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Expression of Heat Shock Protein and Antioxidant Genes in Rice Leaf Under Heat Stress

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ABSTRACT

We have previously investigated the proteome changes of rice leaves under heat stress (Lee et al. in Proteomics 2007a, 7:3369-3383), wherein a group of antioxidant proteins and heat shock proteins (HSPs) were found to be regulated differently. The present study focuses on the biochemical changes and gene expression profiles of heat shock protein and antioxidant genes in rice leaves in response to heat stress (42°C) during a wide range of exposure times. The results show that hydrogen peroxide and proline contents increased significantly, suggesting an oxidative burst and osmotic imbalance under heat stress. The mRNA levels of chaperone 60, HSP70, HSP100, chloroplastic HSP26, and mitochondrial small HSP responded rapidly and showed maximum expression after 0.5 or 2 h under heat stress. Transcript levels of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and Cu-Zn superoxide dismutase (Cu-Zn SOD) showed a rapid and marked accumulation upon heat stress. While prolonged exposure to heat stress resulted in increased transcript levels of monodehydroascorbate reductase, peroxidase, glyoxalase 1, glutathione reductase, thioredoxin peroxidase, 2-Cysteine peroxiredoxin, and nucleoside diphosphate kinase 1, while the transcription of catalase was suppressed. Consistent with their changes in gene expression, the enzyme activities of APX and DHAR also increased significantly following exposure to heat stress. These results suggest that oxidative stress is usually caused by heat stress, and plants apply complex HSP- and antioxidant-mediated defense mechanisms to cope with heat stress.
(**Key words :** Antioxidant, Heat stress, HSP, Rice, Transcriptomic)

I INTRODUCTION

Rice is one of the most important cereals that widely consumed by the large part of the world human population (ProSTAT, 2006). Population is sharply increasing, creating more pressure on agriculture to produce more food, animal protein and livestock feed. Therefore, development of crop with new traits and its alternative use is prime important within our limited resources. Recently in Korea, it has been started to use rice as a forage crop for animal feed. Environmental stresses reduce crop yield including rice greatly. Temperature shifts accelerate the production of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Yang et al., 2006). These activated oxygen species often injure various cellular components including proteins, membrane lipids and nucleic acids (Rice-Evans et al., 1991). In order to protect cells against ROS, plants activate a number of antioxidant mechanisms that detoxify ROS. The primary components of this system include low molecular weight antioxidants (ascorbate, glutathione, carotenoids, flavonoids, and tocopherols), general antioxidative enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP), and peroxidase (POD)) and the enzymes involved in the ascorbate-glutathione (As-GSH) cycle (mono-dehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and ascorbate peroxidase (APX)) (Asada, 1996).

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In our previous study, we found that heat stress significantly increased the levels of several antioxidant proteins, including DHAR, thiorodoxin *h* and/or nucleoside diphosphate kinase 1 (NDPK1) in rice leaves (Lee et al., 2007a). Interestingly, the majority of these antioxidant proteins are involved in ascorbate-glutathione pathways. These results led us to suggest that heat stress may generate ROS in rice leaves. Although transcriptomic analyses of ROS scavenging genes responding to several biotic or abiotic stresses have been conducted extensively by several research groups (Conklin and Last, 1995; Nishikawa et al., 2003), very little attention has been paid to responses to heat stress.

Thus, the present study aims to evaluate whether heat stress generates ROS in leaves, and biochemical parameters in rice leaves were therefore investigated. In addition, the mRNA kinetics of several ROS sensitive antioxidant genes that are involved in the ascorbate-glutathione cycle, as well as other known antioxidant mechanisms and heat shock proteins, were monitored in response to heat stress. In this study, genes with stress-tolerant as well as antioxidant production in rice leaf showed better response to heat stress. Therefore, it would be very promising candidates for rice crop improvement including of human food as well as animal feed production.

II MATERIALS AND METHODS

1. Plant growth and stress treatments

Rice seedlings were grown in a growth chamber maintained at 28°C under fluorescent light with a 16/8-h (light/dark) photoperiod. Two-week old seedlings were subjected to heat stress by increasing the temperature of the growth chambers to 42°C with 60% humidity. Leaf samples were harvested 0.5, 2, 6, 12 and 24 h after treatment. Control plants grown in 28°C were harvested at the same time as the treated ones. Samples were immediately frozen in liquid nitrogen and kept at −)°C until analysis.

