and others 2002), which indicates that expression of a single Na^+/H^+ antiporter gene in plants can be effective in reducing Na^+ toxicity.

In addition, an effective antioxidative response system is at least in part responsible for an increased level of salt tolerance. High concentrations of NaCl seem to impair electron transport and lead to the formation of ROS (reactive oxygen species) in chloroplasts (Asada 1990; Foyer and Noctor 2000; Hernández and others 1995; Pang and others 2005). Salt-tolerant plants have well-developed defense systems against ROS, involving both enzymatic and nonenzymatic mechanisms. SOD (superoxide dismutase) has a central role in the antioxidant defense network. SOD is the key enzyme for diminishing superoxide (Slooten and others 1998). SOD catalyzes the superoxide radicals ($O_2^{-\bullet}$) to yield molecular oxygen and hydrogen peroxide (H_2O_2). The control of the steady-state $O_2^{-\bullet}$ levels by SOD is an important protective mechanism against cellular oxidative damage, because $O_2^{-\bullet}$ acts as a precursor of more cytotoxic or highly reactive oxygen derivatives such as peroxynitrite or $HO^{\bullet}$ (Halliwell and Gutteridge 1999). Therefore, SOD is usually considered the first line of defense against oxidative stress, and increased SOD activity is related to alleviating damage associated with salt stress (Pang and others 2005; Sigaud-Kutner and others 2002).

The Chenopodiaceae C_3 halophyte *Suaeda salsa* is one of the most important halophytes in China and has important economic value because its seeds contain approximately 40% oil, rich in unsaturated fatty acids, which can be easily converted to chemical compounds for industrial and pharmaceutical use (Zhao 1998). *S. salsa* is native to saline soils and even grows in the intertidal zone of the Yellow River Delta, where soil salt content is often higher than 3%. Interestingly, the shoots of *S. salsa* plants grown in the intertidal zone are red-violet, but green at elevation and in land far from the seaside. Our analysis shows that the red pigments in *S. salsa* are not anthocyanins but betacyanins, and biosynthetic regulation of betacyanins in *S. salsa* has also been preliminarily studied in our laboratory (Wang and Liu 2006, 2007; Wang and others 2006, 2007). However, no information exists about the biological roles of betacyanins in salt tolerance of the C_3 halophyte *S. salsa*.

Compared with the green *S. salsa* plants grown at elevation and land far from the seaside, the red-violet *S. salsa* plants grown in the intertidal zone can tolerate coastal seawater salinity and salinity fluctuation resulting from water evaporation and tidal inundation, so the red-violet *S. salsa* has a higher salt tolerance than green plants. To determine whether there is a connection between betacyanin accumulation and salt tolerance in *S. salsa*, we developed high-betacyanin *S. salsa* seedlings for exposure to different levels of salinity. The higher V-ATPase activity, chloroplastic SOD activity, and thylakoid-bound SOD activity demonstrated in seedlings with accumulated betacyanin, but lower in the control, suggest that the accumulation of betacyanin may be involved in the salt tolerance of *S. salsa* under salt stress.

Materials and Methods

Betacyanin Accumulation Induction and Salinity Treatments

Betacyanin accumulation in *S. salsa* was induced as previously described (Wang and Liu 2006). Seeds of the *S. salsa* were collected from the Yellow River Delta. After being sterilized with 0.5% $HgCl_2$ for 3 min, seeds were washed and germinated in plastic plates filled with sand and watered with half-strength Hoagland nutrient solution containing 100 mmol L^{-1} NaCl. In a growth cabinet, seeds were divided into two groups, one was cultured in 24 h darkness and the other one was cultured in 14 h light/10 h dark. The ambient temperature was 28°C, relative humidity was 60%, photon flux density was 200 μmol m^{-2} s^{-1}. Three days later, the red-violet seedlings obtained from seeds cultured in the dark (24 h dark) were used as high-betacyanin seedlings, whereas the green seedlings obtained from seeds cultured in light (14 h light/10 h) were used as the control.

Both the uniform high-betacyanin seedlings and the controls were transplanted to light conditions (14 h light/10 h dark) and cultured in half-strength Hoagland nutrient solution containing different levels of NaCl. NaCl concentrations were stepped up in 50 mmol L^{-1}/3 days increments until final concentrations (0, 100, 200, and 400 mmol L^{-1}) were achieved. The other culture conditions were the same as previously described. To determine the responses of *S. salsa* seedlings to NaCl, the seedlings exposed to different levels of NaCl for 8 days were used for physiologic analysis. To determine the time responses of *S. salsa* seedlings under salt stress, the seedlings exposed to 200 mmol L^{-1} NaCl were used for physiologic analysis at 0, 2, 4, 6, 8, 10, 12, and 14 days thereafter. Each measurement was taken and performed five times.

Isolation and Analysis of Betacyanin

Betacyanin of *S. salsa* seedlings was extracted and measured according to the method of Wang and others (2007).

