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THE OPTIMAL DOSAGE OF ^{60}Co GAMMA IRRADIATION FOR OBTAINING SALT GLAND MUTANTS OF EXO-RECRETOHALOPHYTE *LIMONIUM BICOLOR* (BUNGE) O. KUNTZE

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Abstract

Limonium bicolor (Bunge) O. Kuntze is a typical exo-recretohalophyte with multi-cellular salt glands. It is often used to improve saline-alkali soil. Seeds of *L. bicolor* were treated with different doses of ^{60}Co gamma irradiation to determine the LD_{50} for ^{60}Co gamma irradiation; the goal was to produce a relatively high number of mutants in salt gland development and salt secretion with a relatively low level of mortality. ^{60}Co gamma irradiation did not greatly affect germination, but an increase in gamma dose prevented the development of true leaves and reduced the percentage of seedlings that emerged from soil. The LD_{50} for ^{60}Co gamma irradiation was 120 Gy. Two mutants (*few* and *many*) were obtained under the LD_{50} using the screening methods — differential interference contrast microscope and leaf discs excretion model. Compared with the wild type, *few* and *many* had mutation in salt gland development, and *many* showed lower salt secretion rate per single salt gland than WT. These mutants would provide insight into the molecular mechanisms of salt gland development and salt secretion and into the development of salt-tolerant crop plants.

Introduction

More than 800 million hectares of land are salt-affected worldwide, and although high levels of salt generally reduce plant growth, tolerance to soil salinity differs greatly among plant species (Munns & Tester, 2008). With the increase of the demand for agricultural products and the spread of salinity, understanding how to develop crops in saline environments is increasingly important (Rozema & Flowers, 2008). Halophytes are adapted to growing in saline environments and have substantial potential as vegetable, forage, and oilseed crops. It is also possible that halophytic properties can be developed in crop plants for "saline agriculture". *Limonium bicolor* is a typical exo-recretohalophyte (a halophyte that can excrete absorbed salt to the outside) belonged to *Limonium*, plumbagenaceae and has a typical salt excretory structure called salt gland. *L. bicolor* can improve and desalt saline-alkali soil; furthermore, it is often seen in floral art as decorative flowers and medicinal materials.

The salt-excretory structures (salt glands and salt bladders) are the only visible morphologic characteristics that distinguish recretohalophytes from non-halophytes. To avoid salt stress, *L. bicolor* excretes excess salt ions from salt glands on the leaves and stems. In saline environments, salt excretion by salt glands makes it possible to maintain the ion balance required for normal metabolism. The ultrastructure and excretory mechanism of salt glands have been the focus of many studies nowadays (Wiehe & Breckle, 1990; Bosabalidis, 2010; Semenova *et al.*, 2010).

Understanding the mechanisms controlling salt gland development and salt secretion is important for explaining salt tolerance, but the genes involved are still unclear mainly because of the unclear genetic background of *L. bicolor*. Experiments have indicated that the development of salt-excretory structures was controlled by multiple genes (Yang *et al.*, 2011). Currently, the most widely used tools to study structure and function are over-expression or deletion mutants, which enable researchers to identify essential components affecting specific aspect of plant growth and development, such as salt gland development and salt excretion. Two kinds of mutants would be useful for investigating salt gland structure and function, salt gland density mutants (mutants with few or many salt glands) and salt secretion mutants (mutants in which salt excretion per gland is low or high). These mutants can be obtained by exposing plants to ionizing radiation, such as ^{60}Co gamma ray.

Gamma ray is widely used in ionizing radiation that causes variation of chromosome structure proportional to the dose of the ion beam radiation. The most common variations in chromosomal structural are shifts, inversions, and deletions (Saito *et al.*, 2001). Ionizing radiation is more likely to generate desirable traits in crop breeding than EMS, as has been demonstrated for corn, soybeans, and cotton (Khan & Tyagi, 2009). Gamma irradiation is now used for breeding in many fields, including the breeding of high yield *Saccharomyces cerevisiae* (Lee *et al.*, 2012), the production of high purity medical chitosan (Shen *et al.*, 2011), and high quality blackberry varieties (Basaran & Kepenek, 2011). In the past 30 years, breeding based on gamma irradiation-induced mutation has generated 1,700 crop varieties with increased yield, improved quality, and increased resistance to stress (Brunner, 1995; Zhu *et al.*, 2010).

