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Ca^{2+} significantly enhanced development and salt-secretion rate of salt glands of *Limonium bicolor* under NaCl treatment

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Abstract

The divalent cation, Ca^{2+}, plays crucial roles in plant growth, development and stress resistance. *Limonium bicolor* seedlings were treated with 200 mM NaCl combined with three levels of Ca^{2+} (0 mM, 5 mM and 20 mM) for 15 days to study the effects of Ca^{2+} on development and salt-secretion rates of salt glands. It was shown that the 4th leaf areas of *L. bicolor* seedlings under 20 mM Ca^{2+} treatment were significantly higher than those under 0 mM and 5 mM Ca^{2+} treatments. The total number and the densities of salt glands per leaf increased markedly with increased Ca^{2+} concentrations. The diameters of salt glands increased by 59% and 63% as Ca^{2+} concentration increased from zero to 5 mM and 20 mM, respectively. Under 20 mM Ca^{2+} treatment, the salt-secretion rate per leaf was obviously higher than that treated with 5 mM Ca^{2+}, but there was no significant difference in the salt-secretion rates per salt gland between the two groups. Under 0 mM Ca^{2+} treatment, leaf-cell membrane permeability increased significantly, which led to serious leakage of ions and a significant increase in Na^+ loss rate. The results showed that the increase of Ca^{2+} concentration markedly enhanced development and salt-secretion rates of salt glands in the leaves of *L. bicolor*, the increase of salt secretion per leaf is due to the efficiency of the secretion process per salt gland and the number of salt glands, the salt-secretion rates per salt gland have a relationship with the diameters of salt glands.
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Keywords: Ca^{2+}; Development; *Limonium bicolor*; NaCl; Salt glands; Salt-secretion rate

1. Introduction

The structural mechanisms by which plants adapt to their environment are complicated and different environments lead to different structures of plants (Wang and Wang, 1989). Soil salinity not only affects plant growth but also induces their morphology and cell structure to change (Lu et al., 1995). The most remarkable morphological characteristics of recretohalophytes are the salt-secreting structures (salt glands and salt bladders) that can excrete excessive salts (Zhou et al., 2001). Salt glands and salt bladders are prevalent on stem and leaf surface of plants, they play an important role in regulating ion balance, maintaining the stability of osmotic pressure and enhancing the salinity tolerance (Zhang et al., 2003). The salt-secreting mechanism of salt glands in plants is still not clear: it is only known that ions enter salt glands against an electrochemical concentration gradient. Secretion is an active physiological process, the energy being supplied by metabolism and it is sensitive to hypoxia and hypothermia (Zhao and Li, 1999). It is clear that secretion increases with external concentration of, and time of exposure to salt (Pollak and Waisel, 1970; Liphschitz and Waisel, 1974; Marcum and Murdoch, 1992). However, it is not clear whether or not glandular function is inducible (Thomson et al., 1988). Liphschitz and Waisel (1974) found no increase in the number of glands in *Chloris gayana* over a 5 d exposure to salt, although, interestingly, spraying with benzyl adenine (BA) did increase the number of glands. Rozema and Riphagen (1977) found that the number of salt glands of *Glaux maritime* in high saline environments was significantly higher than that in low-salinity. Ramadan and Flowers (2004) reported BA to increase salt loss in maize plants due to its influence on the number of microhairs and on leaf area, but this loss was unrelated to salt excretion *per se*.

Abbreviations: BA, Benzyl adenine; FW, Fresh weight.

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Increasing evidence indicates that osmotic and salt stress, and most likely other conditions, act through or include an oxidative stress component that is responsible for instantaneous increases in intracellular Ca^{2+} (Taylor et al., 1996; Takahashi et al., 1997). It is widely reported that supplemental Ca^{2+} ameliorates Na^+ toxicity symptoms in different plant species by decreasing Na^+ influx through nonselective cation channels (for review, see Läuchli, 1990; Rengel, 1992; and Hasegawa et al., 2000). Shabala et al. (2006) found an additional mechanism of Ca^{2+} action on salt toxicity in plants: the amelioration of K^+ loss from the cell by regulating (both directly and indirectly) K^+ efflux channels. However, it is not known whether there is an additional mechanism of Ca^{2+} action on the alleviation of salt toxicity in recretohalophyte by enhancing salt-secretion rate of salt glands.

