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## Contribution of Keeping More Stable Anatomical Structure under High Temperature to Heat Resistance of *Rhododendron* Seedlings

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High temperature has been an obstacle to grow *Rhododendron* plants, and there are few reports on anatomical changes of *Rhododendron* plants under heat stress. To identify the mechanism of anatomical adjustment under high temperature, three *Rhododendron* species with different sensitivity to high temperature stress were selected to investigate the effect of heat stress on the anatomical structure. Heat stress resulted in stomatal closure and the decrease of stomatal density, however, the limited stomatal adjustment reached by *R. fortune* and *R. mariesii* may not prevent water loss effectively, of which the leaf thickness was reduced and obvious plasmolysis was observed in the mesophyll cells. *R. simsii* with smaller stomata and higher stomatal density had greater heat resistance than *R. fortune* and *R. mariesii*. Heat stress injured cell membrane structure seriously, and it was found that the nuclear membrane was digested and the nucleolus disappeared. The response of chloroplasts to high temperature was most sensitive, in which thylakoid lamellas became blurred, even degraded in heat-sensitive *Rhododendron* species. The thermal endurance were sequenced as follows: *R. simsii*, *R. mariesii* and *R. fortunei*. Greater heat resistance of *R. simsii* may be associated with stabilizing anatomical structure under high temperature. The results offered cytological evidence of adaptation for heat stress in heat resistant *Rhododendron* species.

**Key words:** chloroplast, heat stress, leaf anatomy, *Rhododendron*

### INTRODUCTION

The genus of *Rhododendron* contains 1000 species distributed in Asia, Europe, and North America, and China has the most diverse *Rhododendron* flora with 571 species (Fang *et al.*, 2005). Many wild *Rhododendron* species in China were introduced directly to Europe and North–America or used as hybrid parents. These wild species and their hybrid offspring are among the most popular landscape plants in Europe and North–America. But the cultivated varieties in China are limited since high temperature in most of China cities has been an obstacle to grow *Rhododendron* plants. Moreover, the global mean temperature continues to rise at a rapid rate, and our climate is likely to warm by 1.1–6.4°C within the next century (IPCC, 2007). On the other hand, few studies on heat tolerance of *Rhododendron* have been reported (Arisumi *et al.*, 1983, 1989; Ranney *et al.*, 1995).

Anatomical adjustment under high temperature was one of the adaptive approaches of plants. The crucial reason why the heat-tolerant cultivars have higher resistance to heat stress than heat-sensitive cultivars is that the former possess a highly stable membrane structure (Ma *et al.*, 2003). Stomata are essential for carbon dioxide acquisition but at the cost of water loss (Beerling *et al.*, 1993). High temperature resulted in the reducing of cell size, closure of stomata and curtailed water loss, increased stomatal densities (Wahid *et al.*, 2007). The

closure of stomata affects the photosynthesis. In grapevine leaves (Greer and Weston, 2010, 2012), the reduction in photosynthesis induced by heat stress was mainly attributed to reduced stomatal conductance (Gs).

At the sub-cellular level, major modifications occur in chloroplasts, leading to significant changes in photosynthesis. The damage of chloroplasts is characterized under heat stress by membrane damage such as membrane of thylakoid expanded and ruptured, destacking of the thylakoid membranes and reduced grana (Xu *et al.*, 2006; Kreslavski *et al.*, 2008). Photochemical reaction in thylakoid lamellae and carbon metabolism in the stroma of chloroplast have been suggested as the primary sites of injury at high temperature (Wise *et al.*, 2004). The response of mitochondria to high temperature was not sensitive compared with chloroplasts. Zhang *et al.* (2005) reported that under heat stress the mitochondrial envelope was digested and cristae were disrupted and became vacuolated. The adverse effect on anatomical structures under high temperature may lead to poor plant growth, even death.

Despite many studies that have investigated plant response to heat stress at different scales, but there are few reports on anatomical changes under heat stress, especially, *Rhododendron* plants. So in this study three *Rhododendrons* species with different sensitivity to high temperature stress were selected to investigate the effect of heat stress on the anatomical structure.