Determination of Na^+, K^+ Content and Osmolarity

Leaves were frozen at −20°C and, after thawing, cell sap was obtained using a filter paper disc-covered syringe. The osmolarity of the cell sap was measured with a cryoscopic osmometer. The cell sap from both seedlings with accumulated betacyanin and the controls was diluted with twofold volumes of distilled water and boiled for 5 min. The filtered solution was used for the determination of Na^+ and K^+ content using an atomic absorption spectrophotometer (Z-8000, Hitachi, Tokyo) as described by Gao and others (2003).

Analysis of P-ATPase, V-ATPase, and V-PPase Hydrolytic Activities

Activities of substrate hydrolysis of P-ATPase, V-ATPase and V-PPase were measured as described by Wang and others (2001). V-ATPase activity was analyzed in the presence of 1 mmol L^{-1} molybdate, 1 mmol L^{-1} azide, and 1 mmol L^{-1} vanadate at 37°C. V-PPase activity was analyzed in the presence of 1 mmol L^{-1} molybdate, 1 mmol L^{-1} azide, 50 mmol L^{-1} nitrate, and 1 mmol L^{-1} vanadate at 37°C. P-ATPase activity was analyzed in the presence of 1 mmol L^{-1} molybdate, 1 mmol L^{-1} azide, and 50 mmol L^{-1} nitrate at 37°C.

Preparation of Leaf SOD Extracts and SOD Activity Assay

One gram of plant material was homogenized at 4°C in 2 ml of medium: 100 mmol L^{-1} K-phosphate buffer (pH 7.8) containing 3 mmol L^{-1} $MgSO_4$, 3 mmol L^{-1} EDTA, and 2% (w/v) polyvinyl polypyrrolidone (PVP). The homogenate was centrifuged at 20,000*g* (Eppendorf Centrifuge 5417R) for 5 min at 4°C. The supernatant was used for determination of SOD and protein content.

In the presence of SOD the photochemical reduction of nitro blue tetrazolium (NBT) is inhibited and the level of inhibition was used to quantify the enzyme. SOD was assayed according to Giannopolitis and Ries (1977) with some modifications. The reaction medium was composed of 50 mmol L^{-1} K-phosphate buffer (pH 7.8), 0.1 mmol L^{-1} EDTA, 15 mmol L^{-1} methionine, 60 μmol L^{-1} riboflavin, 2.25 mmol L^{-1} NBT, and an appropriate aliquot of extract in a final volume of 4 ml. The reaction mixture was illuminated with a photon flux density of 72 μmol m^{-2} s^{-1} for 15 min and turning the lights off stopped the reaction. A control reaction was always performed wherein all the steps and components were exactly the same as described above, except that crude enzyme was replaced with an equal volume of phosphate buffer (pH 7.8). Assays were always performed at 25°C. The reaction was measured at 560 nm. One unit of enzyme activity was defined as the quantity of enzyme that reduced the absorbance reading of samples to 50% in comparison with tubes lacking enzymes.

The protein content was determined by using bovine serum albumin as a standard according to Bradford (1976).

Isolation of Chloroplast SOD and SOD Activity Assay

Chloroplasts were isolated according to the method of Sgherri and others (2000) with some modifications. Twenty grams of *S. salsa* leaves were placed at −15°C for 1 h and then washed with cold deionized water and homogenized in ice-cold grinding medium containing 330 mmol L^{-1} sorbitol, 50 mmol L^{-1} MES (pH 6.1), 10 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} EDTA-Na_2, 0.5 mmol L^{-1} KH_2PO_3, and 2 mmol L^{-1} sodium ascorbate. The homogenate was filtered through eight layers of cheesecloth and centrifuged at 300*g* for 30 s. The supernatant was centrifuged at 1500*g* for 2 min, then the supernatant was discarded and the pellet obtained was suspended in 3 ml suspension medium [330 mmol L^{-1} sorbitol, 50 mmol L^{-1} HEPES (pH 7.6), 10 mmol L^{-1} NaCl, 2 mmol L^{-1} $MgCl_2$, 2 mmol L^{-1} EDTA-Na_2, 0.5 mmol L^{-1} KH_2PO_3, 2 mmol L^{-1} sodium ascorbate]. Three milliliters of chloroplast suspension was loaded onto 4 ml of 40% Percoll and centrifuged at 2500*g* for 60 s. The lowest layer, containing intact chloroplasts, was removed and washed three times with the suspension medium. One fraction was resuspended with 1 ml suspension medium containing 0.1% Triton X-100, which is used in total SOD activity assay. The other fraction was resuspended in a hypotonic medium (the same as the suspension medium except that sorbitol concentration was 4 mmol L^{-1}) to break the chloroplasts and to release the stromal enzyme. After centrifugation at 20,000*g* for 5 min, the supernatant was used as stromal SOD extract and thylakoids were included in the pellet for solubilizing membrane-bound SOD. Thylakoid-bound SOD was solubilized as previously reported in Navari-Izzo and others (1998). Thylakoid-bound SOD was extracted by incubating membranes with 0.1% Triton X-100 and 1.5 mmol L^{-1} DTT for 30 min (Hayakawa and others 1985). All the steps were carried out at 4°C.