Calcium is a central regulator of plant growth and development (Hepler, 2005). Shen et al. (2006) speculate that calcium was a hub in the stomatal development signal regulation network, and other signal transduction pathways regulated stomatal development by influencing or being influenced by calcium signal transduction pathways. Both stomata and salt glands originate in the protodermal cells, but their structure, development and function are distinctly different (Bosabalidis and Thomson, 1984; Larkin et al., 1997). It is still unknown whether calcium affects the development of salt glands.

In the present study, *Limonium bicolor*, a typical recretohalophyte was used to examine the effects of Ca^{2+} on the development and salt-secretion rate of salt glands.

2. Materials and methods

2.1. Plant growth and treatment conditions

L. bicolor seeds were collected from native soil saline–alkaline land in the Yellow River Delta. After being sterilized with 0.1% $HgCl_2$ for 10 min and washed with tap water, plump seeds were selected and sowed in plastic pots filled with fine sand. They were irrigated with tap water. After germination they were irrigated with half-strength Hoagland solution (pH 5.7) in controlled growth chambers (photoperiod: 15 h; light intensity: 600 μmol m^{-2} s^{-1}; relative humidity of 70 ~ 80%; day temperature: 30 ± 2 °C; night temperature: 23 ± 2 °C).When the fourth leaves came out, uniform seedlings were selected and transferred to plastic pots (5 plants in a pot). They were divided into 3 groups (2 pots in a group) and irrigated with Hoagland solution (Table 1) combined with 3 levels of Ca^{2+} (0 mM, 5 mM and 20 mM) for 5 d to acclimate to the Ca^{2+} concentrations. Then they were treated with 200 mM NaCl combined with 3 levels of Ca^{2+} (0 mM, 5 mM and 20 mM) for 15 d. 1 pot in each group was selected and the surfaces of 4th leaves were sprayed with 500 μM Na_3VO_4 once a day for 2 d.

2.2. Measurement of Na^+ lost from leaves

To determine the quantity of Na^+ lost (including the secretion rate of salt glands and leakage from epidermal cells) from the leaves, ions were collected according to Zhou and Zhao (2003). The surface of the 4th leaves was thoroughly washed with distilled water after 15 d. The chosen leaves were washed again with a fixed volume of distilled water (5 ml) after 3 d, and the Na^+ in the washing solution was measured with flame photometer (Flame Photometer 410, Sherwood). Five replicates were determined for each treatment.

Table 1
Elements in Hoagland's solution supplied with different Ca^{2+} concentrations.

Salts	0 mM Ca^{2+}	5 mM Ca^{2+}	20 mM Ca^{2+}
NH_4NO_3	5	0	0
$Ca(NO_3)_2$	0	5	5
$CaCl_2$	0	0	15
KH_2PO_4	1	1	1
KNO_3	5	5	5
$MgSO_4$	2	2	2
H_3BO_3	2.5×10^{-4}	2.5×10^{-4}	2.5×10^{-4}
H_2MoO_4	5×10^{-4}	5×10^{-4}	5×10^{-4}
$CuSO_4$	5×10^{-4}	5×10^{-4}	5×10^{-4}
$ZnSO_4$	2×10^{-4}	2×10^{-4}	2×10^{-4}

pH was adjusted to 6.0.

2.3. Measurement of leaf areas (S)

Leaf area was measured according to Liu (2004). The whole leaf was excised from the plant and the shape was traced on paper and the leaf was weighed. The area was calculated knowing the weight per unit area of the paper. Five replicates were determined for each treatment.