### MATERIALS AND METHODS

#### Plant material and handling

Two-year-old seedlings of three *Rhododendron* species were used as plant materials, including *R. fortune*,

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*R. simsii* and *R. mariesii*. Eight pots of each kind of healthy plants that were about the same size were chosen and transferred into artificial climate incubator. In order to avoid the external water deficit, the potted plants were placed in a tray with water and heat-stressed at 42°C/30°C (day/night) for 24 h and light intensity of 4000 LX/0 LX (day/night). As a control, the other potted plants were placed at 28°C/18°C (day/night) for 24 h. After the heat stress experiments, the plants were moved back to the greenhouse of 23–33°C. Leaf samples were taken 6d after heat stress (HS) and used to observe the changes of anatomical structure.

### Electron microscope scanning

Small pieces from the middle region of tender leaves were fixed in FAA solution (formalin, glacial acetic acid and 50% ethanol, in volume ratio 5:5:90) at 4°C for at least 24 h, and then washed with distilled water, dehydrated by gradient ethanol, dried and sputter-coated with gold, then observed with Hitachi-S450 microscopy and photographed. Epidermis, mesophyll, palisade tissue, spongy tissue, leaf thickness, stomatal apparatus length and width were measured with image ProPlus 6.0 software. The stomatal density ( $S_D$ ) was calculated as follows:  $S_D = N/S$ , where N is the number of stomata, S is the area of observation.

### Transmission electron microscopy (TEM)

To identify the cytological effects of heat stress, small pieces (1 cm × 0.5 cm) of the tip zones of fresh leaves were excised 6d after heat stress and used for TEM. Tissues were fixed with glutaraldehyde solution at 4°C for at least 24 h, and then washed with 0.1 M phosphate buffer (PB) for three times, and 15 min each time. Tissues were post-fixed in 1% osmium tetroxide for 3h and then rewashed with 0.1 PB for three times again, and 15 min each time. The tissues were dehydrated through graded ethanol and acetone and embedded in Epon 812 resin.

## RESULTS

### Leaf anatomical structure

As shown in Fig. 1 and Table 1, *R. simsii* leaf in control had higher abaxial stomatal densities (SD) than *R.*

*mariesii* and *R. fortunei*. Heat stress reduced SD on the abaxial surface of these three *Rhododendron* species. The decrease rate of *R. fortunei* was highest (30.76%), followed by *R. mariesii* (16.67%), the decrease rate of *R. simsii* was the lowest (only 6.48%). And high temperature resulted in stomatal closure of the three species at different degrees, the degree of stomatal closure in *R. fortunei* leaf was most significant. Heat stress did not affect the stomatal length, the changes of stomatal width and the ratio of stomatal length and width were not significantly.

Leaf thickness (LT) of three *Rhododendron* species in control temperature varied from 91.3 to 386.8 μm, with *R. fortunei* having the thickest leaves. As shown in Fig. 2, adaxial epidermis cell, abaxial epidermis cell and mesophyll tissue (palisade and spongy tissue) of *R. fortunei* were thickest, followed by *R. mariesii* and *R. simsii*. Heat stress decreased the leaf thickness of three species, and the decrease trend was similar with SD (*R. fortunei*, 45.14%; *R. mariesii*, 13.04%; *R. simsii*, 2.85%).

