

Full Length Research Paper

# Recovery of antioxidant gene expression in sacred lotus (*Nelumbo nucifera* Gaertn.) embryonic axes enhances tolerance to extreme high temperature

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Sacred lotus (*Nelumbo nucifera* Gaertn.) seed is long-living and have various stress-resistance characteristics. We investigated the protecting mechanisms of lotus seeds against extreme high temperature by comparison of expression patterns of antioxidant genes in embryonic axes between exposure and non-exposure to extreme high temperature. It was shown that viability of seeds did not severely decline after exposure to 90°C for 24 h. Germination and growth were inhibited and H<sub>2</sub>O<sub>2</sub> was accumulated at high level in the lotus embryonic axes germinated after heat treatment. Transcriptional levels of superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), glutathione peroxidase (GPX) and thioredoxin-dependent peroxidase (TPX) encoding genes were induced to rise at late germination stage. Transcriptional levels of APX, POD, GPX and alternative oxidase (AOX) encoding genes were also immediately stimulated and up-regulated after heat treatment. These results suggest that the embryonic axes of sacred lotus maintain a protective and recovery mechanism from heat damage during and after exposure to extreme high temperature. Furthermore, the recovery of antioxidant gene expression enhanced tolerance to extreme high-temperature stress in sacred lotus.

**Key words:** Antioxidant gene, high temperature, seed germination, *Nelumbo nucifera* Gaertn.

## INTRODUCTION

Environmental conditions, including high temperature, are sources of stress to which the plant responds by excessive production of oxygen radicals in plant cells. Reactive oxygen species (ROS) are regarded as the main source of damage to cells under biotic and abiotic stresses (Mittler, 2002; Candan and Tarhan, 2003; Bor et al., 2003; De Gara et al., 2003; Vaidyanathan et al., 2003;

Esfandiari et al., 2007). ROS damage vital cell components such as nucleotides, proteins and lipids and disturb the cell metabolism and ultimately cause cell death (Burke, 2001; Vranová et al., 2002; Sakihama et al., 2002). In the course of evolution, plants have developed complicated antioxidant systems to eliminate or reduce ROS, which are effective in combating different levels of stress-induced deterioration, maintaining the oxidative balance and efficient functioning of the cell (Baek and Skinner, 2003; Dąbrowska et al., 2007; Esfandiari et al., 2007). The intrinsic antioxidant system plays an essential role in controlling the concentration of ROS in plant cells and tissues (Alscher et al., 1997; Mallick and Mohn, 2000; Van Breusegem et al., 2001).

The sacred lotus (*Nelumbo nucifera* Gaertn.) is a large aquatic plant which is primitive angiosperm and one of the most ancient seed plants (Les et al., 1991). sacred lotus is cultivated as a vegetable, medicine and ornamental

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**Abbreviations:** AOX, Alternative oxidase; APX, ascorbate peroxidase; DAB, 3,3'-diaminobenzidine; GPX, glutathione peroxidase; GSH, glutathione; PCR, polymerase chain reaction; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; TPX, thioredoxin-dependent peroxidase; Trx, thioredoxin.

crop in some countries such as China, Japan and India (Liu et al., 2004; Masuda et al., 2006; Rai et al., 2006). Lotus seeds are reported to exhibit strong free radical scavenging activity as medicine (Rai et al., 2006). Sacred lotus seeds have the world record of seed longevity, with 80% viability observed in 200 to 1,300-year-old fruits (Shen-Miller et al., 2002). In order to germinate upon imbibition, lotus seeds after long-term storage must have a system to cope with accrued severe cellular impairment and recover quickly from stress damage. The great ability of sacred lotus seeds to survive in extreme stress conditions has been well documented. The viability of lotus embryos has evidently been little affected by exposure to a total maximum dose of  $\gamma$ -radiation of 3 Gy accumulated over 1300 years (Shen-Miller et al., 2002).

Sacred lotus seeds are found to have superior heat resistance capacity, with those of some cultivars maintained at 13.5 to 100% viability after 24 h treatment in an air oven at an extreme high temperature of 100°C (Huang et al., 2003; Ding et al., 2008). It is suggested that some repair proteins such as L-isoaspartyl methyltransferase, heat-resistant proteins, antioxidant enzymes and annexin could minimize seed damage and help the seed survive harsh temperatures (Oge et al., 2008; Shen-Miller, 2002; Chu et al., 2011). However, the precise molecular basis of the recovery under stress and post-stress is unclear. In this study, we measured the expression levels of genes encoding antioxidant enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), peroxidase (POD), glutathione peroxidase (GPX), thioredoxin-dependent peroxidase (TPX) and Alternative oxidase (AOX) in lotus embryonic axes during the recovering process of the germination after exposure to extreme high temperature. The evaluation of the antioxidant gene expression will provide more detail understanding to the roles of enzymatic antioxidant system in resistance of extreme high-temperature stress in sacred lotus seeds and germinating seedlings.