Results

Betacyanin Content of *S. salsa* Seedlings Under Salt Stress

As we previously described, the shoots of *S. salsa* seedlings obtained from seeds cultured in the dark for 3 days were

red-violet, whereas those from seeds cultured in 14 h light/10 h dark for 3 days were green (see picture in Wang and Liu 2006). The betacyanin content in *S. salsa* seedlings grown in the dark for 3 days was much higher than that in seedlings grown in light for 3 days (Figure 1A). So the red-violet seedlings grown in the dark (24 h dark) for 3 days were used as high-betacyanin seedlings for salt treatments, whereas the green seedlings grown in the light (14 h light/10 h) for 3 days were used as the controls.

Both high-betacyanin seedlings and the controls were transferred to light conditions (14 h light/10 h) and cultured for 8 days in nutrient solution containing different levels of NaCl. After an 8-day culture, the betacyanin content decreased markedly in both high-betacyanin seedlings and the controls under nonsalt stress conditions (Figure 1B, 0 mmol L^{-1} NaCl), but the decreases were obviously suppressed by NaCl treatments (Figure 1B, 100–400 mmol L^{-1} NaCl). The betacyanin contents were proportional to NaCl concentrations, and the betacyanin content in high-betacyanin seedlings remained at higher levels than that in the controls in different NaCl treatments. In addition, betacyanin content gradually decreased at 0-5 days but increased at 6-14 days in both high-betacyanin seedlings and the controls when subjected to salt stress, and the betacyanin contents in high-betacyanin seedlings were much higher than that in the controls throughout the salt treatment (Figure 1C).

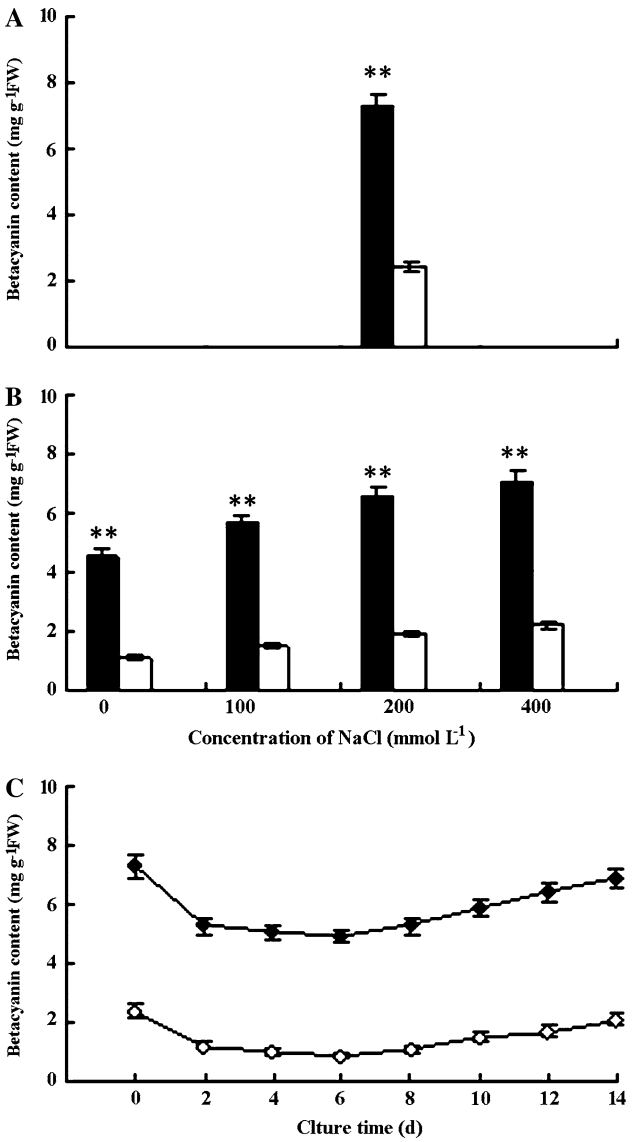

Fig. 1 Betacyanin content in high-betacyanin *S. salsa* seedlings (■) and the controls (□) before NaCl treatments (**A**), exposed to different levels of NaCl for 8 days (**B**), and exposed to 200 mmol L^{-1} NaCl for different days (**C**). Data are mean ± SE (n = 5), and the significant levels of difference between high-betacyanin *S. salsa* seedlings (■) and the control (□) are indicated by ** ($p < 0.01$)

Effects of NaCl Treatments on Growth and Ion Content in *S. salsa*

The growth of *S. salsa* plants was significantly promoted by NaCl treatments (Figure 2). The fresh weight per plant increased significantly with the increase of NaCl concentration up to 200 mmol L^{-1} in both high-betacyanin seedlings and the control. However, the fresh weight per plant of *S. salsa* seedlings grown in 400 mmol L^{-1} NaCl levels remained high in high-betacyanin seedlings but decreased slightly in the control.

Cell sap osmolarity increased with increasing NaCl concentration. At a given salt concentration, the cell sap osmolarity in high-betacyanin seedlings was a little higher than that in the controls, but the difference was not significant (Table 1). To further investigate whether the cell sap osmolarity of *S. salsa* seedlings is related to ion levels, after high-betacyanin seedlings and the controls had been exposed to different concentrations of NaCl for 8 days, total Na^+ and K^+ concentrations in leaves were measured. As shown in Table 1, Na^+ increased while K^+ and the K^+/Na^+ ratio decreased in both high-betacyanin seedlings and the controls following treatments with increased NaCl. So increased Na^+ may contribute to increased cell sap osmolarity under salt stress, but Na^+ content and K^+/Na^+ in high-betacyanin seedlings were similar to that in the controls.