In toxicity studies, LD_{50} (the dose of irradiation to cause the death of 50% of the exposed seedlings) is significant data to further mutagenesis. In the current study, we determined the LD_{50} for gamma radiation of *L. bicolor* seed and under which mutants in salt gland are expected to the maximum number with a minimum of mortality. We individually screened salt gland density mutants of *L. bicolor* by differential interference contrast microscope from tens of thousands of seedlings exposed by ^{60}Co gamma ray compared with wild type (Liu & Meinke, 1998; Ding *et al.*, 2010). Leaf discs excretion model was applied to further obtain salt secretion mutants of *L. bicolor* (Faraday *et al.*, 1986; Dschida *et al.*, 1992). The goal of

current report is to initially obtain mutants in salt gland development and salt secretion of exo-recretohalophytes *L. bicolor* using LD_{50} of gamma radiation. The LD_{50} of gamma radiation in the current report provided an efficient method to generate mutants in *L. bicolor* for the study of molecular mechanisms of salt gland development and salt secretion. Salt-tolerant crops would be developed in order to utilize salt-affected land worldwide.

Materials and Methods

Experimental material and ^{60}Co gamma ray irradiation treatment: In October 2010, seeds of *L. bicolor* were collected from a saline inland environment (N37°20'; E118°36') in the Yellow River Delta, Shandong, China. Dry seeds were stored in a refrigerator at <4ºC for 6 months before being used. Before ^{60}Co gamma irradiation treatments, seeds to be planted to non sterilized soil were not sterilized but seeds to be germinated on plates were surface sterilized in 0.1% $HgCl_2$ for 10 minutes and then thoroughly washed with sterile-distilled water. ^{60}Co gamma irradiation doses were set as 0, 100, 200, 300, 400 and 500 Gy referring to Borzouei *et al.* (2010) with some modifications.

Effect on seed germination and seedling growth: Germination percentage was determined according to Song *et al.* (2008) with some modifications. All seeds were sown in Petri dishes (9 cm diameter) on two layers of filter paper moistened with double-distilled water. All Petri dishes were placed in a paper box with constant darkness in a growth chamber to maintain a relative humidity of 60/80% (day/night) and a temperature of 28 ± 3/23 ± 3ºC (day/night). Germination was recorded daily until no additional germination was detected. Seeds were considered to have germinated when the emerging radicle was at least 1 mm long. Each treatment was replicated four times with 100 seeds per replicate. The root length under different ^{60}Co gamma ray irradiation treatment was measured with 30 replicates each.

For determination of the seedling growth, seeds were sown 2 mm deep in plastic pots (116 cm long × 39 cm wide × 12 cm high respectively) filled with a mixture of muck, vermiculite, and perlite (4:2:1 V/V, 8 cm high) when the germination experiments were started. Each treatment was represented by four replicate pots with 100 seeds per pot. Seeds under all treatments were sown in the same plastic pot to maintain consistent culture conditions. The plants were grown in a growth chamber under natural light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The temperature and relative humidity in the growth room were the same as described in germination part. The number of seedlings in each pot was recorded daily. The percentage of seedling emergence was determined by the formula $a/b \times 100\%$, where a was the number of seedlings and b was the total number of seeds sown in each treatment.

Determination of LD_{50} of ^{60}Co gamma ray irradiation: The LD_{50} for ^{60}Co gamma ray irradiation was based on seedling emergence. The plot of log-transformed emerged percentage vs. irradiation dose was fitted to the linear regression equation $\log Y = a_2 + b_2X$, and the regression was used to determine the LD_{50}.