## MATERIALS AND METHODS

### Plant materials and extreme high-temperature treatment

Seeds of sacred lotus (*Nelumbo nucifera* Gaertn. cv. Ganlian 62) were obtained from Institute of Guangchang White Lotus, Jiangxi Province, China. The mature seeds with intact seed shell were stored in a sealed container with caustic lime which kept lower than 2.6% water content in seeds. To facilitate germination, the top parts of the seeds were held in a pair of pliers and cut to pierce through the dark exterior of the seed shells before treatment. This will allow water to enter the seeds in the subsequent germination assay. For high-temperature treatment, seeds were placed in glass plates and incubated at 90°C for 24 h in an air oven (Chu et al., 2011).

Untreated seeds were used as controls. After treatment the seeds (20 seeds per plates, 5 replicates for each treatment) were then incubated in distilled water at 25°C under 16L/8D conditions in growth chamber to assess their ability to germinate. Samples were taken every 12 h within 60 h at its germination stage for measurement, photograph and 3,3'-diaminobenzidine (DAB) stain. Embryonic axis samples for ribonucleic acid (RNA) preparation were

immediately frozen in liquid nitrogen and stored at -80°C until use.

### Germination assay and embryonic axis elongation assay

Embryonic axes were measured every 12 h at its germination stage. The average lengths were compared using *t*-test to determine whether there was any significant difference between the treated and control embryonic axes. Difference were considered statistically significant when the *p* value was <0.05 and <0.01.

### In vivo detection of H<sub>2</sub>O<sub>2</sub>

The accumulation of H<sub>2</sub>O<sub>2</sub> in embryonic axes was visually detected by using DAB according to Liu et al. (2011). The embryonic axes were immersed in 1 mg/ml solution of DAB, pH 3.8, incubated for 12 h at 25°C. The reaction was terminated by immersion of the embryonic axes in destaining solution (mixture of 20% acetic acid, 20% glycerol and 60% ethanol) and boiled for 10 min, photographed after cooling. The brown spots were characteristic of the reaction of DAB with H<sub>2</sub>O<sub>2</sub>.

### RNA isolation and quantitative real-time PCR analysis

Total RNA was isolated from developing embryonic axes by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The isolated RNA was then subjected to reverse transcription by using the PrimeScript II 1st strand cDNA synthesis kit (TaKaRa, Japan). Primer pairs of *NnMSD1*, *NnCSD1*, *NnAPX1*, *NnAPX2*, *NnPOD*, *NnGPX*, *NnTPX*, *NnAOX1a* and *NnAOX1b* were designed according to complete cDNA or EST sequences deposited in the GenBank (Table 1). Quantitative real-time PCR was performed on each cDNA sample with the SYBR Green Master Mix (TOYOBO, Japan) and analyzed with an IQ<sup>TM</sup>5 Multicolor Real-time PCR Detection System (Bio-Rad) following the manufacturer's instructions. Amplification of *18S rRNA* was used as an internal control (Li et al., 2009). The program was 95°C denaturation for 30 s, 40 cycles of 95°C for 10 s, 57°C for 15 s, and 72°C for 20 s. Each data point has three replicates. The data were analyzed using the IQ<sup>TM</sup>5 Optical System Software (Bio-Rad) by employing an optimized comparative Ct ( $\Delta\Delta$ Ct) value method.

## RESULTS

### Effects of extreme high temperature on germination of sacred lotus seeds

To determine heat resistance capacity, the sacred lotus seeds were subjected to extreme high-temperature treatment at 90°C for 24 h. The sacred lotus seeds were imbibed in distilled water right after treatment. Approximately 86% of sacred lotus seeds remained alive and germinated (Figure 1A). After imbibition in water, the lotus seed shells treated with high temperature were spongier and less tightknit compared with the control, and seedlings showed growth inhibited in the following germination stages (Figure 1B). The treated embryonic axes and cotyledons showed slightly browning in the first 36 h of germination. Retardance of growth could be detected after 12 h of post-stress germination, the

