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Influence of High Temperature during Grain Filling on the Accumulation of Storage Proteins and Grain Quality in Rice (*Oryza sativa* L.)

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The present study was performed to understand the effects of high temperature (HT) during filling on the expression of storage proteins and the quality of rice grains. HT (35/30 °C day/night) reduced the weight, amylose content, and flour gel consistency of grains. It increased the accumulation of all classes of storage proteins at early filling stage but decreased the accumulation of prolamins at maturation. For albumins, the expressions of cyclophilin 2, peroxiredoxin, and HSP16.9 were differentially enhanced by HT. For globulins, HT decreased the accumulation of globulin but increased that of glyoxalase I and peroxiredoxin. HT enhanced the transcription of genes for glutelins, prolamins, globulins, and protein disulfide isomerase at early filling stage but decreased the expression of these genes at a later stage. Low amounts of prolamins and globulins, as well as low pH value, were found in sound, immature, and dead kernels grown under HT. The relationships among HT, storage proteins, and grain quality are discussed.

KEYWORDS: High temperature; *Oryza sativa*; storage protein; prolamin; grain quality

INTRODUCTION

High temperature (HT) is known to reduce rice grain yield and quality. Exposure to HT enhances the rate of grain filling but reduces grain weight and final yield (*1*−*3*). With regard to the milling quality, HT can reduce the ratio of head rice, increase the chalky appearance of grain, and thus degrade its market value (*2*,*3*). Nevertheless, the physiological basis for these HT effects still needs clarification.

During grain filling, changes in temperature could change the chemical ingredients of rice caryopses such as starch and storage proteins and the contents of fatty acid, thus affecting the quality of rice (*4*−*6*). Most of the research has focused on the effects of HT on starch, which is important to the physicochemical properties of rice grains. HT can reduce the content and change the structure of starch grains (*7*). The starch of rice grains grown under HT had high gelatinization temperature and enthalpies (*8*, *9*). Amylose content was reduced, and the chain lengths of the 13−24 fraction of amylopectin were increased by high night-time temperature (*7*). The modification of starch structure might be attributed to the regulation of expression of corresponding enzymes and genes (*10*, *11*).

Protein accounts for 6−10% of dry matter of debraned rice grains and is important for grain quality for nutrition, cooking, and brewing (*12*, *13*). Rice storage proteins are in general classified into glutelins, prolamins, globulins, and albumins, constituting about 70, 3, 7, and 5% of rice grain nitrogen, respectively (*12*, *13*). Glutelins contain the highest amount of sulfur-containing amino acids (*14*). Prolamins were reported to be negatively related to the stickiness of rice flour and eating quality of rice grains, especially for japonica cultivars (*15*). A high ratio of glutelins to prolamins was reported to be negatively correlated with the brewing quality of sake (*16*, *17*). In addition, the content or chemical properties of proteins, such as SH status, may affect the physicochemical characteristics of rice flour (*18*, *19*).

Compared with the effect of HT on starch, that of HT on protein has been much less studied in rice. HT can change the nitrogen or protein content of rice grains, but the extent has been controversial. Although HT can increase the protein content of rice grain (*20*), this may not be significant (*21*). Most reports have focused on the effects of HT on nitrogen or crude protein content. Although storage proteins contain >90% of the nitrogen of rice grains, the influence of HT on different classes of storage proteins has not been documented. The characteristics of storage proteins in defective grains induced by HT are also unknown.

Taiwan is located in the subtropics and is one of the lowest latitude areas where japonica type rice genotypes are mostly cultivated. The daily mean temperature is >30 °C during the grain-filling stage in the main crop season. We have reported the effects of HT on grain yield, milling quality, and some physicochemical properties (*3*). Like others, we consider that HT may be the major climatic factor conferring the chalky trait of rice (*2*, *22*). We further used a proteomic approach to reveal the protein expression patterns of rice grains during development and in response to HT (*23*). In the present study, we focused on the effects of HT on the accumulation of storage proteins of different classes and the transcriptional expression of related genes. We discuss the

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association of analyzed characteristics and grain quality together with our previous findings.

