

Salt secretion in different leaf ages and leaf positions of *Limonium bicolor*

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Abstract. Recretohalophytes can excrete excessive salt from salt glands or salt bladders in order to avoid salt damage. *Limonium bicolor*, belonged to Plumbaginaceae, is a typical exo-recretohalophyte with salt glands. A differential interference contrast (DIC) microscope and leaf discs secretion model was used in the study to explore the salt secretion of different leaf ages in *L. bicolor* at the individual level. For a single mature leaf, more salt glands are distributed in the leaf abaxial than leaf adaxial. The total Na⁺ secretion amount was positively proportional to the leaf age and leaf area. And the Na⁺ secretion rate per salt gland obviously increased at the beginning and kept relatively stable at late period of leaf development. These results suggested that Na⁺ secretion rate of *L. bicolor* were adapted to the leaf age and area.

1. Introduction

There is more and more saline land worldwide, and the soil salinization has become a serious global environmental problem. Most plants can not survive in saline land, but halophytes can complete their life cycle in saline land and have attracted more and more attention [1]. Among these halophytes, exo-recretohalophytes can excrete excessive salt from salt glands to keep the ion balance [2]. Salt glands can secrete many kinds of ion such as Na⁺, K⁺, Ca²⁺, Mg²⁺, Cl⁻ and so on, and studies show that the secretion of Na⁺ and Cl⁻ is obviously higher than others [3].

K⁺/Na⁺ ratio plays an important role in salt tolerance of plant. Recretohalophytes usually decrease their cellular Na⁺ and increase cellular K⁺ to maintain a favorable K⁺/Na⁺ ratio to reduce injury from salt stress. *Limonium bicolor* (Bunge) Kuntze is a typical exo-recretohalophyte and has a typical 20 cells salt gland [4]. Some studies have been done on salt glands and salt secretion of *L. bicolor*. When treated with 200 mM NaCl, Na⁺ secretion rate of *L. bicolor* markedly increased and K⁺ secretion rate decreased, and K⁺ accumulation in cytoplasm and nucleus of salt gland cells was enhanced [5]. Ca²⁺ significantly enhanced development and salt-secretion rate of salt glands of *L. bicolor* under NaCl treatment [6]. Each secretory cell of *L. bicolor* salt gland has four secretory pores and scanning electron microscope micrographs showed that NaCl was secreted through these pores. Studies in the past mainly focus on matured leaves of ion secretion. In this study, we focus on the Na⁺ secretion rate of single salt gland of different leaf age and leaf position in *L. bicolor* and aim to explore the relationship between salt secretion and leaf age in individual level.



2. Materials and methods

2.1. Experimental materials and culture condition

Seeds of *L. bicolor* were collected from the Yellow River Delta, Shandong, China. Dry seeds were stored in refrigerator (4°C) before used. The plump seeds were selected and planted in soil (muck, vermiculite, and perlite in a 4:2:1, proportion, V/V). Three typical growth stages: one month (six-leaf stage), three months (twenty-leaf stage) and five months (fifty-leaf stage) were chosen, respectively expressed as the sixth leaf, twentieth leaf and fiftieth leaf. The plants were grown in a growth chamber with $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity, 60%/80% (day/night) relative humidity and $28 \pm 3^\circ\text{C}/23 \pm 3^\circ\text{C}$ (day/night) temperature.

2.2. Measurement of salt gland density on adaxial and abaxial peels in the expended leaves of three stages of *L. bicolor*

The expended sixth leaf, twentieth and fiftieth leaves were cleared using Hoyer's solution according to Liu and Meinke [7] and salt gland was detected using a differential interference contrast (DIC) microscope (Nikon, ECLIPSE 80i, Japan). The salt gland density on adaxial and abaxial peels in the sixth leaf, twentieth and fiftieth leaves were counted under UV excitation (330-380 nm) at $\times 100$ magnification. Ten fields were randomly selected, and the procedure was repeated with ten leaves of the same leaf region in the expended sixth leaf, twentieth and fiftieth leaves. The SE was calculated with the following formula: SD/\sqrt{n} . The same calculation method of SE and statistical analyses was carried in the following experiments.