2. Determination of *in vivo* H_2O_2 and proline content

The H_2O_2 concentrations of treated and control leaves were measured spectrophotometrically, as described previously (Ahsan et al., 2007). Proline content was determined according to the method of Bates et al. (1973). Briefly, 0.5 g of leaf tissue was ground with liquid nitrogen and homogenized in 10 mL of 3% sulfosalicylic acid. Homogenates were centrifuged at 12,000 rpm for 20 min at 4°C. After the supernatant was added to acetic acid and ninhydrin and boiled for 1 h, its absorbance at 520 nm was measured to determine the amount of proline.

3. RNA extraction and RNA gel blot analysis

The total RNA was isolated from the leaf tissues of treated and control plants using a Plant RNeasy mini kit (Qiagen, CA, USA). Ten micrograms of RNA samples were separated on a 1.2% agarose gel containing formaldehyde. Hybridization probes were prepared by PCR with oligonucleotide PCR primers (Table 1), using rice cDNA as a template. All primers were designated according to the rice cDNA sequences from NCBI GenBank. Gene-specific PCR-products were labeled with [α-^{32}P] dCTP by a random primer labeling kit (Amersham, UK). RNA blot analysis was conducted as described previously (Ahsan et al., 2008).

4. Antioxidative enzyme assays

A total of 0.5 g of the sample of frozen leaves was ground to a fine powder with liquid nitrogen and extracted with an ice-cold 50 mM potassium phosphate buffer (pH 7.0) that contained 5 mM ethylenediaminetetraacetic acid and 10% insoluble polyvinylpolypyrrolidone (w/v). The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C, and the supernatant was used to determine enzyme activities (Kenyon and Duke, 1985). All activities were measured at 25°C in a final volume of 1 ml, using aliquots of the supernatants. The activity of ascorbate peroxidase was measured by monitoring the rate of ascorbate oxidation at 290 nm (E = 2.8 mM^{-}1 cm^{-}1). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 M H_2O_2, 0.5 mM ascorbate and an aliquot of crude enzyme (Nakano and Asada, 1981). DHAR was assayed directly by following the formation of ascorbate at 265 nm, according to Hossain and Asada (1984). The reaction

mixture contained 50 mM potassium phosphate (pH 7.0), 0.5 mM dehydroascorbate, 5 mM glutathione and an aliquot of the crude enzyme.

5. Statistical analysis

The results for the physiological parameters were analyzed statistically using Duncan's multiple range test. Amounts were considered significantly different at $p < 0.05$.

III RESULTS AND DISCUSSION

1. Physiological responses of rice leaves under heat stress

In the present study, physiological parameters, such as H_2O_2 accumulation and proline content, were investigated in rice leaves under heat stress. ROS may be generated by heat stress, which can initiate the peroxidation and destruction of lipids with subsequent membrane damage (Murakami et al., 2000). In support of this hypothesis, H_2O_2 levels in heat stressed rice leaf tissues increased up to 32% (Fig. 1A). However, H_2O_2 levels were lower after 24 h compared with only 12 h of treatment, which might be due to the activation of H_2O_2 scavengers such as antioxidant enzymes. It has also been determined that heat stress induced oxidative stress in plants, which causes lipid peroxidation and membrane injury (Song et al., 2006). In addition, the proline content was significantly increased in rice leaves under heat stress (Fig. 1B). Increased proline content is a general response to various abiotic stresses (Lee et al., 2008) to counter the toxicity generated by the stress, mediated through the generation of ROS. Proline might acts as a cellular osmoregulator, and also exhibits a number of protective effects. It has also been reported that heat stress induce the accumulation of proline in many plants (Chakraborty and Tangden, 2005), and plants with elevated level of proline show enhanced tolerance to abiotic stress (Alcázar et al., 2011).