2.4. Preparation of leaf samples for scanning electron microscopy

Leaf samples were prepared according to Lu et al. (1995). The fresh leaf of *L. bicolor* was cut into 0.2 × 0.2 cm pieces, air-dried and fixed for 24 h with 2.5% glutaraldehyde at room temperature. The material was then washed with 0.1 mM phosphate buffer solution, fixed 1.5 h with 1% OsO_4 and washed again with distilled water, before being dehydrated in alcohol series concentrations (30%, 50%, 70%, 80%, 95% alcohol for 20 min and in 100% alcohol for 3 h). After infiltration for 24 h with isoamyl acetate, samples were embedded in the same isoamyl acetate, which was polymerized for 24 h at 30 °C, 45 °C, and 60 °C, respectively. The material was dried with common critical point drier and platinized with ion sputter (IB-5), then observed and pictured with Hitachi H-580 scanning electron microscope. The diameter of salt glands was observed in images obtained with a scanning electron microscope using a magnification of 1000 fold. Fifty salt glands from five leaves were determined.

2.5. Calculation method of salt-secretion rate and density of salt glands

Leaf-prints were prepared according to the method described by Hilu and Randall (1984). Clear nail varnish was applied to the leaf's surface for about 1 h and then the dry film was peeled off using ultra clear Sellotape and placed on a clean slide. Salt glands were counted with a light microscope (Olympus BX51)

using a magnification of 100 fold (an area of 0.0254 mm^2). Salt glands were counted in 10 fields selected randomly. The density of salt glands was calculated according to the average of these 50 fields from 5 leaves.

The total number of salt glands (the number was represented with *N*) on adaxial surfaces of *L. bicolor* leaves was calculated according to the leaf area and the density of salt glands. The total Na^+ secreted by salt glands (the total Na^+ was represented with *M*) on the adaxial surface of *L. bicolor* leaves for 3d was calculated according to Na^+ concentrations measured with a flame photometer. Na^+ secretion rate per salt gland per day (the rate was represented with *V*) was calculated according to the following formula, $V=M/3N$.

2.6. Membrane permeability determination

Membrane permeability was determined by the method of relative conductivity (Shou et al., 2004). In brief, the 4th leaves were rinsed with distilled H_2O to remove ion contamination on the surface. Leaf discs (7 mm in diameter) were placed in a 20 × 150-mm glass tube containing 15 ml of distilled water. The test tubes were subjected to vacuum to remove air bubbles adhering to the surface of the leaves. The tubes were shaken for 3 h at room temperature. After that, the initial conductivity of the solution was measured by using a conductivity meter (DDS-11A, Shanghai Second Analytical Instruments Factory, China). The solutions together with the leaves were then boiled at 100 °C for 30 min to completely disrupt the cell structure. The electrolyte conductivities of the boiled solution were recorded as the absolute conductivity after cooling at room temperature. The percentage of electrolyte leakage (membrane permeability) was calculated by dividing the initial conductivity by the absolute conductivity.

2.7. Detection of lipid peroxidation

The extent of lipid peroxidation in leaves was estimated by measuring the amount of MDA, a decomposition product of the oxidation of polyunsaturated fatty acids, as described (Lin et al., 1984). Five replicates were determined for each treatment.

2.8. Statistical analysis

Data were subjected to a one-way ANOVA using the SAS™ software (SAS Institute, 1989). Treatment means were compared by LSD at $P=0.05$.

3. Results

3.1. Ca^{2+} significantly expanded the leaf area

As shown in Fig. 1A, the 4th leaf areas of *L. bicolor* treated with 20 mM Ca^{2+} were obviously larger than those treated with 0 mM and 5 mM Ca^{2+}, respectively. The relative areas of 4th leaves treated with 20 mM Ca^{2+} increased to 234% compared with plants treated with 0 mM Ca^{2+}. There was no significant difference in the 4th leaf areas of *L. bicolor* between plants treated with 5 mM Ca^{2+} and those treated with 0 mM Ca^{2+}.