Screening the mutants in salt gland development and salt secretion: The 20th expanded leaves of *L. bicolor* grown for three months under the treatment of the LD_{50} of ^{60}Co gamma irradiation were used to screen salt gland mutants. DIC (a differential interference contrast) microscope (ECLIPSE 80i, Nikon, Japan) was used to determine "salt gland density mutants" and leaf discs excretion model was applied to screen "salt secretion mutants" of *L. bicolor*. The salt gland density of abaxial surface was counted in ten fields at ×100 magnification selected randomly referred to Liu & Meinke (1998) using DIC microscope. The density of salt gland was calculated according to Ding *et al.* (2010).

The excreted salt solution of salt gland was obtained by the method of leaf discs excretion model referred to Faraday *et al.* (1986) and Dschida *et al.* (1992) with some significant modifications. After the leaves were rinsed free of excreted salts, 10 mm diameter discs were cut from the leaves and placed in Petri dishes containing 100 mM NaCl treatment solutions. The abaxial surface downloaded from leaf disc was covered with mineral oil. Within 24 hours under 20ºC, secretion droplets appeared below the oil above each salt gland. The secretion droplets above known number of salt glands were coalesced and collected with a micropipettor (0.2-2 μL) and tested the concentration of Na^+ by flame photometer (Flame Photometer 410, Sherwood). Five leaf discs were repeated of each irradiated seedling. The Na^+ secretion per leaf area (ng/mm^2) and the Na^+ secretion rate per single salt gland (ng/hour) were calculated (Ding *et al.*, 2010).

Statistical analysis: Data for germination percentage and seedling emerged percentage were transformed (arcsine) before statistical analysis to ensure homogeneity of variance. Means of germination percentage and root length among different ^{60}Co gamma irradiation doses were compared using Duncan's multiple range test at the 0.05 significance level (Duncan, 1955). Values of the salt gland density, salt secretion per leaf area, and salt secretion rate per single salt gland of mutants were also compared with those of wild type at $P = 0.05$. All statistical analyses were performed with SPSS Version 16.0 for Windows.

Results

Effects of ^{60}Co gamma ray irradiation on *L. bicolor* germination and seedling growth: ^{60}Co gamma ray irradiation was introduced into our experiment aiming to obtain salt gland mutants to the maximum extent. In order to examine whether ^{60}Co gamma ray irradiation affected germination of *L. bicolor*, effect of six gamma ray doses treatments (0, 100, 200, 300, 400 and 500 Gy) on the germination was measured.

Germination was delayed for irradiated seeds, was not greatly affected by the radiation dose, and reached a maximum of about 90% on day 4 for both control and irradiated seeds (Fig. 1B). Doses higher than 200 Gy repressed seedling growth and especially root length (Fig. 1A, C).

