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Changes in extreme high-temperature tolerance and activities of antioxidant enzymes of sacred lotus seeds

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Sacred lotus (Nelumbo nucifera Gaertn. 'Tielian') seed is long-lived and extremely tolerant of high temperature. Water content of lotus and maize seeds was 0.103 and 0.129 g H_2O [g DW] $^{-1}$, respectively. Water content, germination percentage and fresh weight of seedlings produced by surviving seeds gradually decreased with increasing treatment time at 100℃. Germination percentage of maize (*Zea mays* L. 'Huangbaogu') seeds was zero after they were treated at 100℃ for 15 min and that of lotus seeds was 13.5% following the treatment at 100℃ for 24 h. The time in which 50% of lotus and maize seeds were killed by 100℃ was about 14.5 h and 6 min, respectively. With increasing treatment time at 100℃, relative electrolyte leakage of lotus axes increased significantly, and total chlorophyll content of lotus axes markedly decreased. When treatment time at 100℃ was less than 12 h, subcellular structure of lotus hypocotyls remained fully intact. When treatment time at 100℃ was more than 12 h, plasmolysis gradually occurred, endoplasmic reticulum became unclear, nuclei and nucleoli broke down, most of mitochondria swelled, lipid granules accumulated at the cell periphery, and organelles and plasmolemma collapsed. Malondialdehyde (MDA) content of lotus axes and cotyledons decreased during 0—12 h of the treatment at 100℃ and then increased. By contrast, the MDA content of maize embryos and endosperms increased during 5—10 min of the treatment at 100℃ and then decreased slightly. For lotus seeds: (1) activities of superoxide dismutase (SOD) and glutathione reductase (GR) of axes and cotyledons and of catalase (CAT) of axes increased during the early phase of treatment at 100℃ and then decreased; and (2) activities of ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) of axes and cotyledons and of CAT of cotyledons gradually decreased with increasing treatment time at 100℃. For maize seeds: (1) activities of SOD and DHAR of embryos and endosperms and of GR of embryos increased during the early phase of the treatment at 100℃ and then decreased; and (2) activities of APX and CAT of embryos and endosperms and of GR of endosperms rapidly decreased with increasing treatment time at 100℃. With decrease in seed germination, activities of SOD, APX, CAT, GR and DHAR of axes and cotyledons of lotus seeds decreased slowly, and those of embryos and endosperms of maize seeds decreased rapidly.

activities of antioxidant enzymes, extreme high-temperature tolerance, lipid peroxidation, Nelumbo nucifera seeds, subcellular structure, Zea mays seeds

Seeds of sacred lotus (*Nelumbo nucifera* Gaertn.) (Nelumbonaceae) are the longest-lived seeds known[1–3]. Ohga[4] found Indian sacred lotus fruits in a dry lake bed of Pulandian, Liaoning Province, China. ^{14}C-dated results showed that longevity of these fruits was (1024± 210) a (radiocarbon years)[5]. More than 1000 a old lotus fruits could germinate[3], and 200—1300 a old fruits germinated to 80%[6]. It has been suggested that the factors associated with a long life-span of lotus fruits

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Abbreviation: APX, ascorbate peroxidase; AsA, ascorbic acid; BSA, bovine serum albumin; CAT, catalase; DHA, dehydroascorbic acid; DTT, dithiothreitol; DW, dry weight; EDTA, ethylenediamine tetraacetic acid; g g^{-1}, g H_2O (g DW)$^{-1}$; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HSF, heat shock transcription factor; HSP, heat shock protein; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NBT, nitroblue tetrazolium; $\cdot O_2^-$, superoxide radicle; POD, peroxidase; PVPP, polyvinylpolypyrrolidone; RH, relative humidity; ROS, reactive oxygen species; SOD, superoxide dismutase

include (1) the protecting role of pericarp and heat-resistant proteins; (2) the repair function of a protein-repair enzyme, *L*-isoaspartyl methyltransferase[7]; and (3) the high content of polyunsaturated fatty acid[2].

Another remarkable character of lotus fruits is their high-temperature tolerance. When intact lotus fruits of *China Antique* were treated in water at 90℃ for 2 h, their germination percentage was 50%, and when treated at 80℃ for 4 d, it was 60%[8]. However, little is known about the mechanism of heat-tolerance of lotus fruits. In plants, ROS is continuously produced in chloroplasts, mitochondria, and peroxisomes. Production and removal of ROS are influenced by a number of abiotic stress factors. For example, light, water, temperature, and oxygen stresses cause an unequal balance between production and scavenging of ROS, which thereby results in oxidative damage[9–11]. It has been shown that heat stress results in injury of mitochondria functions and induction of injury caused by lipid peroxidation[12,13]. Tang[14] showed that high-temperature tolerance of lotus seeds was greater than that of its fruits and that pericarp had a side-effect on high-temperature tolerance of lotus seeds. Huang et al.[15] investigated heat tolerance of lotus seeds and changes in activities of SOD, POD and CAT during their development. To our knowledge, changes in subcellular structure and antioxidant enzyme activities during the treatment at extremely high-temperature of lotus seeds have not been reported.

In the present study, sacred lotus (*Nelumbo nucifera* Gaertn. 'Tielian') and maize (*Zea mays* L. 'Hunagbaogu') seeds were used as materials, and the changes in seed germination, subcellar structure, lipid peroxidation and activities of antioxidant enzymes were comparatively studied.

1 Materials and methods

1.1 Plant material

Fruiting receptacles of sacred lotus were collected at maturity in the suburb of Xiangtan, Hunan Province, China, in September, 2005. Fruits (nutlets) were removed from the receptacles, cleaned in water, and dehydrated at (25±2)℃ and 70% RH for 30 d, at which time the water content was (0.103±0.003) g g^{-1}. They were kept at 4℃ until used.

Maize (*Zea mays* L. 'Huangbaogu') fruits were collected at maturity in Menglun, Mengla, Yunnan Province, China in June, 2006. After removal from cobs, caryopses (hereafter seeds) were dehydrated at 15±2℃ and 70% RH for 15 d and to a water content of (0.129±0.004 g g^{-1}). They were kept at 4℃ until used.