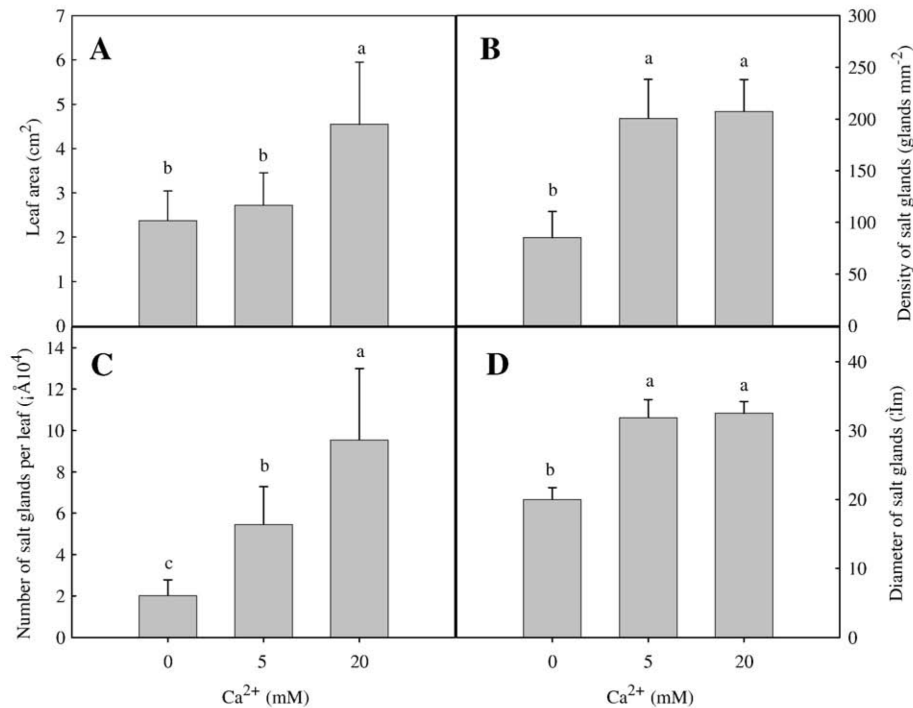

Fig. 1. (A) Effect of Ca^{2+} on the 4th leaf areas of *L. bicolor* under 200 mM NaCl treatment. Means identified by different letters are significantly different at $P<0.05$. (B) Effect of Ca^{2+} on densities of salt glands on adaxial surface of *L. bicolor* leaves under 200 mM NaCl treatment. (C) Effect of Ca^{2+} on total number of salt glands on adaxial surface of *L. bicolor* leaves under 200 mM NaCl treatment. (D) Effect of Ca^{2+} on diameter of salt glands on adaxial surface of *L. bicolor* leaves under 200 mM NaCl treatment. Means identified by different letters are significantly different at $P<0.05$. Vertical bars represent standard deviations, $n=50$ for (B, D) and $n=5$ for (A, C).

3.2. Ca^{2+} increased densities of salt glands and the number of the glands per leaf

As shown in Fig. 1B, the densities of salt glands on adaxial surface of *L. bicolor* leaves under condition of 5 mM and 20 mM Ca^{2+} treatments were significantly greater than those under 0 mM Ca^{2+} treatment. There was, on average, 20% more salt glands per unit area under 20 mM Ca^{2+} treatment than that under 0 mM Ca^{2+} treatment. There was no significant difference in the densities of salt glands between plants grown with 5 mM Ca^{2+} supplement and those under 20 mM Ca^{2+} treatment. As shown in Fig. 1C, the total number of salt glands on adaxial surface increased with increasing Ca^{2+} concentrations. With 20 mM Ca^{2+} treatment, the total number per leaf increased to 610% compared with plants treated with 0 mM Ca^{2+}, the difference was significant.

3.3. Ca^{2+} markedly increased the diameter of salt glands

L. bicolor shows glands surrounded with a "flower-like" structure of 5 ~ 8 epidermal cells (Fig. 2). Ten salt glands were taken from each leaf print randomly to average diameters and 5 leaves were taken from each group. As shown in Figs. 1D and 3, the diameters of salt glands under 5 mM or 20 mM Ca^{2+} increased to 159% and 163% compared with those under 0 mM Ca^{2+}, but there was no significant difference in the diameters between plants treated with 20 mM Ca^{2+} and those treated with 5 mM Ca^{2+}.