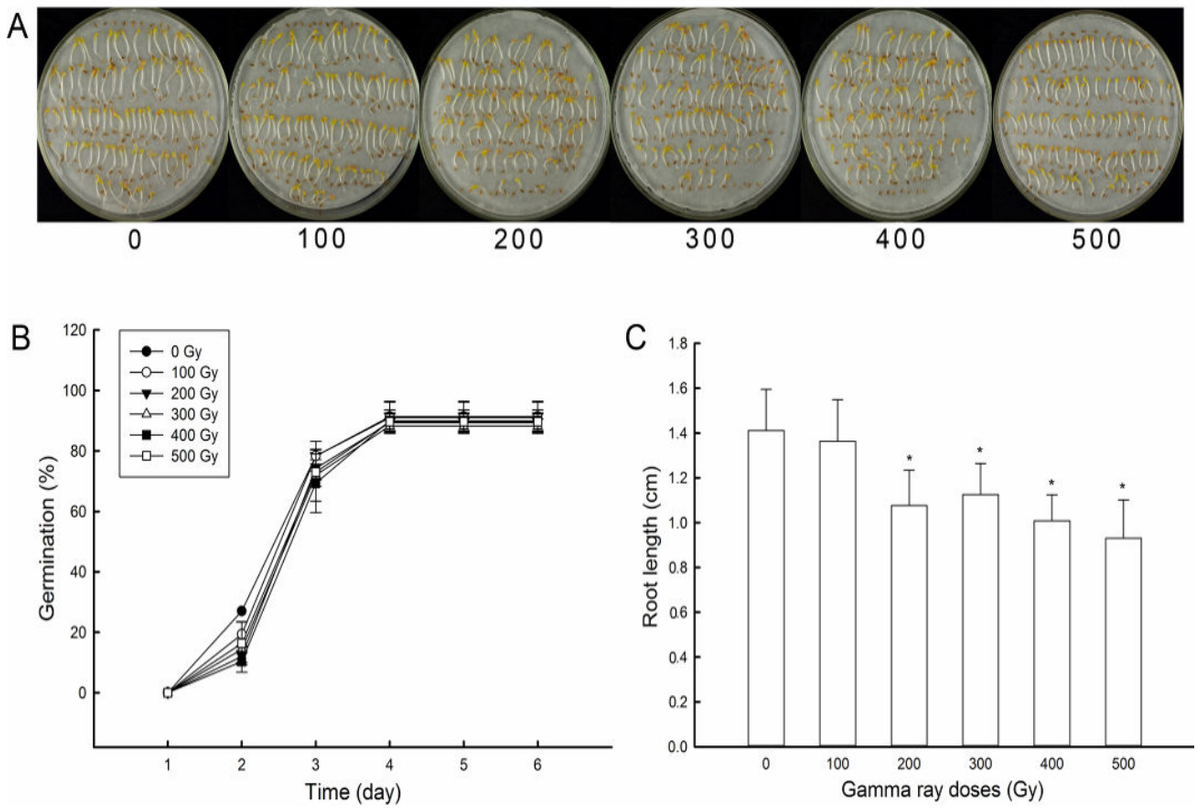

Fig. 1. Effect of ^{60}Co gamma irradiation doses (0 to 500 Gy) on the appearance of *L. bicolor* seeds after 4 days (A), germination percentage over time (B), and root length after 4 days (C). In B, values are the means ± SD of four replicates. In C, values are the means ± SD of 30 replicates, and bars with asterisk (*) mean significantly different compared with 0 Gy treatment at P=0.05 according to Duncan's multiple range test.

Because the percentage of seedlings that germinated was not greatly affected by ^{60}Co gamma ray irradiation, germination was not suitable for determining the LD_{50} of the irradiation. Therefore, we examined the effect of irradiation on seedling emergence and growth in soil. As the irradiation dose increased, seedling emerged percentage decreased (Fig. 2A). For the control, the number of seedlings that emerged and survived increased until day 15 (when true leaves appeared) and then remained stable (Fig. 2B). For irradiated treatments, the number of seedlings survival first increased and then decreased as some of the emerged seedlings died after day 15. Survival of seedlings in the 100 Gy treatment stabilized after day 24, but most seedlings in treatments with higher doses failed to generate true leaves and the cotyledons reddened, which caused a continuing decline in survival. Results indicated that ^{60}Co gamma ray irradiation treatment significantly reduced seedling emerged percentage compared with the control and the seedlings of *L. bicolor* were sensitive to gamma ray irradiation in all doses tested.

Because emerged seedlings on day 30 decreased gradually with the increase in ^{60}Co gamma radiation dose, these data were used to calculate the LD_{50}. Based on linear regression of the logarithm of emerged seedling (day 30) vs. gamma radiation dose, the LD_{50} for ^{60}Co gamma ray irradiation was 120 Gy (Fig. 3).

