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Photosynthesis and ultrastructure of photosynthetic apparatus in tomato leaves under elevated temperature

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Abstract

The microstructure of leaves and ultrastructure of chloroplasts were examined in tomato (*Lycopersicon esculentum* L.) plants treated with elevated temperature. Plants were exposed to 35ºC for 30 d after florescence. The plants grown continuously under 25ºC served as controls. Compared with the controls, the net photosynthetic rate (P_N) in stressed plants decreased significantly. Stomatal conductance, intercellular CO_2 concentrations, the rate of transpiration, and the limitation of stomatal conductance showed that the decrease in P_N was caused mainly by nonstomatal restrictions. Meanwhile, stomata density increased significantly in the stressed plants. The stomata status of opening and closing became disorganized with a prolonged 35ºC exposure. The damage of chloroplast membrane occurred earlier and was more serious in the plants under elevated temperature. At the same time, the thylakoids were loosely distributed with lesser grana, but the number of lipid droplets increased in chloroplasts. The number of starch grains in chloroplasts increased first and then decreased. In addition, the length of the main nerve in leaves increased and the main vein showed distortion in the plants stressed by 35ºC. An increase was observed in the number of cells on the abaxial side of the main vein and these cells were overly congregated. The thickness of a vertical section became thinner in the stressed leaves. The cells of the upper epidermis thinned, and the ratio of palisade tissue to spongy tissue decreased. Generally, the photosynthetic apparatus of tomato changed significantly and the changed chloroplast ultrastructure might be one of the important reasons that caused the decrease of P_N under 35ºC.

Additional key words: chloroplast; gas exchange; heat stress; *Lycopersicon esculentum* L; microstructure; photosynthetic apparatus.

Introduction

Temperature is one of the critical factors in plant life. Low- and high-temperature regimes might be harmful to plants. Tomato is one of the most common vegetables grown in protected horticulture. The optimum range of daytime temperatures for the growth and development of tomato is between 25 and 30ºC, with the upper limit of 35ºC (Zhang, 2010). Many studies have been carried out to study adverse effects of environmental alterations on the growth and development of tomato (Karim *et al*. 1999, 2000, Camejo *et al*. 2005, Stobrawa and Lorenc-Plucińska 2007). Such conditions can induce diverse changes in plant morphology, anatomy, and physiology that are manifested from the whole-plant to the cellular or subcellular levels (Holá *et al*. 2008). However, studies focused on extremely high

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Abbreviations: C_i – intercellular CO_2 concentrations; CK – control; DAE – days after exposure; E – transpiration rate; HT – high-temperature; g_s – stomatal conductance; L_s – the limitation of stomatal conductance; P_N – net photosynthetic rate; SEM – scanning electron microscpe; T – temperature; TEM – transmission electron microscope.

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temperatures, such as 40ºC (Wu *et al*. 2001, Camejo *et al*. 2005, Mao *et al*. 2005). It is an extreme stress to most C_3 plants and it can impose great injury in a short time. The same extent of damage from moderately high temperatures becomes evident after a longer time. Little information is available on the effects of moderately high temperatures on the photosynthetic characteristics of tomato in greenhouse. We chose a daytime temperature of 35ºC as the elevated temperature for tomato, since such temperature has been shown to be detrimental to the growth and development of plants (Zhang *et al*. 2011).

High-temperature stress has strong influence on chloroplasts and the changes in chloroplast ultrastructure are often used as the evidence of stress (Miao *et al*. 1994, Braun *et al*. 2002, Xu *et al*. 2006, Chen *et al*. 2012, Grigorova *et al*. 2012). In other words, chloroplasts act as the major cellular sensors of adverse environment (Xu *et al*. 2006, Holá *et al*. 2008). It is usually considered that the changes of photosynthetic characteristics are consistent with the changes of chloroplast ultrastructure. For example, chloroplast membranes are ruptured and general loss of structural integrity follows (Pfeiffer *et al*. 2005). Heat-induced changes of chlorophyll (Chl) fluorescence and the damage of the photosynthetic apparatus were reported (Xu *et al*. 2006). Upon temperature stress, photoinhibition of PSII, rearrangement of membranes and production of heat-shock proteins may occur (Braun *et al*. 2002). Miao *et al*. (1994) found that grana were disorganized with lower grana stacks after high-temperature stress in cabbage. The increased number of lipid droplets is a sign of the senescence of chloroplast (Holzinger *et al*. 2007, Wang *et al*. 2010). In addition, the number of pubescent leaves, the number of stomata and their status of opening, and the arrangement of palisade tissue often change under heat stress (Li and Wang 2002).