2.3. Salt secretion determination at different leaf ages in the sixth leaf, twentieth and fiftieth leaves

With the leaf growth, the area of the blade extended continuously and eventually expanded in full. To measure the salt gland distribution in leaves with different ages, we traced the leaf area changes in the development of one leaf. The leaf area was measured. The area of the sixth leaf growing from one month expanded to more than 300 mm^2 ; therefore the sixth leaf can be divided into eight sections (from i to viii) according to the leaf area $\{i \in (0, 20] (0 < \text{leaf area} \leq 20 \text{ mm}^2, \text{ the same below}), ii \in (20, 50], iii \in (50, 90], iv \in (90, 150], v \in (150, 200], vi \in (200, 250], vii \in (250, 300], viii \in (300, 500)\}$. Ranging from the youngest to fully expanded leaves, the division methods were the same in the twentieth leaf growing from three months (from I to IX) $\{I \in (0, 40], II \in (40, 200], III \in (200, 300], IV \in (300, 500], V \in (500, 800], VI \in (800, 1000], VII \in (1000, 1400], VIII \in (1400, 2000], IX \in (2000, 2500)\}$ and the fiftieth leaf growing from five months (from I to X) $\{I \in (0, 150], II \in (150, 350], III \in (350, 500], IV \in (500, 700], V \in (700, 1000], VI \in (1000, 1300], VII \in (1300, 1700], VIII \in (1700, 2500], IX \in (2500, 4000], X \in (4000, 5500)\}$. To guarantee the repeatability and reproducibility, more than 300 identical seedlings were kept for further experiments. Taking the sixth leaf as an example, we marked the sixth leaf since it appeared, and measured the blade area each day. In each leaf age, the sixth leaf was drawn for clearing, ten repetitions were performed in each leaf stage, and so forth in the remaining leaf stages of the sixth leaf age. After drawing the leaves in all of the ages of the sixth leaf, we counted the salt gland density of each leaf under UV excitation (330-380 nm) at $\times 100$ magnification; we used ten randomly selected fields and leaves for each age. The total number of salt glands on the abaxial surfaces of *L. bicolor* leaves with different ages was calculated according to the leaf area and the density of salt glands [6].

The excreted salt solution from salt glands was obtained by the leaf discs secretion model method [6]. The sixth leaf with different leaf ages, the twentieth leaf and the fiftieth leaf were rinsed to free them of secreted salts. 10-mm-diameter discs were cut from the leaves and placed in Petri dishes containing 100 mM NaCl treatment solutions. The abaxial surface of the leaf disc was covered with mineral oil. Within 24 hours under 24°C , secretion droplets appeared below the oil above each salt gland. The secretion droplets above corresponding number of salt glands were collected with a micropipettor (0.2-2 L) and the concentration of Na^+ was measured with a flame photometer (Flame

Photometer 410, Sherwood). Five leaf discs were repeated for each leaf stage of the sixth, twentieth and fiftieth leaves.

2.4. Statistical analysis

One-way ANOVA procedure was performed to evaluate data in the experiment. Duncan's test was used to compare differences in means between treatments and considered significant at $P = 0.05$. All statistical analyses were performed with the SPSS 13.0 (SPSS Software Inc., USA).

3. Results

3.1. Salt gland density on the adaxial and abaxial in the expended sixth leaf, twentieth and fiftieth leaves

Leaves at three typical growth stages were chosen for the experiments. There were more salt glands on abaxial surface than adaxial in the sixth leaf, and the same trend was found in twentieth and fiftieth leaves. What's more, the salt gland density of different leaf positions (the sixth leaf was considered as lower position and followed by the twentieth and fiftieth leaf) had no significant difference (Fig. 1).

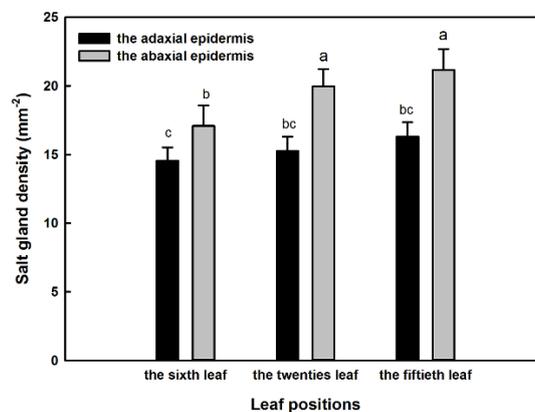


Fig 1. Salt gland density on adaxial and abaxial peels in the expended sixth leaf, twentieth and fiftieth leaves of *L. bicolor*. Values are the means \pm SE of 10 replicates

