

Different effects of night versus day high temperature on rice quality and accumulation profiling of rice grain proteins during grain filling

Haixia Li · Zhen Chen · Meixia Hu ·
Zhenmei Wang · Hua Hua · Changxi Yin ·
Hanlai Zeng

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Abstract High temperature has adverse effects on rice yield and quality. The different influences of night high temperature (NHT) and day high temperature (DHT) on rice quality and seed protein accumulation profiles during grain filling in *indica* rice ‘9311’ were studied in this research. The treatment temperatures of the control, NHT, and DHT were 28°C/20°C, 27°C/35°C, and 35°C/27°C, respectively, and all the treatments were maintained for 20 days. The result of rice quality analysis indicated that compared with DHT, NHT exerted less effect on head rice rate and chalkiness, whereas greater effect on grain weight. Moreover, the dynamic accumulation change profiles of 61 protein spots, differentially accumulated and successfully identified under NHT and DHT conditions, were performed by proteomic approach. The results also showed that the different suppressed extent of accumulation amount of cyPPDKB might result in different grain chalkiness between NHT and DHT. Most identified isoforms of proteins, such as PPDK and pullulanase, displayed different accumulation change patterns between NHT and DHT. In addition, compared with DHT, NHT resulted in the unique accumulation patterns of stress and defense proteins. Taken together, the mechanisms of seed protein accumulation profiles induced by NHT and

DHT during grain filling should be different in rice, and the potential molecular basis is discussed in this study.

Keywords Rice · Night versus day high temperature · Rice quality · Grain proteins accumulation · Grain filling

Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
APX	Ascorbate peroxidase
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid
DHT	Day high temperature
DAF	Days after fertilization
DTT	DL-Dithiothreitol
HSP	Heat shock protein
LTQ	Linear ion trap quadrupole
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MS	Mass spectrometry
NHT	Night high temperature
PPDK	Pyruvate phosphate dikinase
Prxs	Peroxiredoxins
PUL	Pullulanase
SOD	Superoxide dismutase
TCTP	Translationally controlled tumor protein
Trx h	Thioredoxin h

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H. Li · Z. Chen · M. Hu · Z. Wang · H. Hua · C. Yin ·
H. Zeng (✉)
Crop Physiology and Production Center (CPPC),
Key Laboratory of Huazhong Crop Physiology,
Ecology and Production, Ministry of Agriculture,
Huazhong Agricultural University, Wuhan 430070, China
e-mail: zenghl@mail.hzau.edu.cn

Introduction

Temperature is one of the main ecological factors that affect plant growth and development. It is well known that

the accelerated global warming has become a growing problem owing to the rapid increase in atmospheric greenhouse gas concentrations. Thus, high temperature becomes one of the most important environmental factors that can reduce the yield and quality of crops (Peng et al. 2004; Lin et al. 2005). As an important crop, rice (*Oryza sativa* L.) provides staple food for more than half of the world population (Sasaki and Burr 2000). The rice yield and quality are also impaired by the elevated temperature, especially during grain-filling stage. This has been the consensus generated in the academia.

Previous studies revealed that the typical symptoms resulted from high temperature stress during rice grain filling included increased grain-filling rate, decreased grain weight, low amylose content, poor milling quality and increased chalkiness degree (He et al. 1990; Lin et al. 2005; Lisle et al. 2000; Umemoto and Terashima 2002; Yamakawa et al. 2007). In addition, high temperature could decrease the expression of several starch synthesis-related genes, such as branching enzymes, but increase the expression of starch-consuming α -amylases gene (Jiang et al. 2003; Yamakawa et al. 2007). This can explain the reduced amylose content caused by high temperature at the gene expression level. Recently, *cyPPDKB* has been identified as the causative gene for *floury endosperm-4* (*flo-4*) mutant with a severely chalky appearance (Kang et al. 2005). Moreover, grains of the *amylose-extender* (*ae*) mutant, which was deficient in starch-branching enzyme IIb (*BEIIb*) gene, revealed a severely chalky opaque appearance, but, the chalkiness of the grains could be recovered when the wild-type *BEIIb* gene was transferred into the mutant (Nishi et al. 2001; Tanaka et al. 2004). The expression of *cyPPDKB* and *BEIIb* was down-regulated by high temperature and resulted in the increase of rice grain chalkiness (Yamakawa et al. 2007). These results suggested that *BEIIb* and *cyPPDKB* may be two of the candidate genes triggering for the grain chalkiness. Furthermore, under high-temperature stress, the induction of small heat shock proteins (sHSP) and the reduction of allergen-like proteins might affect grain starch structure or storage proteins and thereby change the physicochemical properties of rice grains (Lin et al. 2005; Schaeffer and Sharpe 1997).

In the past century, the increasing speed of the night (daily minimum) temperature was faster than that of the day (daily maximum) temperature (Karl et al. 1991; Easterling et al. 1997; Peng et al. 2004). However, most of the studies concerning the effects of high temperature on rice were based on daily mean temperature or day temperature. And there are only a few studies on the different effects of night and day high temperature on rice yield and quality. Peng and his colleagues (2004) reported that rice grain yield declined by 10% for each 1°C increase in night temperature, whereas the effect of day temperature on yield

was insignificant. In addition, a previous result (Dai et al. 2009) suggested that compared with increased day temperature, increased night temperature exerted similar but less effects on milling quality, appearance quality and nutritional quality; however, effects of both treatments on starch structure, eating and cooking quality were different.

In this paper, we aimed to contribute to the basic theoretical research for a better understanding of the different responsive mechanisms of proteins in rice filling grains under DHT and NHT. Thus, a further study on the different effects of night versus day high temperature on rice quality and accumulation profiling of rice grain proteins during grain filling was carried out. The possible function of some identified proteins was discussed further.

Materials and methods

Plant materials and sampling

The *indica* rice (*Oryza sativa* L.) variety ‘9311’ was used in this research. Rice plants were cultured during rice growing season (May to September) under natural conditions in Wuhan (30°47'N, 114°35'E) in enamel pots (16 cm diameter × 20 cm height) with usual fertilizer (with NPK ratio of 15:15:15) and water management. There were three rice plants in each enamel pot, and each plant was restricted to the primary tillers. The panicles were labeled for subsequent sampling when half of the spikelets were flowering, and on the next day, the rice plants in which ovaries were fertilized were transferred to plant incubators (HP1500GS-B, Ruihua) with fluorescent light (300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and relative humidity of 80%. Control plants were grown at 28°C/20°C (12-h-day/12-h-night cycles), while plants in the DHT and NHT treatments were subjected to 35°C/27°C, 27°C/35°C, respectively. And then all the rice plants were replaced to natural conditions (with the average temperature of 24°C, relative humidity of 70% and natural light in mid-to-late September, Wuhan) from 20 days after fertilization (DAF) to maturity, because of the development process at about 20 DAF belonging to the end of the late stage of rice grain filling (Ishimaru et al. 2003; Xu et al. 2008). During the course of grain filling, developing grains were harvested 5, 10, 15 and 20 DAF and divided into two parts: one part for protein extraction, and the other part for RNA isolation and then immediately frozen in liquid nitrogen and stored at -80°C until use.

Grain weight and rice quality analysis

Fresh weight was monitored during grain development. Fresh weight was measured right after sampling. Rice seeds were harvested at the maturity stage and dried for

3 months in natural draft drying house for rice quality analysis. The rice quality indexes including brown rice rate, milled rice rate, head rice rate, chalky degree, amylose content and gel consistency were measured by Food Quality Supervision and Testing Center (Wuhan), Chinese Ministry of Agriculture.

Protein extraction and 2-DE

Samples of rice grains (0.5 g) for each developmental stage were ground with prechilled mortar and pestle in liquid nitrogen to fine powder, soluble proteins extracted with 2.5 ml Mg/NP-40 buffer through the method described by Kim et al. (2001) and the supernatant precipitated for 30 min at 4°C with four volumes of cold acetone at –20°C. Following centrifugation at 3,000 rpm for 10 min, the pellets were air-dried and stored at –80°C for protein analysis by 2-DE.

Before 2-DE, protein pellets were dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% bio-lyte and 0.002% bromophenol blue, followed by centrifugation at 12,000 rpm at 10°C for 1 h. Protein concentration was determined according to the Bradford method using BSA as standard (Bradford 1976).

Protein samples (250 µg) were applied to 17 cm liner IPG strips (Bio-rad, pH 4–7). After 16-h passive rehydration at 18°C, isoelectric focusing was performed using PROTEAN IEF Cell (Bio-rad) at 18°C according to the protocol described by Wang et al. (2010) with few modifications: 250 V for 30 min, 500 V for 1 h, 1,000 V for 2 h, 10,000 V for 5 h, focused for 60,000 Vh. Then IPG strips were equilibrated for 15 min in an equilibration buffer (6 M urea, 20% glycerol, 0.375 M Tris–HCl (pH 8.8), and 2% SDS) containing 2% DTT, followed by 15 min in an equilibration buffer with 2.5% iodoacetamide.