3.4. Salt-secretion rate and sodium leakage

Since secretion is an active physiological process, plasmalemma ATPase may play an important role in salt secretion. Na_3VO_4, an inhibitor of plasmalemma ATPase, was sprayed on the 4th leaf surface to compare secretion rate of salt glands with leakage rate of epidermal cells. As shown in Fig. 4A, Na^+ loss rate (including the secretion rate of salt glands and leakage from epidermal cells) of plants unsprayed with Na_3VO_4 under 0 mM Ca^{2+} treatment was significantly higher than those under 5 mM and 20 mM Ca^{2+} treatment, Na^+ loss rate under 20 mM Ca^{2+} treatment was significantly higher than that under 5 mM Ca^{2+} treatment. However, in plants sprayed with Na_3VO_4, there was no significant difference in Na^+ loss rate under 0 mM Ca^{2+} treatment compared with those unsprayed with Na_3VO_4, but

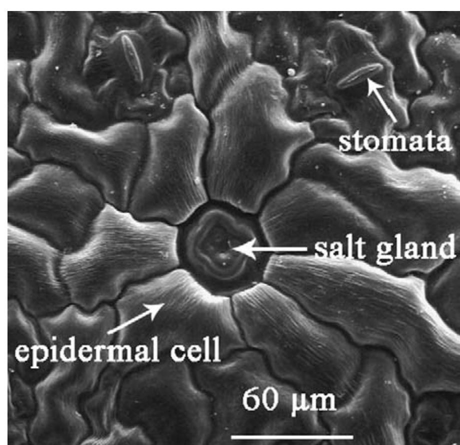

Fig. 2. An image of salt gland on adaxial surface of 4th leaf of *L. bicolor* grown in Hoagland, viewed using a scanning electron microscope. Bars 60 µm.

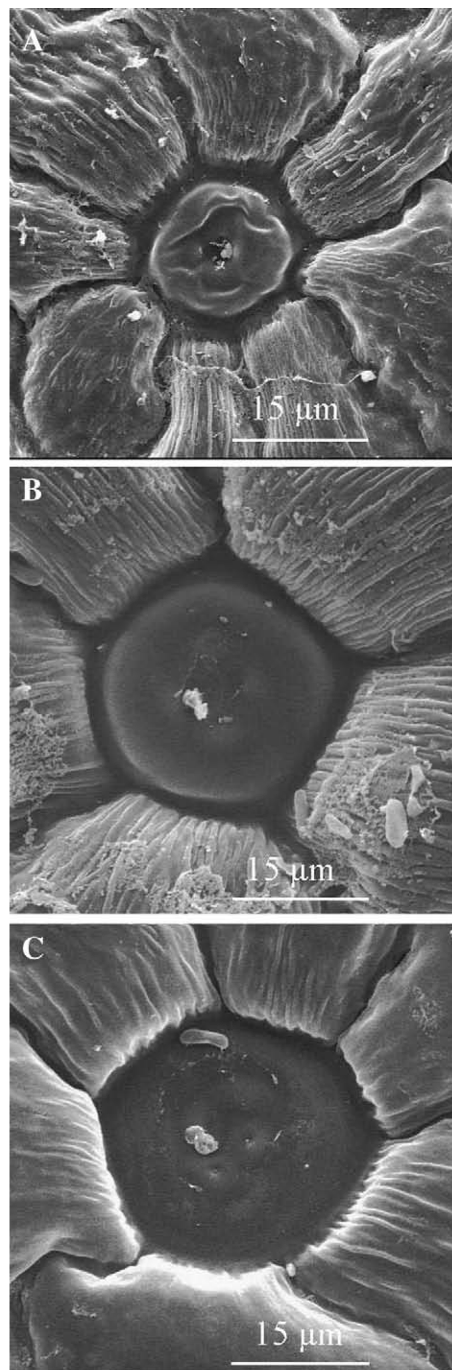

Fig. 3. Scanning electron microscopy of salt glands on adaxial surface of the fourth leaves of *L. bicolor* plants treated with different concentration of Ca^{2+}. (A) 0 mM Ca^{2+} treatment. (B) 5 mM Ca^{2+} treatment. (C) 20 mM Ca^{2+} treatment. Bars 15 µm.