1.2 Extreme high-temperature treatment of seeds

Lotus seeds, with pericarp removed from nutlets, were treated in air at 100℃ (hereafter at 100℃) for 0, 2, 4, 8, 12, 16 and 24 h, and maize seeds for 0, 5, 7.5, 10, 15 and 20 min.

1.3 Water content

Water content of lotus axes and cotyledons and of maize embryos and endosperms was determined gravimetrically (103℃ for 17 h). Water content is expressed on a dry mass basis (g g^{-1}).

1.4 Germination

After the treatment at 100℃ for different periods of time, seeds of lotus and maize were germinated on two layers of the filter paper moistened with 10 mL of distilled water in 9-cm–diameter Petri dishes at (25±1)℃ in darkness for 5 d. Radicle protrusion of 2 mm was used as the criterion for the completion of germination.

1.5 Relative conductivity

Relative electrolyte leakage of 5 axes of lotus seeds was measured using a Model 4310 conductivity meter (Jenway Ltd, Essex, England). Five axes were placed in 4 mL distilled water, and conductivity was measured immediately (A_0); and then these axes were maintained in distilled water at (25±1)℃ for 2 h. The solution was shaken five times before the second measurement (A_1). Finally, these axes in solution were boiled at 100℃ for 30 min and cooled in tap water. Then, a third conductivity measurement (A_2) was taken. The relative electrolyte leakage = $(A_1–A_0) / (A_2–A_0) \times 100\%$.

1.6 Chlorophyll content

After lotus seeds were treated at 100℃ for different periods of time, the axes were excised and their total chlorophyll content was measured immediately according to the method of Arnon[16].

1.7 Subcellular structure

After lotus seeds were treated at 100℃ for different periods of time, the hypocotyls were excised, and cut into 1-2 mm^3 pieces, and fixed in 3% (*v*/*v*) glutaraldehyde in 100 mmol/L phosphate buffer (pH 7.2) for 120 h.

After rinsing three times (20-min each) in the above-mentioned phosphate buffer, these materials were re-fixed in 0.5% (*w/v*) aqueous osmium tetroxide for 4 h, followed by routine dehydration, embedding, and sectioning. After staining with lead citrate, sections were viewed and photographed with a transmission electron microscope (Model JEM-1230, JEOL, Ltd).

1.8 MDA content

Thirty seeds each of lotus and maize were imbibed on two layers of filter paper moistened with 10 mL of distilled water at 25±1℃ in darkness for 48 h after treatment at 100℃ for different periods of time. MDA content nmol (g DW) $^{-1}$ of axes and cotyledons of lotus seeds and of embryos and endosperms of maize seeds was measured as described by Kumar and Knowles[17].

1.9 Assay of SOD, APX, CAT, GR and DHAR activities

After treatment at 100℃ for different periods of time, 50 seeds each of lotus and maize were imbibed on the filter paper moistened with distilled water at 25±1℃ in darkness for 48 h. Axes and cotyledons of sacred seeds, and embryos and endosperms of maize seeds were homogenized to a fine powder with a mortar and pestle under liquid nitrogen, and then these powders were kept at −60℃ until used.

Extraction and assay of SOD (EC 1.15.1.1) were based on the method of Beauchamp and Fridovich[18], who measured the inhibition of the photochemial reduction of NBT at 560 nm. The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.8), 13 mmol/L methionine, 75 μmol/L NBT, 16.7 μmol/L riboflavin, 0.1 mmol/L EDTA and enzyme extract (ca. 25 μg protein). An illuminated blank without protein gave the maximum reduction of NBT, and therefore, the maximum absorbance at 560 nm. SOD activity is presented as (absorbance of sample) by (absorbance of blank) × 100, giving the percentage of inhibition. One unit of SOD is defined as the amount required to inhibit the photoreduction of NBT by 50%. The specific activity of SOD was expressed as unit of SOD (mg protein)$^{-1}$.

For extraction and assay of APX, CAT, GR and DHAR, the fine powders mentioned above were extracted by grinding in 5 mL of 50 mmol/L Tris-HCl (pH 7.0) containing 20% (*v/v*) glycerol, 1 mmol/L AsA, 1 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L GSH, 5 mmol/L $MgCl_2$ and 1% (*w/v*) PVPP. After two centrifugation steps (6 min at 12 000×*g* and 16 min at 26 900×*g*), the supernatant was stored at −60℃ for subsequent determinations of APX, CAT, GR and DHAR activities.

APX (EC 1.11.1.7) was assayed as the decrease in absorbance at 290 nm (2.8 (mmol/L)$^{-1}$ cm^{-1}) due to AsA oxidation[19]. The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.0), 1 mmol/L AsA, 2.5 mmol/L H_2O_2 and enzyme extract (ca. 25 μg protein). Enzyme activity was expressed as nmol AsA (mg protein) $^{-1}$ min^{-1}.

CAT (EC 1.11.1.6) activity was determined by directly measuring the decomposition of H_2O_2 at 240 nm (0.04 (mmol/L)$^{-1}$ cm^{-1}) as described by Aebi[20]. The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.0), 10 mmol/L H_2O_2 and enzyme extract (ca. 25 μg protein). Enzyme activity was expressed as nmol H_2O_2 (mg protein)$^{-1}$ min^{-1}.

GR (EC 1.6.4.2) was determined as the decrease in absorbance at 340 nm (6.2 (mmol/L)$^{-1}$ cm^{-1}) due to the oxidation of NADPH[21]. The reaction mixture contained 50 mmol/L Tris-HCl buffer (pH 7.5), 5 mmol/L $MgCl_2$, 0.5 mmol/L GSSG, 0.2 mmol/L NADPH and enzyme extract (ca. 25 μg protein). Enzyme activity was expressed as nmol NADPH (mg protein)$^{-1}$ min^{-1}.

DHAR (EC 1.8.5.1) was assayed directly by following the formation of AsA at 265 nm (14 (mmol/L)$^{-1}$ cm^{-1})[22]. The reaction mixture contained 50 mmol/L phosphate buffer (pH 7.0), 0.5 mmol/L DHA, 2.5 mmol/L GSH and enzyme extract (ca. 50 μg protein). Enzyme activity was expressed as nmol AsA (mg protein)$^{-1}$ min^{-1}.

1.10 Protein assay

Protein was measured following the procedure of Bradford[23], using BSA as a standard.