SDS–PAGE was performed with 12% polyacrylamide gels using the PROTEAN II XL Multi-Cell apparatus (Bio-rad) for 1 h at 50 V/gel and then 200 V/gel until the dye front reached the gel bottom. The molecular mass and pI of the proteins were determined by the molecular mass marker and liner pH arrangement of IPG strips, respectively. Protein spots were visualized by silver staining according to the method of Blum et al. (1987).

Image analysis

Staining gels were imaged by Gel Explorer (Ultra-Lum, USA). Protein spots were quantified based on their spot quantities and normalized to total spot density in gel image under PPM ($\times 1,000,000$) scaling step by PDQuest software (Bio-rad) according to the PDQuest user guide. When detecting spots, the Gaussian model during test and

Smoothing ContraMean 5×5 were selected, and the parameter of sensitivity, size scale, min peak, vertical streak and horizontal streak was 3.91, 9, 4,078, 85 and 160, respectively. After automated spot detection and matching, the spots were edited and matched manually. Then the quantity data of well-separated spots were exported to excel and statistically analyzed by one-way ANOVA ($p < 0.05$) using SPSS. Only those with significant and reproducible changes were considered to be differentially accumulated proteins. Three to five gels for each sample were used for image analysis.

Destained gels containing protein spots

Differentially accumulated protein spots were excised from gels, destained for 20 min in 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1 v/v), and washed in distilled water until the gels were destained.

Protein identification by MS

Some destained protein spots were analyzed through MALDI-TOF MS according to the procedure described by Zhang et al. (2008). The PMFs obtained from MALDI-TOF MS were searched against National Center for Biotechnology non-redundant (NCBI nr 20070216) rice protein database (136,222 sequences) using the MASCOT software (Matrix Science Ltd, London, UK). The proteins were identified according to their MOWSE score and functions as documented in NCBI database.

Most destained protein spots were analyzed through MALDI-TOF/TOF MS according to the previously described procedure (Pan et al. 2009; Wang et al. 2010). The combined data set of PMF and MS/MS using Flex-Analysis was submitted to MASCOT and searched against NCBI nr with *Oryza sativa* (rice, 20100325, with 139,485 sequences) as taxonomy for protein identification. Only identified proteins with protein score confidence interval (CI) $\geq 95\%$ were accepted.

Some protein spots were further analyzed through LTQ-ESI-MS/MS using a Finnigan LTQ mass spectrometer (ThermoQuest, San Jose, CA, USA) coupled with a Surveyor HPLC system (ThermoQuest) according to the procedure described by Wang et al. (2010). Protein identification using MS/MS raw data was carried out with the SEQUEST program in the BioWorks™ 3.1 software suite (University of Washington, licensed to Thermo Finnigan) based on the database of NCBI nr.

Real-time quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from 0.1 g of rice grains at 5 DAF and 10 DAF, using TRNzol Reagent (Tiangen).

First-strand cDNA was synthesized from 5 µg of total RNA using the RevertAid™ first strand cDNA synthesis kit (MBI, Lithuania) according to the manufacturer's instruction. Gene-specific primers (Table 1) were designed with the Beacon Designer (Bio-Rad) based on the sequences in GenBank. A twenty-microlitre-reaction system was carried out with the iQ™ 5 Multicolor Real-Time PCR Detection System (Bio-Rad) by using the SYBR Green RealMaster Mix kit (Tiangen) according to the manufacturer's protocol. Actin was amplified as an endogenous control to normalize expression between different samples. The relative quantification method ($2^{-\Delta\Delta C_t}$) was used to evaluate quantitative variation between samples examined.

Statistical and bioinformatic analysis

Data were processed by means of Excel 2003. Statistical significance was defined at $p < 0.05$ using SPSS.

Functional analysis of the identified proteins was performed as follows: First, the corresponding InterPro and GO (Gene Ontology) annotation of some proteins could be got directly from NCBI protein and InterProScan (<http://www.ebi.ac.uk/Tools/InterProScan>) databases. Second, for proteins without definite functional annotation, the amino acid sequences were obtained from NCBI and searched for their homologs with BLASTP (<http://www.ncbi.nlm.nih.gov/Blast>), whose functional domains were also predicted by InterProScan. Then, these GO annotations were plotted using the Web Gene Ontology Annotation Plotting tool (WEGO, <http://wego.genomics.org.cn>).

The chromosome loci of the protein-encoding genes were searched in Rice Genome Annotation Project BLAST

Search (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml), and the loci with highest scores were considered positive results (Xu et al. 2008).

In order to better show the differences of accumulation changes of rice grain proteins between DHT and NHT treatments, the accumulation data was analyzed as follows: First, to eliminate the developmental accumulation changes, the control quantity of each spot at each development stage was set to "1". The values of the spots that changed more than 1.5 times were considered differentially accumulated under NHT and DHT treatments. Second, the log base 2 was used to transform value of each spot; in this case, all of the normalized control value of each spot was "0". Third, we denoted the normalized fold value of NHT minus value of DHT of each protein spot at each stage as "NHT-DHT". If the absolute value of "NHT-DHT" was less than or equal to 0.585 ($\log_2 1.5 = 0.585$), we thought that there was no accumulation change between NHT and DHT. Therefore, only the absolute value of "NHT-DHT" greater than 0.585 was regarded as the meaningful value.

Results

Different effects of night versus day high-temperature treatments on rice quality and fresh weight of rice grain at grain-filling stage

In the study, the grains from those '9311' plants which were exposed to DHT and NHT after the fertilization of the ovaries matured faster than that from the control plants. After the beginning of the treatments, the fresh weights of

Table 1 Specific primers used in real-time quantitative RT-PCR

Gene ^a	Accession number ^b	Primer (5' → 3')	T _m (°C)	Product length (bp)
Trx h	NM_001065604	F: 5'-GATCGCCTGCCACAACAAG-3' R: 5'-CCACACCAGGAAGCAGTGAA-3'	60	99
APX	NM_001067276	F: 5'-TGAAGGCCGTCTTCTGATG -3' R: 5'-GTCCAGGCTCCCTCAAAGC-3'	60	159
2-Cys Prx	AL606453.2	F: 5'-GCATTCTACCATTAACAACCTTGCTA-3' R: 5'-CCGGGTTTTCTTGGACATACTG-3'	60	92
Cu/ZnSOD	AP004587.3	F: 5'-TGCATATCAACAGGACCACATTTT-3' R: 5'-CACCTTCAGCATTGGCAACA-3'	60	112
HSP70	X67711.2	F: 5'-TGCCAATGGTATCCTGAACGT-3' R: 5'-TGCTGAGCCTGCCCTTGT-3'	60	90
Actin	X16280	F: 5'-GAGACCTTCAACACCCCTGCTA-3' R: 5'-ATCACCAGAGTCCAACACATTACCT-3'	60	99

^a *Trx h* Thioredoxin h-type protein; *APX* Ascorbate peroxidases; *Prx* Peroxiredoxin, *Cu/ZnSOD* Cu/Zn Superoxide dismutase; *HSP70* Heat shock protein 70

^b Accession number of the gene is from NCBI database corresponding to the matched protein

both DHT and NHT treated grains increased faster than that of the control (Fig. 1). The grain weight increase of NHT and DHT nearly ceased by 10 DAF and 15 DAF, respectively, and the development began into desiccation stage from 15 DAF. Although the fresh weight at 15 and 20 DAF showed no significant difference, the weight of control grains continued to increase steadily until at least 20 DAF.

The grains ripened under DHT and NHT showed poor rice quality (Table 2). The two treatments both could cause the reduction of the grain maturity weight, brown rice rate, milled rice rate, head rice rate, amylose content and gel consistency, while increasing chalkiness degree. Compared with DHT, NHT exerted more serious effects on the grain maturity weight, brown rice rate, milled rice rate and amylose content. Especially the latter two parameters, the decreasing range of which reached to about 2.0 and 2.6 fold relative to the control, respectively. While, compared with NHT, DHT had greater influence on head rice rate, chalkiness and gel consistency, particularly chalkiness, the increasing range of which achieved up to 6.7 fold relative to the control.

Protein identification by 2-DE and MS

To investigate the differentially accumulated changes of the rice grain proteins between DHT and NHT treatments relative to the control at grain-filling stage, sample proteins

5, 10, 15 and 20 DAF were separated by 2-DE. In the 5 DAF sample collected, silver staining presented nearly 550 protein spots in the gel (Fig. 2). However, approximately 900 spots could be monitored with PDQuest software in all of samples, in which about 400 protein spots were well detected in two to five replicates of each sample. A total of 70 protein spots were reproducibly and differentially accumulated at least at one time point. All of these spots were excised from silver-stained gels and submitted to MS after destaining and in-gel digestion.

