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Distribution pattern and salt excretion rate of salt glands in two recretohalophyte species of *Limonium* (Plumbaginaceae)

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ABSTRACT

Salt glands are the unique salt-excreting structures of recretohalophytes. The most important strategy by which recretohalophytes tolerate salinity is to excrete excess salt via salt glands. However, little is known about how the salt gland distribution pattern and salt excretion rate of dicotyledon recretohalophytes are correlated with salinity. This work focuses on the salt gland distribution patterns and excretion rates in two *Limonium* species of Plumbaginaceae under control conditions and NaCl stress. In the two dicotyledonous recretohalophytes, *Limonium bicolor* and *Limonium gmelinii*, the majority of the salt glands and veins were separated by no more than one epidermal cell, and the percentage of salt glands near veins of *L. bicolor* was greater than that of *L. gmelinii*. No salt glands were located alongside one another in either of the *Limonium* species and the salt glands and stomata were usually separated by more than one epidermal cell. The salt gland density on the adaxial epidermis of *L. bicolor* was greater than that of the abaxial epidermis whereas *L. gmelinii* showed the opposite trend. The optimal NaCl concentrations for maximal biomass were 100 mM for *L. bicolor* and 50 mM for *L. gmelinii*. Salt gland and stomata density increased when the NaCl concentration was higher than the optimal concentration. The Na^+ excretion rate of a single salt gland in both *Limonium* species increased with increasing NaCl concentrations. These results suggest that salt gland distribution patterns and excretion rates are positively correlated with salt tolerance.

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1. Introduction

Soil salinization is attracting increasing attention around the globe. More than 800 million hectares, accounting for 6% of the total land area of the world, are salt-affected (Munns and Tester, 2008). Salt-affected soils impact nearly 50% of all irrigated land in the world (Ruan et al., 2010). Few crops can survive in salt-affected regions, leading to substantially reduced crop production and often further degradation and desertification of soils (Flowers and Colmer, 2008). Halophytes are considered promising species for use in the improvement of saline land (Song and Wang, 2014). Halophytes can survive and complete their life cycle in environments with ≥200 mM NaCl and play an important role in ecological protection and restoration of saline soils (Flowers and Colmer, 2008). Therefore, studies on the salt-resistant mechanism of halophytes are of great significance. Halophytes are divided into euhalophytes, recretohalophytes and pseudohalophytes according to their physiological mechanism of salinity tolerance, morphologic structures and ecological characteristics (Flowers et al., 2010).

Over the last several decades, much work has been conducted on the salt-tolerant mechanism of euhalophytes. Halophytes need to accumulate essential nutrients (particularly K) in the presence of high concentrations of the ions generating salinity (Na) and limit the entry of these saline ions into the transpiration stream (Flowers et al., 2010, 2014). In the process of ion accumulation for osmotic adjustment, some ions can 'leak' into the transpiration stream (Yeo et al., 1987; Munns, 2005). Under saline conditions, Na^+ competes with K^+ for its intracellular binding sites as well as for uptake across plant plasma membranes via K^+-selective ion channels (Mark and Romola, 2003). Thus, salinity decreases the K^+/Na^+ ratio in the cell and alters the osmotic balance, in addition to interfering with protein synthesis (Rao, 2011). Therefore, such leaks must be minimized if the aerial parts of plants are not to be swamped by ions.

Recretohalophytes excrete salt through salt glands or salt bladders. Salt glands and salt bladders, which originate from dermatogen cells, are excretory organs specially adapted for dealing with ionic homeostasis in the cells of recretohalophytes (Yuan et al., 2015). A salt bladder is usually composed of a stalk and an enlarged bubble-shaped cell (Zhao and Li, 1999). Twelve families have been found to have salt gland structures: Scrophulariaceae, Frankeniaceae, Primulaceae, Myrsinaceae, Rhizophoraceae, Acanthaceae, Sonneratiaceae, Verbenaceae, Convolvulaceae, Plumbaginaceae, Tamaricaceae and Poaceae (Yuan et al.,