1.11 Statistical analysis

All data were analyzed using a one-way ANOVA model from the SPSS 12.0 package for Windows (SPSS Inc).

2 Results

2.1 Effects of high temperature on water content, germination percentage and seedling fresh weight

Water content of lotus axes and cotyledons and of maize seeds was 0.109, 0.104 and 0.129 g g^{-1}, respectively. Water content ($P \leqslant 0.001$), germination percentage ($P \leqslant$

0.001) and seedling fresh weight ($P \leqslant 0.001$) gradually decreased with increasing treatment time at 100℃ (Figures 1 and 2). Germination percentage of maize seeds treated for 15 min at 100℃ was zero(Figure 1(b)), and that of lotus seeds treated for 24 h was 13.5% (Figure 1(a)). The time in which 50% of lotus and maize seeds were killed at 100℃ (T_{50}) was 14.5 h and 6 min, respectively. Fresh weight of seedlings produced by surviving lotus and maize seeds decreased rapidly with increasing treatment time. For example, compared with control fresh weight of seedlings of lotus seeds treated at 100℃ for 8 h decreased by 65% (Figure 1(a)), and that of maize seeds treated at 100℃ for 10 min decreased by 79% (Figure 1(b)).

2.2 Changes in relative electrolyte leakage and total chlorophyll content of lotus axes

Relative electrolyte leakage of lotus axes increased ($P \leqslant 0.001$), and total chlorophyll content of lotus axes markedly decreased ($P \leqslant 0.001$) with increasing time at 100℃. For example, relative electrolyte leakage of lotus seeds treated at 100℃ for 24 h increased by 184.9%, and total chlorophyll content decreased by 37.2% compared with control (Figure 3).

2.3 Changes in subcellular structure of lotus hypocotyl

After the treatment at 100℃ for 8, 12, 16 and 24 h, the subcellular structure of hypocotyl cells of lotus seeds

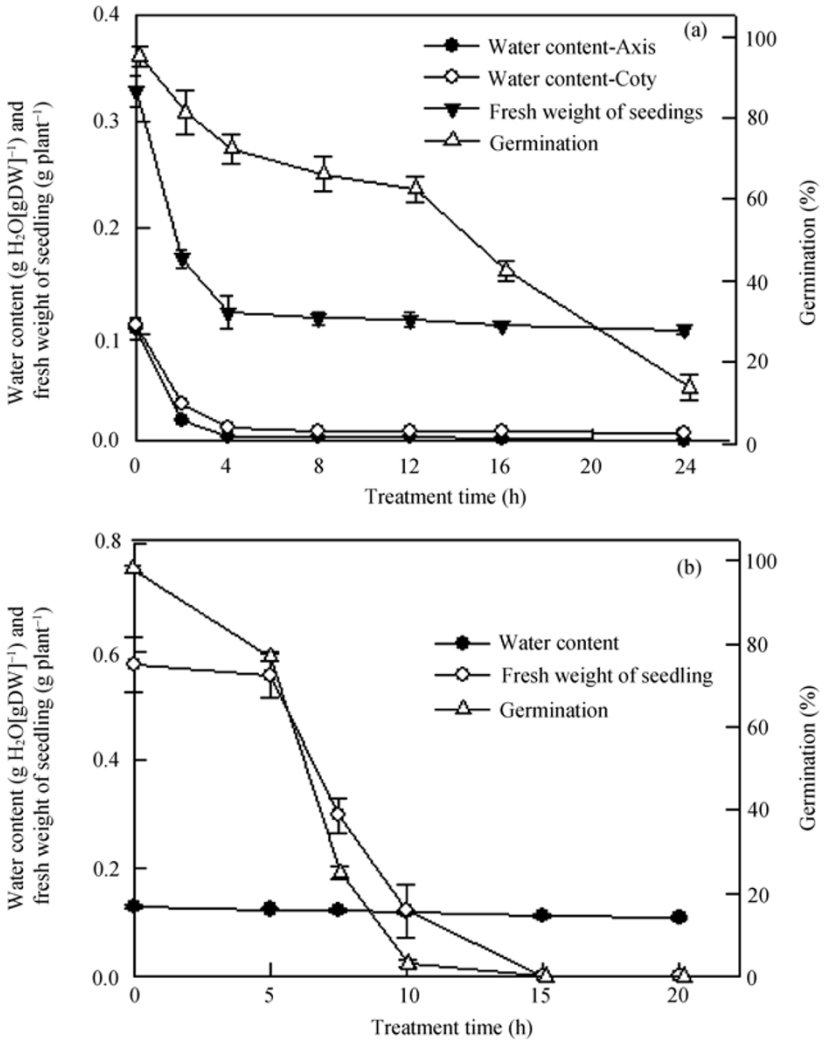

Figure 1 Changes in water content, germination percentage, fresh weight of seedling produced by surviving seeds during treatment of sacred lotus (a) and maize (b) seeds at 100℃. Seeds with radicle emerged 2 mm were scored as germinated. Fresh weight of seedlings did not include cotyledons or endosperm. Coty, cotyledon. All values are means ± SD of four replicates of 25 seeds each.

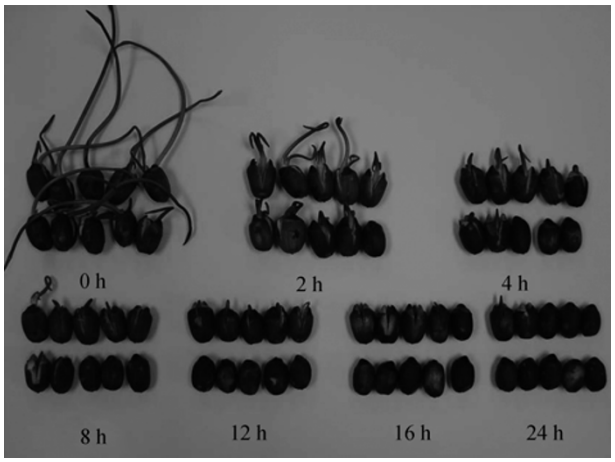

Figure 2 Effects of treatment at 100℃ on germination of sacred lotus seeds. Seeds were treated at 100℃ for indicated time in the figure and then germinated at 25℃ and in the dark for 5 d.

exhibited marked difference from those of nonheated control (Figure 4). Integrity of subcellular structure of the hypocotyl, e.g., numerous mitochondria, normal nuclei and nucleoli, and clear endoplasmic reticulum (Figure 4(c)—(e) were maintained in the seeds treated at 100 ℃ for less than 12 h. Most of the plasmalemmas were adpressed against the cell wall, except that plasmolysis occurred in part of the cells treated at 100℃ for 12 h, and plasmodesmi also could be observed (Figure 4(e)). However, when seeds were treated at 100℃ for longer than 12 h, the organelles were damaged by high temperature (Figure 4(f)—(h)). Thus, plasmolysis occurred, endoplasmic reticulum became unclear, nuclei and nucleoli broke-down, most of the mitochondria swelled, and lipid granules accumulated at cellular periphery (Figure 4(f)—(h)). Organelles and plasmalemmas collapsed (Figure 4(h)) in the seeds treated at 100℃ for 24 h.