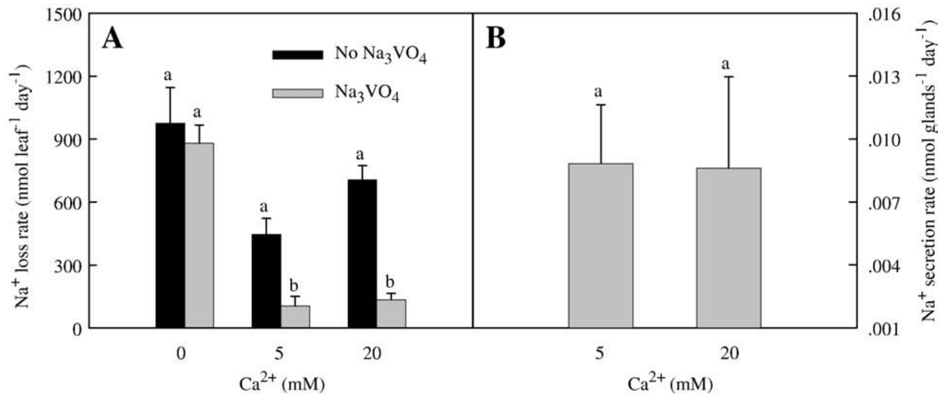

Fig. 4. (A) Effect of Ca^{2+} on Na^+ loss rates per leaf on adaxial surface of the fourth leaves of *L. bicolor* plants under 200 mM NaCl treatment. (B) Effect of Ca^{2+} on salt-secretion rate per salt gland on adaxial surfaces of the fourth leaves of *L. bicolor* plants under 200 mM NaCl treatment. For Fig. 4. (A), means at the same calcium level with the different letters are significantly different at $P<0.05$; For Fig. 4. (B), means identified by the same letters are not significantly different at $P<0.05$. Vertical bars represent standard deviations ($n=5$).

Na^+ loss rates under 5 mM and 20 mM Ca^{2+} treatment were significantly lower than those unsprayed with Na_3VO_4. Obviously, Na^+ loss rate was the leakage of epidermal cells under 0 mM Ca^{2+} and the leakage rate was significantly higher than that under 5 mM and 20 mM Ca^{2+} treatment. However, Na^+ loss rates chiefly based on the secretion of salt glands under 5 mM and 20 mM Ca^{2+} treatment and there was no significant difference in their Na^+ leakage rates. In order to compare the Na^+ secretion rate between 5 mM and 20 mM Ca^{2+} treatment, the leakage of epidermal cells was ignored and Na^+ secretion rates were considered as Na^+ loss rates, Na^+ secretion rate of salt glands on the adaxial surface under 20 mM Ca^{2+} treatment was significantly higher than that under 5 mM Ca^{2+} treatment. These results indicated that the leakage of epidermal cells of the plants grown under 0 mM Ca^{2+} condition is a mainly passive process and not effected by Na_3VO_4, while the decrease of Na^+ loss rates of the plants grown under 5 mM and 20 mM Ca^{2+} conditions is mainly due to the decrease of Na^+ secretion rate.

Na^+ secretion rates of individual salt glands were figured out, as shown in Fig. 4B. No significant difference in Na^+ secretion rates of individual salt glands on the adaxial surface between 5 mM Ca^{2+} treatment and 20 mM Ca^{2+} treatment was detected.

3.5. Leaf membrane permeability and MDA contents

It is well known that Ca^{2+} plays an important role in maintaining the biomembrane integrity. As described above, Na^+ loss rate under 0 mM Ca^{2+} treatment was significantly higher than that under 5 mM and 20 mM Ca^{2+} treatment. To examine the effect of Ca^{2+} on membrane permeability and MDA content, leaf membrane permeability and MDA content were measured.

As shown in Fig. 5A, leaf membrane permeability under 0 mM Ca^{2+} was really significantly higher than that under 5 mM and 20 mM Ca^{2+} treatment. There was no significant difference in leaf membrane permeability between 5 mM Ca^{2+} treatment and 20 mM Ca^{2+} treatment.