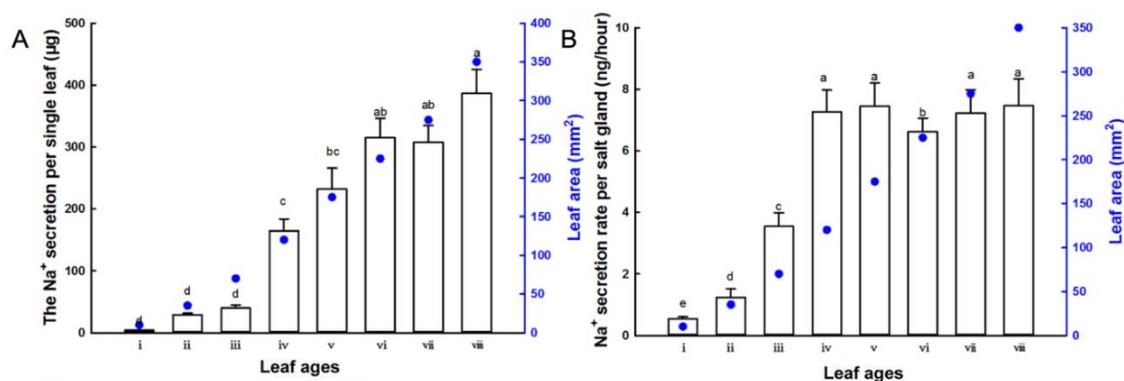


Fig 2. Salt secretion of salt glands in the sixth leaf of *L. bicolor* at different leaf ages. (A) Na⁺ secretion per single leaf at different leaf ages; (B) The Na⁺ secretion rate per salt gland per unit of time at different leaf ages. Blue dotted lines represent the trend of leaf areas at different leaf ages. Values are the means \pm SD of 5 replicates

3.2. The salt secretion of the sixth, twentieth and fiftieth leaves of *L. bicolor*

With the increase in the leaf area, the total Na⁺ secretion amount per single leaf increased significantly (Fig. 2A) and the Na⁺ secretion rate per single salt gland increased obviously at the beginning until leaf area iv (7.27 ng/hour), then, it maintained relatively constant (Fig. 2B). Although the density of

salt glands in leaf area i was much higher than that in other areas, the Na^+ secretion rate per salt gland was the lowest.

The same trends can be found in the salt secretion of the twentieth and the fiftieth leaves of *L. bicolor* at different leaf ages (Fig. 3 and Fig. 4). Correspondingly, the stable Na^+ secretion rate per single salt gland of area VII of the twentieth leaf was 8.59 ng/hour. The stable Na^+ secretion rate per single salt gland of area VII of the fiftieth leaf was about 8.62 ng/hour.

4. Discussion

The observation methods using leaf discs and the autofluorescence of salt glands in our study was consistent with our previous study [8], and also widely used in *Arabidopsis* and *rice* to assess structure of patch-clamped membranes. As shown in the study, the salt gland density on adaxial peel was significantly less than that on abaxial peel in the expanded sixth leaf, twentieth and fiftieth leaves of *L. bicolor*. This may result from that the stomata density on abaxial peel is much more than that on adaxial peel. Salt taken by transpiration pull needs to be excreted so accordingly the salt gland density on the abaxial peel increases. Of course, these assumptions need to be further examined.