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2016). The evolution of salt glands is uncertain (Céccoli et al., 2015). Structures of salt glands vary among different species. Salt glands of dicotyledonous recretohalophytes are generally multicellular. For example, in *Limonium bicolor* (Bag.) Kuntze, the salt glands consist of sixteen cells, with four groups each consisting of outer cup cells, inner cup cells, accessory cells and secretory cells (Ding et al., 2010; Feng et al., 2015; Yuan et al., 2015, 2016). Salt glands of monocotyledonous recretohalophytes are usually bicellular. Species of Poaceae, such as *Aeluropus littoralis* (Gouan) Parl., *Sporobolus* R. Br., *Spartina* Schreb. and *Zoysia* Willd., have bi-cellular salt glands (Ramadan and Flowers, 2004; Chen et al., 2009; Semenova et al., 2010; Céccoli et al., 2015).

There are several studies on salt gland distribution in monocotyledonous recretohalophytes, such as in Poaceae (Céccoli et al., 2015). In *Spartina gracilis* Trin. and *Spartina pectinata* Link, salt glands are located in rows above the rows of stomata on ridges walls on the adaxial surfaces, while on the abaxial surfaces the salt glands were found to be in rows, often alternating with the stomata (Otani et al., 2009). In *A. littoralis* (Willd) Parl., salt glands appeared as bulbous epidermal extrusions on both leaf surfaces, and their density increased from 4200 glands cm^{-2} in control plants to reach a maximum of 6900 glands cm^{-2} in plants subjected to 400 mM NaCl (Barhoumi et al., 2007, 2008). In Salt Range ecotype of *Cynodon dactylon* (Linn.) Pers, the development of vesicular (bladder) hairs increased to adapt to high level of NaCl (Hameed et al., 2013). It has been reported that salinity tolerance is positively correlated with salt excretion and salt gland density in species of *Zoysia* (Marcum et al., 1998; Marcum, 2008; Rao, 2011).

Much research has been conducted on the growth and physiological responses to salinity stress of dicotyledonous recretohalophytes and the anatomical structures of salt glands are examined by paraffin sections or with an electron microscope (Storey and Thomson, 1994; Sobrado and Greaves, 2000; Tan et al., 2010; Feng et al., 2015; Yuan et al., 2015). However, little is known about salt gland distribution patterns correlating with leaf veins, stomata, and epidermal cells of dicotyledonous recretohalophytes or salt excretion rates under control and NaCl conditions.

In the present study, two species of dicotyledonous recretohalophytes [*L. bicolor* and *Limonium gmelinii* (Willd.) Kuntze] were used to study in detail the patterns of salt gland distribution and the possible role in their salt tolerance. In addition, responses of salt excretion rate to different concentrations of NaCl were also examined to verify that the salt excretion rate is positively correlated to salt tolerance. *L. bicolor* grows on saline soils in the Yellow River Delta area, China, and is a typical recretohalophyte with multicellular salt glands in the epidermis of its leaves and stems (Yuan et al., 2013). *L. gmelinii* is one of the main halophytes in saline soils of Xinjiang, China and has many multicellular salt glands on the leaves (Zhou et al., 2005; Shi and Zhang, 2011). Our aims were to explore the roles of salt gland distribution and the salt excretion rate of a single salt gland in the salt tolerance of both species of *Limonium*.

2. Materials and methods

2.1. Sampling and cultivation

Seeds of *L. bicolor* were collected from a saline inland environment (N37°20′; E118°36′) on the Yellow River Delta, Shandong, China. Seeds of *L. gmelinii* were obtained from Fukang (N43°45′; E87°46′), Xinjiang, China. All dry seeds were stored in a refrigerator at <4 °C for 6 months before use.

The seeds were planted in a plastic pot (16 cm in diameter, 12 cm high) containing soil (a mixture of muck, vermiculite, and perlite in a 4:2:1 ratio, V/V) or in clean river sand (NaCl treatment). The seedlings planted in soil were watered once a day with 1/2 strength Hoagland's solution (pH 6.2) for the following experiments.