Two mutants of *L. bicolor* were obtained under the LD_{50} of ^{60}Co gamma ray irradiation: The density of salt gland was calculated at ×100 magnification under DIC microscope. Fig. 4A (WT: right) showed the salt gland at ×600 magnification was composed of sixteen cells, four pairs of secretory cells, accessory cells, inner cup cells and outer cup cells. We initially obtained two mutants in salt gland density under the LD_{50} of ^{60}Co gamma Irradiation, mutants with few and many salt glands (abbreviated as *few* and *many* below).

The salt gland density of *few* was significantly less than WT, and *many* had much more salt gland than WT (Fig. 4B). However, the salt secretion per leaf area (Fig. 4C) and salt secretion rate per single salt gland (Fig. 4D) of *few* showed no significant difference with WT. *few* may have mutant in the genes involved in the development of salt gland. It is amazing that the salt secretion rate per single salt gland (Fig. 4D) of *many* was significantly lower than WT. *many* may have mutant in genes involved in both salt gland development and salt secretion. *few* mutant can be used to study the mechanism to control the development of salt gland, and *many* is significantly important to reveal the pathway involved in salt secretion.

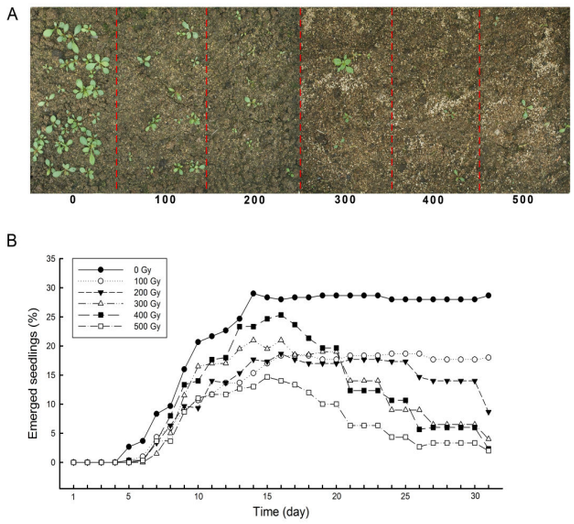

Fig. 2. The effect of ^{60}Co gamma radiation doses (0 to 500 Gy) on the appearance of *L. bicolor* seedlings after 30 days (A) and emerged seedling over time (B). In B, values are means of four replicates with 100 seeds per replicate.

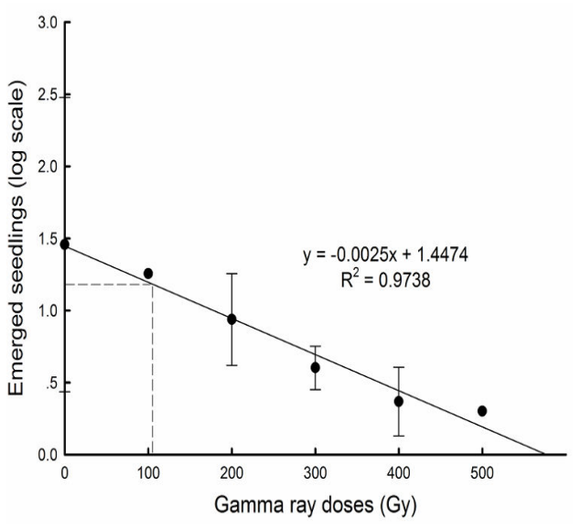

Fig. 3. Linear regression of the logarithm of *L. bicolor* seedling emergence (%) 30 days after sowing in soil vs. ^{60}Co gamma radiation dose. The LD_{50} for ^{60}Co gamma ray irradiation was 120 Gy. Values are the means ± SD of four replicates with 100 seeds per replicate.

Discussion

This article is a new report of mutants in salt gland development (*few*) and salt secretion (*many*) of exo-recretohalophyte *L. bicolor* under the LD_{50} of ^{60}Co gamma Irradiation. The structure of salt gland by DIC microscopy is consistent with that by scanning electron microscopy (Ding *et al.*, 2010), and obvious sixteen-cell structure was observed. DIC microscope and leaf discs excretion model are first applied to count the salt gland density and directly measure secreted salt ions. The optimal dosage of ^{60}Co gamma ray radiation is also determined for the first time.