MATERIALS AND METHODS

Plant Materials. Two rice cultivars, an indica type (Taichung Native 1, TN 1) and a japonica type (Tainung 67, TNG 67), were used in this study.

Table 1. Primers Used for Gene Expression Analysis

gene name	primer sequence (5′−3′)
prolamin 7 (*Pro7*)	F: ATGAAGATCATTTTCGTCTTTGCT R: AACTTCCGAACCACTATACTGT
glutelin (*Glu*)	F: CTAGTCGTGCTGATTCATACAA R: TTCGGACTCATTCGATAATCCT
19 kDa globulin (*Glb*)	F: GGAGATGAGGTTCAGGGACA R: CCTCGTAGCTCCTCACCATC
protein disulfide isomerase (*PDI*)	F: TGATGCTGCAGTTGAGAG R: CCTCAGCCCAAAGTACTG
starch synthase (*SSII*)	F: GCGGTAGAAGAGGAGACGTG R: GGGTAAAGCACCTGCAACAT
granule bound starch synthase, waxy gene (*GBSS*)	F: GACGTCCAGATCGTTCTTCT R: TCAAGGAGCAGCCACGTTCT
cyclophilin 2 (*Cyp2*)	F: GAACACGAGGGTGTTCTTC R: AGAACTGGGACCCGTTAG
peroxiredoxin family (*PRDX*)	F: AGAAGGTCGTCCTCTTCG R: TGTTTGCAACAGTAACCTTG
peroxiredoxin 6 (*RAB24*)	F: GGCGACACCTATGTCATC R: GTACAGGAAGCTCAGCTT
16.9C heat shock protein (*16.9c HSP*)	F: CCGACCTCCCCGGCGTCA R: GTGCCACTTGTCGTTCTTGTC
glyoxalase I (*GLOI*)	F: GGACACCAACTTTGCACT R: TCCTCAGCAGCTTCATAC
eukaryotic elongation factor 1A (*eEF1A*)	F: ATGCTCTCCCCCATGCTATC R: TCTTCCTTGCTCATCCTGTC

TNG 67 is a high-yield cultivar, but sensitive to HT, and has a high chalky grain rate under HT. Seedlings at the 3-leaf stage were transplanted into plastic Wagner's pots (1/2000 a), with 3 seedlings per pot. For each pot, 2.0 g of ammonium sulfate, 3.5 g of superphosphate, and 1.0 g of potassium chloride were applied as basal dressing, and 0.5 g of ammonium sulfate was added as top dressing at panicle initiation stage. Rice plants were grown with natural light in a phytotron at 25/20 °C (day/night) and 75−90% relative humidity. For HT treatment, plants were transferred to 35/30 °C (day/night) at heading stage. Spikelets were labeled at anthesis when at least half the spikelets of a panicle were flowering. The labeled spikelets were harvested at 3, 6, 9, 12, 15, 20, and 30 (maturation) days after anthesis (DAA) and stored in liquid nitrogen. The maturation stage of a panicle was defined as only three to five spikelets remaining green on the bottom region of a panicle. To avoid developmental variation among spikelets within a panicle, only labeled spikelets of a panicle were harvested for analysis.

For analyzing the expression patterns of storage proteins in grains with different appearances, mature grains were harvested, dehusked, and passed through a single-grain rice inspector (RN-600, Kett, Japan). Dead, immature, and sound grains were separated for analysis of storage protein and flour pH value.

Grain Weight, Amylose Concentration, Protein Concentration, and Gel Consistency. Mature panicles and grains were weighed. Grains were husked, ground into flour, and passed through a sieve with 100 mesh. Analysis of amylose, protein, and gel consistency was performed at the Rice Quality Laboratory of the Taichung District Experimental Station, Taiwan, according to established routine procedures. Amylose concentration was determined by use of an autoanalyzer (AAII system, Technicon, U.K.) as described (*24*). Protein concentration was measured by use of near-infrared reflectance spectrometry (Bran and Lubbe InfraAlyzer 500, Germany).