Effects of NaCl Treatments on V-ATPase, V-Ppase, and P-ATPase Activity in *S. salsa*

Measurement of the hydrolytic activities of V-ATPase, V-Ppase, and P-ATPase revealed no obvious differences in these enzyme activities between high-betacyanin seedlings and the control under non-salt-stress conditions (Figure 3).

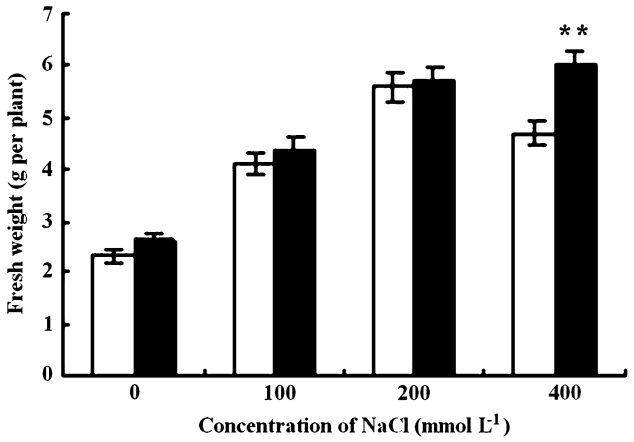

Fig. 2 Fresh weight of high-betacyanin *S. salsa* seedlings (■) and the controls (□) exposed to different levels of NaCl for 8 days. Data are mean ± SE ($n = 5$), and the significant levels of differences between high-betacyanin *S. salsa* seedlings (■) and the control (□) are indicated by ** ($p < 0.01$)

However, V-ATPase hydrolytic activity increased dramatically with increasing NaCl up to 200 mmol L^{-1} in both high-betacyanin seedlings and the controls. When the NaCl concentration exceeded 200 mmol L^{-1}, V-ATPase activity increased slightly in high-betacyanin seedlings and decreased a bit in the controls. The increases in V-ATPase activity in high-betacyanin seedlings were much higher than that in the controls when seedlings were subjected to 200 and 400 mmol L^{-1} NaCl (Figure 3A). For example, the increase in V-ATPase hydrolytic activity in high-betacyanin seedlings was 47 μmol mg^{-1} tonoplast protein h^{-1} compared to 24 μmol mg^{-1} tonoplast protein h^{-1} in the controls when grown in 400 mmol L^{-1} NaCl. Furthermore, V-ATPase hydrolytic activity increased with time under salt stress (Figure 4A).

In contrast, the activities of V-PPase and P-ATPase in the shoots were much lower than that of V-ATPase, and no marked differences in the two enzyme activities could be seen between high-betacyanin seedlings and the controls in different levels of NaCl treatments (Figure 3B, C). The activity of V-PPase increased at 0–4 days but decreased slowly at 5–14 days (Figure 4B), whereas the activity of P-ATPase remained constant in both high-betacyanin seedlings and the controls during the culture period of 14 days (Figure 4C). These results support the idea that high-betacyanin seedlings have higher potential energized by the electrochemical gradient, particularly by V-ATPase, which may lead to higher salt tolerance than the control.

Effects of NaCl Treatments on Leaf and Chloroplast SOD Activity

It is known that there are Mn-SOD and Cu/Zn-SOD isoforms in *S. salsa* and no novel SOD isoenzyme appeared under salt stress (Zhang and others 2005). Therefore, in this study activity rather than the number and type of SOD was detected to determine the response of *S. salsa* to salinity. Leaf SOD activity in high-betacyanin seedlings was similar to that in the control under non-salt-stress conditions. Leaf SOD activity did not show a distinct increase in either high-betacyanin seedlings or the controls grown below 200 mmol L^{-1} NaCl, but it was significantly enhanced by 400 mmol L^{-1} NaCl. The increase of leaf SOD activity in high-betacyanin seedlings was much higher than that in the controls when grown in 400 mmol L^{-1} NaCl (Figure 5A). In addition, leaf SOD activity gradually increased with time (Figure 6A).

To examine the response of chloroplast SOD activity to NaCl stress, total chloroplast SOD activity, thylakoid-bound SOD activity, and stroma SOD activity were determined, respectively. As shown in Figure 5B, C, and D, no obvious differences in these enzyme activities were detected under non-salt-stress conditions. Similar changes were observed in chloroplast SOD and leaf SOD activity when *S. salsa* seedlings were subjected to different levels of NaCl. Both leaf SOD activity and chloroplast SOD activity were markedly enhanced with increasing NaCl or with time (Figures 5B and 6B). The increases in chloroplast SOD activity were much higher in high-betacyanin seedlings than in the controls in different NaCl treatments.