**Table 1.** Primers used in this study.

Gene name	Primer	Accession numbers
<i>NnMSD1</i>	Forward primer (5'-3'): TTATGCGGCTCCATCACCA <sup>a</sup> Reverse primer (5'-3'): TAACCACTGCGGACGAATC <sup>a</sup>	AY934858
<i>NnCSD1</i>	Forward primer (5'-3'): TACTTTACCGAGGAAGAAGATGGAT Reverse primer (5'-3'): CTTTGCTTTGAGGATTGAAATGTGG	GQ149102
<i>NnAPX1</i>	Forward primer (5'-3'): GTCCCCATCACCTGCTACTCAT Reverse primer (5'-3'): GGCTTCCCCATCCACTACGCTC	EF419885
<i>NnAPX2</i>	Forward primer (5'-3'): ACATCCTCAAAGTGGGCGAAG Reverse primer (5'-3'): TCAAACACCGTGTGGTAGCTG	EE987078
<i>NnPOD</i>	Forward primer (5'-3'): ACTGGGAAGGCGTGATGGGC Reverse primer (5'-3'): GCGAGAGGTGAAGGAGGTGC	EE986736
<i>NnGPX</i>	Forward primer (5'-3'): TCAGGAGCCAGGGACTAATGAGCAGA Reverse primer (5'-3'): GCCCTCTTTGTCAACCAGGAACCTT	EF421198
<i>NnTPX</i>	Forward primer (5'-3'): GGTCTCCATTCCTCCCTCGCTGTCCG Reverse primer (5'-3'): TAGGTCTTCGCCCATGCCTTCATCAC	EF421194
<i>NnAOX1a</i>	Forward primer (5'-3'): GAGTCTTCCTGTTTTCTTCATGC Reverse primer (5'-3'): CCCTTCTCTCTTTTTCTTTGTT	AB491175
<i>NnAOX1b</i>	Forward primer (5'-3'): GGGTGGAGATGAACAAACGC Reverse primer (5'-3'): GCCCCAGTAACCTTACGATT	AB491176
<i>18S rRNA</i>	Forward primer (5'-3'): CCATAAACGATGCCGAC <sup>a</sup> Reverse primer (5'-3'): CACCACCCATAGAATCAAGA <sup>a</sup>	

<sup>a</sup>Primer sequences according to Li et al. (2009).

embryonic axis elongation of treated seeds was significantly suppressed after 36, 48, and 60 h (Figure 2A) ( $p < 0.05$ ,  $p < 0.01$ ). Seeds with severely retarded or inhibited germination were associated with obvious cotyledon damage and putrescence, but the embryonic axes still maintained vivid greenness in the first few days after imbibition. Therefore inhibition of germination and growth may be due to failure in nutrition supply from damaged cotyledons.