2. mRNA and protein expression profiles of heat shock protein genes under heat stress

To investigate the transcript levels of a number of HSPs in rice leaves upon exposure to heat stress, RNA gel blot analysis was carried out. RNA gel blot analysis showed

Table 1. Primer sequences used for cDNA synthesis for RNA blot analysis

Gene name	Forward primer	Reverse primer
2-Cys Prx	CTACATCGGGAAGAAGTACG	GGTCCTCATGGTCTCATCGA
APX	AACTTCCCATCCTCTCCTAC	CAGCATATTTCTCCACCAGT
CAT	TCTCCTACTCCGACACGCAG	GTGCGTCGATCCATCTCTTG
CPN 60	ACACTGGGACCAAAGGGAAG	CATCCGCAAGCTCACTGTCT
ChlHSP26	CCCATATGCAGGAGAACAGGG	TAGGATCCATTCGTCTACTGG
CuZnSOD	GTGAAGGCTGTTGCTGTGCT	GCCAATGATGGAGTGTGCTC
DHAR	GTCGAAACCCAAAATCTTCTCTTCC	GTGCACACTTGCAACAACCATAT
HSP70	GGA GAA GTA CAA GGC GGA GG	AAA AGC TTC ATC GCA CGA AA
HSP100	GGAAGCCTTGGTTACGATCC	TTGAACTAGCAAGGGAAATTGTG
GR	TTGGTGCATCAATGTGTGGA	CCGTTCCAGGTAGAATCCCT
Glyoxlase 1	ACGGTTACATGTTTGAGCTT	CAAAATCTTTCCCCCTAGTT
MDHAR	TCACTGACTTTGGTGTTCAA	AATGCATCAGTCTTGATTCC
MtHSP	AAGATAAGCACAGTTACGCAG	ACTTATGCGATTGATCTCGT
NDPK1	GCAACCTTCACCCTTGGA	CCCATCACATCCAATCCA
POD	CTTAGCTTAATGCTGCTGGT	GTTGTAGATCCTGTCCCTGA
TPx	CAACAGAGAAGCATTTCCTC	GGGTCGAAGTAGGACAGC

2-Cys Prx (AM039889); APX (XM_479627); CAT (AY339372); CPN 60 (NP_910308); ChlHSP26 (AB020973); CuZnSOD (L19434); DHAR (AY074786); HSP70 (NP_915417); HSP100 (XP_468773); Glyoxalase 1 (AB017042); GR (D78136); MDHAR (XM_483751); MtHSP (XM_467890); NDPK1 (AP004051); POD (AF014467); TPx (XM_464429).

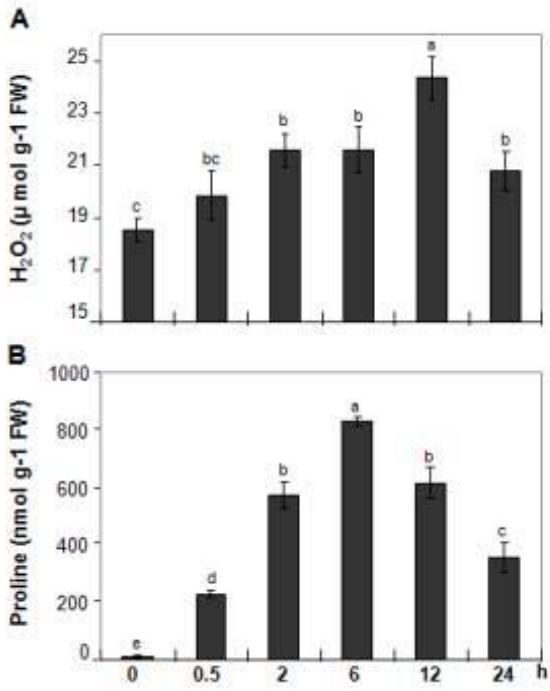

Fig. 1. Biochemical responses of rice leaves under heat stress. (A) H_2O_2 concentration; (B) proline accumulation in control (0 h), and heat stress-treated (0.5-24 h) rice leaves.

that a number of HSPs were rapidly and strongly induced under heat stress (Fig. 2A). These included chaperone 60, HSP70, HSP100, chloroplastic HSP26, and mitochondrial small HSP. Overall, the induction of HSPs was higher than the control, and these HSPs rapidly accumulated within 0.5 to 2 h of heat treatment. However, except for chaperone 60, all HSPs showed a declining pattern prior to treatment time. The transcript of chaperone 60 was strongly induced after 0.5 h under heat stress and showed a constant level until 24 h under heat stress. Transcripts of HSP100, chloroplastic HSP26, and mitochondrial small HSP were not detected under the control conditions, but accumulated greatly after heat stress. In contrast, the transcripts of chaperone 60 and HSP70 were barely detected under the control conditions. Rapid accumulation of transcripts of HSPs suggests that these HSPs play a crucial early role in heat stress or during heat acclimation. Proline might acts as a cellular osmoregulator, and also exhibits a number of protective effects. It has also been reported that heat stress induce the accumulation of proline in many plants (Chakraborty and Tangden), and plants with elevated level of proline show enhanced tolerance to abiotic stress (Alcázar et al., 2010).

