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Salt excretion through the cuticle without disintegration of fine structures in the salt glands of Rhodes grass (*Chloris gayana* Kunth)

Takao Oi, Hiroshi Miyake, Mitsutaka Taniguchi *

Laboratory of Plant Resources and Environment, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

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ABSTRACT

Some salt-tolerant plants belonging to the Poaceae possess bicellular salt glands that excrete salt-containing water. To clarify the excretion process from the outer cell of the bicellular salt gland (cap cell), unwashed and washed fresh leaves of Rhodes grass (*Chloris gayana* Kunth), Poaceae, were cryo-fixed rapidly, and the surface fine structures of the leaves were observed by cryo-scanning electron microscopy with high resolution. The cuticle on the cap cell did not have any pores or signs of rupturing. The cuticle of the cap cells lacks the epicuticular waxes that cover most surfaces of leaf epidermis. After excreted droplets on the salt glands were completely removed by washing with water, re-excreted droplets were observed 3 h later. These findings indicate that the bicellular salt glands of Rhodes grass excrete salt-containing water continuously through the wax-free cuticle of the cap cell without disintegration of the cuticular structure.

Introduction

Many plants have glands to export specific substances, such as sugars, polysaccharides, secondary metabolites (e.g. resin, oil, and terpenes), and inorganic salts (Lüttge, 1971). "Salt glands" are specialized epidermal cells or trichomes to excrete salts from the leaves (Fahn, 1988; Waisel, 1972). Active salt excretion is considered to be one of the salt-tolerance mechanisms, which regulate the ion balance in leaf tissues (Naidoo and Naidoo, 1998a).

Salt glands occur in genera of at least 12 plant families (Waisel, 1972) and are divided into three types on the basis of their structure: the bladder cells of the Chenopodiaceae, the multicellular glands of other dicotyledonous families, and the bicellular glands of the Poaceae (Kobayashi, 2008; Thomson et al., 1988). The bladder cells accumulate salts in their vacuoles and eventually the cells rupture releasing the salts to the outside of leaf surfaces (Esau, 1977; Fahn, 1988). The multicellular glands are composed of varying numbers of cells, from 6 to 40 or more, and excrete salts directly through pores in the surface cuticle (Esau, 1977; Thomson et al., 1988). The bicellular glands of the Poaceae consist of an inner basal cell and an outer cap cell (Thomson et al., 1988). The basal cell is assumed to be the salt-collecting cell, and the cap cell is assumed to be the salt-excreting cell (Kobayashi, 2008; Liphschitz and Waisel, 1974). However, the excretion pathway of salts via the bicellular salt glands is not well understood.

Rhodes grass (*Chloris gayana* Kunth), which belongs to the subfamily Chloridoideae in the Poaceae, is known to possess bicellular salt glands (Liphschitz et al., 1974; Oi et al., 2012). This grass is cultivated for fodder and is considered particularly useful in saline areas because of its salt tolerance (Suttie, 2000). Recently, we studied the distribution and morphology of salt glands of Rhodes grass by scanning electron microscopy (SEM) on leaf specimens processed by chemical fixation and critical point drying (Oi et al., 2012). The salt glands of Rhodes grass are distributed above the small veins on the adaxial surface and between the veins on the abaxial surface. The cap cell of a salt gland is smooth and globular in shape, and its diameter is 10–15 μm. Moreover, we examined droplets excreted from the salt glands by SEM in a low-vacuum mode (LV-SEM): Oi et al. (2013). The LV-SEM allows observing fresh, hydrated specimens without chemical fixation and critical point drying, and thus maintains soluble attachments on the specimen surfaces. Our LV-SEM observation showed that the bicellular glands excrete droplets just above their cap cells without rupturing the cuticle on the cap cell (Oi et al., 2013).

Our observation by SEM and LV-SEM showed that there is no rupturing scar or porous structure at the surfaces of the cap cells (Oi et al., 2012, 2013). However, there is a possibility that these techniques cannot detect minute openings or preserve fine structures on the specimen surfaces. It was reported that LV-SEM observation

* Corresponding author.
E-mail addresses: i062004b@gmail.com (T. Oi), miyake@agr.nagoya-u.ac.jp (H. Miyake), taniguti@agr.nagoya-u.ac.jp (M. Taniguchi).

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reduces the resolution and stability of specimens at high magnification (Pathan et al., 2008). On the other hand, surface fine structures might be damaged during specimen preparation by conventional SEM (Pathan et al., 2008). To clarify the salt excretion process through bicellular salt glands in Rhodes grass, it is necessary to observe the surfaces of the structurally intact glands at higher magnification.

Cryo-SEM (low-temperature SEM) is a method for observing frozen-hydrated specimens at low temperature in the scanning electron microscope (Read and Jeffree, 1991). It is considered superior to other techniques in the preservation of plant surface details (Jørgensen et al., 1995; Pathan et al., 2008; Read and Jeffree, 1991). Cryofixation is more rapid and less damaging than chemical fixation, and cryo-SEM can visualize with higher resolution than LV-SEM.

In this study, we revealed the fine surface structures of the salt glands in Rhodes grass by cryo-SEM and discuss the excretion process of the salt-containing water through the cuticle of the cap cell.