2.4 Changes in MDA content

Malondialdehyde content of lotus axes and cotyledons declined during the early phase of the treatment at 100℃ to 12 h of treatment, and then rose ($P \leqslant 0.001$), whereas that of maize embryos and endosperms increased during the early phase of treatment at 100℃ and then decreased ($P \leqslant 0.001$) (Figure 5). However, the MDA content of maize embryos was much higher than that of endosperms during the treatment at 100℃ (Figure 5).

2.5 Changes in activities of SOD, APX, CAT, GR and DHAR

Activities of SOD of lotus axes and cotyledons and of maize embryos increased during the early phase of the treatment at 100℃ and then rapidly decreased, while those of maize endosperm decreased with increasing treatment time at 100℃ ($P \leqslant 0.001$, Figure 6). Superoxide dismutase activities of lotus cotyledons were higher than those of axes, but SOD activities of maize embryos were higher than those of endosperms (Figure 6).

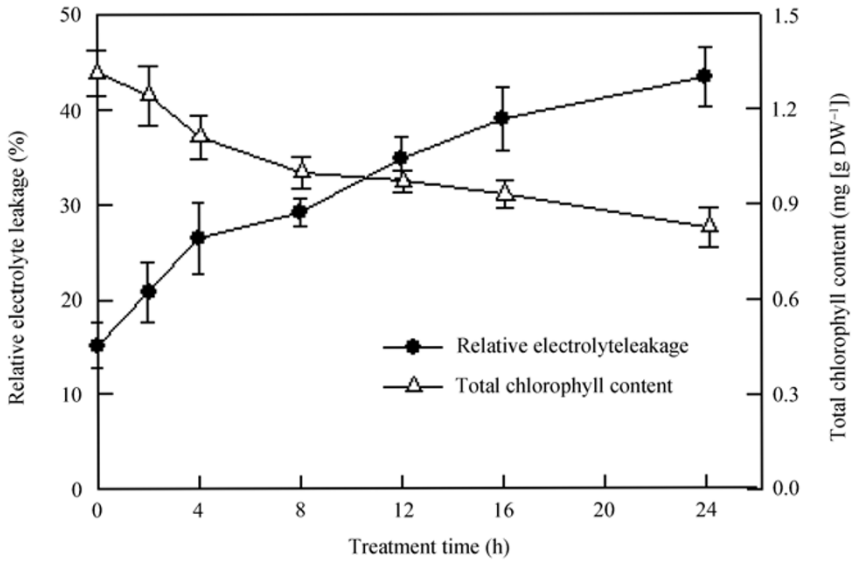

Figure 3 Changes in relative electrolyte leakage and total chlorophyll content of axes of sacred lotus seeds treated at 100℃. After the treatment at 100℃ for indicated time, axes were removed from seeds. All values are means ± SD of four replicates of 5 axes each.