Irradiation cuts a portion of the chromosome or the entire chromosome resulting in chromosome translocation or deletion; ^{60}Co gamma ray irradiation is generally used for producing useful mutations (Saito *et al.*, 2001), which was applied in the mutagenesis of *L. bicolor* because salt gland is controlled by multiple genes and the mutants in single genes may not affect salt gland development or salt secretion. In this paper, gamma ray irradiation did not adversely affect germination on plates (same to Ikram *et al.*, 2010) or in soil (data not shown) but reduced seedling emergence in soil and the survival of seedlings after emergence. Death of emerged seedlings was associated with the failure to produce true leaves. These results indicated that ^{60}Co gamma ray radiation had little effect on the key genes involved in *L. bicolor* germination but reduced seedling survival by preventing the formation of true leaves. Linear regression indicated that treatment of *L. bicolor* seeds with 120 Gy of ^{60}Co gamma irradiation caused 50% mortality. The LD_{50} of gamma irradiation differs among different plant species (Borzouei *et al.*, 2010; Basaran & Kepenek, 2011).

The results presented above should be useful for generating mutations in *L. bicolor* relevant to salt-stress tolerance. To our knowledge, this is the first report of the LD_{50} for ^{60}Co gamma irradiation (120 Gy) for the exo-recretohalophyte *L. bicolor*. We expect to obtain mutants that have an abnormally large or small density of salt gland (e.g. *few*) and an abnormally large or small quantity of salt secretion (e.g. *many*). Using LD_{50} of ^{60}Co gamma irradiation, we initially obtained two mutants in salt gland for the first time, and more mutants in salt gland development and salt secretion would be further screened by the methods of DIC and leaf discs excretion model.

Mutants in salt glands of *L. bicolor* will be compared with the wild type to identify differentially expressed genes involved in salt gland development and salt secretion. Forward genetic methods can be combined with reverse genetic means to investigate the molecular mechanism of salt gland development and salt secretion. Crops transformed with these genes are expected to produce salt glands on their leaf or stem surfaces and may thus be able to grow normally in saline soil. Given that there are 800 million hectares of salt-affected land worldwide, the development of salt-tolerant crops will significantly contribute to the world's food supply.

Conclusions

In conclusion, LD_{50} (120 Gy) is first applied in *L. bicolor* to generate mutants, and differential interference contrast microscope and leaf discs excretion model are reported in screening salt gland mutants for the fist time. Two mutants in salt glands (*few* and *many*) were obtained under the LD_{50} using the screening methods. Compared with the wild type, *few* and *many* had mutation in salt gland development, and the latter showed lower salt secretion rate per single salt gland than WT. More mutants in salt gland development and salt secretion would be screened by these means. These mutants would provide insight into the molecular mechanisms of salt gland development and salt secretion and into the development of salt-tolerant crop plants. Salt-affected land will be improved using salt-tolerant crops transformed with these genes involved in salt gland development and salt secretion.