At the beginning, ten of protein spots, randomly selected, were subjected to MALDI-TOF MS tentatively to see if the successful rate was acceptable. Unfortunately, only just one protein spot (no. 30) was successfully identified. The success rate was only 10%. Then, all of the other 69 protein spots were submitted to MALDI-TOF/TOF-MS, which successfully identified 57 of the spots. It was worth noting that protein spots 30, 31, 32 and 33 were closely and horizontally inline (Fig. 3a). Spots 30, 31 and 32 were identified to be pyruvate orthophosphate dikinase (PPDK); nevertheless, spot 33 failed to be identified, which we speculated was also PPDK. To verify this idea, spot 33 and other two spots with high abundance were further analyzed by LTQ-ESI-MS/MS. As a result, all of three spots were successfully identified. The result of spot 33 was as expected. Taken together, out of 70 protein spots, 61 proteins were successfully identified (Table 3), and marked in gels (Figs. 2, 3a, 4a).

Fig. 1 Changes in the fresh weight of rice grains during grain-filling with different high-temperature treatments. Significant differences at the 0.05 probability level determined by ANOVA are indicated by different *lowercase letters*. C, control; DHT, day high temperature; NHT, night high temperature

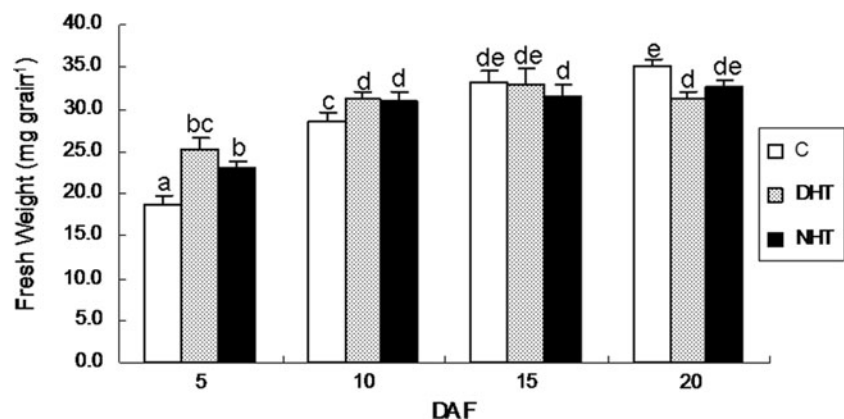
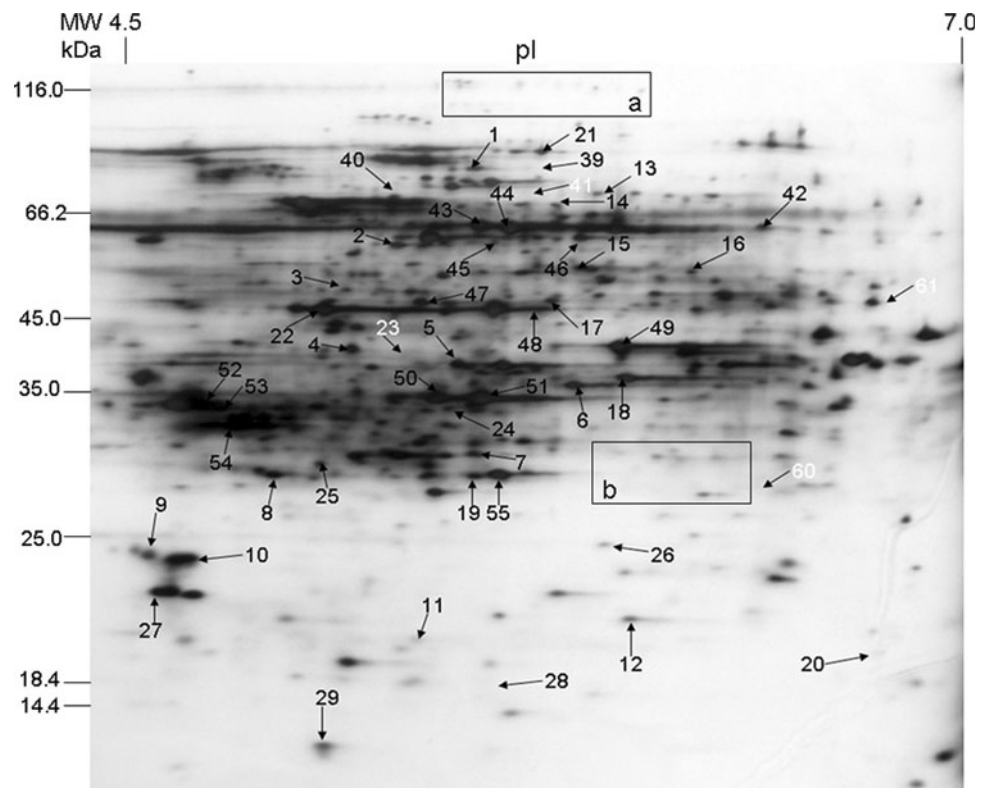


Table 2 Effects of high temperature at grain-filling stage on rice quality

Treatment	AT (°C)	Grain fresh weight (Maturity) (mg)	Brown rice rate (%)	Milled rice rate (%)	Head rice rate (%)	Chalkiness (%)	Amylose content (%)	Gel consistency (mm)
C	24.0	31.0	79.1	69.3	65.9	1.3	13.6	70
DHT	31.0	27.2	75.0	63.7	16.3	12.1	12.8	61
NHT	31.0	25.7	73.0	57.9	29.4	2.9	11.5	64

C control; DHT day high temperature; NHT night high temperature; AT average temperature

Fig. 2 A representative 2-D gel image of rice control grain proteins 5 DAF with silver staining. Regions a and b of the image are presented in more detail in Figs. 3a and 4a. Spots marked by *black numbers* are the proteins that are detectable on the image, while spots marked by *white numbers* are those which are undetectable on the image. The detailed information of proteins marked by *arrows* is provided in Table 3



Functional analyses and categories of identified proteins

By bioinformatic analysis, 61 identified proteins represented the products of 46 different genes, wherein 41 had at least one matched GO. According to WEGO analysis, all of these GOs were classified into specific groups based on their cellular component, molecular function and biological process (Fig. 5). Viewed from cellular component, eight genes (17.4%) and six genes (13.0%) were located in cell part and macromolecular complex, respectively, accounting for the larger proportion. Furthermore, a large number of the identified genes (65.2%) were involved in metabolic processes, and an overwhelming majority of genes (69.6%) had catalytic activities, including eight genes (17.4%) with transferase activity or oxidoreductase activity, seven genes (15.2%) with lyase activity, five genes (10.9%) with hydrolase activity, two genes (4.3%) with isomerase activity and one gene (2.2%) with ligase activity. In addition, more than half of the genes (58.7%) had binding functions.

Among 61 identified protein spots, 9 different proteins were found with 2–5 isoforms possessing the same accession number, for example, spots 25 and 56 (Table 3). In this case, the query item was treated as just one when analyzed using WEGO. Considering this, the functional pie chart of all identified protein spots was generated (Fig. 6). The functional categories were represented by

carbohydrate metabolism followed by stress and defense proteins, amino acid metabolism, signal transduction, unknown function and others function. The others functional group contained energy metabolism (spots 2, 26, 48 and 61), protein synthesis and destination (spots 6, 12 and 18), miscellaneous (spots 40 and 41), transcription (spot 9), nitrogen and sulfur metabolism (spot 42) and nucleotides metabolism (spot 22).

Compared with DHT, the accumulation of NHT-responsive grain proteins revealed five accumulation change patterns during grain-filling stage

In order to better evaluate the accumulation changes between NHT and DHT, only the meaningful values of the protein accumulation data were considered. After analysis, five distinct accumulation change patterns between NHT and DHT could be revealed (Table 3): i, compared with DHT, proteins accumulation of NHT repressed at early stage, whereas enhanced at middle or late stages, showing a positive slope with advancing development (9 spots); ii, contrary to i-type, proteins accumulation of NHT increased at early stages, while decreased at middle or late stages, showing a negative slope (7 spots); iii, proteins accumulation of NHT was up-regulated compared with DHT at just one stage (9 spots), two stages (5 spots) or three stages (spot 56); iv, proteins accumulation of NHT was down-regulated compared with DHT at only one stage (13 spots)