### Ultrastructural changes under high temperature

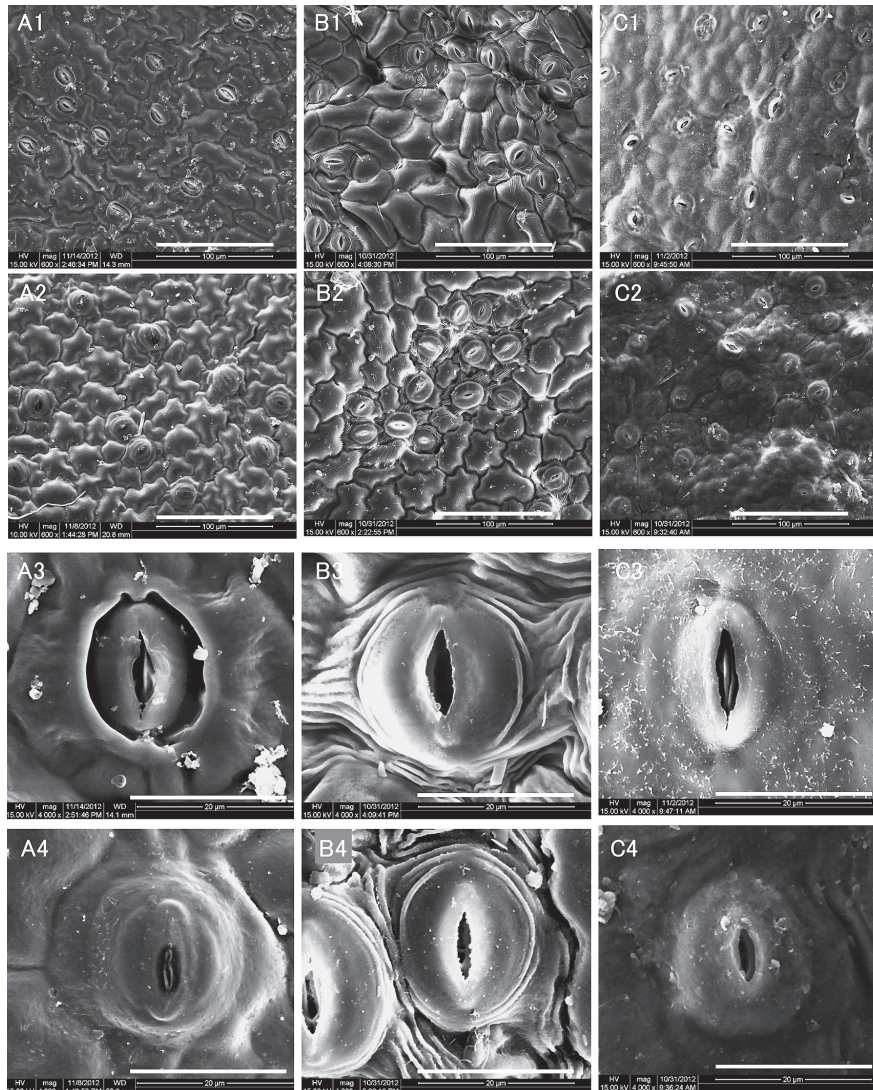
It was shown that in leaves of *R. simsii* (Fig. 3:1–3), which was grown at normal condition, the mesophyll cells maintained an intact ultrastructure. Chloroplast was oblong and against the cell wall with the thylakoid lamellae arranged orderly and tightly, without starch granule and osmiophilic globuli. Mitochondria appeared to be round and against the cell wall. There were a few cell bodies in the central vacuole. After heat stress, slight injury appeared in the ultrastructure of mesophyll cells such as degenerated cytoplasm, broken tonoplast membrane and pyknotic nuclei. Chloroplast was nearly against the cell wall with little starch granules observed, some chloroplasts became swollen with loosely arranged thylakoid lamellae. The plasma membrane maintained normal, plasmolysis was not observed. Mitochondria did not change obviously.

In contrast, mitochondria and nucleus of *R. mariesii* were intact. The normal chloroplasts appear to be oblong with a clear inner structure, and large starch grains were observed in chloroplasts. But few plasma membranes were slightly detached from the cell wall. After heat stress, the plasma membranes were seriously detached from the cell wall. After heat stress, serious injury