Gel consistency was measured as described (*25*). Rice flour was put in a test tube, mixed with thymol blue and 0.2 N potassium hydroxide solution, heated in a water bath for 10 min, allowed to stand for 5 min, and cooled at 0 °C for 20 min. The test tube was set horizontally for 30 min, and the gel length in the tube was measured and classified as hard (H), ≤35 mm; medium (M), 36−49 mm; or soft (S), ≥50 mm.

pH Value of Brown Rice Grains. The pH value of brown rice grains was measured as described (*26*). Brown rice grains with different appearances were ground and mixed thoroughly with 200 μL of distilled water, and the pH value was measured with a pH-meter (XP-701, SUNTEX, Taiwan).

Extraction, Quantification, Electrophoresis, and Densitometry Analysis of Storage Proteins. Four classes of storage proteins were extracted sequentially as described (*27*). In total, 100 mg of brown rice powder was defatted with 2 volumes of ethyl ether and vacuum-dried before storage proteins were extracted. Albumins were extracted by

Table 2. Effect of High Temperature on Agronomic and Physicochemical Related Characteristics[a]

cultivar and treatment	panicle weight (g/panicle)	thousand spikelet weight (g)	amylose (% db)[b]	protein[c] (% db)	gel consistency[d] (mm)
TN 1					
25/20 °C	1.74 ± 0.37	23.0 ± 0.6	28.7 ± 0.4	11.1 ± 0.3	25 H
35/30 °C	1.62 ± 0.30	19.4 ± 0.5	21.2 ± 0.4	11.3 ± 0.5	17 H
TNG 67					
25/20 °C	2.91 ± 0.62	22.1 ± 1.7	22.1 ± 0.2	9.1 ± 0.2	80 S
35/30 °C	1.75 ± 0.42	19.9 ± 0.4	15.5 ± 0.1	8.8 ± 0.3	48 M

[a] Data are means ± standard errors of four biological replicates. Panicle weight and thousand spikelet weight were measured at maturation stage. [b] Percentage of dry weight base. [c] Crude protein. [d] H, ≤ 35 mm; M, 36−49 mm; S, ≥50 mm.

Table 3. Storage Protein Levels in Response to High Temperature[a]

cultivar and treatment	glutelin (mg/g db)	prolamin (mg/g db)	globulin (mg/g db)	albumin (mg/g db)	glutelin/prolamin ratio
TN 1					
25/20 °C	90.2 ± 7.3	7.3 ± 0.6	35.4 ± 5.6	17.3 ± 0.2	12.4
35/30 °C	92.4 ± 6.8	4.8 ± 0.5	16.7 ± 4.2	19.5 ± 0.3	19.3
TNG 67					
25/20 °C	65.3 ± 6.5	7.6 ± 1.2	27.6 ± 4.3	30.8 ± 3.2	8.4
35/30 °C	69.5 ± 7.4	5.5 ± 0.8	18.7 ± 3.6	27.4 ± 2.5	12.6

[a] Data are means ± standard errors of four biological replicates and expressed on a dry weight basis.