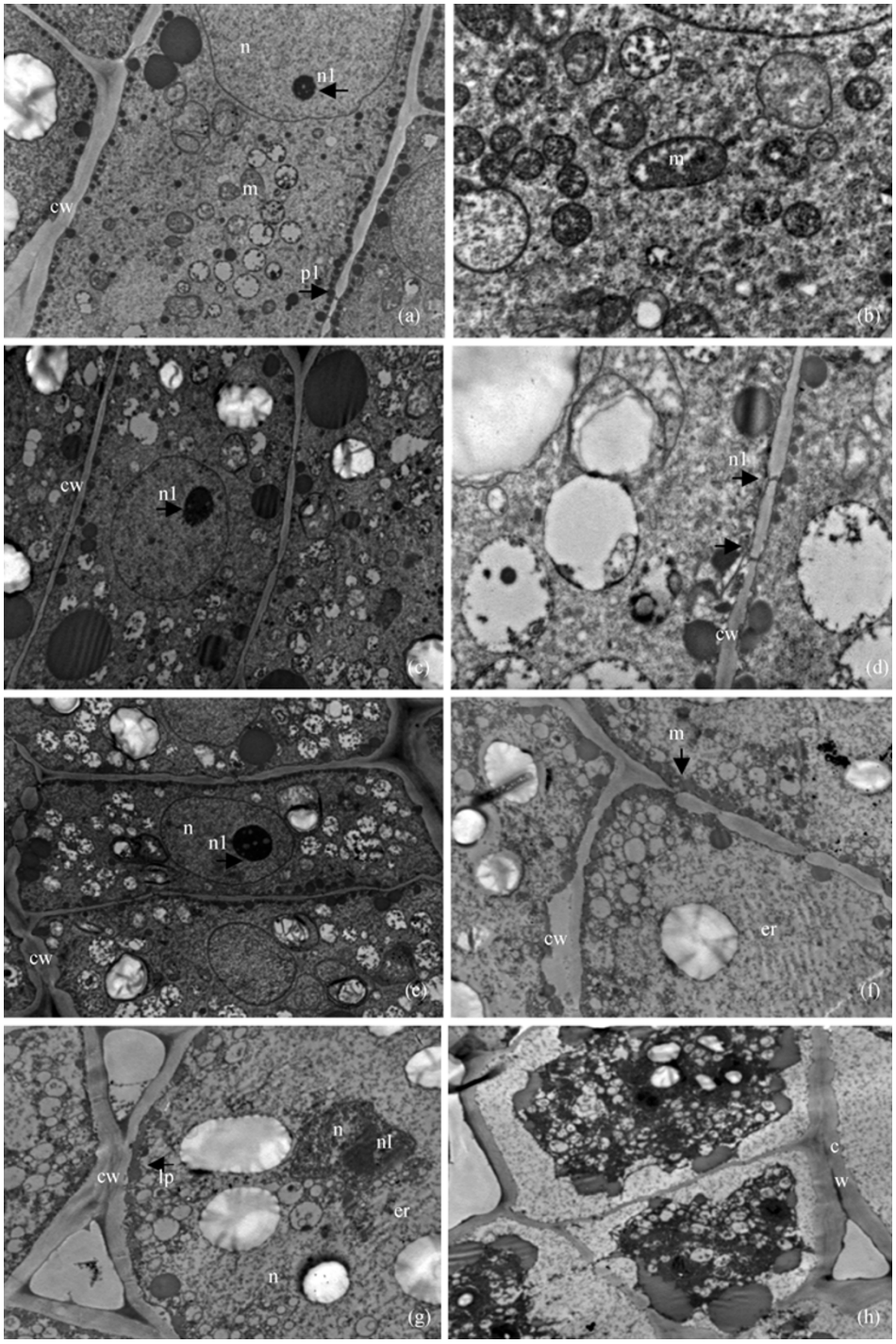

Figure 4 Changes in subcellular structure of sacred lotus hypocotyls. After the treatment at 100℃ for indicated time, lotus seeds were placed in 100% RH at 25℃ for 48 h and then examined with an electron microscope. (a) and (b), 0 h, show relatively few and small vacuoles, abundant undifferentiated mitochondria (m), intact nuclei (n), nucleoli (arrow, nl), starch (s), and plasmodesmi (arrow, pl) ((a), 5000×; (b), 20000×). (c), (d) and (e), 8, 8 and 12 h, respectively, show that ultrastructure was fully maintained, e.g. mitochondria (m), nuclei (n), nucleoli (arrow, nl), plasmodesmi (pl), and plasma membrane, which is adpressed against the cell wall (cw) except in E, where it is partly separated ((c), 5000×; (d), 15000×; (e), 5000×). (f), (g) and (h), 16, 16, and 24 h, respectively, in contrast to the above cells, show the damaged organelles such as plasma membrane separated from cell wall (cw), endoplasmic reticulum (er) with unclear membrane structure, unintegrated nuclei (n), nucleoli (arrow, nl), swollen mitochondria (m), and peripheral accumulation of lipid (arrow, lp); especially in (h), organelle and plasmolemma apparently are collapsed ((f), 5000×; (g), 5000×; (h), 5000×).

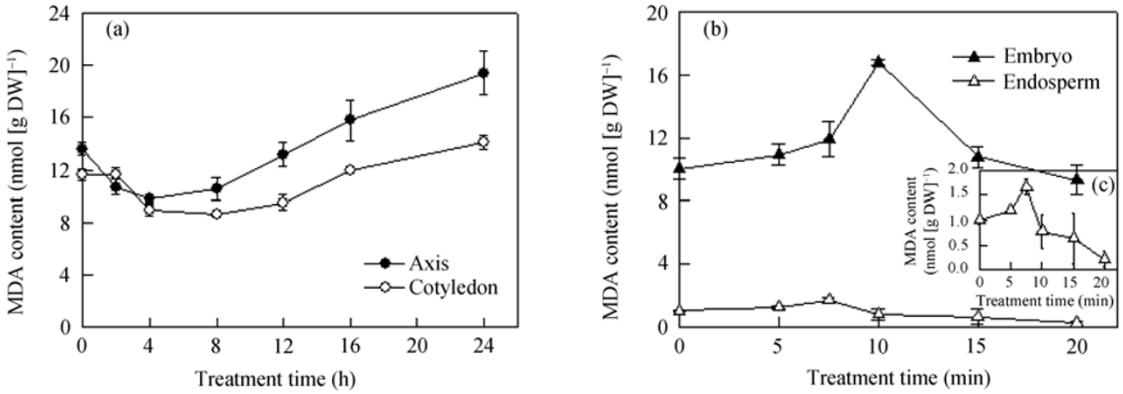

Figure 5 Changes in MDA content. After the treatment at 100℃ for indicated time, sacred lotus and maize seeds were imbibed on filter paper moistened with distilled water for 48 h, and then the MDA content of axes and cotyledons of lotus seeds and of embryo and endosperm of maize seeds were measured. (a) lotus seed; (b) maize seed; (c) maize endosperm. The MDA content of axes and cotyledons of dry lotus seeds (control, untreated at 100℃, same below) was 16.27±0.16 and 12.61±0.86 nmol [g DW]$^{-1}$, respectively; that of embryos and endosperms of dry maize seeds (control) was 12.15±3.75 and 1.11±0.04 nmol [g DW]$^{-1}$, respectively. All values are means ± SD of five replicates of 30 seeds each.

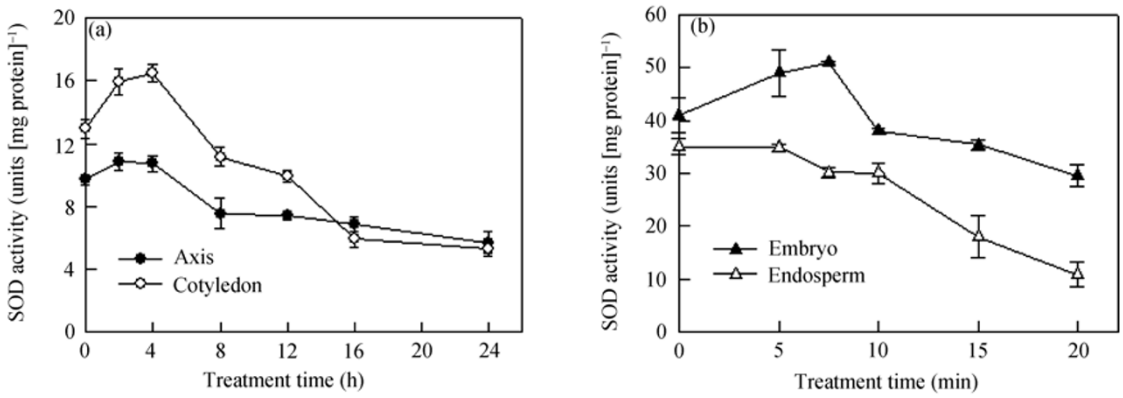

Figure 6 Changes in SOD activity. After the treatment at 100℃ for indicated time, seeds of sacred lotus and maize were imbibed on the filter paper moistened with distilled water for 48 h, and then SOD activity of axes and cotyledons of lotus seeds and of embryos and endosperms of maize seeds was measured. (a) lotus seed; (b) maize seed. Superoxide dismutase activities of axes and cotyledons of dry lotus seeds (control) were 6.41±0.54 and 7.61±0.62 units [mg protein]$^{-1}$, respectively; those of embryos and endosperms of dry maize seeds (control) were 36.69±6.65 and 25.10±2.21 units [mg protein]$^{-1}$, respectively. All values are means ± SD of four replicates of 50 seeds each.