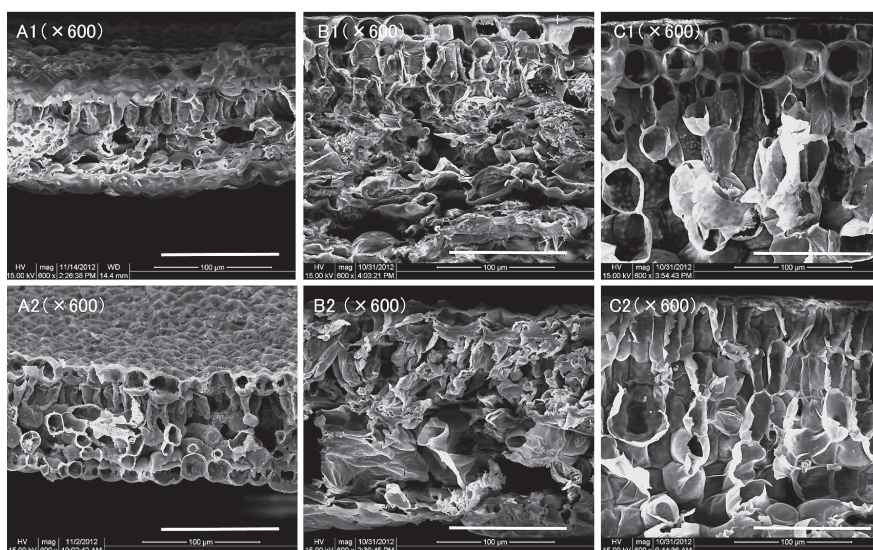
**Table 1.** Leaf stomatal characteristics and leaf thickness of three *Rhododendron* species.

Species	Treatment	Density (mm <sup>-2</sup> )	Stomatal length (μm)	Stomatal width (μm)	Length/width	Leaf thickness (μm)
<i>R. simsii</i>	Control	308.8 <sup>a</sup>	19.2 <sup>b</sup>	15.4 <sup>b</sup>	1.25 <sup>a</sup>	91.3 <sup>d</sup>
	Heat stress	288.8 <sup>ab</sup>	21.9 <sup>b</sup>	20.1 <sup>ab</sup>	1.09 <sup>a</sup>	88.7 <sup>d</sup>
<i>R. mariesii</i>	Control	279.5 <sup>ab</sup>	23.3 <sup>a</sup>	19.1 <sup>ab</sup>	1.22 <sup>a</sup>	216.7 <sup>b</sup>
	Heat stress	232.9 <sup>b</sup>	22.8 <sup>b</sup>	20.8 <sup>a</sup>	1.1 <sup>a</sup>	188.5 <sup>c</sup>
<i>R. fortunei</i>	Control	242.2 <sup>b</sup>	24.0 <sup>a</sup>	19.8 <sup>ab</sup>	1.21 <sup>a</sup>	386.8 <sup>a</sup>
	Heat stress	167.7 <sup>c</sup>	24.6 <sup>a</sup>	21.9 <sup>a</sup>	1.12 <sup>a</sup>	212.2 <sup>b</sup>

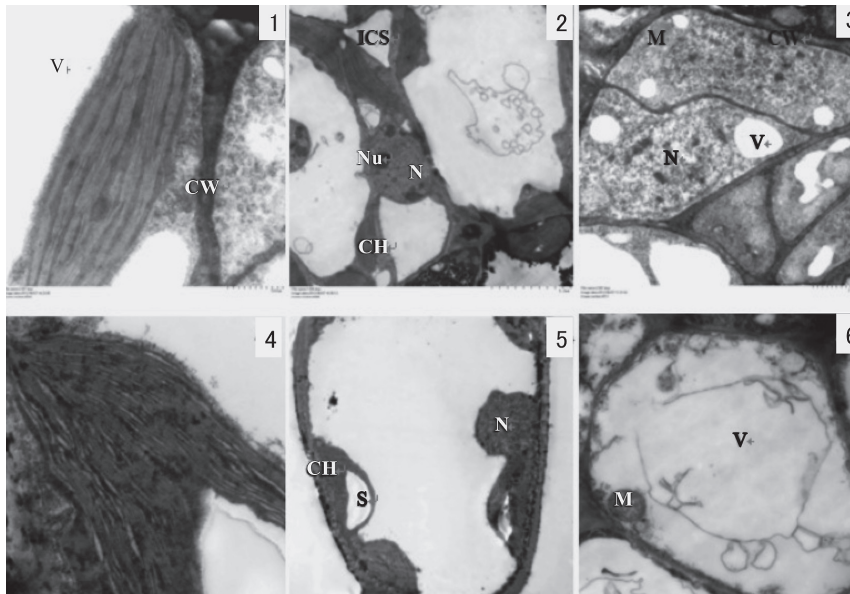
Statistical analyses were carried out by using SPSS17.0 software. The Duncan's multiple range test (DMRT) was applied to compare significant differences among treatments. Different letters in the column indicate statistical difference ( $p < 0.05$ ).