3. mRNA expression profiles of antioxidant genes in rice leaves under heat stress

The effects of various environmental stresses in plants are known to be mediated, at least partially, by an enhanced generation of ROS or imposed oxidative stress. In the present study, physiological analysis of rice leaves in response to heat stress clearly revealed that heat stress generates ROS, which creates oxidative stress in rice leaves. Plants contain a complex antioxidant system to detoxify ROS (López-Gómez et al., 2007). The improvement in stress tolerance, including heat stress tolerance, is often related to the enhanced activities of the antioxidant systems in plants. Ascorbate and GSH are essential plant metabolites and powerful regulators of major cell functions, and they play a pivotal role in antioxidant defense (Foyer et al., 1994). Therefore, the present study was undertaken to investigate the mRNA kinetics of the antioxidant genes of rice leaves in response to heat stress, and possible functions of the antioxidant genes examined in this study are discussed.

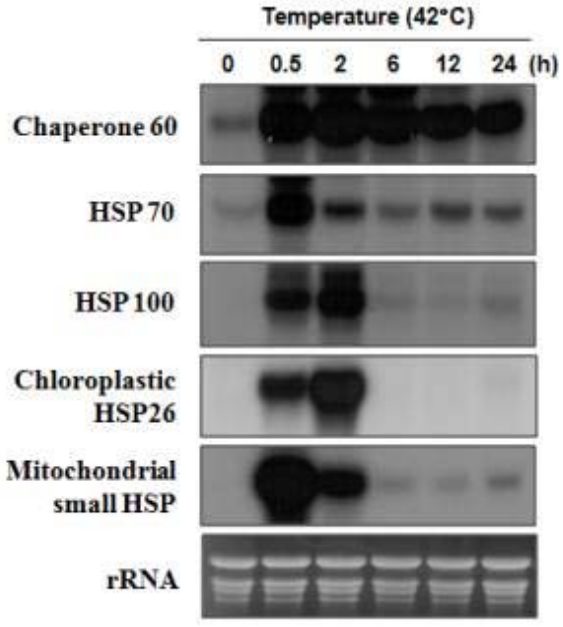

Fig. 2. Expression profiles of HSP genes in response to heat stress.

4. Responses of ascorbate cycle-dependent genes to heat stress

In this study, the transcription pattern of genes involved in the ascorbate cycle, including APX, MDHAR, and DHAR, was investigated in rice leaves exposed to heat stress (Fig. 3A). As a major antioxidant, ascorbate directly scavenges free radicals and is considered to be of paramount importance as an electron donor for H_2O_2 detoxification *via* APX in plant cells (Noctor and Foyer, 1998). APX uses two molecules of ascorbate to reduce H_2O_2 to water by generating two molecules of MDHA. In the present study, a marked accumulation of the APX mRNA transcript was observed at the initial stage of heat stress and showed a constant pattern prior to the time point examined (Fig. 3A). The mRNA, protein and activity of APXs increase in response to oxidative stress in plants, including heat (Rizhsky et al., 2002; Gómez et al., 2004).

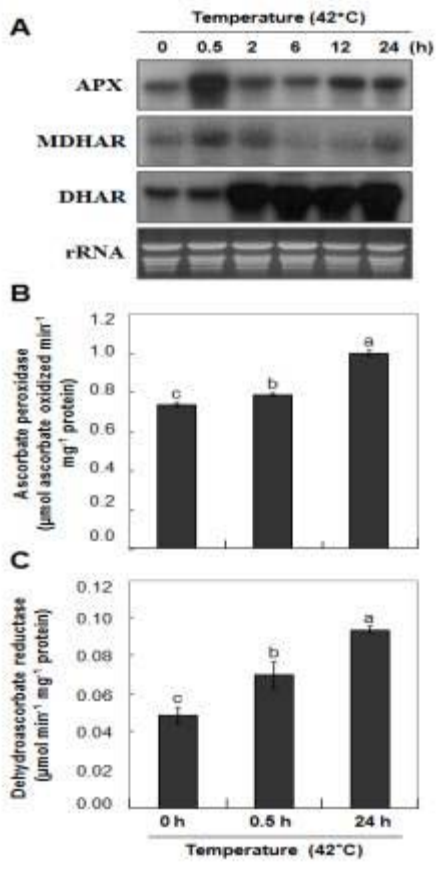

Fig. 3. Gene expression (A) and enzymatic activity (B and C) of ascorbate-cycle related genes under heat stress.