Decline in SOD activities was similar to that in germination percentage of lotus and maize seeds. Germination percentage of maize seeds decreased to zero, and SOD activities of embryos and endosperms decreased by 13.7% and 48.8%, respectively, compared with control; germination percentage of lotus seeds decreased to 13.5%, and SOD activities of axes and cotyledons decreased by 41.2% and 59%, respectively, compared with control (Figures 1 and 6).

Ascorbate peroxidase activities of lotus axes and of maize embryos and endosperms decreased with increasing treatment time at 100℃, but those of lotus cotyledons slightly increased during the early phase of the treatment at 100℃ and then decreased ($P \leqslant 0.001$, Figure 7). Compared with control, APX activities of maize embryos and endosperms decreased by 78.5% and 96.2%, respectively, when seeds were treated at 100℃ for 15 min; those of lotus axes and cotyledons decreased by 62.1% and 35.7%, respectively, when seeds were treated at 100℃ for 12 h (Figure 7). APX activities of maize embryos were higher than those of endosperm, and APX activities of lotus axes were higher than they were in cotyledons (Figure 7).

Catalase activity of lotus axes increased during the early phase of the treatment to 4 h at 100℃, and then markedly decreased. CAT activities of lotus cotyledons, which were much lower than those of axes, decreased with increasing treatment time ($P \leqslant 0.001$, Figure 8(a)).

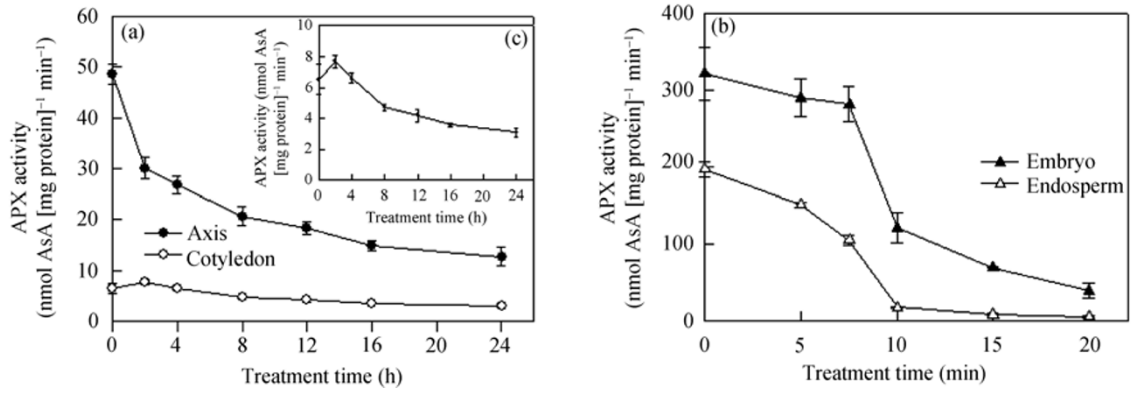

Figure 7 Changes in APX activity. Treatment of sacred lotus and maize seeds was the same as in Figure 6. (a) lotus seed; (b) maize seed; (c) lotus cotyledon. Ascorbate peroxidase activities of axes and cotyledons of dry lotus seeds (control) were 72.26±9.37 and 7.40±2.00 nmol AsA [mg protein]$^{-1}$ min^{-1}, respectively; those of embryos and endosperms of dry maize seeds (control) were 46.42±3.21 and 3.60±0.10 nmol AsA [mg protein]$^{-1}$ min^{-1}, respectively. All values are means ± SD of four replicates of 50 seeds each.

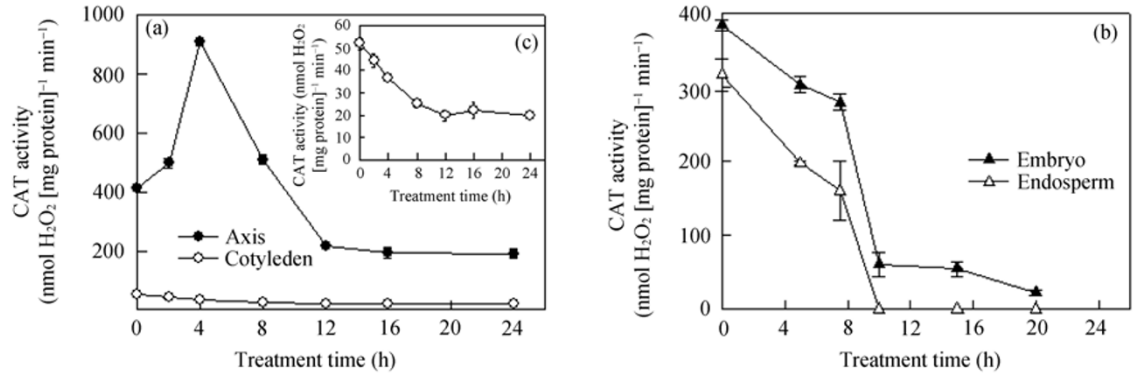

Figure 8 Changes in CAT activity. Treatment of sacred lotus and maize seeds was the same as in Figure 6. (a) lotus seed; (b) maize seed; (c) lotus cotyledon. Catalase activities of axes and cotyledons of dry lotus seeds (control) were 298.24±7.89 and 47.40±4.43 nmol H_2O_2 [mg protein]$^{-1}$ min^{-1}, respectively; those of embryos and endosperms of dry maize seeds (control) were 263.01±24.69 and 115.75±8.68 nmol H_2O_2 [mg protein]$^{-1}$ min^{-1}, respectively. All values are means ± SD of four replicates of 50 seeds each.