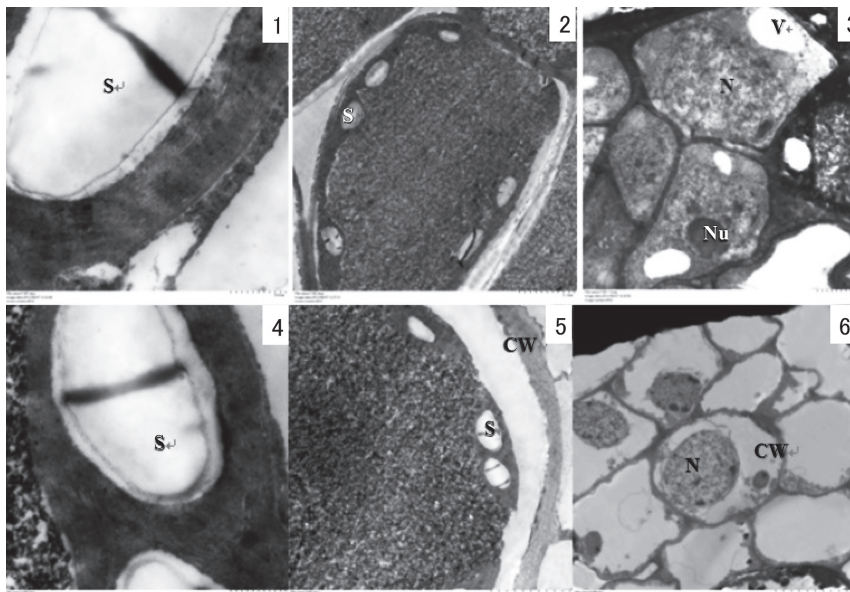
**Fig. 1.** Abaxial epidermis and leaf stomatal characteristics of *R. simsii* (A), *R. mariesii* (B), and *R. fortunei* (C) from heat-stressed (2 and 4) and control (1 and 3) seedlings. Scale bars in A1, B1, C1, A2, B2 and C2 are 100  $\mu\text{m}$ , and those in A3, B3, C3, A4, B4, and C4 are 20  $\mu\text{m}$ .



**Fig. 2.** Leaf cross sections of *R. simsii* (A), *R. mariesii* (B), and *R. fortunei* (C) from heat-stressed (A2, B2 and C2) and control (A1, B1 and C1) seedlings. Scale bars are 100  $\mu\text{m}$ .



**Fig. 3.** Ultrastructure of mesophyll cell in response of *R. simsii* to heat stress. Leaf samples were taken 6d after heat stress (4, 5 and 6) and control temperature (1, 2 and 3). Magnification: 1 and 4,  $\times 7.0k$ ; 2 and 5,  $\times 1.0k$ ; 3 and 6,  $\times 3.0k$ . CH: chloroplast; CW: cell wall; ICS: intercellular space; M: mitochondria; N: nucleus; Nu: nucleolus; S: starch granule; V: vacuole.