The transcript of MDHAR was slightly increased after 0.5 h of heat treatment, and then decreased with time (Fig. 3A). MDHAR is critical in maintaining the proper concentration of ascorbate in cells by reducing the monodehydroascorbate radical directly to ascorbate at the expense of an NAD(P)H (Asada, 1996). Eltayeb et al. (2007) showed that over-expression of MDHAR in tobacco confers enhanced tolerance to environmental stresses. DHAR catalyzes the reduction dehydroascorbate to ascorbate using reduced glutathione. The antioxidant activity of ascorbate is dependent upon its regeneration ability, and DHAR catalyzes the re-reduction of dehydroascorbate to ascorbate using glutathione (Kato et al., 1997). In the present study, the transcript levels of DHAR increased markedly and gradually with the time under heat stress (Fig. 3A). In addition, the enzymatic activities of APX and DHAR also correspond to their transcript levels (Fig. 3B). Consistent with our result, it has also been determined that DHAR activity rapidly increases in rice during heat stress (Urano et al., 2000). Taken together, these results suggest that the ascorbate-GSH pathway plays a great role in protecting plants against high temperature-induced oxidative stress.

5. Responses of GSH-cycle dependent genes to heat stress

Among the genes involved in the GSH cycle, the mRNA kinetics of SOD, CAT, POD, glyoxalase 1 and GR were investigated (Fig. 4). SOD is the first line of defense against injury caused by ROS, catalyzing the dismutation of O_2^- to H_2O_2 and molecular oxygen (Gómez et al., 2004). The mRNA transcripts of copper-zinc SOD (CuZnSOD) increased significantly in rice leaves under heat stress (Fig. 4). The increased activities of SODs have also been investigated in plant responses to several environmental stresses (Gómez et al., 2004). These results suggest that CuZnSOD detoxifies the heat stress-induced superoxides in rice leaves. CAT catalyzes the oxidation of H_2O_2 (Asada, 1992). In the present study, the transcript level of CAT declined rapidly in response to heat stress (Fig. 4). A similar observation was also noted by Liu et al. (2008): CAT activity decreased in Kentucky bluegrass under heat and drought stress. Thus, a decrease in CAT activity can

result in H_2O_2 accumulation, which can react with O_2^- to produce hydroxyl-free radicals *via* the Herbert-Weiss reaction (Elstner, 1982). POD is known to utilize substrates to metabolize H_2O_2 (Polle et al., 1994). We observed that POD increased rapidly in response heat stress and showed a constant level prior to the time the sample was examined (Fig. 4). POD decomposes H_2O_2 by the oxidation of co-substrates, such as phenolic compounds and/or antioxidants (Blikhina et al., 2003). Glyoxalase I is the key enzyme of the glyoxalase pathway, which plays a pivotal role in the detoxification of methylglyoxal in the presence of the glutathione formed from several abiotic stresses (Espartero et al., 1995). In the present investigation, we also examined glyoxalase I transcripts, which showed a gradual increase in response to heat stress (Fig. 4). Up-regulation of glyoxalase I at the proteome level has also been monitored in plants exposed to several abiotic stresses (Khan et al., 2005; Ahsan et al., 2007). Based on these observations, we propose that glyoxalase I may play a pivotal role in detoxifying the toxic substances induced by thermal stress in plants. GR is the key enzyme both in eukaryotes and prokaryotes that catalyzes the reduction of oxidized glutathione disulfide (GSSG) to GSH, using NADPH as an electron donor. The GSH pool maintained by GR is necessary for active protein function. In the present study, it was found that the mRNA level of GR increased slightly in the heat-treated leaves and showed a time dependent response to heat stress (Fig. 4).