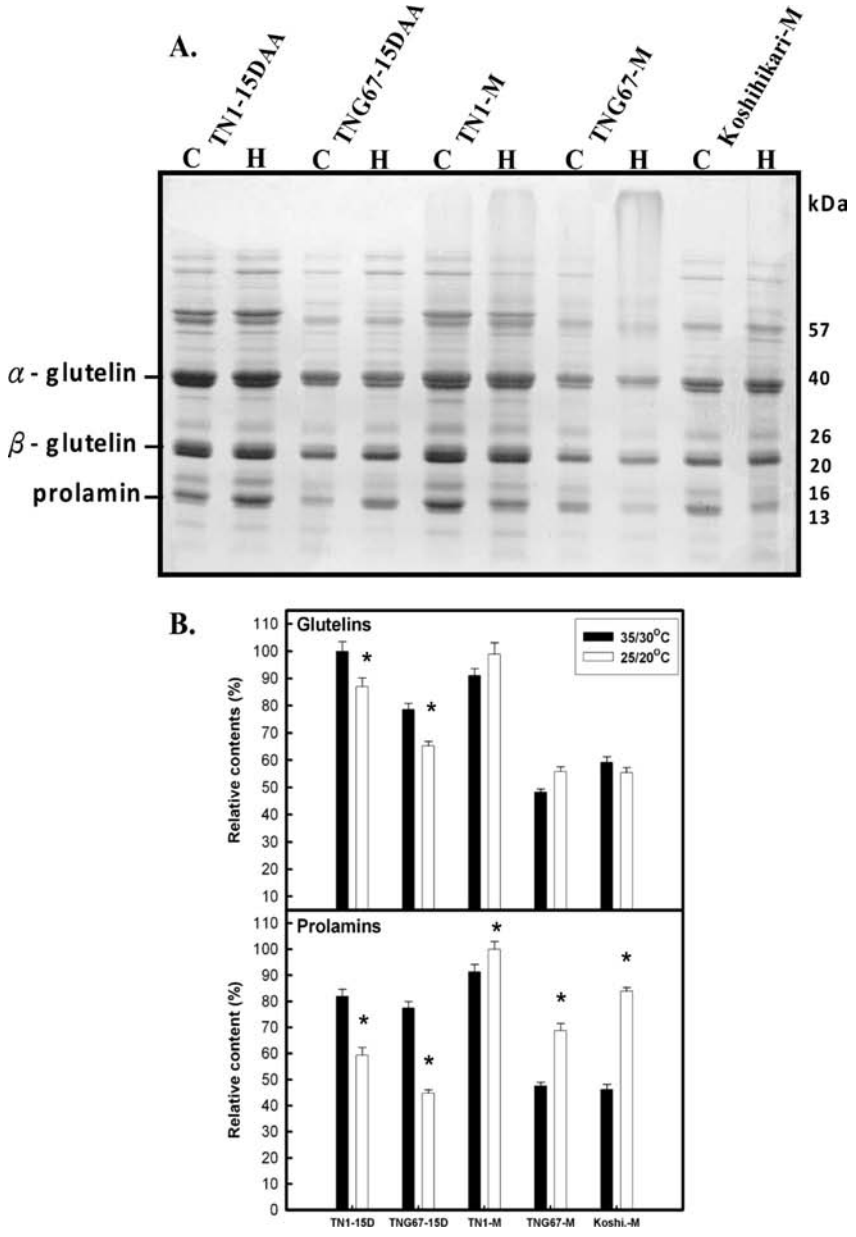

Figure 1. SDS-PAGE analysis of glutelins and prolamins of kernels of cultivars TNG 67, TN 1, and Koshikari in response to temperature treatments. (**A**) Gel patterns of SDS-PAGE; (**B**) relative contents of kernel proteins of three cultivars at two different development stages calculated from four biological repeats. Proteins were extracted with 100 mg of brown rice flour, and 20 μL of the protein extract was loaded in each lane. Kernels were harvested at 15 days after anthesis (DAA) and maturation (M). $*$, $P \leq 0.05$ between temperature treatments.

solution A (10 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetate), glutelins were extracted by solution B (solution A with 2% SDS and 0.6% 2-mercaptoethanol), and prolamins were extracted by solution A with 60% 1-propanol. Each extraction was repeated three times, and supernatants were collected. Protein fractions were precipitated with cold acetone at −20 °C, vacuum-dried, and stored at −20 °C. Different classes of storage proteins extracted from every sample were weighed or used for electrophoresis.

Total storage proteins for electrophoresis were extracted by the SDS−urea attraction method (*27*, *28*). Samples were ground into powder. An amount of 30 mg was placed into a 2.0 mL Eppendorf tube to which was added 0.8 mL of SDS−urea solution (4% SDS, 8 M urea, and 20% glycerol dissolved in 1× PBS, pH 6.8; when being used, 5% β-ME was added). The mixture was shaken for 2 h at 60 °C and 1400 rpm by a Thermomixer (Eppendorf, Hamburg, Germany) and then centrifuged for 5 min at 20 °C and 13000*g*. Then the supernatant fluid was moved into a 0.2 mL Eppendorf tube; 20 μL of supernatant fluid was used for the electrophoresis. Electrophoresis was conducted with 13.5% SDS-PAGE. The gels were stained with Coomassie Brilliant Blue R250.