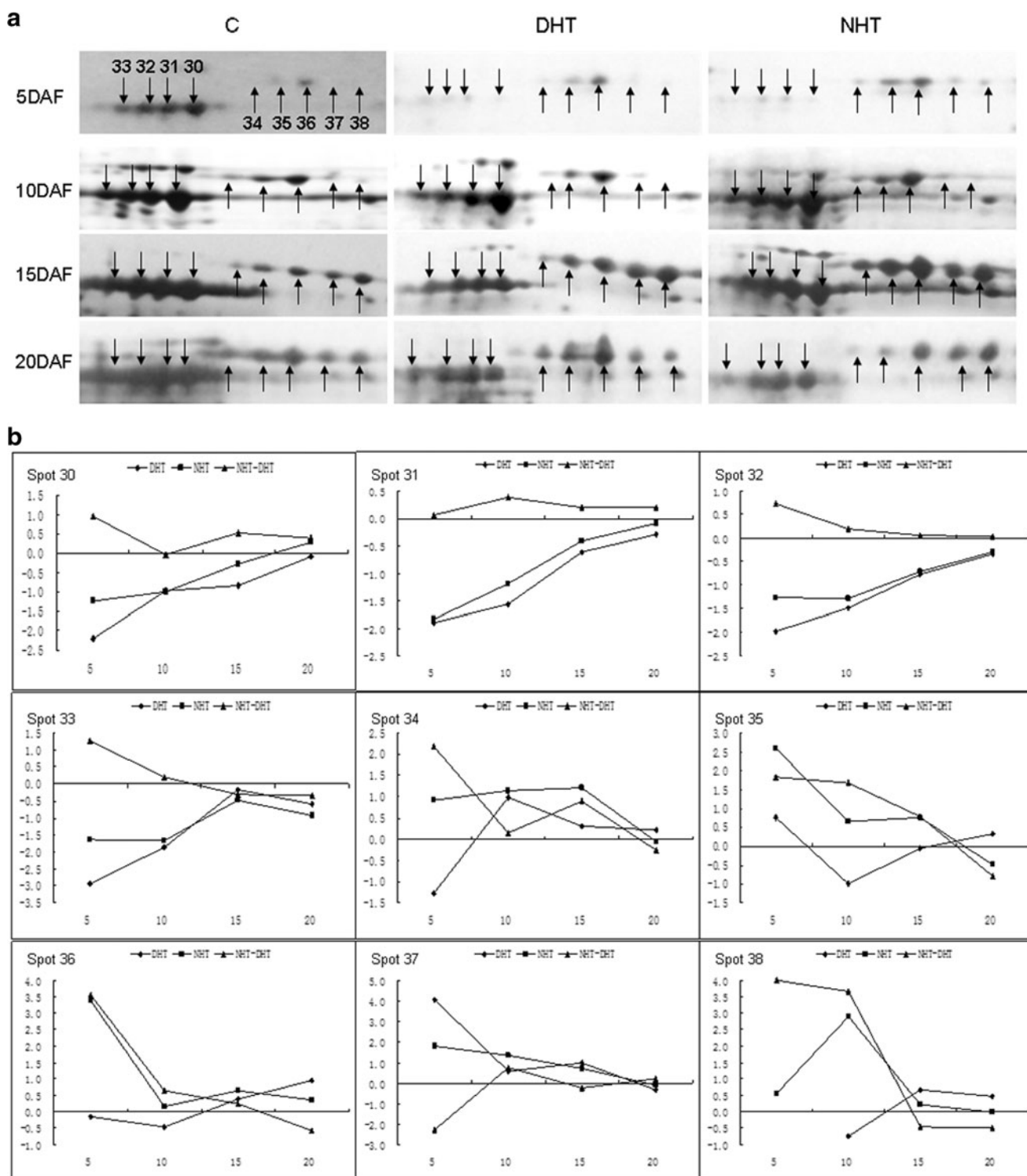


Fig. 3 Accumulation profiles of isoforms of PPDK and pullulanase during rice grain development with different high-temperature treatments. **a** Close-up views of gel images corresponding to region a in Fig. 2. **b** Accumulation variations of isoforms of PPDK and pullulanase with DHT and NHT treatments. The vertical axis shows the normalized fold calculated as described under “Materials and

methods”, and the horizontal axis shows the grain development stage 5, 10, 15 and 20 DAF. Each point represents the average of two to five replicates. Spots 30–33 and 34–38 show the isoforms of PPDK and pullulanase, respectively. C, control; DHT, day high temperature; NHT, night high temperature

Table 3 List of selected differentially accumulated rice seed proteins in response to heat stress during grain-filling stage identified by MS

Spot no ^a	Homologous protein	Accession no./ Chromosome locus	Experimental, theory MW(kDa)/pI	PM/ Cov. (%) ^b	Protein score	Group ^c
Carbohydrate metabolism						
5	Chloroplastic aldolase	gil218155 Os08g02700	38.8/5.50 42.1/7.60	8/21.9	89	iv
15	Enolase	gil90110845 Os10g08550	53.6/5.90 47.9/5.41	11/33.2	86	i
16	Enolase	gil90110845 Os10g08550	52.6/6.20 47.9/5.41	11/30.5	97	iv
43	Enolase, putative, expressed	gil110288669 Os10g08550	60.4/5.64 51.5/5.84	14/38.3	516	v
44	Enolase, putative, expressed	gil110288669 Os10g08550	60.3/5.75 51.5/5.84	15/40.6	548	v
17	Isocitrate lyase and phosphorylmutase family protein	gil115458044 Os04g42580	47.9/5.80 41.3/5.66	14/45.2	538	iv
19	Triose phosphate isomerase, cytosolic	gil115434516 Os01g05490	28.2/5.60 27.0/5.38	6/34.4	84	iii
55	Triose phosphate isomerase, cytosolic	gil115434516 Os01g05490	29.9/5.75 27.0/5.38	11/56.5	587	iv
21	Putative transketolase	gil28190676 Os06g04270	82.8/5.80 80.0/6.12	19/30.8	336	i
13	Pyruvate phosphate dikinase	gil115463815 Os05g33570	70.1/6.00 102.7/5.98	13/16.4	63	i
30 ^d	Pyruvate phosphate dikinase	gil2443402 Os05g33570	105.3/5.80 102.7/5.98		119	iii
31	Pyruvate phosphate dikinase	gil2443402 Os05g33570	106.1/5.69 102.7/5.98	26/31.2	342	v
32	Pyruvate phosphate dikinase	gil2443402 Os05g33570	106.3/5.65 102.7/5.98	26/31.6	385	iii
33 ^e	Pyruvate phosphate dikinase	gil2443402 Os05g33570	106.5/5.61 102.7/5.98	4/4.0		iii
34	Pullulanase	gil262345489 Os04g08270	109.2/5.84 102.5/5.44	20/25.3	163	iii
35	Pullulanase	gil262345489 Os04g08270	108.8/5.89 102.5/5.44	17/21.4	278	ii
36	Pullulanase	gil262345489 Os04g08270	107.4/5.97 102.5/5.44	19/24.6	238	iii
37	Pullulanase	gil262345489 Os04g08270	107.8/6.04 102.5/5.44	16/18.1	158	i
38	Pullulanase	gil262345489 Os04g08270	107.1/6.10 102.5/5.44	20/24.1	148	iii
39	Phosphoglucomutase	gil115454931 Os03g50480	75.9/5.84 62.9/5.40	13/27.3	179	i
46	UDP-glucose pyrophosphorylase	gil114848914 Os09g38030	58.4/5.94 51.6/5.59	18/47.8	520	v
47	Succinyl-CoA synthetase subunit beta	gil115447367 Os02g40830	49.9/5.47 45.1/5.98	15/32.2	150	iii
49 ^e	Malate dehydrogenase, cytoplasmic	gil75141370 Os10g33800	43.2/6.10 35.6/5.75	8/24.7		iv

Table 3 continued

Spot no ^a	Homologous protein	Accession no./ Chromosome locus	Experimental, theory MW(kDa)/pI	PM/Cov. (%) ^b	Protein score	Group ^c
Amino acid metabolism						
3	Putative precursor chloroplastic glutamine synthetase	gil19387272 Os04g56400	49.1/5.20 49.4/6.18	10/35.3	155	i
7	Putative ornithine carbamoyltransferase	gil41053142 Os02g47590	29.8/5.60 39.6/6.37	5/20.2	222	ii
14	Ketol-acid reductoisomerase, chloroplast precursor	gil115465569 Os05g49800	66.6/5.90 62.3/6.01	5/14.0	99	iv
23	Cysteine synthase, mitochondrial precursor	gil115442595 Os01g74650	39.9/5.40 41.8/6.28	9/35.5	191	ii
24	Cysteine synthase	gil4574135 Os12g42980	33.8/5.50 33.8/5.39	10/48.3	181	ii
25	Methylenetetrahydrofolate reductase, 3-partial	gil37718877 Os03g60090	29.1/5.10 41.8/6.10	6/18.5	86	ii
56	Methylenetetrahydrofolate reductase, 3-partial	gil37718877 Os03g60090	32.1/6.10 42.2/6.10	5/14.4	102	iii
Stress and defense proteins						
1	Heat shock protein 70	gil21664287 Os12g38180	76.8/5.60 71.0/5.17	16/32.9	394	i
8	Ascorbate peroxidases	gil115474285 Os07g49400	28.3/5.00 27.1/5.21	7/35.9	298	iv
10	2-Cys peroxiredoxin	gil38344034 Os04g33970	23.1/4.70 18.1/4.67	4/35.9	98	i
28	Putative superoxide dismutase[Cu–Zn],chloroplast precursor	gil42408425 Os08g44770	14.9/5.80 20.5/5.79	5/43.4	303	iii
29	Thioredoxin H-type	gil115470941 Os07g08840	9.9/5.10 13.2/5.16	5/46.7	141	iii
45	Hsp20 domain containing protein	gil115452113 Os03g15960	57.6/5.70 17.9/5.80	2/16.2	73	ii
50	Glyoxalase I	gil16580747 Os08g09250	36.1/5.54 32.5/5.51	8/30.9	148	iv
51	Glyoxalase I	gil16580747 Os08g09250	35.8/5.68 32.5/5.51	7/24.4	76	v
59	1-Cys peroxiredoxin	gil158517776 Os07g44430	30.0/6.49 24.1/5.97	12/62.3	334	ii
60	1-Cys peroxiredoxin	gil158517776 Os07g44430	29.9/6.69 24.1/5.97	7/37.3	110	iii
Signal transduction						
11	Serine/Threonine protein kinases, catalytic domain	gil115473147 Os07g40550	19.4/5.40 78.8/9.54	15/26.7	62	v
52	14-3-3 protein	gil115446909 Os02g36974	35.6/4.82 29.7/4.71	14/62.2	363	iv
53	14-3-3 protein	gil115446909 Os02g36974	34.9/4.88 29.7/4.71	10/44.7	250	v
54	14-3-3 protein	gil125561626 Os08g33370	33.5/4.91 28.8/4.78	6/30.1	108	v