Table 1 Osmolarity and Na^+ and K^+ concentration of leaf cell sap of high-betacyanin *S. salsa* seedlings (B) and the control exposed to different levels of NaCl for 8 days

Concentration of NaCl (mmol L^{-1})	Osmolarity (mmol kg^{-1})		Na^+ (mmol L^{-1})		K^+ (mmol L^{-1})		K^+/Na^+	
	B	Control	B	Control	B	Control	B	Control
0	678 ± 35 a	543 ± 28 a	91 ± 4.6 a	95 ± 4.8 a	113 ± 5.8 a	121 ± 6.1 a	1.24 ± 0.071 a	1.27 ± 0.073 a
100	967 ± 48 b	813 ± 41 b	319 ± 16 b	301 ± 14 b	69 ± 34 b	73 ± 37 b	0.21 ± 0.001 b	0.24 ± 0.001 b
200	1519 ± 75 c	1334 ± 66 c	767 ± 38 c	701 ± 35 c	51 ± 2.5 c	55 ± 2.6 c	0.066 ± 0.0005 c	0.078 ± 0.0005 c
400	2038 ± 101 d	1843 ± 91 d	1197 ± 51 d	998 ± 49 d	35 ± 1.6 d	36 ± 1.6 d	0.029 ± 0.0001 d	0.036 ± 0.0001 d

Data are mean values ± SD ($n = 5$). Different letters in the same column indicate that differences are significant at 1% level ($p < 0.01$)

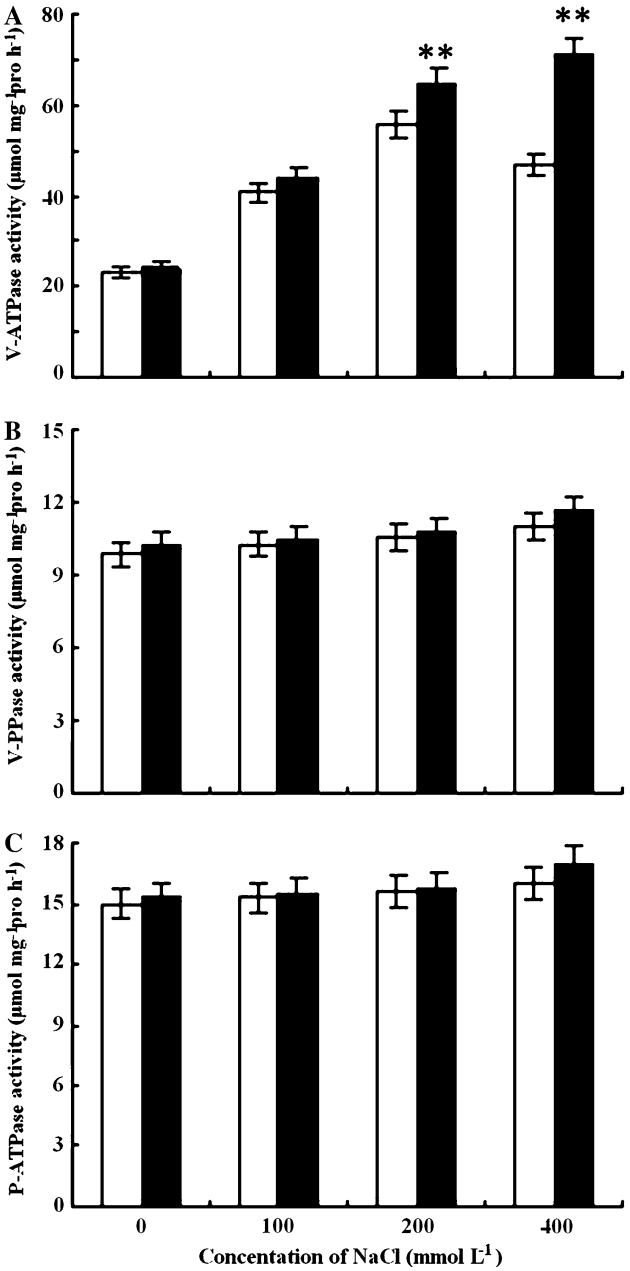

Fig. 3 Activities of V-ATPase (**A**), V-PPase (**B**), and P-ATPase (**C**) in leaves of high-betacyanin *S. salsa* seedlings (■) and the controls (□) exposed to different levels of NaCl for 8 days. Data are mean ± SE ($n = 5$), and the significant levels of difference between high-betacyanin *S. salsa* seedlings (■) and the control (□) are indicated by ** ($p < 0.01$)