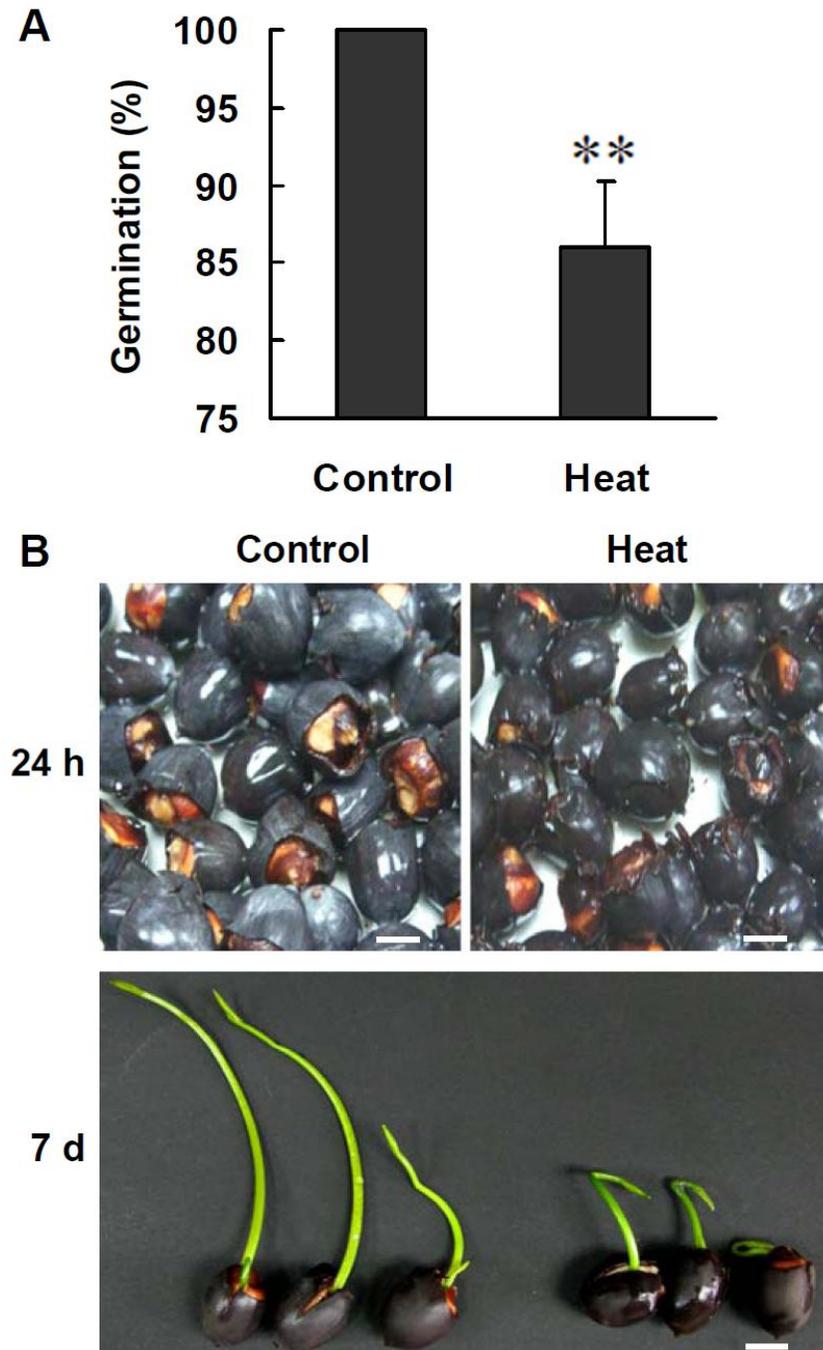
### Effects of high temperature on the H<sub>2</sub>O<sub>2</sub> accumulation

Heat-stress usually causes ROS, such as H<sub>2</sub>O<sub>2</sub> generation. To examine H<sub>2</sub>O<sub>2</sub> accumulation after heat-stress and subsequent elimination events in treated tissues, embryonic axes were subjected to DAB staining for detection of H<sub>2</sub>O<sub>2</sub>. As shown in Figure 2B, under normal conditions, the axes without treatment still had a few brown H<sub>2</sub>O<sub>2</sub> spots within the total area. The seeds germinated after high-temperature stress exhibited more brown areas within embryonic axes than those of the

corresponding control plants. After 12 to 24 h germination, both of the embryonic axes with or without high-temperature treatment showed increasing brown staining, and brown areas in treated tissues are much deeper than control. After 36 h, the H<sub>2</sub>O<sub>2</sub> level in both embryonic axis tissues gradually decreased. Though the appearance of treated embryonic axes were still a little browner than normal embryonic axes, the tissues of treated seeds after 60 h germination showed very low H<sub>2</sub>O<sub>2</sub> content. These results suggest that during germination the antioxidant system was activated and efficiently eliminated H<sub>2</sub>O<sub>2</sub> produced under stress and post-stress repairing.

### Changes in expression of antioxidant genes

The expression of the protective antioxidant genes in embryonic axes tissues treated by high temperature was investigated and compared with untreated control. During germination, the expression of two *Nelumbo nucifera* SOD encoding genes *NnMSD1* and *NnCSD1* in embryonic axes kept at relatively low level of expression