As shown in Fig. 5B, MDA contents in *L. bicolor* leaves kept at 0 mM Ca^{2+} increased significantly compared with those under 5 mM and 20 mM Ca^{2+} treatment, MDA contents in

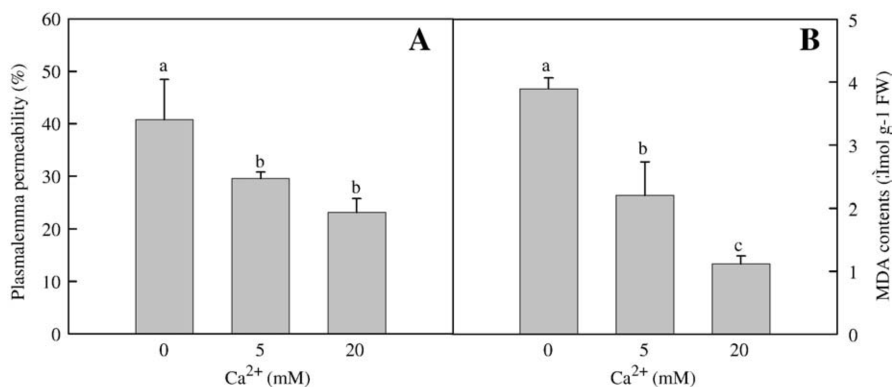

Fig. 5. Effect of Ca^{2+} on plasmalemma permeability and MDA contents of the fourth leaves of *L. bicolor* plants under 200 mM NaCl treatment. Means identified by different letters are significantly different at $P<0.05$. Vertical bars represent standard deviations ($n=5$).

L. bicolor leaves at 20 mM Ca^{2+} decreased significantly compared with those at 5 mM Ca^{2+}.

4. Discussion

The experiments on the effects of extracellular Ca^{2+} on plant development focused on the researches on pollen germination and pollen tube elongation (for review, see Steer and Steer, 1989; Taylor and Hepler, 1997; Malhó, 1998; Franklin-Tong, 1999; Malhó et al., 2006; and Cheung and Wu, 2007), which indicated a possible involvement of Ca^{2+} influx in pollen germination and tube growth. We firstly reported that exogenous Ca^{2+} significantly enhanced the growth and development of salt glands in recretohalophyte *L. bicolor* leaves. Since Ca^{2+} treatments increased leaf areas, Ca^{2+} could lead to bigger cell sizes and thus lower frequencies of specialized cell types. However, the total number and the diameters of salt glands on adaxial surfaces under condition of 5 mM and 20 mM Ca^{2+} treatments increased significantly compared with that under 0 mM Ca^{2+} treatment. Obviously, Ca^{2+} caused more dermatogen cells to develop into salt glands and Ca^{2+} enhanced the development of individual salt glands as well. Generally, salt glands in recretohalophytes lack a large central vacuole but contain many microvacuoles, these microvacuoles may have a relationship with the active metabolism of salt glands (Ziegler and Lüttge, 1967; Shimony and Fahn, 1968; Vassilyev and Stepanova, 1990). Perhaps the diameters of salt glands increased due to the great accumulation of microvacuoles, and the secretion rates increased with the number of microvacuoles. Clearly, a further study on the ultrastructure of salt glands of *L. bicolor* will be required to provide direct evidence. There was no significant difference in the diameters of salt glands between 20 mM Ca^{2+} treatment and 5 mM Ca^{2+} treatment. Therefore, there was no significant difference in the number of microvacuoles in their secretary cells, so that there was no significant difference in salt-secretion rate of individual salt glands. Interestingly, no significant difference was detected in the leaf area between 5 mM Ca^{2+} treatment and 0 mM Ca^{2+} treatment, but the increase of the density of salt glands in the plants treated with 5 mM Ca^{2+} caused the total number of salt glands on adaxial surfaces to increase significantly. The density of salt glands was not significantly affected by the increase of Ca^{2+} concentration above 5 mM, but leaf areas increased significantly, which caused an increase in the total number of salt glands on adaxial surfaces. Leaf areas and density of salt glands under 20 mM Ca^{2+} treatment were significantly higher than those under 0 mM Ca^{2+} treatment. It is reported that salt glands of tamarix and limonium arose from dermatogen cells (Campbell and Strong, 1964; Wiehe and Breckle, 1990). The total number of salt glands on adaxial surfaces increased with increasing Ca^{2+} concentrations, suggesting that Ca^{2+} induced more dermatogen cells to develop into salt glands during the process in which dermatogen cells developed into epidermal cells and salt glands. In other words, development of the dermatogen cells into salt glands in the *L. bicolor* leaves is Ca^{2+}-dependent under salinity. How calcium affects the development of the dermatogen cells into salt glands in the *L. bicolor* leaves needs to be studied in more detail.