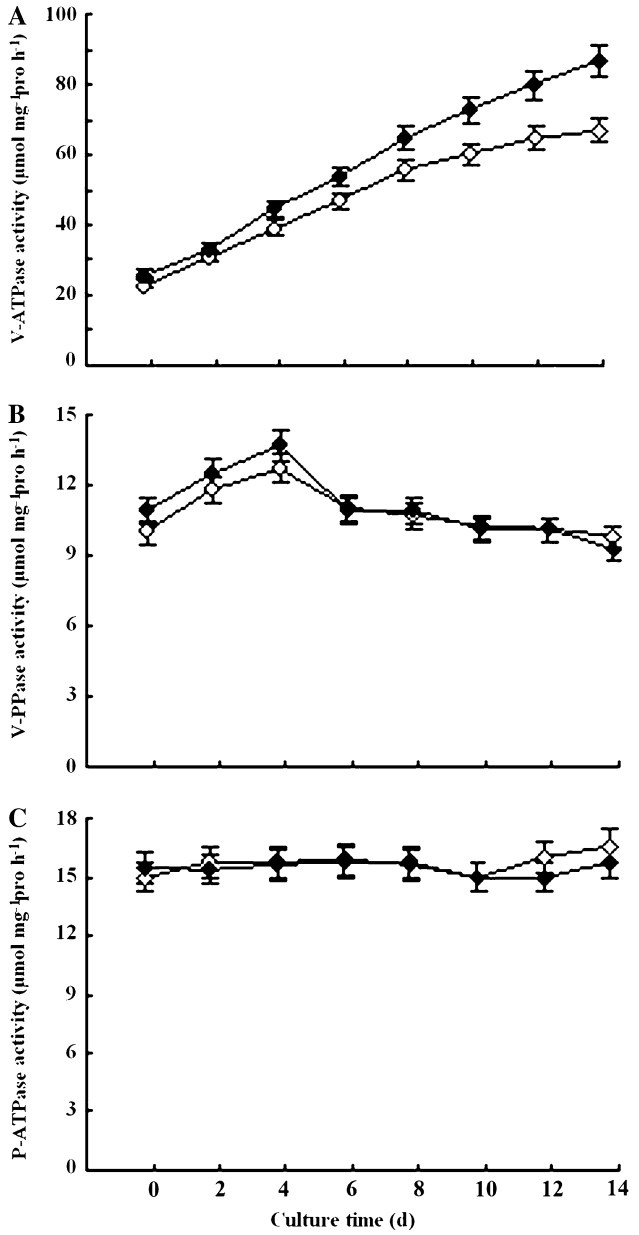

Fig. 4 Activities of V-ATPase (**A**), V-PPase (**B**), and P-ATPase (**C**) in leaves of high-betacyanin *S. salsa* seedlings (■) and the controls (□) exposed to 200 mmol L^{-1} NaCl for different days. Data are mean ± SE ($n = 5$)

Similar to chloroplast SOD activity, the activities of both thylakoid-bound SOD and stroma SOD increased with increasing NaCl concentrations (Figure 5C, D) or with time (Figure 6C, D). The thylakoid-bound SOD activity was much higher than the stroma SOD activity in both high-betacyanin seedlings and the controls under salt stress conditions. For example, the activity of thylakoid-bound SOD was 48 U mg^{-1} pro h^{-1}, whereas the activity of stroma SOD was 10 U mg^{-1} pro h^{-1} in high-betacyanin seedlings when grown in 200 mmol L^{-1} NaCl. Furthermore, the increases in the thylakoid-bound SOD activity in high-betacyanin seedlings were much higher than in the controls under salt stress. For example, the increase in thylakoid-bound SOD activity was 42 U mg^{-1} pro h^{-1} in high-betacyanin seedlings, whereas it was 26 U mg^{-1} pro h^{-1} in the control when grown in 400 mmol L^{-1} NaCl. These results indicate that high-betacyanin seedlings have a higher ability to scavenge ROS *in situ* produced in the chloroplasts than the controls.

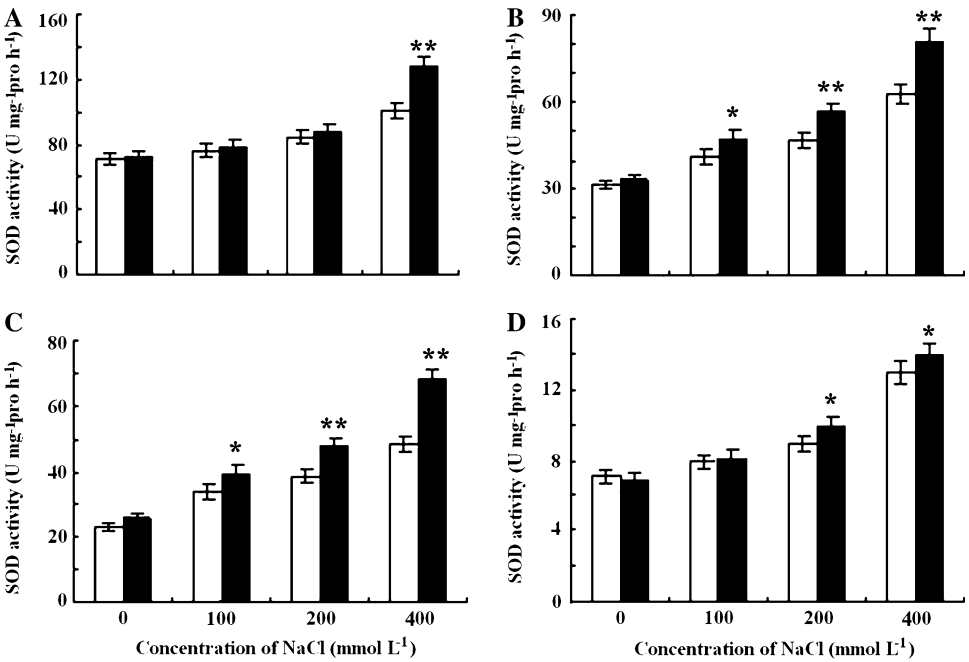

Fig. 5 Leaf SOD activity (**A**), total chloroplast SOD activity (**B**), thylakoid-bound SOD activity (**C**), and stroma SOD activity (**D**) of high-betacyanin *S. salsa* seedlings (■) and the controls (□) exposed to different levels of NaCl for 8 days. Data are mean ± SE ($n = 5$), and the significant levels of difference between high-betacyanin *S. salsa* seedlings (■) and the control (□) are indicated by * ($p < 0.05$) and ** ($p < 0.01$)