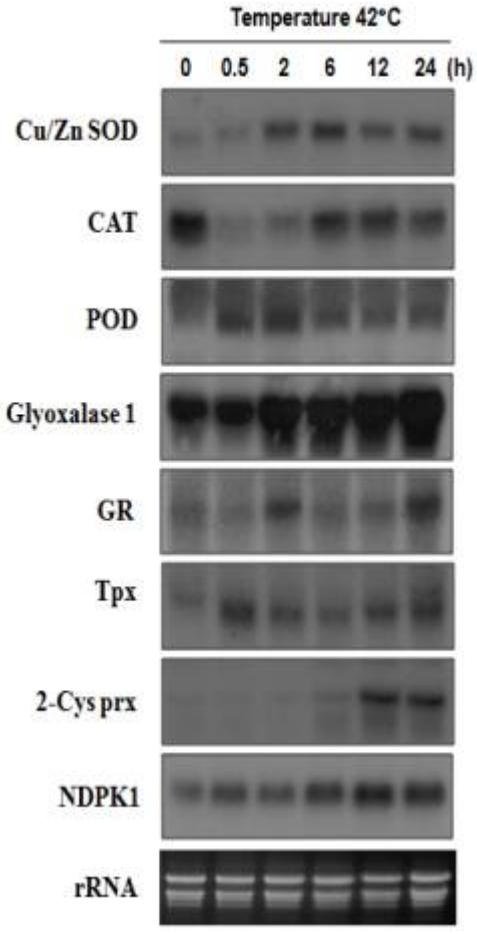

Fig. 4. RNA blot analysis of several antioxidant genes in response to heat stress.

6. Response of other genes involved in antioxidant defense mechanisms

In addition to ascorbate-GSH cycle genes, we also examined the transcript levels of some other genes that are involved in antioxidant defense mechanisms, such as thioredoxin peroxidase (TPx), 2-cysteine peroxiredoxin (2-Cys Prx), and NDPK1. The TPx are 2- Cys Prxs, which have been shown to reduce H_2O_2 with the use of electrons from the thioredoxin system (thioredoxin, thioredoxin reductase and NAD(P)H (Henkle-Dúhrsen and Kampkötter, 2001). The mRNA transcript of thioredoxin peroxidase increased 0.5 h after treatment and showed a constant pattern prior to the end point of treatment (Fig. 4). Increased accumulation of thioredoxin peroxidase at the mRNA or/and protein levels has also been demonstrated under several environmental stresses including heat (Rizhsky et al., 2002; Lee et al., 2007b, Ahsan et al., 2008). On the other hand, the mRNA transcript of 2-Cys Prx was barely detected within 6 h; however, it increased to a maximum level after 12 and/or 24 h of heat treatment (Fig. 4). Two- Cys Prxs are classified as novel members of the Trx-fold super family. It has been reported that during the exposure of cells to strong oxidative or heat stress conditions, 2-Cys Prxs change their protein structures from low-molecular weight to high-molecular weight complexes to trigger their functional switching from peroxidases to molecular chaperones (Jang et al., 2006). These results suggest that 2-Cys Prx may play a chaperone role in the defense of cells in later stages of heat treatment. The mRNA level of NDPK1 gradually increased in rice leaves under heat stress (Fig. 4). NDPKs

are believed to be housekeeping enzymes that maintain the intracellular levels of all (d)NTPs used in biosynthesis, except ATP. The expression of NDPK1 in rice or other plant species is known to be induced by several environmental stresses, including heat shock (Lin et al., 2005). In our previous studies, NDPK1 was also identified as an up-regulated protein in rice leaves in response to thermal and oxidative stresses (Lee et al., 2007a; Lee et al., 2007b, Ahsan et al., 2008). Taken together, these results suggest that rice NDPK1 has a crucial role in protecting cells in response to thermal stress.

Ⅳ CONCLUSION

The generation of ROS in response to heat stress has already been noted; therefore, we intended to verify whether heat stress also generates ROS in plants. The increased H_2O_2 and proline concentrations in rice leaves suggest that oxidative stress is generated by heat stress. Moreover, comparative transcriptomic analysis of several antioxidant genes suggests that plants engage in complex antioxidant defense mechanisms to cope with thermal stress. Transcripts of chaperone 60, HSP70, HSP100, chloroplastic HSP26, and mitochondrial small HSP responded to heat stress rapidly and strongly. The rapid accumulations of APX, DHAR and SOD in response to heat stress suggest these genes provide an initial defense against heat-induced ROS. Furthermore, the enzyme activities of APX and DHAR increased significantly under heat stress. This result suggests that these antioxidant genes may be involved in the detoxification of heat stress-induced toxic substances and the equilibration of several important metabolites.

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