Although the above-described changes of chloroplast structure can be considered as the general symptoms of heat stress, the specific way of their manifestation depends on several other factors as well. For example, short-term or long-term exposure of plants to high temperature result in different changes of chloroplast ultrastructure (Holá *et al*. 2008). However, little information is available about the effect of long-term exposure to moderately high temperature.

In previous studies, we noted that elevated temperature (35ºC) changed the parameters of Chl fluorescence in tomato leaves. This method has always been shown to be reliable for the detection of temperature-induced changes in the photosynthetic apparatus (Zhang *et al*. 2011). In the present study, tomato plants were exposed to an elevated temperature of 35ºC for 30 d. Photosynthesis, stomata status, chloroplast ultrastructure, and leaf microstructure were examined. Our objective was to study the effects of a prolonged elevated temperature on photosynthesis and ultrastructure in tomato leaves. The mechanisms responsible for the changes in photosynthesis in plants under the elevated temperature were investigated.

Materials and methods

Plants and treatments: Plants of the tomato cultivar 'Liaoyuanduoli' were used in this experiment. Seedlings were sown in plastic pots and grown in a greenhouse at Shenyang Agricultural University, China. Plants were transferred to 33.7 cm (diameter) × 40 cm (height) plastic pots at the stage of five true leaves, and they were placed in a climate chamber. Then all plants were grown at 25ºC during a 12-h photoperiod with a 12-h dark period at 15ºC.

When the first inflorescence opened, a group of plants (n = 36) was transferred to another climate chamber and exposed to 35ºC (HT plants) during daytime for 30 d (DAE). A group of plants (n = 36) kept at 25ºC served as control (CK). The night temperature for all plants was 15ºC. Leaves of the first node above the first inflorescence were examined every 5 d for their microstructure and ultrastructure. The plants (n = 4) of each treatment were randomly chosen for the analysis of chloroplast ultrastructure.

Gas-exchange measurements: The measurements were made using a portable *Li-6400 meter* (*LI-COR Inc*., Lincoln, NE, USA). During the measurements, the light intensity was set at 600 μmol(photon) m^{-2} s^{-1}, but the temperature and air humidity during the measurements were not controlled and kept the condition inside the climate chambers. The indices tested included P_N, intercellular CO_2 concentration (C_i), stomatal conductance (g_s), and the rate of transpiration (E). The limitation of stomatal conductance (L_s) was calculated using the equation: $L_s = 1 - C_i/C_a$ (Farquhar and Sharkey 1982).

Specimen preparation: The ultrastructure of chloroplasts was studied in mesophyll cells of mature leaves of the first node above the first inflorescence of tomato plants. Leaf samples were taken at the beginning of the photoperiod every time. Several small pieces (approximately 4 mm^2) were cut from the middle third of each leaf blade and served as the samples for the electron microscopy. They were prepared according to the standard procedure (Kutík *et al*. 2004, Holá *et al.* 2008): the double fixation with 2.5% glutaraldehyde followed by 1% osmic acid treatment, and the dehydration through ethanol/acetone series.

Some of these samples were substituted by isoamyl acetate ester. After drying by CO_2, they were installed in object stage and were sprayed by the conductive coating. Then the stomata of these samples were observed by *S-450* scanning electron microscope (*S-450 SEM*, *Hitachi Ltd.*, Japan). At least 15 visual fields of each sample were observed.