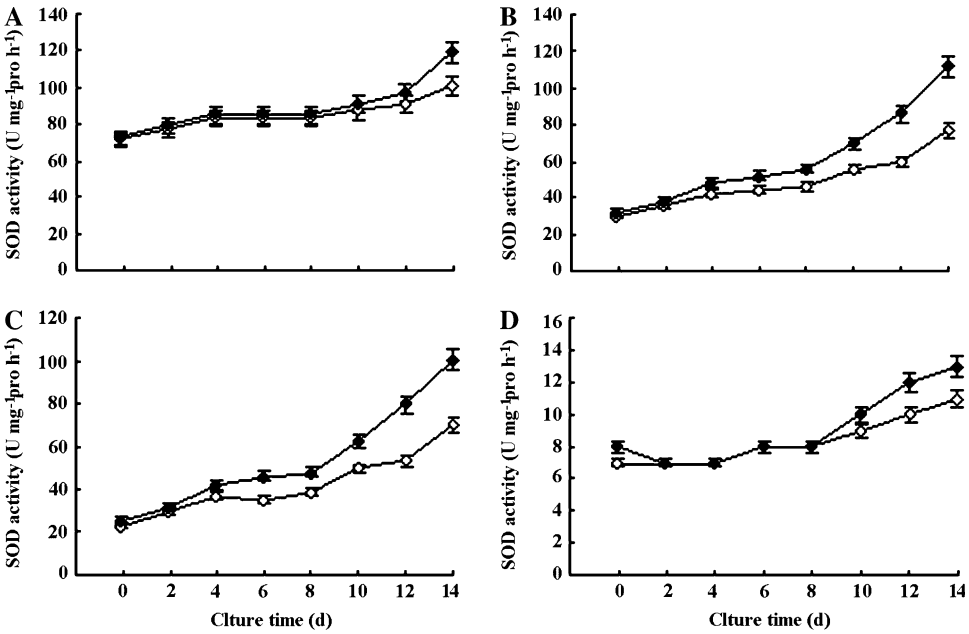

Fig. 6 Leaf SOD activity (**A**), total chloroplast SOD activity (**B**), thylakoid-bound SOD activity (**C**), and stroma SOD activity (**D**) of high-betacyanin *S. salsa* seedlings (■) and the controls (□) exposed to 200 mmol L^{-1} NaCl for different days. Data are mean ± SE ($n = 5$)

Discussion

S. salsa is an important euhalophyte exhibiting a high degree of salt tolerance with leaf succulent characteristics. Considering the fresh weight per plant as an indicator of plant growth capacity, it is obvious from the results obtained in the present study that the growth of *S. salsa* was significantly enhanced by salt treatments (Figure 2). When grown in 400 mmol L^{-1} NaCl, the higher fresh weight of high-betacyanin *S. salsa* seedlings compared to the controls indicated that these seedlings were better able to cope with high salinity.

Betacyanins (red-violet pigments), together with betaxanthins (yellow pigments), are a group of chromoalkaloids known as betalains. These pigments are synthesized from tyrosine and constitute a class of secondary metabolites found in the species of the Caryophyllales, with the exception of the Caryophyllaceae and Molluginaceae families, whose members accumulate anthocyanins (Clement and Mabry 1996; Stafford 1994; Steglich and Strack 1990). In *Salicornia europaea* L., pigment synthesis was induced as a result of salt stress (Bothe 1976), suggesting that betalains may function as osmolytes to uphold physiologic processes. Similarly, it has been suggested that

betaxanthins may serve as osmoregulators either directly or indirectly by modulating the amino acid pool through betaxanthin cleavage or betaxanthin synthesis in Opuntia species (Stintzing and others 1999, 2002). In the present study, the difference of cell sap osmolarity between high-betacyanin *S. salsa* seedlings and the controls was not significant (Table 1), so the betacyanin in *S. salsa* may not be an effective osmoregulator.

To cope with salt stress, plants have developed the mechanisms of ion homeostasis, including Na^+ extrusion system or sequestering of Na^+ into the vacuole and regulating the K^+/Na^+ ratio (Blumwald 2000). Theoretically, the ability to compartmentalize sodium may result from a stimulation of the proton pumps that provide the driving force for increased sodium transport into the vacuole via Na^+/H^+ exchangers (Parks and others 2002). In contrast to some other halophytic plants, *S. salsa* does not have salt glands or salt bladders on its leaves. Thus, this plant must compartmentalize the toxic Na^+ in the vacuoles (Wang and others 2001). The V-PPase and the V-ATPase, which establish an electrochemical H^+ gradient across the tonoplast that energizes the transport of Na^+ against the concentration gradient, should play an important role in salt tolerance of *S. salsa*.