Three-leafed seedlings of both *L. bicolor* and *L. gmelinii* were selected for NaCl treatment. Each treatment group contained five pots. Control plants were irrigated with full-strength Hoagland nutrient solution (0 mM NaCl). The NaCl was dissolved in the nutrient solution described above. To avoid osmotic shock, the NaCl concentration was stepped up by 50 mM per day until the final concentration was achieved. To avoid salt accumulation in the sand due to evaporation, each pot was flushed with 2000 ml nutrient solution containing the respective concentrations of NaCl twice daily and allowed to drain. The experiment was terminated 30 days after the final salinity concentrations had been reached and the fully expanded sixth leaf from each plant was harvested for experiments.

All the plants were grown in a growth chamber. The light intensity was approximately 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$, the relative humidity was 60%/80% (day/night) and the temperature was 28 ± 3 °C/23 ± 3 °C (day/night).

2.2. Salt gland distribution

The sixth expanded leaf of the plant was collected and fixed in a mixture of ethanol and acetic acid (3:1; v/v) (Kuwabara and Nagata, 2006). They were then rinsed in ethanol (70%) for decolorization and cleared in Hoyer's solution (7.5 g of gum arabic, 100 g of chloral hydrate, 5 ml of glycerol in 30 ml of water) as described by Meinke (1994). The cleared leaves were examined with a differential interference contrast microscope (DIC, ECLIPSE 80i, Nikon, Japan) or a fluorescence microscope (ECLIPSE 80i, Nikon, Japan) at ×200 magnification (Yuan et al., 2013). DIC microscopy can focus on different dimensions of the cleared leaves, while the salt glands and leaf veins are distributed in different dimensions. Photos under DIC and fluorescence of salt glands (Fig. 1A and B) and veins (Fig. 1C and D) were obtained. Then, combined images (Fig. 1E and F) were drawn by Photoshop CS6, and salt glands were marked in solid red circles to determine the relationship between salt glands and leaf veins. According to the spacing of the epidermal cells between salt glands and leaf veins, two groups were classified: Group 1 (spacing of no more than one epidermal cell); Group 2 (spacing of two or more cells). Five leaves of the same size were examined in both the adaxial and abaxial epidermis of *L. bicolor* and *L. gmelinii*, and the salt glands were counted in five fields selected randomly but at the same general position on each leaf.

The distances in epidermal cell number between nearby salt glands were similarly measured in both adaxial and abaxial epidermis of *L. bicolor* and *L. gmelinii*. In each field, one central salt gland was locked as the target. Then, the numbers of spacing epidermal cells were counted relative to the surrounding salt glands. The salt gland with the minimum number of spacing cells was recorded. Five leaves with the same size were examined, and the salt glands were counted in five fields selected randomly but at the same general position on each leaf. In addition to the minimum spacing between salt glands and stomata, the diameter of a single salt gland, the density of salt glands and stomata, and the areas of epidermal cells were measured in both adaxial and abaxial epidermis of *L. bicolor* and *L. gmelinii*.

2.3. The fresh weight, water content and salt excretion rate under different NaCl concentrations in L. bicolor and L. gmelinii

The fresh weight of plant and the water content under different NaCl concentrations were measured. The salt excretion rate was determined by using previous methods (Yuan et al., 2013). 10-mm-diameter disks were cut from the leaves rinsed by deionized water and placed in Petri dishes containing 100 mM NaCl and the abaxial surface of the disk was on the top. The leaf disks were then covered with mineral oil. Within 24 h under 24 °C, the secretion droplets above the abaxial (whose salt glands were counted) were collected with a micropipettor (0.2–2.0 μl), and the concentration of Na^+ in the fluid was determined with a flame photometer (Flame Photometer 410, Sherwood). Five leaf disks were examined for each seedling. Based on counted salt gland density, the Na^+ excretion rate per single salt gland (pmol $gland^{-1}$ h^{-1}) was calculated (Ding et al., 2010).

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