Materials and methods

Plant growth conditions and salt treatment

Caryopses of Rhodes grass (*Chloris gayana* Kunth cv. Katambora) were germinated on culture soil in 500-mL pots in a growth chamber. The cultivation condition was controlled at 28/20 °C (light/dark), relative humidity of 60%, 14-h photoperiod, and light intensity of 500 μmol m^{-2} s^{-1}. Following germination, two seedlings per pot were left by thinning and were grown and watered with tap water. After 7 d of growth, salt treatment was conducted by supplying 150 mL of 100 mM NaCl solution every other day for 14 d. After 14 d of the treatment, the seventh leaves were used for observations. The seventh leaves had not appeared at the beginning of NaCl treatment and were fully expanded at day 7 of the treatment.

Cryo-SEM observation

Small segments (approx. 3 mm × 5 mm) were excised from the middle portion of the leaf blades and mounted on a stub with adhesive carbon tape; the margins of the segments were pressed tightly to the tape with tweezers. Then the specimens were immersed in liquid nitrogen (−196 °C) for cryo-fixation, and immediately transferred to a field emission scanning electron microscope (S-4200K, Hitachi, Tokyo, Japan) equipped with a cryo-stage (−150 to −120 °C). The surfaces of the specimens, without any metal coating, were observed at an accelerating voltage of 15 kV for low magnification (under 1000×) and 3 kV for high magnification (at and over 1000×), respectively.

To remove excreted materials from the leaf surfaces, the leaves were washed with running tap water for 30 s and rinsed with distilled water three times. After the excess water was absorbed with paper towels, washed leaves were also cryo-fixed and observed by cryo-SEM as above.

Other observations

The leaves of Rhodes grass were also examined by conventional SEM to compare with the surface fine structures in cryo-SEM. Small segments of the leaves were fixed in glutaraldehyde, dehydrated in ethanol, critical-point dried in CO_2, and coated with gold, according to Oi et al. (2012). The specimens were observed with a field emission scanning electron microscope (S-4200K, Hitachi) equipped with a standard specimen stage at 15 kV accelerating voltage.

To confirm the continuous excretion by the salt glands, fresh leaves of Rhodes grass were examined with a digital microscope (VHX-1000, Keyence, Osaka, Japan) on the identical leaf surface before and after washing the leaf. The leaf sheathes were cut at 20 mm from the base of leaf blades, and the cut ends were immersed in distilled water in 10-mL flasks. The detached leaves were taped to the specimen stage of the digital microscope, and the adaxial surfaces of the middle portion of the leaf blades were observed. Then the leaves were washed with running tap water for 30 s and rinsed with distilled water three times. After the excess water was absorbed with paper towels, the washed leaves were observed again with the digital microscope.

Results

Excreted droplets were observed on the unwashed leaf surfaces by cryo-SEM (Fig. 1A and B). These droplets were globular, and their diameters were 75 ± 20 μm. The droplets and macro-hairs on the leaves were distributed on the same lines, parallel with veins. Salt glands were hardly observed on the unwashed leaf surfaces. The droplets were removed by washing the leaves with water (Fig. 1C and D), and thereafter the salt glands were visible on the washed leaf surfaces. The head of a *Chloris* salt gland (the cap cell) is globular in shape, and its diameter is approximately 15 μm (Figs. 1D and 2B). The salt glands on the washed leaves showed the same distribution as the droplets on the unwashed leaves: the glands were positioned on lines parallel with veins along which macro-hairs were also distributed. Droplets and salt glands on the leaves of Rhodes grass without NaCl-treatment were not much different in shape and size from those of the NaCl-treated plants (data not shown). Although the droplets on the salt glands disappeared from leaf surfaces just after the washing (Fig. 1A–D), they appeared again on the salt glands 24 h later (Fig. 1E and F). These droplets covered almost all of the salt glands and their diameters were 25 ± 5 μm.

Cryo-SEM observation at low electron dose (3 kV accelerating voltage) showed the surface fine structures of the leaves in detail (Fig. 2A–C). Cap cells of the salt glands have smooth surfaces, without any pores or rupturing scars on the cuticle (Fig. 2B). The surfaces of epidermal cells are covered with microstructures of epicuticular waxes (Fig. 2A and C). In contrast, the surfaces of the cap cells are smooth and lacking such wax structures (Fig. 2B). Conventional SEM observation did not show the wax microstructures on the entire leaf surfaces, neither on the epidermal cells (Fig. 2D). Cryo-SEM was able to show these fine surface structures on the leaves distinctly better than conventional SEM.

To elucidate the positional relation between the droplets and the salt glands, unwashed leaf surfaces were observed from an angle. Most of the salt glands were completely hidden under the droplets (average diameter: 75 μm) and were not seen on the leaves of Rhodes grass grown for three weeks (data not shown). However, the salt glands under the droplets were seen on the leaves of Rhodes grass grown for two weeks and observed in oblique view (Fig. 3). The droplets (average diameter: 45 μm) were spherical and did not spread onto the surfaces of the epidermal cells (Fig. 3A and B). An enlarged view showed that the droplet covered the upper region of the cap cell (Fig. 3C). Another view showed that the droplet contacted the top of the papilla of the epidermal cell (Fig. 3D). The positional relation between the droplets and the salt glands is shown in Fig. 4 giving a scheme of the ultrastructure of the cells as described in detail by Oi et al. (2012).

On the washed leaf surfaces, droplets were observed again on the salt glands 24 h after the leaf washing (Fig. 1). To confirm the continuous excretion, identical leaf surfaces of Rhodes grass were observed with a digital microscope for 6 h after washing (Fig. 5). Before washing, large droplets (diameter: 75 ± 20 μm)