The amount of protein was determined by densitometry analysis (*28*). Gel images were taken by scanner (Epson perfection V750 PRO), and signal intensities of target polypeptide bands were measured by Scion Image software (Scion Image Beta 4.02). The background intensity of each gel was measured as we previously described (*23*). Net signal intensities of the target bands were measured and averaged from at least four biological replication gels.

Protein Identification by Tandem Mass Spectrometry. Protein identification was as we previously described (*23*). Protein bends were excised from three to five replicate gels, destained, and then subjected to in-gel reduction, alkylation, and tryptic digestion. The digestion mixture was loaded onto a 150 × 0.5 mm PE Brownlee C18 column with 5 μM particle diameter and 300 Å pore size (Perkin-Elmer). A gradient of 5−65% (v/v) acetonitrile in 0.1% (v/v) formic acid was generated over 60 min at a flow rate of 5 μL/min, eluted chromatographed peptides was loaded directly into the electrospray source of a LCQ ion trap mass spectrometer. The mass spectrometer was programmed to acquire successive sets of three scans. The MS scan determined ion intensities from m/z 395 to 1605. The most abundant ions were analyzed by zoom scan and MS/MS scan.

The resulting MS/MS spectra of the peptides were interpreted by database correlation with SEQUEST Browser software. Protein identification was conducted using the UniProt Knowledgebase (http://www.uniprot.org/), NCBI (http://www.ncbi.nlm.nih.gov/), KOME (http://cdna01.dna.affrc.go.jp/cDNA/), and MSU/TIGR databases (http://rice.plantbiology.msu.edu/).

Semiquantitative RT-PCR. The RNA expression of target proteins was analyzed by semiquantitative RT-PCR (*29*, *30*). Total RNA was isolated from 1 g of caryopses endosperm. First-strand cDNA synthesis involved 5 μg of total RNA with the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer's instructions. PCR amplification of selected genes was performed with VioTaq Taq DNA Polymerase (Viogene) and the Veriti Thermal Cycler (ABI) with primers obtained or designed from the rice genomic database (**Table 1**). The amplification cycle for each selected gene was based on our preliminary logarithmic/exponential tests. PCR products were separated by 1% agarose gel electrophoresis and visualized by 0.05% EtBr staining. Images of gels were taken using a Video Gel Image System (VGIS-1, TOPBOI, Taiwan), and densitometry analysis of target product bands involved the use of Scion Image Beta 4.02 (Scion Corp.). *eEF1A* was used as a normalization control.

Statistical Analysis. Statistical differences between measurements ($n = 4$) for different treatments or different times were analyzed following Student's t test or Duncan's multiple-range test. A $P < 0.05$ was considered to be statistically significant.