Other samples were embedded in epoxy resin and were sliced into ultraslices (thickness of about 60–90 nm) by an

ultramicrotome (*EM UC7, Leica*, Germany), stained with both uranyl acetate and lead citrate, and the ultrastructure of chloroplasts was examined with a transmission electron microscope (TEM) *JEM-100 CX* (*JEM-100 CX-II TEM*, *Hitachi Limited*, Japan). At least 15 visual fields were used in the experiment and all of them entered the stage of results analyzed.

In addition, the scanning electron microscope (SEM) was used to compare the characteristics of main vein in tomato leaves. The main vein of the same position in HT plants and CK were sampled after 30 d of elevated-temperature exposure. The arrangement of cells on the abaxial and the vertical section of leaves were observed after samples were treated by the methods that were used to analyze the ultrastructure of chloroplasts.

SEM was used at the primary magnification of about 300 ×. The number of stomata and stomatal status were examined. Then the stomata density was calculated.

On the microphotographs from TEM, the volume densities of main chloroplast compartments, *i.e.,* grana and thylakoids, starch grains, and stroma were evaluated using stereological grids with regularly distributed points. At the same time, the integrity of chloroplast membrane was evaluated compared with controls.

Statistical analysis: All results were reported as means ± SE of 5 or 15 independent measurements in each treatment. Data were compared using a variance analysis for repeated measurement, followed by *Student – Newman – Keuls*'s test. Differences were considered statistically significant at the 5% or 1% level (P<0.05 or P<0.01).

Results

P_N decreased in the HT plants by 12.1 and 17.8% after 5 and 25 DAE, respectively (Fig. 1). P_N in HT plants showed a rapid decreasing trend after 15 DAE, with a significant difference between treatments (Fig. 1).

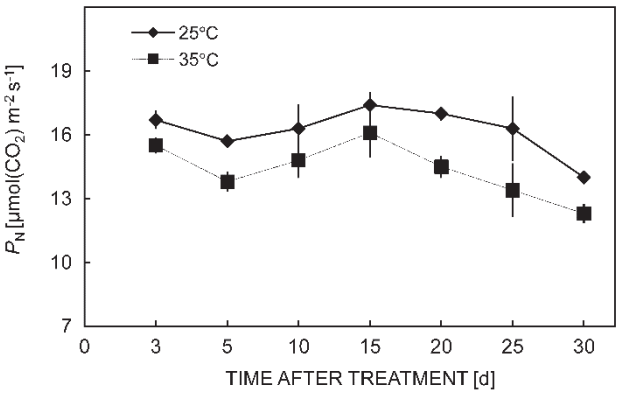

Fig. 1. Effect of the elevated temperature on the net photosynthetic rate (P_N) in tomato leaves. All values are means ± SE (n = 5).

Changes in P_N in HT plants were accompanied by alterations in the values of g_s, C_i, E, and L_s. The decreased P_N was accompanied by a decline in L_s and increases in g_s, E, and C_i. The differences in the g_s, E, and L_s between treatments were significant (Table 1). Compared with CK, the length of main nerve increased and the main vein showed distortion and thinner in HT plants stressed for 30 DAE (Fig. 2*A,B*). Meanwhile, the arrangement of cells on the abaxial side of the main vein was in disorder. An increase in the number of cells was observed in HT plants and these cells were overly congregated compared to CK (Fig. 2*B,D*). The cells in CK were uniform in size and arranged orderly according to the SEM observation (Fig. 2*C*).

The thickness of vertical section in HT leaves became thinner than in CK (Fig. 3). A further SEM observation proved that the upper epidermis cells became thinner, the length of cells in palisade tissue became shorter, but the thickness of spongy tissue showed no obvious change compared with CK. Thus, the ratio of palisade/spongy tissue decreased. Beyond that, the arrangement of palisade tissue became loosening in HT plants (Fig. 3).