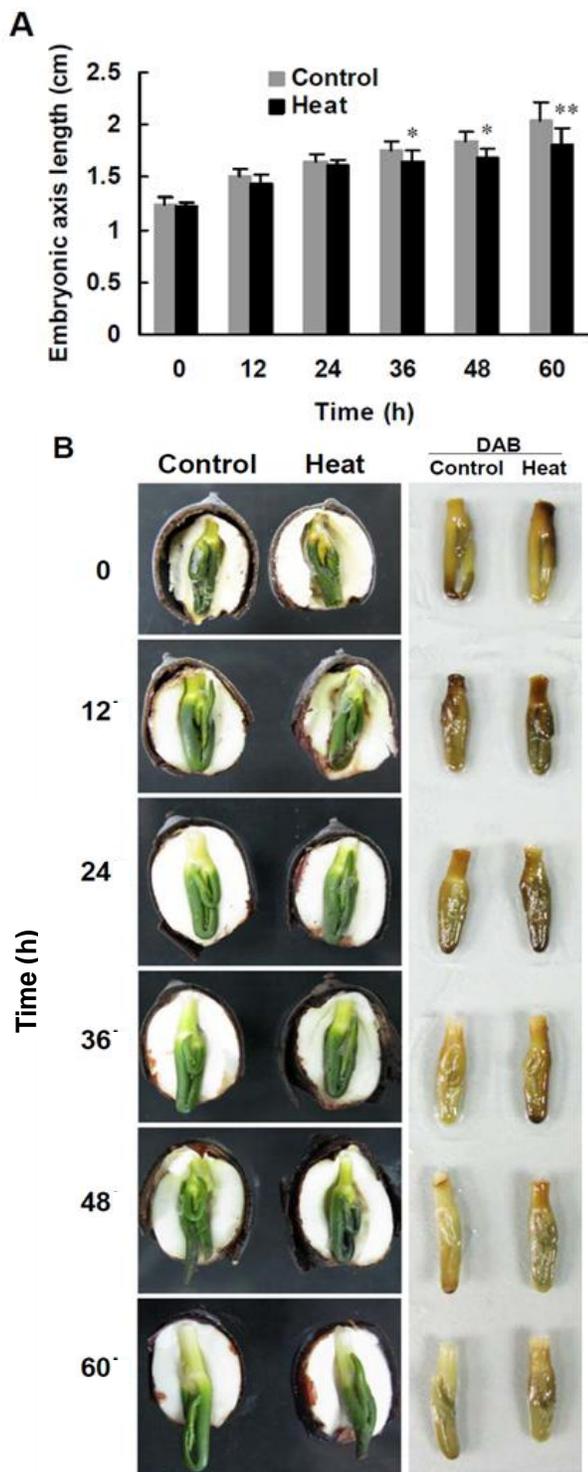


**Figure 1.** Germination assays of sacred lotus seeds. (A) Quantitative analysis of germination. (B) sacred lotus germinating seeds and germinated seedlings. Germination rates were calculated 7 days after high-temperature treatments. Percentages are means of five replicates of 100 seeds ( $5 \times 100$ ) (mean  $\pm$  SD). \*\* indicates extremely significant at  $P < 0.01$  (Student's *t*-test). Bar = 1.0 cm.

in the first 48 h of germination under high-temperature stress treatment. However, at 60 h germination, the expression of *NnMSD1* and *NnCSD1* were shown to increase 3.38-fold and 3.44-fold, respectively (Figure 3). It is indicated that transcription of these two *SOD* genes

increased subsequently rather than immediately increase by heat-stress treatment.

Expression analysis of two APX encoding genes *NnAPX1* and *NnAPX2* revealed that the expression of both genes had been stimulated and rose immediately



**Figure 2.** Effects of extreme high temperature on elongation of sacred lotus embryonic axes and H<sub>2</sub>O<sub>2</sub> accumulation detection by DAB stain. (A) Lotus seeds embryonic axis elongation measurement. Each column represents an average of three replicates and each with 10 seeds (mean±SD). \* and \*\* indicate significant differences from the corresponding control at  $p < 0.05$  and  $p < 0.01$  respectively. (B) Lotus seeds germinated in distilled water for H<sub>2</sub>O<sub>2</sub> accumulation detection. Browning of cotyledons and retardance of growth could be detected after post-stress germination.

after the extreme high-temperature treatment in embryonic axes compared with the control. In the following 36 h, the expression of APXs fluctuated at a relatively low level and then bounced to higher level than the control at 48 h (*NnAPX1* expression increased 4.55-fold and *NnAPX2* expression increased 4.53-fold) and 60 h (*NnAPX1* expression increased 1.12-fold and *NnAPX2* expression increased 3.11-fold) after germination (Figure 4).