Table 3 continued

Spot no ^a	Homologous protein	Accession no./ Chromosome locus	Experimental, theory MW(kDa)/pI	PM/Cov. (%) ^b	Protein score	Group ^c
Others						
9	Nascent polypeptide associated complex alpha chain	gil32352154 Os05g31000	23.5/4.60 22.3/4.34	1/6.3	68	i
22	Adenosine kinase	gil21698922 Os02g41590	46.3/5.10 32.2/5.29	14/66.2	788	iv
42	Putative alanine aminotransferase	gil14018051 Os10g25130	60.2/6.61 52.7/6.23	23/58.1	465	v
6	Elongation factor EF-2	gil115446385 Os02g32030	36.2/5.90 94.0/5.85	11/21.1	358	iv
12	Eukaryotic initiation factor 4A	gil303844 Os06g48750	20.3/6.10 46.0/5.29	15/43.3	338	iv
18	11-S plant seed storage protein family protein	gil115461739 Os05g02520	37.2/6.10 38.2/5.81	13/52.1	428	iv
2	Putative ATP synthase beta subunit	gil56784991 Os01g25065	57.5/5.40 45.9/5.33	19/65.4	1030	iv
26	23 kDa polypeptide of photosystem II	gil1398999 Os07g04840	23.8/6.00 27.0/9.54	3/16.7	133	iii
48	Photosystem I reaction center subunit IV/PsaE family protein	gil255677718 Os07g25430	48.3/5.80 11.3/7.04	2/26.2	86	iv
61	Nad-dependent formate dehydrogenase	gil4760553 Os06g29180	47.4/6.80 41.2/6.87	14/47.1	328	iv
40 ^e	Putative chaperonin 60 beta precursor	gil7248401 Os06g02380	66.1/5.39 64.1/5.60	7/13.1		v
41	Germin-like protein 1	gil4239821 Os08g35760	65.9/5.74 22.0/6.01	2/15.5	119	v
Unknown						
4	OSEYA1	gil6015432 Os06g02028	40.2/5.20 31.8/4.92	10/50.7	266	iv
20	Unknown protein	gil57863891 Os05g01050	19.1/6.80 14.7/6.84	4/38.8	195	iii
27	Translationally controlled tumor protein	gil115486523 Os11g43900	21.5/4.60 18.9/4.51	7/50.6	232	v
57	Stress-responsive A/B Barrel Domain-containing protein	gil125559135 Os07g41810	32.0/6.33 23.7/5.78	4/21.8	251	iii
58	Stress-responsive A/B Barrel Domain-containing protein	gil125559135 Os07g41810	31.1/6.32 23.7/5.78	4/21.8	158	iv

^a Protein spots were listed with the same identifiers correspond to the 2-DE gels in Fig. 2

^b Number of peptides matched (PM)/sequence coverage percentage

^c Compared with day high temperature (DHT), accumulation change patterns of protein spot in response to night high temperature (NHT): i, repressed at early stage, enhanced at middle or late stages; ii, contrary to i-type; iii, up-regulated; iv, down-regulated; v, no expression changes between NHT and DHT

^d The protein spot was identified using MALDI-TOF-MS

^e The protein spot was identified using LTQ-ESI-MS/MS, others without marks were identified using MALDI-TOF/TOF-MS

or two stages (5 spots); and v, proteins showed no accumulation changes between NHT and DHT (12 spots), although the proteins with NHT and DHT treatments were differentially accumulated relative to the control, respectively.

From the combination of functional categories and accumulation change patterns between NHT and DHT, we found that proteins belonging to one functional category, such as stress and defense proteins, could show different

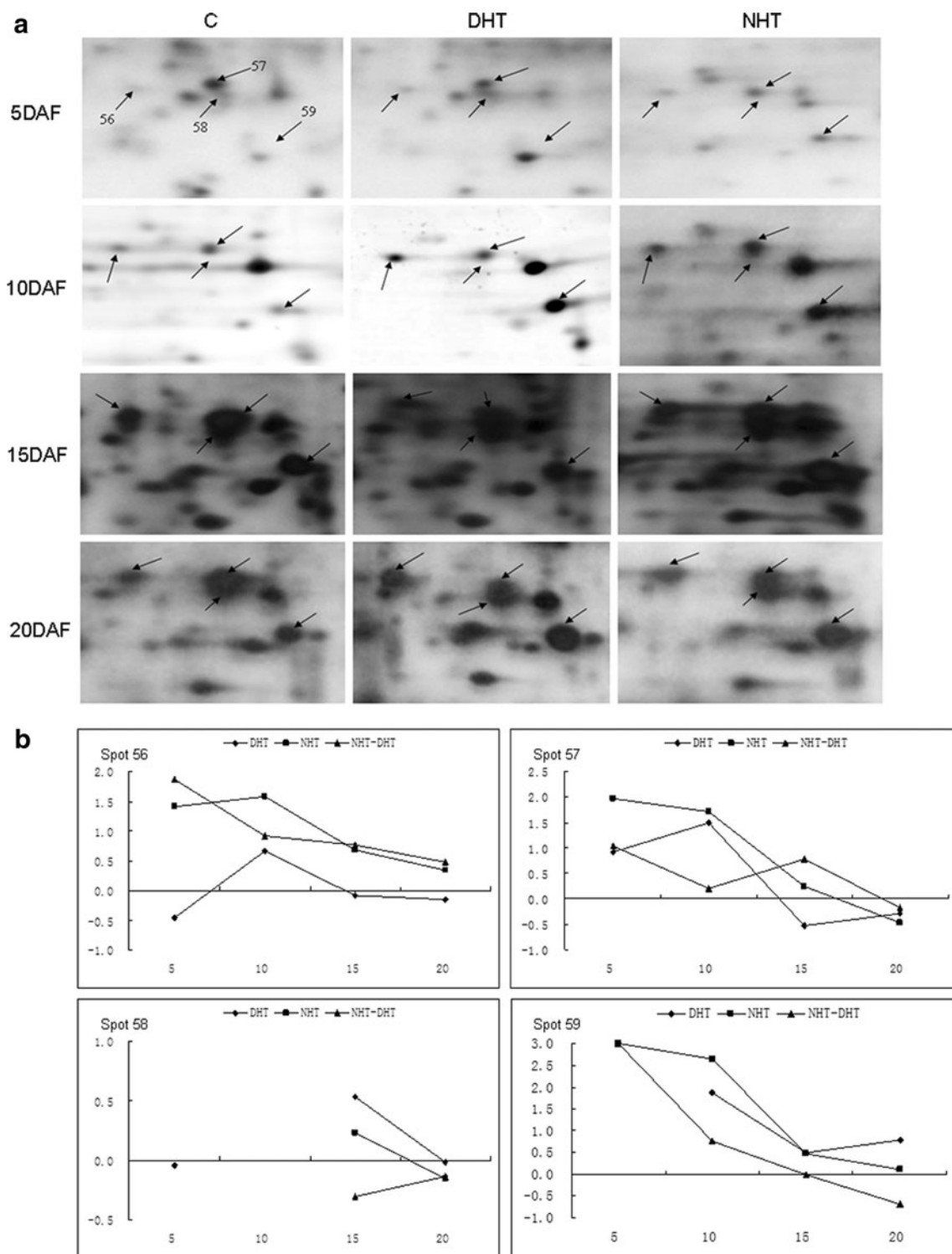


Fig. 4 Accumulation profiles of proteins in region b in Fig. 2 during rice grain development with different high-temperature treatments. **a** Close-up views of gel images corresponding to region b in Fig. 2. **b** Accumulation variations of proteins with DHT and NHT treatments. The vertical axis shows the normalized fold calculated as described under “Materials and methods”, and the horizontal axis shows the grain development stage 5, 10, 15 and 20 DAF. Each point

represents the average of two to five replicates. Owing to spot 59 undetected for control and DHT at 5 DAF, the point of spot 59 for NHT and NHT-DHT at 5 DAF was represented by the maximum scale value (3.0). The detailed information of proteins marked by arrows is provided in Table 3. C, control; DHT, day high temperature; NHT, night high temperature