Table 1. Effects of elevated temperature on stomatal conductance (g_s), intercellular CO_2 concentration (C_i), the rate of transpiration (E), and stomatal limitation (L_s) in tomato leaves. Means values (n = 5) in the same column followed by the same *lowercase letters* are not significantly different at P<0.05 according to the Student–*Newman–Keuls*'s test.

Parameter	T [°C]	Time after treatment [d]						
		3	5	10	15	20	25	30
g_s [mmol(H_2O) m^{-2} s^{-1}]	25	0.158^b	0.125^a	0.238^b	0.271^a	0.302^b	0.283^b	0.485^a
	35	0.268^a	0.156^a	0.359^a	0.229^a	0.518^a	0.559^a	0.501^a
C_i [μmol mol^{-1}]	25	331^a	184^a	323^a	355^a	345^a	375^a	312^b
	35	358^a	193^a	323^a	365^a	371^a	359^a	421^a
E [mmol(H_2O) m^{-2} s^{-1}]	25	3.05^b	3.38^b	2.04^b	2.04^b	2.35^b	2.32^b	3.23^a
	35	3.73^a	4.19^a	4.30^a	4.96^a	4.57^a	5.04^a	3.41^a
L_s	25	0.193^a	0.558^a	0.307^a	0.286^a	0.289^a	0.230^a	0.293^a
	35	0.127^b	0.456^b	0.236^a	0.307^a	0.264^a	0.161^b	0.161^b

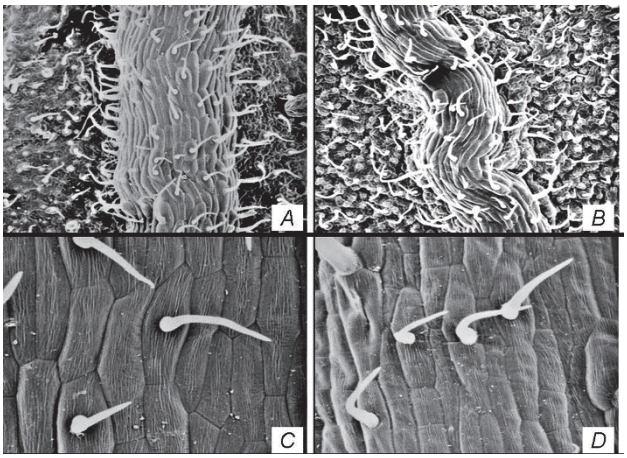

Fig. 2. Effect of the elevated temperature on main nerve of tomato leaves under the scanning electrone microscope. (*A*) The main nerve in controls after 30 d of treatment (40 ×); (*B*) the main nerve in the stressed plants after 30 d of treatment (40 ×); (*C*) the cells on the underside of main nerve in controls after 30 d of treatment (200 ×); (*D*) the cells on the underside of main nerve in the stressed plants after 30 d of treatment (200 ×).

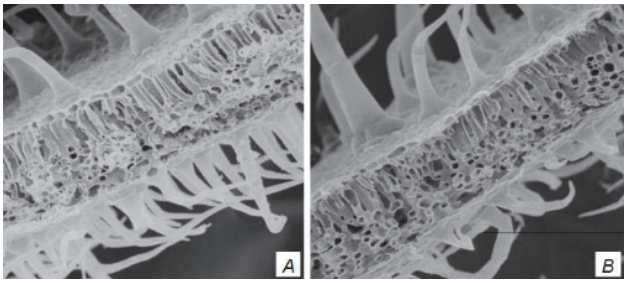

Fig. 3. Effect of the elevated temperature on the vertical section of tomato leaves under the scanning electrone microscope (200 ×). (*A*) the vertical section in controls after 30 d of treatment; (*B*) the vertical section in the stressed plants after 30 d of treatment.