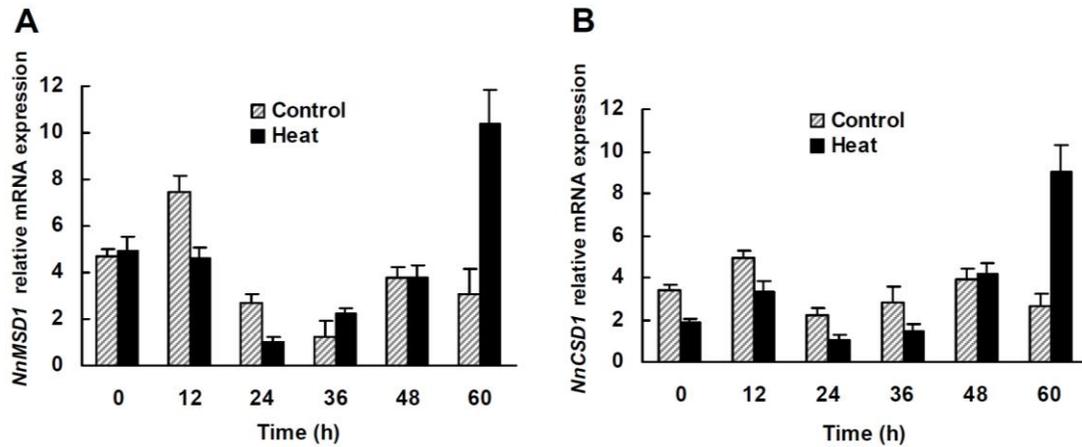
In heat-treated embryonic axes, the *NnPOD* expression was increased at 0, 24, 48 and 60 h (1.42-, 2.59-, 2.23- and 4.06-fold compared with control respectively) (Figure 5). *NnGPX* expression was upregulated at 0 and after 48 h (2.72-, 2.33- and 3.94-fold compared with the control at 0, 48 and 60 h respectively) (Figure 6A). Expression changes of *NnTPX* in heat-stressed embryonic axes were mostly subsided except at 12 and 60 h (Figure 6B). These results indicated that the expression of *POD* and *GPX* genes have similar up-regulated patterns as *APXs* under stress treatment, but transcriptional regulation of *TPX* expression was different.

Expression level of two *AOX* genes *NnAOX1a* and *NnAOX1b* were also analyzed considering *AOX* serve a general function in plant by limiting mitochondrial ROS formation (Maxwell et al., 1999). The results revealed that in embryonic axes the expression of both *AOX* encoding genes increased immediately after extreme high-temperature treatment compared with control (*NnAOX1a* expression increased 2.32-fold and *NnAOX1b* expression increased 3.56-fold). But in the following germination stage the expression of *AOXs* tended to fluctuated. After 48 h of germination the expressions of *NnAOX1a* were at similar low levels as control, while expression levels of *NnAOX1b* were slightly up-regulated (Figure 7).

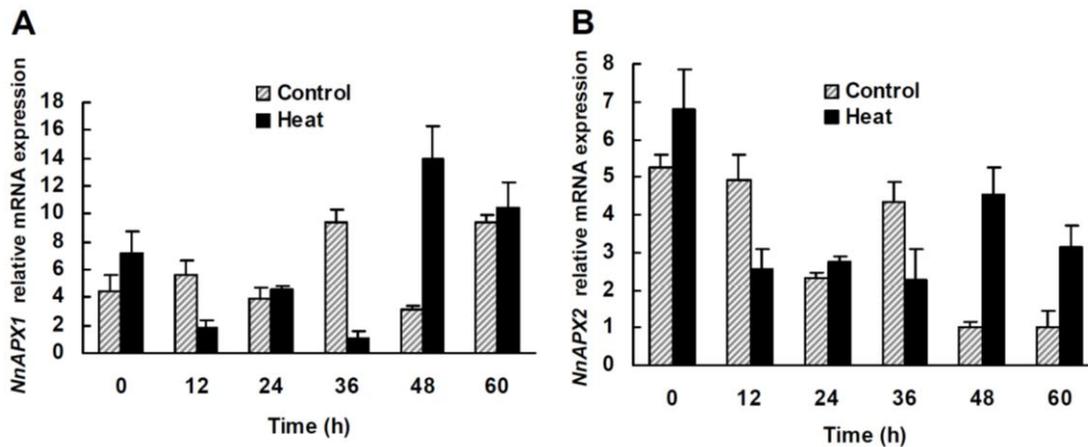
## DISCUSSION

The superior tolerance to extreme high temperature of sacred lotus seeds may partially be due to its heat resistant antioxidant system. The developmental transcriptional levels of different antioxidant genes in control embryonic axes showed some temporal changes, and these may be caused by the effects of germination stage and photoperiod. Analyses of the developmental and extreme high temperature-mediated changes in the expression of different antioxidant genes showed that more than half of the genes analyzed had slightly upregulated immediately after high-temperature treatment, and slipped in the following 12 to 36 h. In stressed embryonic axes, the transcriptions of antioxidant genes except *AOXs* underwent a retarded increase process, and reached compensatory higher level than the control at 48 to 60 h germination, indicating a repairing process during the recovery from high-temperature stress.