Fig. 5 The category distribution of the identified rice grain proteins in response to high-temperature treatment during grain-filling stage analyzed by WEGO (Web Gene Ontology Annotation Plotting tool)

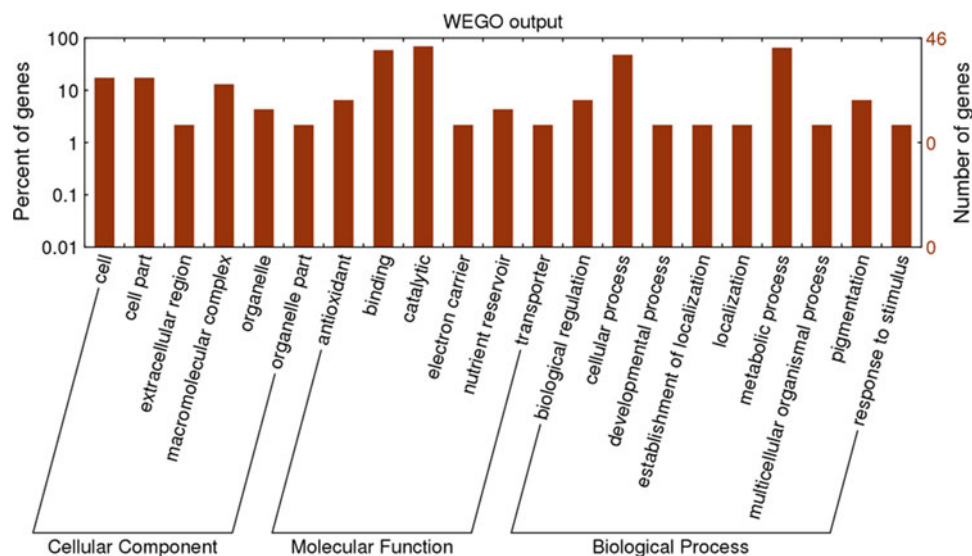
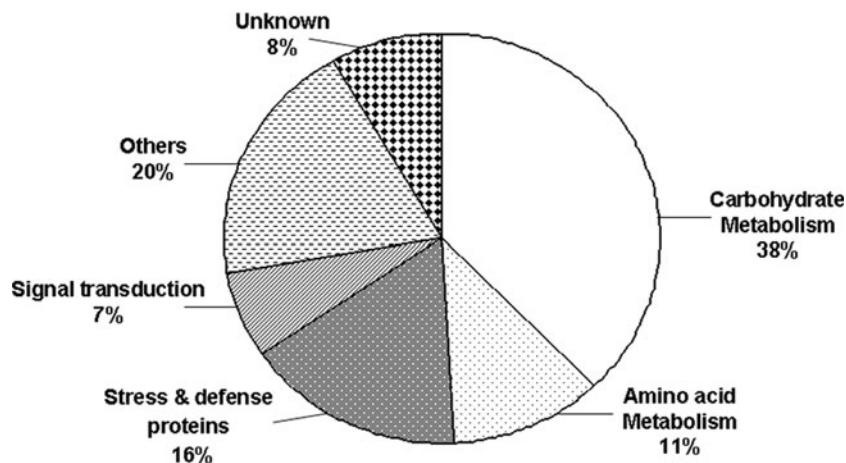


Fig. 6 Functional categories of the identified rice grain proteins in response to high-temperature treatment during grain-filling stage



accumulation change patterns; likewise, proteins representing the similar pattern, such as iv-type proteins, were classified as different functional categories.

Accumulation of isoforms in response to high temperature during rice grain-filling stage

Among all of the identified protein spots, 10 proteins with 29 identities, ranging from 2 to 5 identities, were represented and identified (Table 3). These isoforms possessed similar molecular weights but different pI values, or both different, involved in carbohydrate metabolism (enolase, triose phosphate isomerase, PPK, and pullulanase [PUL]), amino acid metabolism (methylenetetrahydrofolate reductase and cysteine synthase), signal transduction (14-3-3 protein) and, stress and defense response (glyoxalase I and 1-Cys peroxiredoxin [1-Cys Prx]). In addition, two proteins (spots 57 and 58) were identified as stress-responsive alpha-beta

(A/B) barrel domain containing protein, whose function was still unknown.

Most of the isoforms identified displayed different accumulation change patterns between NHT and DHT. Five PPK isoforms were identified (Fig. 3a; Table 3) with three types: i-type (spot 13), iii-type (spots 30, 32 and 33), and v-type (spot 31). Under control, DHT and NHT conditions, the accumulation of four isoforms (spots 30, 31, 32, and 33) showed similar accumulation tendencies (Fig. 3a; Supplemental Table S1): began to increase at 5 DAF, peaked at 10 DAF or 15 DAF and decreased thereafter. Excluded spot 31 owing to no accumulation changed between NHT and DHT, the accumulation of spots 30, 32 and 33 of NHT were up-regulated than DHT at 5 DAF or 10 DAF (Fig. 3b).

Five isoforms of PUL were also showed in rice developing seeds (Fig. 3a; Table 3) with three types: i-type (spot 37), ii-type (spot 35) and iii-type (spots 34, 36 and 38). Under control, DHT and NHT conditions, the temporal accumulation patterns of five isoforms revealed similar

(Fig. 3a; Supplemental Table S1), as most were increased in parallel with seed development, reached peak level at 15 DAF or 20 DAF, except that spots 37 and 38 with relative smooth variation from 5 to 10 DAF. Compared with the control, high temperature, especially NHT, would increase the protein accumulation of most of PUL isoforms at different developmental stages (especially from 5 to 15 DAF). From their responses to high temperature at the initiation stage (5 DAF), the five isoforms of PUL were distinct under DHT and NHT treatments. Spot 37 and spot 36 was the fastest responder to DHT and NHT, respectively (Fig. 3b).

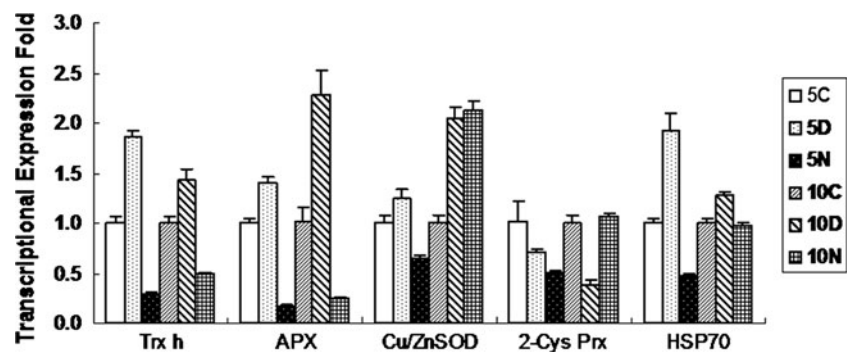
Moreover, spots 57 (iii-type) and 58 (iv-type) revealed two different accumulation patterns (Fig. 4). While the former accumulated in all of the grain-filling stages and three temperature treatments, the latter disappeared at 5 N (NHT treatment at 5 DAF) and 10 DAF.

Effect of high temperature on the accumulation of stress and defense proteins during grain filling

Spot 29 was identified as Trx h (Table 3), which was the homolog of OsTrx23 with the same locus (Os07g08840). The accumulation of Trx h was suppressed under DHT, whereas enhanced under NHT (Supplemental Table S1).

Two members of Prxs, 1-Cys Prx (spots 59 and 60, with ii-type and iii-type, respectively) and 2-Cys Prx (spot 10, with i-type), were detectable on the 2-DE gels. The accumulation of the two isoforms of 1-Cys Prx were almost undetectable at 5 DAF, began to increase at 10 DAF and peaked at late developmental stage (Supplemental Table S1). However, there was an exception that spot 59 of NHT could be detected at 5 DAF, indicating that NHT may trigger the early accumulation of spot 59 (Fig. 4). In addition, the accumulation of 2-Cys Prx approximately declined concomitant with seed development (Supplemental Table S1). Compared with the control, high temperature could increase their accumulation at almost developmental stages with various accumulation patterns in response to DHT and NHT (Table 3; Supplemental Table S1).

Fig. 7 Changes in the relative abundance of selected genes at 5 and 10 DAF with different high-temperature treatments by qRT-PCR. The value of 5C and 10C was set to “1” for each gene. 5C, control at 5 DAF; 5D, day high temperature at 5 DAF; 5N, night high temperature at 5 DAF; 10C, control at 10 DAF; 10D, day high temperature at 10 DAF; 10N, night high temperature at 10 DAF



Spot 28 was identified as Cu/ZnSOD chloroplast precursor (Table 3), the increase of whose accumulation under NHT was much greater than that under DHT (Supplemental Table S1).

Another enzyme involved in detoxication of hydrogen peroxide, APX (spot 8, Table 3), was also identified, whose down-regulated accumulation extent of NHT was greater than that of DHT. During grain filling, the enzyme was present only at 5 and 10 DAF, and undetectable at 15 and 20 DAF (Supplemental Table S1).