In addition, the stomata density in CK showed little change and remained relatively stable during the period of treatment, while the stomata density in HT plants increased after only 3 DAE under elevated temperature and increased dramatically after 5 DAE. The stomata density was 2.4 times higher in HT plants than that in CK at that time (Fig. 4). There were significant differences between treatments. Under the same magnification and field of view, the number of stomata in HT plants increased rapidly compared with CK after 5 DAE (Fig. 5*A,B*). It could be found through careful observation that the size of these stomata were not uniform and there was a great number of newly formed small stomata. In contrast, the stomata in CK were uniform in their size (Fig. 5*A,C*). In addition, there was a sharp decline in stomata density in HT plants after 10 DAE because of the expanding of leaves, and it remained stable after 15 DAE. But it was still significantly greater than CK (Fig. 4).

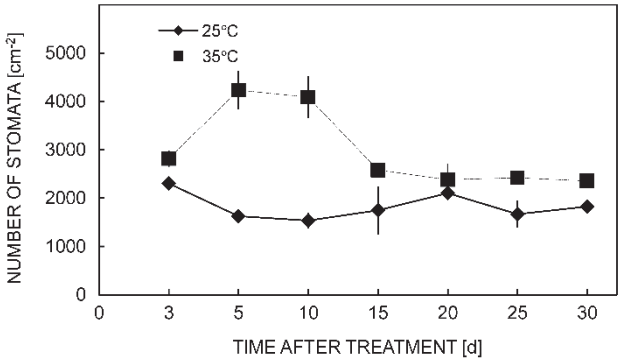

Fig. 4. Effect of the elevated temperature on stomata density in tomato leaves. All values are means ± SE (n = 15).

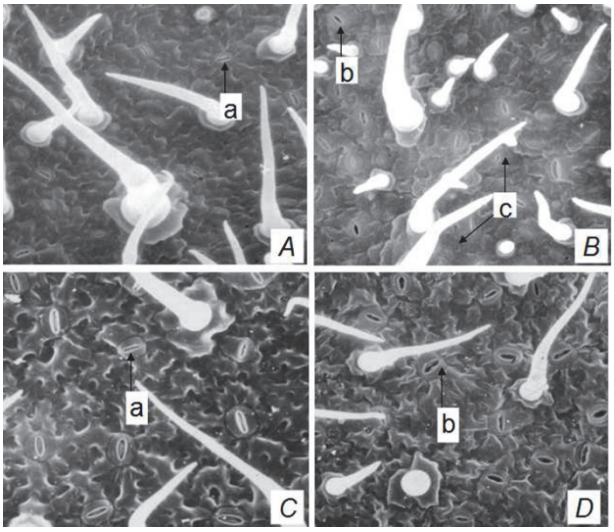

Fig. 5. Effect of the elevated temperature on stomata in tomato leaves (300 ×). (*A*) Stoma in the controls after 5 d of treatment; (*B*) stoma in the stressed plants after 5 d of treatment; (*C*) stoma in the controls after 20 d of treatment; (*D*) stoma in the stressed plants after 20 d of the treatment. a – the closed stoma; b – the opened stoma; c – the newly formed stoma.

Moreover, the opening and closing of stomata aperture were observed by SEM. In HT plants, stress induced the stomata opening while the stomata were closed in CK at the beginning of photoperiod after 5 DAE (Fig. 5*A,B*). With the increasing treatment duration, the portion of opened stomata increased gradually in HT plants; nearly all stomata were opened after 20 DAE (Fig. 5*D*). At this point, the stomata in CK remained still closed (Fig. 5*C*).

TEM was used to study the chloroplast ultrastructure. The TEM micrographs showed differences in chloroplast structure between CK and HT plants (Figs. 6, 7, 8). In HT plants, the thylakoids were loosely distributed with lesser grana, but the lipid droplet number increased, especially, after 15 DAE. The difference was significant between both treatments (Figs. 6*A,C*, 7). Compared with CK, the number of starch grains in chloroplast decreased in HT leaves within 15 DAE, then increased, and the difference between

treatments was significant after 30 DAE (Figs. 6*B*,8). The chloroplast membrane was damaged and disintegrated partly and became blurred in HT plants. In contrast, chloroplast membrane was clearly visible without obvious damage in CK. Meanwhile, the grana lamellae changed into disarray arrangement and blurring in HT plants compared with in CK (Fig. 7).