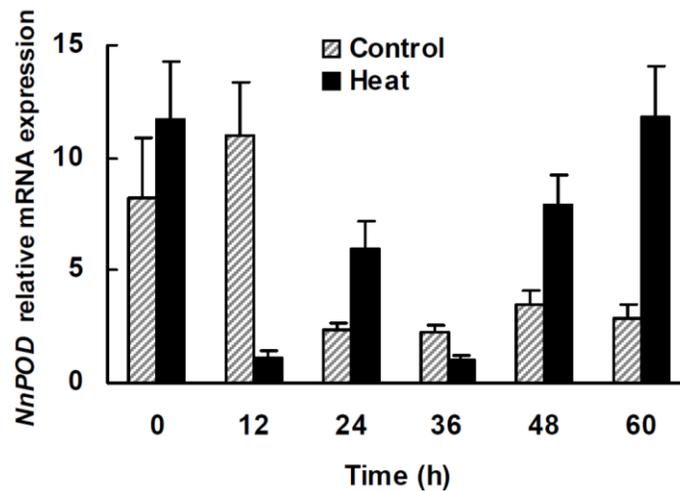
SOD is the key enzyme in the active oxygen-scavenging system because it catalyzes the dismutation of super-oxide free radical into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub>. According to



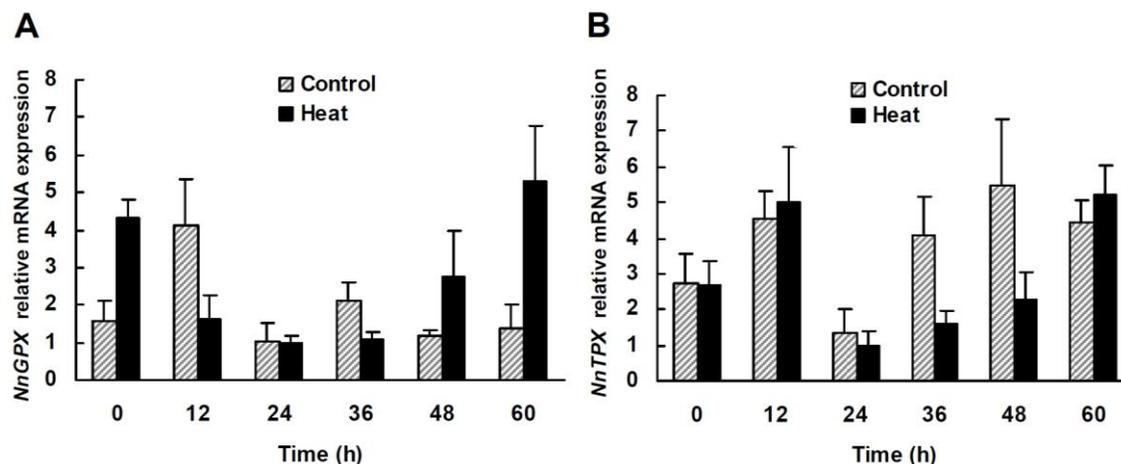
**Figure 3.** Expression pattern of *NnMSD1* and *NnCSD1* genes in lotus embryonic axes. Error bars indicate SD values of triplicate measurements.



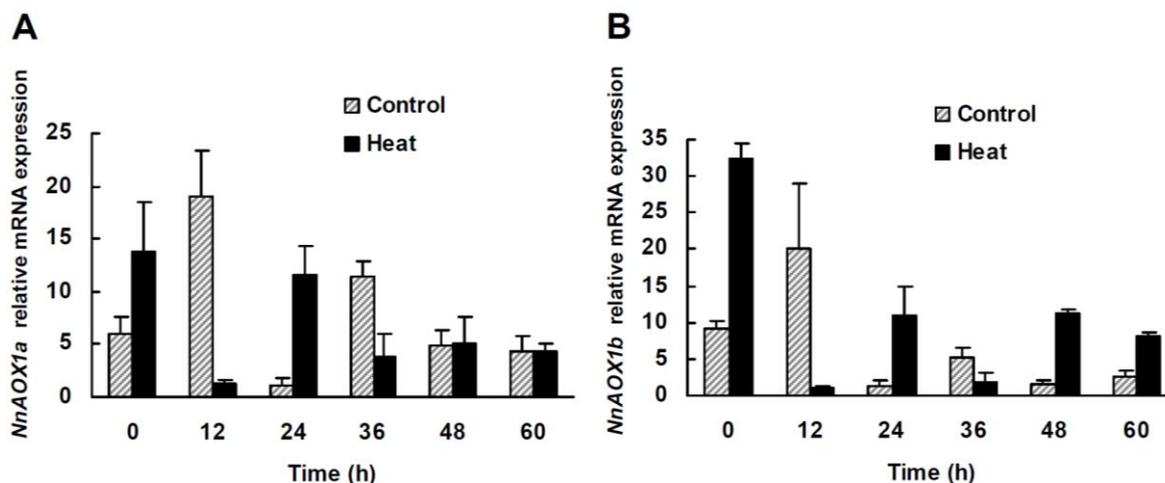
**Figure 4.** Expression pattern of *NnAPX1* and *NnAPX2* genes in lotus embryonic axes. Error bars indicate SD values of triplicate measurements.