One HSP70 protein (spot 1, Table 3) showed elevated accumulation level under DHT and NHT at most developmental stages, although their increase extent was different. Moreover, one Hsp20 domain containing protein (spot 45, Table 3) was also identified, which may protect itself from heat denatured.

In addition, two spots 50 and 51 corresponded to glyoxalases I (Table 3; Supplemental Table S1). Compared with control, the accumulation tendency of two isoforms of glyoxalases I was similar, suppressed by high temperature at 5 and 10 DAF, whereas enhanced at 15 and 20 DAF. Furthermore, there was no significant difference between DHT and NHT, except that the up-regulated accumulation degree of spot 50 under NHT at 15 DAF was smaller than that under DHT.

Transcript analysis by qRT-PCR

Transcript analysis was used to verify the changed expression of genes corresponding to high-temperature-responsive proteins at the mRNA level. Five selected stress and defense transcripts including thioredoxin h-type protein (Trx h, spot 29), ascorbate peroxidases (APX, spot 8), 2-Cys peroxiredoxin (2-Cys Prx, spot 10), Cu/Zn Superoxide dismutase (Cu/ZnSOD, spot 28) and heat shock protein 70 (HSP70, spot 1) for qRT-PCR analysis are shown in Fig. 7. According to the method of protein analysis, expression change patterns of selected genes between NHT and DHT were analyzed at 5 and 10 DAF. The following descriptions were about the gene expression

changes of NHT compared with DHT. HSP70 was repressed at 5 DAF, with no change at 10 DAF, consistent with its protein accumulation pattern. Expression of APX and 2-Cys Prx in mRNA level was similar to that of corresponding proteins at 10 DAF, down-regulated and up-regulated, respectively, but inconsistent at 5 DAF. Cu/ZnSOD and Trx h had opposite expression patterns between mRNA and protein; both repressed in mRNA level, while enhancing in protein level at 5 DAF. The presence of inconsistent expression patterns between mRNA and protein level demonstrated the importance of proteomic studies.

Discussion

Temperature choice

The objective of our study was to contribute to the basic theoretical research for a better understanding of the different responsive mechanisms of proteins in rice filling grains under DHT and NHT. In Yangtze River rice growing region in China (including Wuhan), the average temperature for growing rice is 22–24°C, and the optimal temperature for grain filling is 22–28°C (<http://www.hbnw.gov.cn/other/tqyb4ny/qh/z.htm>). According to the natural temperature variation in rice growing season, the temperature difference was 8°C (Yuan 1994). Thus, the average temperature of the control and heat treatment was set to 24 and 31°C, respectively, and the temperature difference was set to 8°C. In addition, the highest temperature may reach 35–40°C during rice grain-filling stage in Wuhan. So, the temperature regime for DHT was set to 35°C day/27°C night, which also conformed to the local climate during rice grain filling according to the meteorological records in Wuhan (<http://hubei.weather.com.cn/xztq/index.shtml>). In addition, Peng and his colleagues (2004) reported that the effect of increasing night temperature on rice grain yield was greater than day temperature. Thus, to verify this result, in addition to the DHT treatment, we also designed the NHT treatment to compare the different effects of high temperature under light and dark conditions on rice quality and accumulation profiling of rice filling grain proteins. Therefore, under the premise of the same average temperature and temperature difference, the temperature regime for NHT was set to 27°C day/35°C night. In nature, there was no such temperature regime in which the night temperature was higher than the day temperature. However, to study the different effects of high temperature under light and dark conditions on a certain physiological process for the basic research in plant, the night temperature could be higher than the day temperature, such as the studies of Yang et al. (2005) and Dai et al. (2009). So,

although it was the ideal condition, the temperature regime (27°C day/35°C night) was acceptable for our study purpose.

Different effects of DHT and NHT on grain quality

Previous studies proved that excellent experimental reproducibility could be attained by using the incubator system to study the rice grain-filling characteristics under high temperature (Yamakawa et al. 2007; Lin et al. 2005). In this study, plant incubators were also used to offer the suitable, stable and repeatable experimental conditions for three thermal regime treatments (DHT, NHT and control).

The increased rice grain-filling rate, decreased grain weight, low amylose content, poor milling quality and increased chalkiness degree were the common symptoms of the NHT and DHT ripened grains (Fig. 1; Table 2). The different effects between NHT and DHT were just the change ranges of the observed symptoms. Viewing from the grain weight, we could find that relative to the control, the decreasing extent of NHT (17.1%) was larger than that of DHT (12.3%). This result was consistent with the conclusion that the effect of NHT on crop yield was greater than that of DHT (Peng et al. 2004). Furthermore, compared with DHT, NHT exerted less effect on head rice rate and chalkiness, which was in agreement with observations in one previous study (Dai et al. 2009). However, contrary to the research of Dai et al. (2009), the milled rice rate of NHT grains showed lower than that of DHT. The contradictory result indicated that further detailed research would be needed to determine the different effects of NHT and DHT on rice yield and quality using many more rice varieties under the reproducible temperature treatments.

Accumulation of isoforms of PPDK and pullulanase in response to high temperature during rice grain filling

PPDK

PPDK, a C_4 plant enzyme, is expressed in rice developing caryopses with multiple PPDK isoforms and in high abundance (Xu et al. 2008; Lin et al. 2005; Chastain et al. 2006). The most expressed in non-photosynthetic rice tissues is cytosolic OsPPDK (cyOsPPDK), which includes cyOsPPDKA and cyOsPPDKB, encoded by *OsPPDKA* and *cyOsPPDKB*, respectively (Imaizumi et al. 1997; Moons et al. 1998; Yamakawa et al. 2007). In the present study five PPDK isoforms were identified, all of which were cyOsPPDKB. The accumulation tendency of PPDK was consistent with the results in some other studies (Kang et al. 2005; Méchin et al. 2007).

Time-course analysis by 2-DE revealed that the accumulation of most of these isoforms was repressed by high

temperature. The expression of *cyOsPPDKB* was also down-regulated at transcripts level under heat stress in rice grains (Yamakawa et al. 2007). Furthermore, the differential accumulation of PPDK under comparable DHT and NHT treatments mainly occurred at 5 DAF or 10 DAF during grain filling, and it was precisely the golden time when PPDK played important role (Chastain et al. 2006; Kang et al. 2005; Méchin et al. 2007). Remarkably, at early stage, the suppressed extent of accumulation amount of PPDK isoforms and grain chalkiness degree were in the same order: DHT > NHT > control. The consistency might be explained at least in part by the conclusion that *cyPPDKB* may be one dark horse of the candidate factors triggering for grain chalkiness (Kang et al. 2005; Yamakawa et al. 2007). It led us to propose a putative molecular mechanism that the different suppressed extent of accumulation amount of *cyPPDKB* may result in the different grain chalkiness degrees between NHT and DHT. However, the underlying mechanism of how down-regulation of *cyPPDKB* causing chalky appearance in rice grain is still unclear. Further thorough research is needed to confirm the effect of accumulation of *cyPPDKB* on the chalkiness degrees of rice grains under different high-temperature treatments (DHT and NHT). Furthermore, to clarify the precise molecular mechanisms of generation and function of differentially accumulated isoforms in developing rice seeds under heat stress or other stresses, further robust research is necessary.

PUL

PUL is one type of starch-debranching enzymes involved in amylopectin biosynthesis (Kubo et al. 1999). Now, it is considered that PUL has a dual function in both starch synthesis during grain ripening and starch degradation during germination in rice seeds (Li et al. 2009; Yamasaki et al. 2008).

Five isoforms of PUL were shown in developing rice seeds. This same result was also reported by other researchers (Xu et al. 2008). However, to the best of our knowledge, the dynamic accumulation profiles of PUL isoforms in response to high temperature were displayed by proteomic approach for the first time.

The monitored temporal expression or accumulation tendency of PUL coincided with other research studies at transcript level or protein level with only slight variations in the peak stage under natural conditions (Duan and Sun 2005; Xu et al. 2008; Li et al. 2009). This may be caused by the different rice varieties or variations in growing conditions.

Compared with the wild type, except that the short chains ($DP \leq 13$) of amylopectin in *PUL* mutants were increased, the α -glucan composition and the structure of

amylose and amylopectin were essentially the same in *PUL*-deficient mutants (Fujita et al. 2009). In our study, since the structure and content of amylopectin were not analyzed, plus no one published study about the influence of over-expression *PUL* on the starch composition and structure in cereals, the function and molecular mechanism of *PUL* up-regulated under high temperature and distinct thermal responses to DHT and NHT could not be postulated and interpreted legitimately. Therefore, a more precise research is required urgently.

Stress and defense proteins

As expected, high-temperature stress stimulated the differential accumulation of some stress and defense proteins in rice filling grains, including Trx h, peroxiredoxins (Prxs), Cu/ZnSOD, APX, heat shock proteins (HSPs) and glyoxalases I. However, the accumulation patterns of stress and defense proteins induced by DHT and NHT treatments during rice grain-filling stage were distinct.