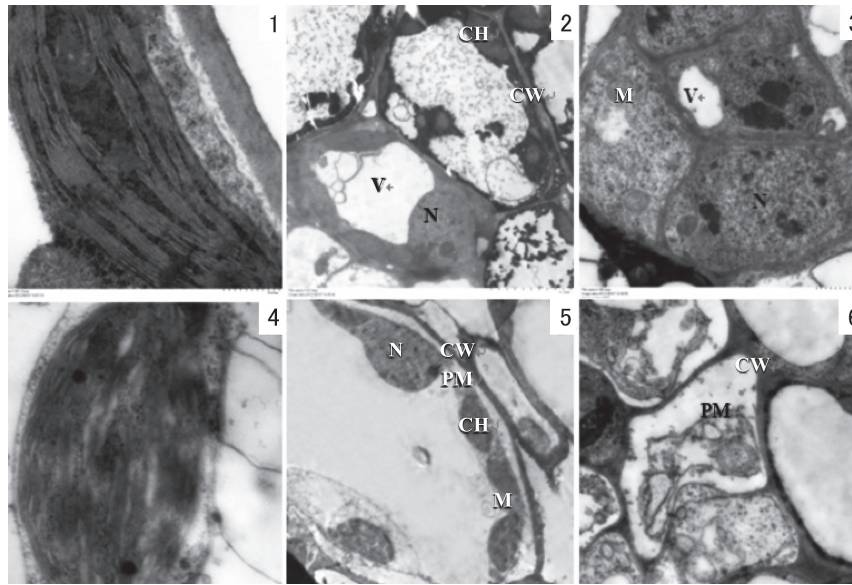


**Fig. 4.** Ultrastructure of mesophyll cell in response of *R. mariesii* to heat stress. Leaf samples were taken 6d after heat stress (4, 5 and 6) and control temperature (1, 2 and 3). Magnification: 1 and 4,  $\times 7.0k$ ; 2,  $\times 0.7k$ ; 3,  $\times 3.0k$ ; 5 and 6,  $\times 1.0k$ . CW: cell wall; N: nucleus; Nu: nucleolus; S: starch granule; V: vacuole.

appeared in the ultrastructure of chloroplasts and mitochondria, as shown in Fig. 4, it was found that thylakoid lamellas became blurred, the cristae of the mitochondria were dissolved. The nuclear membrane was digested and the nucleolus disappeared.

The mesophyll cells of *R. fortune* grown in control temperature maintained an intact ultrastructure. Chloroplasts have a typical structure consisting of an ellipsoidal shape with well-arranged thylakoid membranes, and no starch granules were present. Large quantity of

osmiophilic globuli could be observed in vacuole. After heat stress, the mesophyll cells of *R. fortunei* were also severely damaged, the plasma membranes were seriously detached from the cell wall, thylakoid lamellas became blurred and digested partly, cytoplasm degenerated and more osmiophilic globuli could be observed in plasma membrane. But no significant changes were observed in the mitochondria and nucleus after heat stress.



**Fig. 5.** Ultrastructure of mesophyll cell in response of *R. fortune* to heat stress. Leaf samples were taken 6d after heat stress (4, 5 and 6) and control temperature (1, 2 and 3). Magnification: 1 and 4,  $\times 7.0k$ ; 2 and 5,  $\times 1.0k$ ; 3 and 6,  $\times 3.0k$ . CH: chloroplast; CW: cell wall; M: mitochondria; N: nucleus; PM: plasmalemma; V: vacuole.

## DISCUSSION

### Correlation between leaf anatomical structure and heat resistance

It is well known that plants have a suite of morphological and anatomical adaptations that allow them to survive heat stress and the degree of adaptation to high temperature may vary considerably between species. Stomatal density and leaf area are plastic in their response to environmental changes (Fraser *et al.*, 2009). In the present study, the significantly negative correlation was found between stomatal size and stomatal density in three *Rhododendron* species, and *R. simsii* with smaller stomata and higher stomatal density had greater heat resistance than *R. fortune* and *R. mariesii* (Table 1). It was consistent with the findings of Cai *et al.* (2014), who also suggested *Rhododendron* species with denser but smaller stomata may have a stronger ability to adapt to environmental stresses. A leaf with many, small stomata can reduce potential conductance and increase water-use efficiency (Poulos *et al.*, 2007). Stomatal adjustment through decreasing transpiration rate can maintain the plant water status under stressed conditions. Heat stress induced stomatal closure and decreased stomatal density (Fig. 1 and Table 1), however, the limited stomatal adjustment reached by the plants may not prevent water loss and maintain leaf turgor (Ba'non *et al.*, 2004). After heat stress, the leaf thickness was reduced due to the water loss (Fig. 2), and obvious plasmolysis was observed in the mesophyll cells of *R. fortune* and *R. mariesii* (Fig. 4 and Fig. 5).