CAT activities of maize embryos and endosperms de creased by 85.8% and 100%, respectively, ($P \leqslant 0.001$) with increasing treatment time at 100℃, in seeds treated for 15 min at 100℃ (Figure 8(b)).

Glutathione reductase activities of lotus axes and cotyledons increased by 17.6% and 28.2%, respectively, at 2 h of the treatment at 100℃ and then decreased ($P \leqslant 0.001$, Figure 9). GR activities of maize embryos increased during the early phase of the treatment to 8 min and then decreased ($P \leqslant 0.001$). However, GR activities of maize endosperms decreased by 89.3% with increasing treatment time ($P \leqslant 0.001$) for seeds were treated at 100℃ for 15 min (Figure 9).

Dehydroascorbate reductase activities of lotus axes and cotyledons gradually decreased with increasing treatment time at 100℃ ($P \leqslant 0.001$). However, DHAR activities of axes were much higher than those of cotyledons, and they rapidly decreased during the treatment (Figure 10(a)). DHAR activities of maize embryos and endosperms increased during the early phase of the treatment at 100℃ and then decreased ($P \leqslant 0.001$). DHAR activities of embryos were much higher than those of endosperms (Figure 10(b)).

3 Discussion

One-hundred degrees Celsius is an extremely high temperature for life on earth. Germination percentage of maize seeds treated at 100℃ for 15 min was zero (Fig-

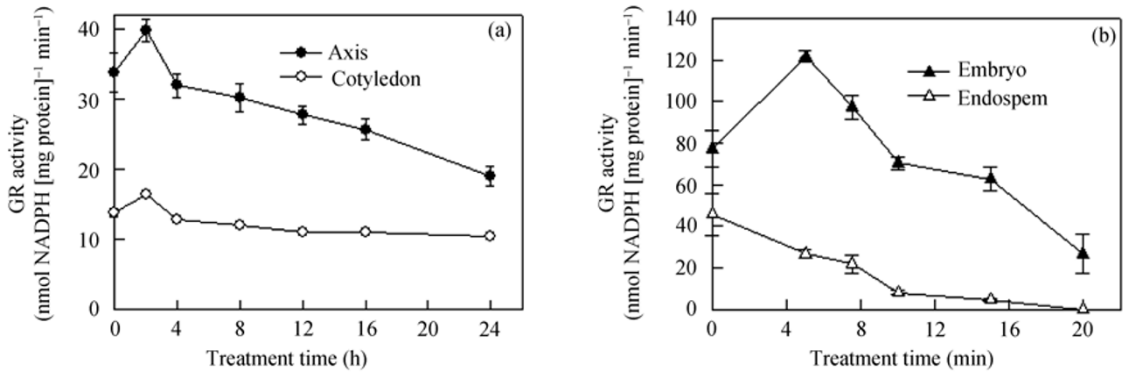

Figure 9 Changes in GR activity. Treatment of sacred lotus and maize seeds was the same as in Figure 6. (a) lotus seed; (b) maize seed. Glutathione reductase activities of axes and cotyledons of dry lotus seeds (control) were 21.25±2.87 and 11.60±0.07 nmol NADPH [mg protein]$^{-1}$ min^{-1}, respectively; those of embryos and endosperms of dry maize seeds (control) were 53.19±2.04 and 38.76±2.57 nmol NADPH [mg protein]$^{-1}$ min^{-1}, respectively. All values are means ± SD of four replicates of 50 seeds each.

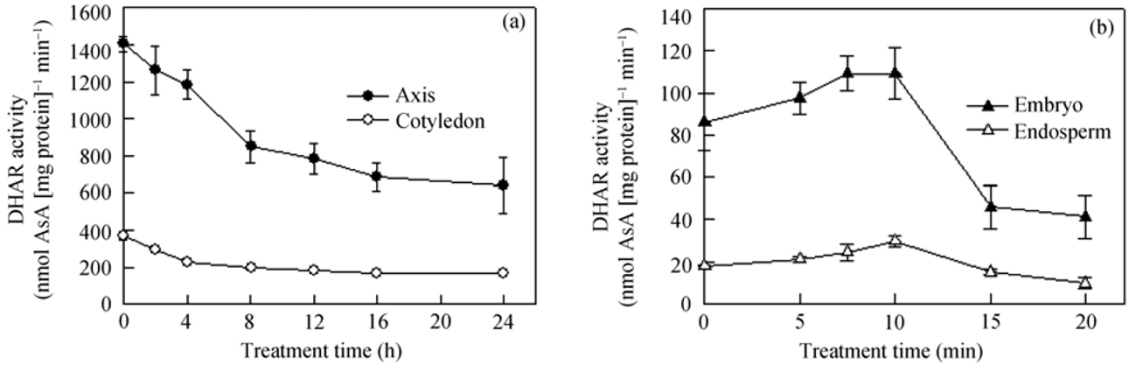

Figure 10 Changes in DHAR activity. Treatment of sacred lotus and maize seeds was the same as in Figure 6. (a) lotus seed; (b) maize seed. Dehydroascorbate reductase activities of axes and cotyledons of dry lotus seeds (control) were 946.38±117.54 and 361.75±31.43 nmol AsA [mg protein]$^{-1}$ min^{-1}, respectively; those of embryos and endosperms of dry maize seeds (control) were 53.41±15.10 and 11.41±1.89 nmol AsA [mg protein]$^{-1}$ min^{-1}, respectively. All values are means ± SD of four replicates of 50 seeds each.

ure 1(b)), but that of lotus seeds treated at 100℃ for 24 h was 13.5% (Figures 1(a) and 2). High-temperature tolerance for germination of lotus 'Tielian' seeds in our study was different from that of Huang et al.[15], who found that germination percentage of lotus 'Gan 62' seeds was 100% after treatment in air at 100℃ for 24 h (water content of seeds was 2.1%, on a fresh weight basis). The time in which 50% seeds were killed at 100℃ for lotus and maize seeds was 14.5 h and 6 min, respectively, showing that sensitivity of maize seeds to high-temperature stress is much higher than that of lotus seeds. Our experiments also indicated that high-temperature tolerance of lotus seeds was much higher than that of their fruits (data not shown). These results were similar to those of Tang[14]. However, the reason why pericarp has an effect on high-temperature tolerance of seeds is unknown. The lotus pericarp has a hard and compact structure, which makes it difficult for water and oxygen to penetrate it[7]. We found that the water content of seeds of lotus fruits treated at 100℃ for 2—12 h was higher than that of lotus seeds alone treated at 100℃ for same time (data not shown). At this temperature, fruits with higher water content might more easily be injured than seeds with lower water content. Furthermore, lotus seed was also highly desiccation tolerance. For example, germination percentage of seeds was 72.5% when axes were dehydrated to a water content of 0.003 ±0.000 g g^{-1} (treated at 100℃ for 4 h) (Figure 1).