Ramadan and Flowers (2004) found that BA increased salt loss in maize plants due to its influence on the number of microhairs and leaf area, but not due to its effect on the efficiency of the secretion process per microhair. Are the salt-secretion rates of individual salt glands induced by Ca^{2+}? Na^+ loss rate per leaf under 0 mM Ca^{2+} was significantly higher than those under 5 mM and 20 mM. The total number of salt glands on adaxial surfaces under 0 mM Ca^{2+} decreased significantly compared with that under 5 mM and 20 mM Ca^{2+} treatments, the diameters of salt glands also decreased but MDA contents and leaf membrane permeability increased significantly. Taking all results into account, the increase of Na^+ loss rate in the Ca^{2+}-deficient plants was caused by the serious damage of the plasmalemma. Na_3VO_4 is a specific inhibitor on the plasma membrane H^+-ATPase (Dschida et al., 1992). There was no significant difference in Na^+ loss rates under 0 mM Ca^{2+} treatment compared with those unsprayed with Na_3VO_4 and the leakage of epidermal cells was a passive process and not affected by being sprayed with Na_3VO_4, so only the secretion of salt glands was inhibited. It suggested that Na^+ loss was mostly due to the leakage of epidermal cells at 0 mM Ca^{2+} and the secretion of salt glands was extremely weak. There was no significant difference in leaf membrane permeability between 5 mM Ca^{2+} treatment and 20 mM Ca^{2+} treatment, but Na^+ loss rate under 20 mM Ca^{2+} treatment was significantly higher than that under 5 mM Ca^{2+} treatment, which indicated that Na^+ secretion rate per leaf under 20 mM Ca^{2+} treatment was significantly higher than that under 5 mM Ca^{2+} treatment and the leakage of epidermal cells under 5 mM Ca^{2+} treatment and 20 mM Ca^{2+} treatment was little. The total number of salt glands on adaxial surfaces under 20 mM Ca^{2+} treatment was significantly more than that under 5 mM Ca^{2+} treatment while there was no significant difference in Na^+ secretion rates per gland. It suggested that at modest Ca^{2+} concentrations Na^+ secretion rate per leaf increased due to the increase of secretion rate per gland and the number of salt glands, while at high Ca^{2+} concentrations Na^+ secretion rate per leaf increased due to the increase of the number of salt glands per leaf.

An increase in external Ca^{2+} concentration can stimulate plasma membrane H^+-ATPase activity via Ca^{2+}-calmodulin-dependent protein kinases (Klobus and Janicka-Russak, 2004). Under salt stress conditions, increased plasma membrane H^+-ATPase activity is necessary for the repolarization of membrane voltage (after depolarization by Na^+ influx), maintaining membrane integrity and ionic homeostasis. Na^+ efflux by the SOS1 Na^+–H^+ antiporter ultimately relies on H^+-ATPase activity (Vitart et al., 2001). Ca^{2+} stimulation of the SOS3–SOS2 pathway also appears to enhance vacuolar Na^+ sequestration by Na^+–H^+ antiporters (Zhu, 2003). Therefore, We suggested that at modest Ca^{2+} concentrations Na^+ secretion rate per salt gland increased due to increased plasma membrane H^+-ATPase activity and enhanced Na^+ sequestration of microvacuoles by Na^+–H^+ antiporters.

Summarizing, the enhanced development and salt secretion of glands in the *L. bicolor* leaf surface by salinity operate in a

Ca^{2+}-dependent mode and the plasmalemma H^{+}-ATPase plays an important role in salt secretion. Further research aimed at finding the targets through which Ca^{2+} exerts its influence and the signaling and response pathways that coordinate the development of glands and salt secretion will be important for revealing the molecular mechanism underlying this process, which could have important ramifications for crop engineering.

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