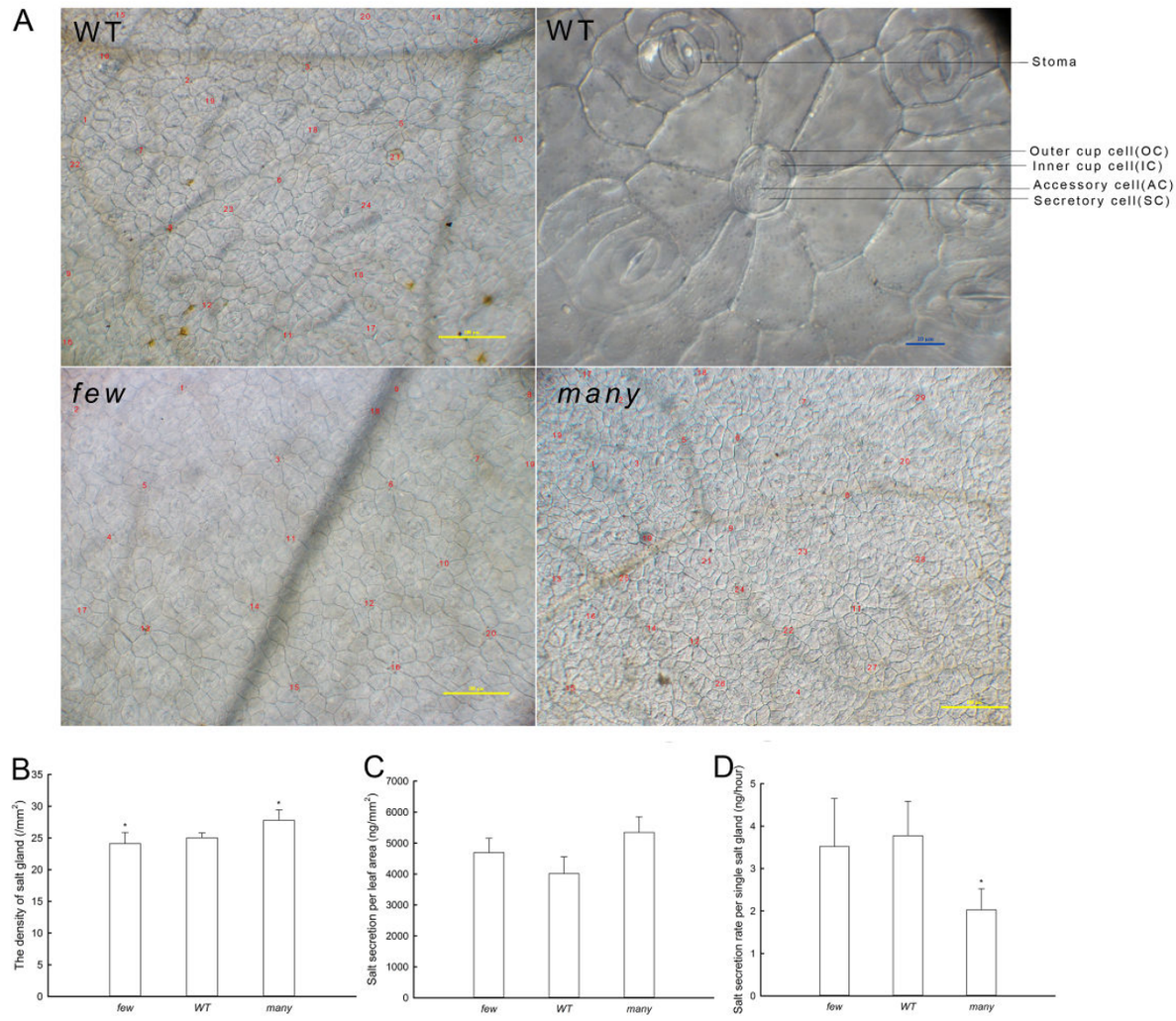

Fig. 4. The DIC images (A), the density of salt gland (B), the salt secretion per leaf area (C) and salt secretion rate per single salt gland (D) of two different salt gland density mutants (*few* and *many*) compared with WT. In A, WT shows the density of salt gland of wild type of *L. bicolor* at ×100 magnification (left, bar=100 μm) and the salt gland at ×600 magnification (right, bar=10 μm). Obvious sixteen cells of salt gland can be observed, four pairs of secretory cells, accessory cells, inner cup cells and outer cup cells. *few* and *many* show the density of salt gland of mutants (bar=100 μm). Values in B are means ± SD of ten fields at ×100 magnification selected randomly. The data in C and D are the means ± SD of five leaf discs. Bars with asterisk (*) mean significantly different at P=0.05 according to Duncan's multiple range test.

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