Our results suggest a decrease in leaf SD in heat stressed seedlings of three *Rhododendron* species in agreement with the findings of Ciha and Brun (1975) in soybean, while the same parameters increased in *Lolium perenne* (Ferris and Taylor, 1995). In the experiment of

*Lotus creticus* plants, SD significantly decreased with temperature on the abaxial surface in deficit irrigation conditions, but there was no effect on the adaxial surface (Ba'non *et al.*, 2004). Higher SD is not an adaptive mechanism but a consequence of lesser leaf area in stressed plants. In our experiment, we only observed the abaxial SD, it may be different between adaxial and abaxial stomata. In cotton, the adaxial and abaxial stomata differ in their responses to light, ambient temperature and water stress (Sharpe, 1973). Compared with stomatal length, stomatal density is relatively plastic and potentially adaptive to environmental change (Richardson *et al.*, 2001). In the present study, heat stress did not affect the stomatal length and width significantly.

### Correlation between ultrastructure and heat resistance

The mesophyll cells were damaged under heat stress at different degrees, the results indicated *R. simsii* was the most heat-tolerant *Rhododendron* species while *R. fortune* was the most sensitive to heat temperature stress. Ultrastructural change due to heat injury usually occurred in chloroplasts, mitochondria and plasma membrane (Zhang *et al.*, 2005). The most sensitive organelle to heat stress in *Rhododendron* mesophyll cells was the chloroplast. Chloroplasts have been suggested as the primary sites of injury at high temperature (Wise *et al.*, 2004). The damage of chloroplasts is characterized under heat stress by membrane damage such as membrane of thylakoid expanded and ruptured, destacking of the thylakoid membranes and reduced grana (Xu *et al.*, 2006; Kreslavski *et al.*, 2008). In the present study, under heat stress the thylakoids were expanded and thylakoid lamellae became blurred, even degraded in heat-sensitive *Rhododendron* species such as *R. fortune* and *R. mariesii* (Fig. 4 and Fig. 5). The damage of mitochon-

dria was observed in *R. mariesii* mesophyll cells, whose mitochondria cristae were dissolved. Heat stress did not affect the ultrastructure of nucleus significantly.

It was found that heat stress resulted in the decrease of net photosynthesis ( $P_N$ ) in our study (data not shown), which should be caused mainly by changing the structural organization of thylakoids (Karim *et al.*, 1997 and Vani *et al.*, 2001). Rice seedling to heat stress of 40°C for 24 h did not cause any change in protein or pigment content of thylakoids, but produced major disorganization of chloroplast ultrastructure (Vani *et al.*, 2001). Such effects on photosynthesis apparatus were suggested to be associated with the production of reactive oxygen species (Camejo *et al.*, 2006; Guo *et al.*, 2006). It is well known that chloroplast is one of the important intracellular generators of reactive oxygen species (ROS) responsible for the damage to cell membranes under abiotic stresses (Sairam and Srivastava, 2002; Perez *et al.*, 2002; Meloni *et al.*, 2003). The chloroplast injury induced by heat stress led to higher malondialdehyde (MDA), hydrogen peroxide ( $H_2O_2$ ) and superoxide radical concentrations (Chen *et al.*, 2012). And rigidification of thylakoid membranes seems to involve altered expression profiles of heat-shock genes (Horvath *et al.*, 1998), and changes the heat tolerance of plants.

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