Trx h

Trx h is one type of thioredoxins, which are small proteins (around 12 kDa) and ubiquitous in all organisms (Gelhay et al. 2004). Based on their redox activity, Trx h proteins are involved in many stress responses, such as hormone stress, oxidative stress, heat stress and cold stress (Nuruzzaman et al. 2008; Serrato and Cejudo 2003; Lee et al. 2007; Xie et al. 2009). In rice, nine genes were predicted to encode Trx h, and *OsTrx23* was one of them (Nuruzzaman et al. 2008). In the present study, Trx h identified is the homolog of *OsTrx23*. *OsTrx23* was initially identified in rice phloem sap, which contains several motifs, particularly the N-terminal MAAEE and C-terminal RKDD motif, critical for the cell-to-cell movement (Ishiwatari et al. 1995; Ishiwatari et al. 1998). Both motifs are also present in Trx h identified, proving this protein is also phloem sap protein and can move from cell to cell.

The potential Trx h target proteins detected using proteomic or other approaches were listed in one previous review (Gelhay et al. 2004). Some differentially accumulated proteins exposed to high temperature, including Cu/ZnSOD, 1-Cys Prx, glyoxalase, triose phosphate isomerase, enolase, aldolase, PPDK, alanine aminotransferase and malate dehydrogenase, were precisely the potential Trx h target proteins. Thus, we speculated that during rice grain filling, according to its involvement in the transfer of nutrients and other compounds from the plant to the developing seed (Serrato and Cejudo 2003) and reversible redox activity (Wong et al. 2003), Trx h possibly acted as a regulator and/or participant that could interact with the target proteins and maintained a relative balance

to catalyze the normal storage material biosynthesis; however, when exposed to high temperature, the accumulation of Trx h was suppressed (DHT) or enhanced (NHT); then, the balance was broken down, leading to the abnormal storage materials biosynthesis and finally affecting the rice yield and quality. However, the molecular mechanism of different effects of DHT and NHT on the accumulation of Trx h, and the confirmation of Trx h interacting with its target proteins will need further research.

Prxs

Prxs are ubiquitous enzymes that could decompose peroxides by their highly reactive cysteine residues under unfavorable conditions (Wood et al. 2003). Two members of Prxs (1-Cys Prx and 2-Cys Prx) were detectable on the 2-DE gels and also observed in other proteomic studies (Finnie et al. 2002; Laino et al. 2010). It is reviewed that 1-Cys Prx and 2-Cys Prx are produced due to a gene duplication event taking place prior to the evolution of eukaryotes (Aalen 1999). High temperature may increase the accumulation level of the two isoforms of 1-Cys Prx at most developmental stages. The result could prove that 1-Cys Prx would protect nuclear components of immature embryos and the aleurone layers against free radicals (Stacy et al. 1999; Aalen 1999). The 2-Cys Prx is a chloroplastic protein reduced by most thioredoxins in vitro involved in the photosynthetic antioxidant network (Dietz 2003). It was presumed that 2-Cys Prx originated from the pericarp, which is green and photosynthetic before maturation of the seed. The 2-Cys Prx could mediate peroxide detoxification in the plastids during the dark phase (König et al. 2002). Thus, this protein may be associated with the resistance of NHT in early developing rice seeds.

Other stress and defense proteins

One Cu/ZnSOD chloroplast precursor was identified. SOD could rapidly convert superoxide to hydrogen peroxide and molecular oxygen, constituting the first line of defense against highly toxic superoxide radicals (Shin et al. 2005). The accumulation of Cu/ZnSOD was up-regulated under high temperature, which was in contrast with one previous study (Lee et al. 2007). These might indicate that the thermal response of Cu/ZnSOD showed the various accumulation patterns in different tissues and/or treatment methods. Moreover, the up-regulated accumulation extent of Cu/ZnSOD under NHT was much greater than that of Cu/ZnSOD under DHT, suggesting Cu/ZnSOD may play a more important role under NHT.

During grain filling, APX (spot 8) was present only at 5 and 10 DAF. The detectable accumulation character of

APX was consistent with other proteomic studies on barley and maize seeds (Finnie et al. 2002; Méchin et al. 2004).

HSPs act as molecular chaperones that could stabilize protein conformation and aid in the renaturation of heat-denatured proteins (Wang et al. 2004). And, under high-temperature treatment, the increase of HSP70 was associated with a decrease in wheat dough quality (Blumenthal et al. 1998). Thus, the enhanced accumulation of HSP70 was possibly related with the poor rice quality under DHT and NHT.

It has been reported that glyoxalases I could confer tolerance to heat stress (Laino et al. 2010). In the present study, there was no significant difference between DHT and NHT at most developmental stages. This may in part indicate that the response of glyoxalases I to DHT and NHT was similar in filling seeds.

Transcript analysis

To verify the changed expression of genes corresponding to five stress and defense proteins at the mRNA level, transcript analysis was used. However, not all the protein levels were correlated with their corresponding transcript levels. To date, there is a consensus that numerous proteins have faint correlation with their corresponding mRNAs in expression profiles in many organisms (Pradet-Balade et al. 2001; Greenbaum et al. 2003; Schmidt et al. 2007). This is because gene expression is controlled at multiple levels. Some researchers thought that this faint association between mRNA and protein levels suggested the importance of posttranscriptional control (Xu et al. 2008; Wu et al. 2008). Taking Cu/ZnSOD for example, the transcript level and protein level of Cu/ZnSOD were consistent under DHT, whereas contrary under NHT. Sunkar et al. (2006) reported that miR398 was important for posttranscriptional chloroplastic Cu/ZnSOD mRNA accumulation and oxidative stress tolerance (negative correlation). Thus, it was postulated that the different regulatory mechanisms of miR398 under DHT and NHT may lead to the distinct expression patterns of Cu/ZnSOD between the transcript level and protein level. Of course, further research is needed to validate this speculation.

Proteins of unknown function

In this study, some proteins for which the function, or precisely the specific function in seeds, were unknown, were identified. OSEYA1 (spot 4) was encoded by *OSEya1*, the homolog of the *eyes absent (eya)* gene in animals. *OSEya1* expressed in the embryo, shoot apex, and caryopsis in rice, but not in leaves (Takeda et al. 1999). The effect of high temperature on accumulation of OSEYA1 was mainly at 5 and 10 DAF (Table 3;

Supplemental Table S1), which was possibly explained by OSEYA1 regulating the morphogenesis of lateral organs as a subunit of a transcription factor (Takeda et al. 1999).

Spot 20 (unknown protein) was a DNA-binding protein according to the annotations from NCBI and InterProScan databases. The accumulation of spot 20 was enhanced and decreased by NHT at 5 and 20 DAF, respectively; however, there was no significant difference between DHT and the control (Table 3; Supplemental Table S1). Thus, we postulated that this protein might be more sensitive to NHT at the initiate and late grain-filling stages than DHT, and that it may play an unknown important role in rice seed development.

Spot 27 was a homolog of translationally controlled tumor protein (TCTP). In Arabidopsis, TCTP is an important regulator of growth throughout plant tissues and developmental stages (Berkowitz et al. 2008). Some studies showed that differential expression of TCTP was observed under abiotic stresses, such cold stress (Lee and Lee 2003). In our study, the effect of high temperature on accumulation of rice TCTP was monitored. And there was no significant difference between DHT and NHT, maybe proving that the expression of TCTP was light-independent (Berkowitz et al. 2008).

Two proteins (spots 57 and 58) with distinct accumulation patterns were identified as the isoforms of stress-responsive A/B barrel domain-containing protein. There is little information on stress-responsive A/B barrel domain; only InterPro annotation (<http://www.ebi.ac.uk/interpro/ISearch?query=IPR013097>+) can be obtained, whose function is unknown. To the best of our knowledge, this is the first report on stress-responsive A/B barrel domain-containing protein accumulated in rice grains. The physiological function of this protein and the cause of its isoforms' different responses to high temperature in rice seeds await more detailed study.

Conclusion

In summary, to the best of our knowledge, for the first time, we analyzed the dynamic changes in protein accumulation profiles during rice grain filling from 5 to 20 DAF with comparable DHT and NHT treatments by combined methods of 2-DE and MS. And the different effects of NHT versus DHT treatments on rice quality were also evaluated. Our results suggested that the different suppressed extent of accumulation amount of cyPPDKB may result in the different grain chalkiness degrees between NHT and DHT. The dynamic accumulation profiles of isoforms of several proteins, such as PPKD and pullulanase, in response to high temperature (DHT and NHT) were displayed by proteomic approach for the first time in rice filling grains.

Most of the isoforms identified showed different accumulation change patterns between NHT and DHT. Especially, when NHT and DHT treatments were compared, stress and defense proteins displayed distinct accumulation change patterns, indicating that different molecular mechanisms involved in the resistance to NHT and DHT stress during grain filling. In conclusion, this result could help understand the molecular basis of physiochemical processes governing rice yield and quality under night or day high-temperature condition during rice grain filling.

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Conflict of interest The authors have declared no conflict of interest.

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