Discussion

Exposure to elevated temperature reduced P_N in tomato leaves. Temperature of 35ºC is a moderately high for tomato plants. Sharkey (2005) reported that moderate heat stress could reduce P_N to near zero. In our case, the continuous HT treatment caused a great adverse effect on photosynthesis (Fig. 1). At the same time, an increase in g_s, C_i, E, and a decrease in L_s were observed in HT plants (Table 1). According to Farquhar and Sharkey (1982), changes in the P_N reflect alterations in both g_s, which results in stomatal limitation, and/or the photosynthetic capacity of the mesophyll. We supposed that the decrease in P_N was mainly caused by nonstomatal limitation and the alterations in mesophyll capacity, which depend on the activity of Rubisco and on the capacity of photosynthetic electron transport (Crafts-Brandner *et al*. 1997, Camejo *et al*. 2005).

Photosynthesis is one of the most heat-sensitive processes in plants and it can be completely inhibited by high temperatures before any symptoms are detected (Berry and Bjorkman 1980, Camejo *et al*. 2005). Changes in chloroplast structure and function are usually regarded as a good indicator of plant stress and various features of chloroplasts have been described as distinctive signs of the exposure of a plant to unfavourable environmental factor (Holá *et al.* 2008). In our case, both the microstructure and ultrastructure changed in tomato leaves under stress of elevated temperature.

Leaf structure is closely related to the photosynthesis (Tang and Shi 1997,Taiz and Zeiger 2002). Exposure to elevated temperature of 35ºC caused the extension of leaf veins. There were more cells in the main veins and these cells were overly congregated in HT plants (Fig. 2). This might be one of the reasons that induced the extension and distortion of the main veins (Fig. 2*B*). Furthermore, the mesophyll cells linked with the vein presented lesser extension. Thus, the leaves of HT plants were curled downwards and were not flat. Whether the elevated temperature stimulated the production of cytokinins in veins and this promoted the cell division and enlargement, it should be investigated further. Moreover, in our case, the epidermal cells in HT leaves thinned and the ratio of palisade tissue/spongy tissue decreased. It was consistent with the report of Miao *et al*. (1994). This was caused by shortening the length of cells in the palisade tissue but the thickness of the spongy tissue showed no obvious change compared with CK controls (Fig. 3).

Stomata density affects gas exchange, g_s, and instantaneous water-use efficiency (Woodward and Bazzaz 1988,

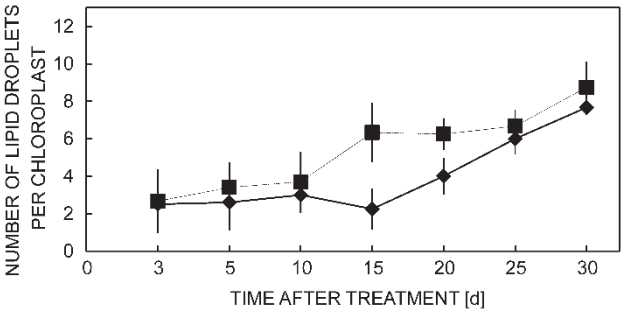

Fig. 6. Effect of the elevated temperature on the number of grana, starch grains, and lipid droplets per chloroplast in tomato leaves during treatment. All values are means ± SE (n = 15). (*A*) The number of grana per chloroplast; (*B*) the number of starch grains per chloroplast; (*C*) the number of lipid droplets per chloroplast.

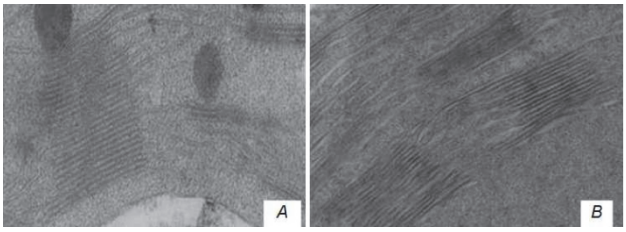

Fig. 7. Effect of the elevated temperature on the grana in chloroplast of tomato leaves. (*A*) The chloroplast in controls (10,000 ×); (*B*) the chloroplast in the stressed plants (10,000 ×).