**Figure 5.** Expression pattern of *NnPOD* gene in lotus embryonic axes. Error bars indicate SD values of triplicate measurements.



**Figure 6.** Expression pattern of *NnGPX* and *NnTPX* genes in lotus embryonic axes. Error bars indicate SD values of triplicate measurements.



**Figure 7.** Expression pattern of *NnAOX1a* and *NnAOX1b* genes in lotus embryonic axes. Error bars indicate SD values of triplicate measurements.

Ushimaru et al. (2001), there were at least 7 isozymes of SOD in lotus seeds detected by activity gel staining. And SODs in lotus seeds is very stable after high-temperature treatment in vitro (Huang et al., 2003). Previous study indicated that the *NnMSD1* expression was inhibited in embryonic axes during the first 48 h of germination after heat shock treatment with 42°C (Li et al., 2009). Our results also showed that the expression of *NnMSD1* in high-temperature treated embryonic axes kept at relatively low level of expression compared with the control in the first 48 h, but subsequently up-regulated at 60 h. The dramatically up-regulated expression of lotus SOD genes in extreme high-temperature treated axes at late germination stage suggest an enhancement of ROS-scavenging ability after recovering from heat-stress suppression.

APX is a key defense enzyme against H<sub>2</sub>O<sub>2</sub> toxicity in developing seeds and decreases in APX occurring during the aging of seeds can be correlated with the onset of a biochemical pathway leading to morphological anomalies of seedlings and to the loss of seed germination capacity (Arrigoni et al., 1992; De Gara et al., 1997). POD can catalyze the oxidation of many substrates (for example, phenolic compounds) at the expense of H<sub>2</sub>O<sub>2</sub> (Asada, 1999; Lee and Lee, 2000). GPX involved in the detoxification of H<sub>2</sub>O<sub>2</sub> and organic hydroperoxides by reducing them to water and the corresponding alcohol to protect the organism from oxidative damage (Fischer et al., 2009). TPX shares high sequence identity with GPX and specifically uses Trx, but not GSH, in catalyzing the reduction of hydroperoxides (Jung et al., 2002). The quick responses in transcriptional up-regulation of *NnAPXs*,

*NnPOD* and *NnGPX* genes at the early stage after exposure to extreme high temperature and after 48 h of germination may play a significant role in eliminating the toxic H<sub>2</sub>O<sub>2</sub> in embryonic axes tissue cells.

AOX is a very important enzyme that catalyzes the alternative pathway respiration and acts as a terminal oxidase in the electron transport chain. Energy is released as heat via this alternative respiration. AOX plays a central role in flowers of sacred lotus for regulating their temperature with remarkable precision (between 30 and 35°C) against fluctuations at ambient temperature (Watling et al., 2008; Grant et al., 2009). AOX has also been reported as an important ROS scavenger (Maxwell et al., 1999). AOX respiration acts to reduce the generation of ROS in plant mitochondria by dampening the leak of single electrons from the electron transport chain to O<sub>2</sub> (Cvetkovska and Vanlerberghe, 2012). AOX encoding gene expression levels had an immediately up-regulation after extreme high-temperature treatment indicated that *NnAOXs* may also involved in ROS-scavenging in sacred lotus embryonic axes tissues, especially at the very early stage after heat treatment.

Based on these analyses, we propose that sacred lotus embryonic axes had a protection system which can maintain and quickly recover activities of vital biomolecules such as nucleotides during and after extreme high-temperature stress. The survival of sacred lotus seeds from extreme heat stress involved ROS scavenging and detoxification. And the antioxidant system in sacred lotus embryonic axes may play a vital role of enhancing plant stress tolerance through the recovery of antioxidant gene expression.

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