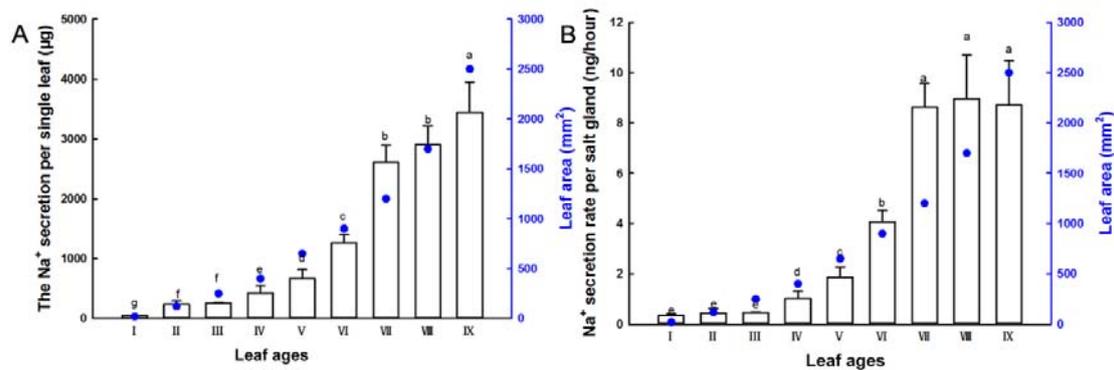


Fig 3. Salt secretion of salt glands in the twentieth leaf of *L. bicolor* at different leaf ages. (A) Na^+ secretion per single leaf at different leaf ages; (B) Na^+ secretion rate per salt gland per unit of time at different leaf ages. Blue dotted lines represent the trend of leaf area at different leaf ages. Values are the means \pm SD of 5 replicates

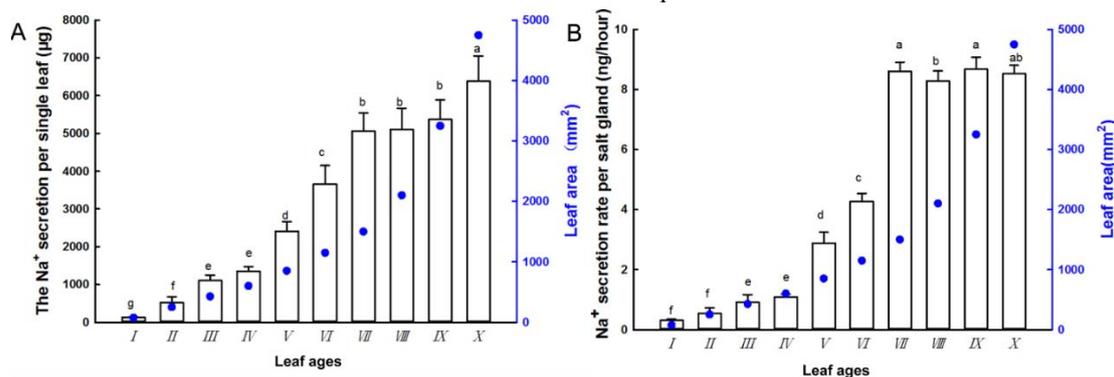


Fig 4. Salt secretion of salt glands in the fiftieth leaf of *L. bicolor* at different leaf ages. (A) Na^+ secretion per single leaf at different leaf ages; (B) Na^+ secretion rate per salt gland per unit of time at different leaf ages. Blue dotted lines represent the trend of leaf area at different leaf ages. Values are the means \pm SD of 5 replicates

When leaves matured, the Na^+ secretion rate per salt gland became stable, which showed no significant difference among three leaf position. What's more, the salt gland density of different leaf positions had no significant difference no matter on adaxial peel or abaxial. The total Na^+ secretion amount per single leaf was positively proportional to the leaf area. These results indicate that when

one leaf was fully expended, the salt gland density would not change apparently and the capability of salt secretion of salt gland would be relatively stable, regardless of the leaf position. *L. bicolor* plants maintained a stable Na^+ concentration mainly by increasing salt gland number (leaf area) rather than the density of salt glands and the Na^+ secretion rate per salt gland, which is different from that of stomata. For the stomata distribution, Lugg and Sinclair [9] found that on the adaxial surface of leaves of two soybean (*Glycine max*) cultivars, the stomatal density progressively increased from the lowest nodes to a maximum at node seven and progressively declined to the uppermost nodes for both cultivars.

Our results indicate that the salt secretion capacity of *L. bicolor* is adapted to the leaf area and leaf age and transpiration, and the Na^+ secretion rate per salt gland of leaves on different leaf positions has no significant differences. Specifically, the larger the leaf area and the older the leaf age, the higher the salt secretion. However, the detailed mechanism needs to be further explored.

5. Conclusion

In summary, the following conclusions can be drawn:

- 1) There are more salt glands in the abaxial side of mature leaves than adaxial side whether in the lower leaf position or higher ones.
- 2) The total Na^+ secretion amount of a single leaf was positively proportional to the leaf age and leaf area.
- 3) The Na^+ secretion rate per salt gland obviously increased at the beginning and kept relatively stable when salt glands mature.

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