With increasing treatment time at 100℃, fresh weight of seedlings produced by surviving lotus and maize seeds (Figure 1) and total chlorophyll content of lotus axes (Figure 3) gradually decreased, and relative elec-

trolyte leakage of lotus axes progressively increased (Figure 3). These results showed that during treatment at 100℃ lotus and maize seeds were gradually damaged, and that loss of high-temperature tolerance is a quantitative feature. When treatment at 100℃ was less than 12 h, the integrity of subcellular structures of hypocotyl were maintained. When treatment was longer than 12 h, plasmolysis occurred, endoplasmic reticulum became unclear, nuclei and nucleoli broke-down, most of the mitochondria swelled, and lipid granules accumulated at cellular periphery. Treatment for 24 h resulted in collapse of organelles and plasmalemmas (Figure 4). This gradual degredation of subcellular structures indicated that loss of high-temperature tolerance of lotus seeds was a quantitative feature.

Malondialdehyde is the final products of lipid peroxidation, and its amount could express the degree of lipid peroxidation. In addition, MDA is poisonous to plant cells. MDA content of lotus axes and cotyledons declined within 12 h of treatment at 100℃ and then rose (Figure 5). This shows that lotus axes and cotyledons could effectively decrease lipid peroxidation during the early phase of treatment at 100℃, and lipid peroxidation increased with increasing treatment time. MDA content of maize embryos and endosperms increased during the early phase of treatment at 100℃ and then decreased (Figure 5), showing that maize embryos and endosperms had a low high-temperature tolerance than lotus axes and cotyledons, and that lipid peroxidation easily occurred. However, the reason why MDA content of maize embryos and endosperms decreased during the late phase of treatment is not known. Enhancement of lipid peroxidation might be a principal cause of seed deterioration at 100℃[24]. Loss of viability and declining vigour were closely associated with increase in lipid peroxidation in rapidly aged soybean seeds[25].

The $\cdot O_2^-$ and H_2O_2 are synthesized at very high rates in the cells even under optimal conditions[9]. The chief toxicity of $\cdot O_2^-$ and H_2O_2 is thought to reside in their ability to initiate a cascade of reactions that result in production of hydroxyl radicals and of other destructive species such as lipid peroxides[26]. Enzymatic ROS scavenging mechanisms in plants include SOD, APX and CAT. SODs act as the first line of defense against ROS, dismutating superoxide to H_2O_2. APX and CAT subsequently detoxify H_2O_2. APX requires an ascorbate and glutathione regeneration system. GR can regenerate GSH from GSSG using NAD(P)H as a reducing agent[9, 24, 26].

Activities of lotus axis and cotyledon SOD (Figure 6(a)) and GR (Figure 9(a)) and lotus axis CAT (Figure 8(a)) increased during the early phase of treatment at 100℃ and then decreased. These results indicated that activities of these enzymes could be induced during the early phase of treatment at 100℃, but they decreased with increasing treatment time. This correlated with MDA content which decreased and then increasd (Figure 5), and with a higher germination percentage of seeds and an intact subcellular structure of hypocotyls within 12 h of treatment at 100℃ (Figures 1(a), 2 and 4). Activities of lotus axis and cotyledon APX (Figure 7(a)) and DHAR (Figure 10(a)) and lotus cotyledon CAT (Figure 8(a)) gradually decreased with increasing treatment time at 100℃, showing that these enzymes were very sensitive to treatment at 100℃. Activities of maize embryo and endosperm SOD (Figure 6(b)) and DHAR (Figure 10(b)) and embryo GR (Figure 9(b)) increased during the early phase of treatment at 100℃, and then decreased, which suggested that activities of these enzymes could be induced during the early phase of treatment at 100℃. However, they decreased with increasing treatment time. Activities of maize embryo and endosperm APX (Figure 7(b)) and CAT (Figure 8(b)) and endosperm GR (Figure 9(b)) rapidly decreased with increasing treatment time at 100℃, showing that these enzymes were also very sensitive to treatment at 100℃. Tsang et al.[27] found that cytosolic CuZn-SOD was conspicuously induced by high temperature stress. CAT is indispensable for oxidative stress tolerance because transgenic tobacco plants with suppressed CAT have enhanced ROS levels in response to both abiotic and biotic stresses[28]. CAT activities in most plants obviously decrease under high-temperature stress[29,30]. Mohan et al. [31] reported that GR acts by preventing oxidation of enzymes and membranes under high- and low-temperature stresses.

It was especially noted that with increasing treatment time at 100℃, decrease in germination percentage of lotus and maize seeds were closely related to decline in activities of SOD, APX, CAT, GR and DHAR; changes

in activities of these enzymes were a feature of organ and species. Compared with maize seeds, activities of SOD, APX, CAT, GR and DHAR of lotus axes and cotyledons decreased slowly, and those of maize embryos and endosperms rapidly declined during high-temperature treatment (Figures 6－10).

Lotus seeds, which have great longevity and extremely high-temperature tolerance, are a good material for studying seed deterioration and high-temperature stress tolerance. Increasing evidences has shown that high-temperature stress tolerance in plants is not only associated with antioxidant system[10,11] but also with HSP[7,10], especially with low-molecular weight HSP[32]. Suzuki and Mittler[33] suggested that heat stress-response signal transduction pathways and defense mechanisms involving HSF and HSP are intimately associated with ROS and that HSFs are involved in the sensing of ROS.

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