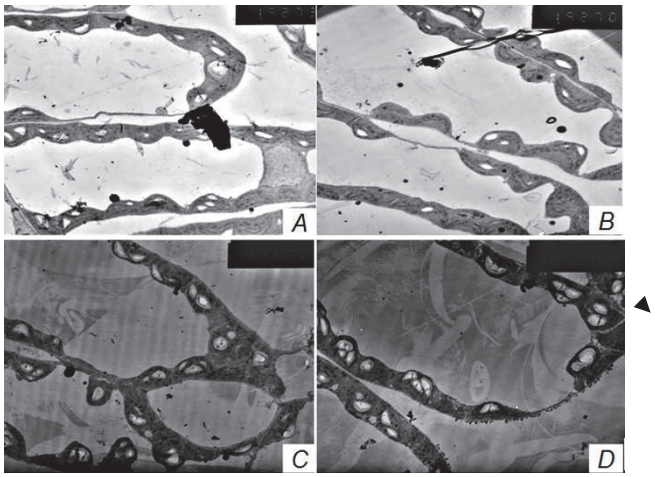

Fig. 8. Effect of the elevated temperature on the starch grains in chloroplast of tomato leaves (4,000 ×). (*A*) Starch grains in controls after 5 d of treatment; (*B*) starch grains in the stressed plants after 5 d of treatment; (*C*) starch grains in the controls after 30 d of treatment; (*D*) starch grains in the stressed plants after 30 d of treatment.

Fu *et al*. 2011). In our case, the number of stomata increased significantly, especially in the early stage of exposure and the stomata status also changed compared with CK (Figs. 1, 5). It was considered that many newly formed stomata appeared in HT plants, thus the plants could increase transpiration to alleviate the damage of high-temperature stress. It could be a mechanism for plants to adapt to the environment. The number of stomata did not changed after 10 DAE as the leaves became adapted to the stress. Long-term exposure to the elevated temperature influenced also stomata movement, blocked normal stomata opening and closure, thus they remained in the open status. It was different from other reports (Li and Wang 2002, Xu *et al*. 2006). It might be caused by the damage of cellulose microfibrils in the guard-cell walls during the long-term stress. The alignment of cellulose microfibrils plays an essential role in the opening and closing of the stomata pore.

The ultrastructural change can reflect directly the state of photosynthetic apparatus. Under 35ºC stress, chloroplast ultrastructure changed significantly in HT plants compared with CK, particularly after a long-term exposure. According to Taiz and Zeiger (2002), more granal thylakoids and stacks mean higher photosynthetic capacity. In our case, the grana lamellae changed into disarray arrangement and blurred, while the chloroplasts of CK maintained the ultrastructure with tightly stacked lamellae of grana (Figs. 6*A*, 7). The enhanced presence of lipid droplets is the symbol of chloroplast senescence (Xu *et al*. 2006, Wang *et al*. 2010). The lipid droplet number increased significantly, especially after 15 DAE in HT plants (Fig. 6*C*).

Photosynthetic products are transported mainly to sink organs in the form of sucrose *via* the phloem, meanwhile some photosynthates are accumulated in leaves in the form of starch (Lalonde *et al*. 1999, Taiz and Zeiger 2002). In our case, the number of starch grains in chloroplasts decreased first and then increased (Figs. 6*B*, 8). This could be due to lowered photosynthesis and lesser amount of photosynthates produced, while the respiration increased under the 35ºC. Thus, the accumulation of starch in chloroplasts decreased compared with CK during the early phase of treatment. While, the transport of photosynthates was affected with the prolonging exposure to high temperature., and it ultimately led to the accumulation of starch grains in chloroplasts. This was consistent in the study of Sam *et al*. (2001).

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