As an analog of anthocyanins, betalains are water-soluble pigments and accumulate in the large central vacuole of plant cells. Even though a few studies have addressed the transport processes of these secondary metabolites across the vacuolar membrane, our knowledge of the mechanisms involved is still scarce (Martinoia and others 2000). It has been suggested that at least two different transport mechanisms are responsible for the sequestration of secondary compounds in plants, H^+-antiport and ABC (ATP-binding cassette)-like vacuole transporter (Frangne and others 2002; Klein and others 2001). Thus, betacyanins and Na^+ may share a similar mechanism for transporting into the vacuole, and betacyanin accumulation in *S. salsa* leaves may induce increases in tonoplast H^+-ATPase activity (V-ATPase) rather than plasma membrane H^+-ATPase (P-ATPase) activity under salt stress. It is still an open question why V-PPase activity is not enhanced by betacyanin accumulation. Thus, a lot of work has been done in the last few years to try to find physiologic conditions that would require greater activity of V-PPase than V-ATPase. However, changes in a variety of growth conditions in different experimental plants led to a variety of responses of V-PPase and V-ATPase, and no clear correlative pattern of activation or deactivation of both proton pumps has been found to date (Binzel and Ratajczak 2001).

In this article, similar to the case of other salt-tolerant plants (Barklaz and others 1995; Parks and others 2002), Na^+ accumulated in *S. salsa* seedlings concurrently with increased V-ATPase activity induced by salt treatment (Table 1, Figure 3A), and no clear pattern was detected for NaCl-dependent P-ATPase and V-PPase activity changes (Figure 3B, C). Thus, P-ATPase and V-PPase seem to be less important physiologically than V-ATPase in *S. salsa* under salt stress. This result supported the idea that plants adapt to high Na^+ levels in part by increasing the activity of proton pumps (Blumwald 2000; Parks and others 2002). Furthermore, the increases in V-ATPase hydrolytic activity in high-betacyanin *S. salsa* seedlings were much higher than that in the controls (Figure 3A) under salt stress, which may lead to higher salt tolerance.

Oxidative stress is a key component of salt stress, and increased radical scavenging activity is correlated with increased protection from damage associated with oxidative stress (Foyer and Noctor 2000; Hernández and others 1995). Betacyanins are a class of compounds with antioxidant and radical scavenging activities (Butera and others 2002; Cai and others 2003). The production of betacyanins in *S. salsa* in response to H_2O_2 treatments suggests that the pigments may function as a ROS scavenger, limiting the oxidative stress caused by environmental stressors (Wang and others 2007). ROS-scavenging enzymes, including catalase (CAT), superoxide dismutase (SOD), and peroxidase (POD), are activated during oxidative stress (Mittler 2002). A detoxification mechanism of H_2O_2 involving a flavonoid-peroxidase of leaf extract has been described (Yamasaki and others 1997). We previously detected that betacyanin accumulation in *S. salsa* was paralleled by increases in SOD activity and CAT activity (Wang and others 2007). The present data showed that both the leaf SOD activity and the chloroplast SOD activity in *S. salsa* increased with the increase of salt concentration (Figure 5A, B) or with time (Figure 6A, B). This implied that the enhanced SOD-scavenging $O_2^{-\bullet}$ radicals protect cells from cellular oxidative damage under salt stress.

Two important sources of oxidative stress in photosynthetic organisms are the chloroplast and the mitochondria. In chloroplasts, the presence of electron flux, high oxygen concentrations, and metal ions can enhance the generation of ROS, particularly $O_2^{-\bullet}$ and O_2^1 (Foyer 1996). The corresponding results were obtained that the activity of chloroplast SOD was increased with increasing NaCl or with time (Figures 5A, 6A). Thylakoids are considered to be one of the major sites of superoxide production because of the simultaneous presence in chloroplasts of a high oxygen level and an electron transport system (Sgherri and others 2000). Our results proved that the activity of thylakoid-bound SOD constituted the major part of total chloroplast SOD activity and was effectively enhanced by salt stress (Figure 5B–D). Consequently, the enhanced SOD activity in chloroplasts of *S. salsa*, especially the thylakoid-bound SOD activity, possibly plays an important role in the resistance to oxidative stress induced by high

salinity. Furthermore, the increases in total chloroplast SOD activity and the thylakoid-bound SOD activity were both higher in high-betacyanin *S. salsa* seedlings than those in the controls under salt stress, which may lead to higher salt tolerance of high-betacyanin seedlings than controls.

In conclusion, the high-betacyanin *S. salsa* seedlings have a higher V-ATPase activity, leaf SOD activity, and chloroplast SOD activity, especially thylakoid-bound SOD activity, than controls under NaCl stress, which are required to energize the tonoplast for ion uptake into the vacuole and scavenge $O_2^{-\bullet}$ *in situ* produced in the chloroplast and thus lead to a higher salt tolerance. This suggests that the presence of the second metabolite betacyanin is positively related to increased salt tolerance of *S. salsa*, but the molecular mechanism needs to be studied further.

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