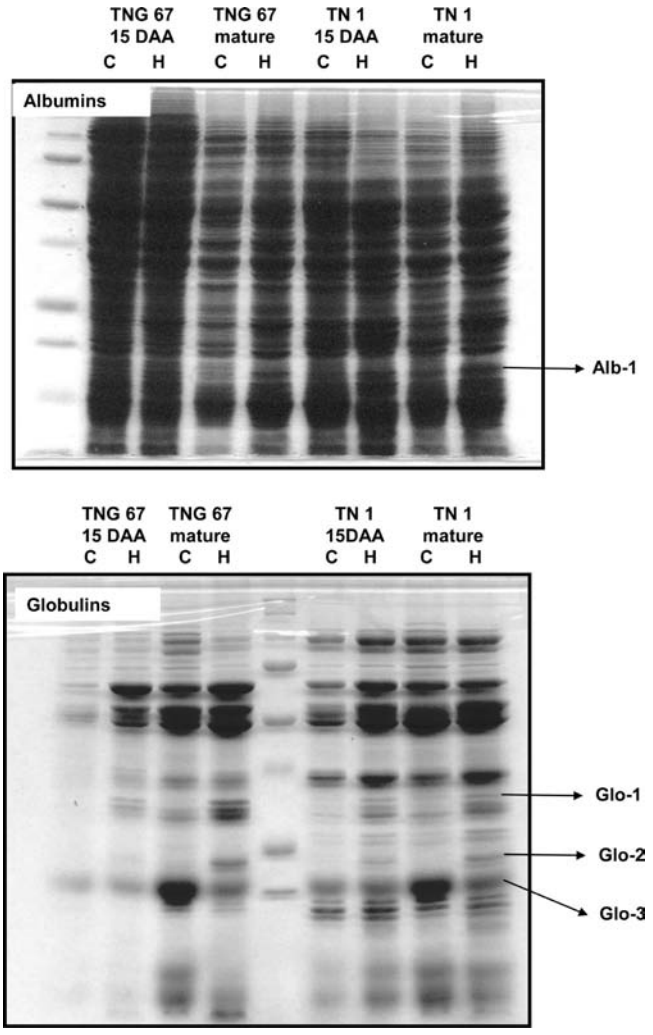

Figure 2. SDS-PAGE analysis of albumins and globulins of kernels of cultivars TNG 67 and TN 1 in response to temperature treatments. Proteins were extracted with 100 mg of brown rice flour, and 20 μL of the protein extract was loaded in each lane. Arrows indicate differentially expressed bands with temperature treatments. 15 DAA, 15 days after anthesis; mature, seeds harvested at maturation; C, control treatment; H, high-temperature treatment.

RESULTS AND DISCUSSION

Panicle and Grain Weight. The panicle weight was measured at the maturation stage. As depicted in **Table 2**, HT decreased the panicle weight and thousand grain weight of both cultivars, which was attributed to the decrease in fertility of spikelets and dimension of kernels caused by HT (*2*, *3*, *23*).

Amylose, Gel Consistency, and Crude Protein Concentration. HT substantially reduced amylose concentration (**Table 2**). HT decreased the gel spreading length of TN 1, the indica cultivar, but the grade remained within the hard (H) category. HT decreased the gel spreading length of TNG 67, the japonica cultivar, and the grade changed from soft (S) to medium (M). HT did not significantly change protein concentration in either cultivar.

As was found previously, HT can decrease the amylose concentration (*10*), whereas crude protein concentration is less sensitive to HT (*20*, *31*). The reduced amylose may result in a softer starch gel, as shown in **Table 2** (*21*). Although amylose concentrations were similarly reduced for both cultivars, the degree of response in gel consistency was distinct. A similar phenomenon was observed with HT for rapid viscosity analysis (data not shown), in which TN 1 (indica type) was less sensitive than TNG 67 (japonica type). Other factors may also be involved in the effects of HT on grain flour characteristics. Storage proteins contribute to the second largest number of macromolecules in rice grains and are important for physicochemical properties and palatability of the grains (*13*, *18*, *19*). Although crude protein concentration was not changed by HT, we further investigated the effect of HT on the accumulation of individual classes of storage proteins.

Concentrations of Prolamin and Globulin of Rice Grains Reduced by HT. The effect of HT on the level of each of the four classes of storage proteins is shown in **Table 3**. Concentrations of glutelin and albumin were not significantly affected by HT in either cultivar, but those of prolamin and globulin were reduced. The ratio of glutelin to prolamin was also increased.

Most storage proteins in rice grains are glutelins. Stable accumulation of glutelins and albumins under HT could explain

Table 4. Identification of Differentially Expressed Proteins by LC-MS/MS[a]

class of storage protein	M_r (kb)	p*I*	homologous protein	score	sequence covered (%)	accession no. (NCBI)
globulin						
Glo-1	32.53	5.51	glyoxalase I	412	24	gil50941905
Glo-2	24.03	5.96	putative RAB24 protein	272	32	gil50939487
Glo-3	21.04	7.51	19 kDa globulin precursor	78	13	gil20159
albumin						
Alb-1	18.32	8.61	cyclophilin 2	101	17	gil600769
Alb-1	17.28	5.58	peroxiredoxin	166	23	gil34911078
Alb-1	14.38	6.23	heat shock protein 16.9C	117	30	gil295501

[a] Differentially expressed protein bands indicated in **Figure 2** were dissected from gels and subjected to LC-MS/MS identification.

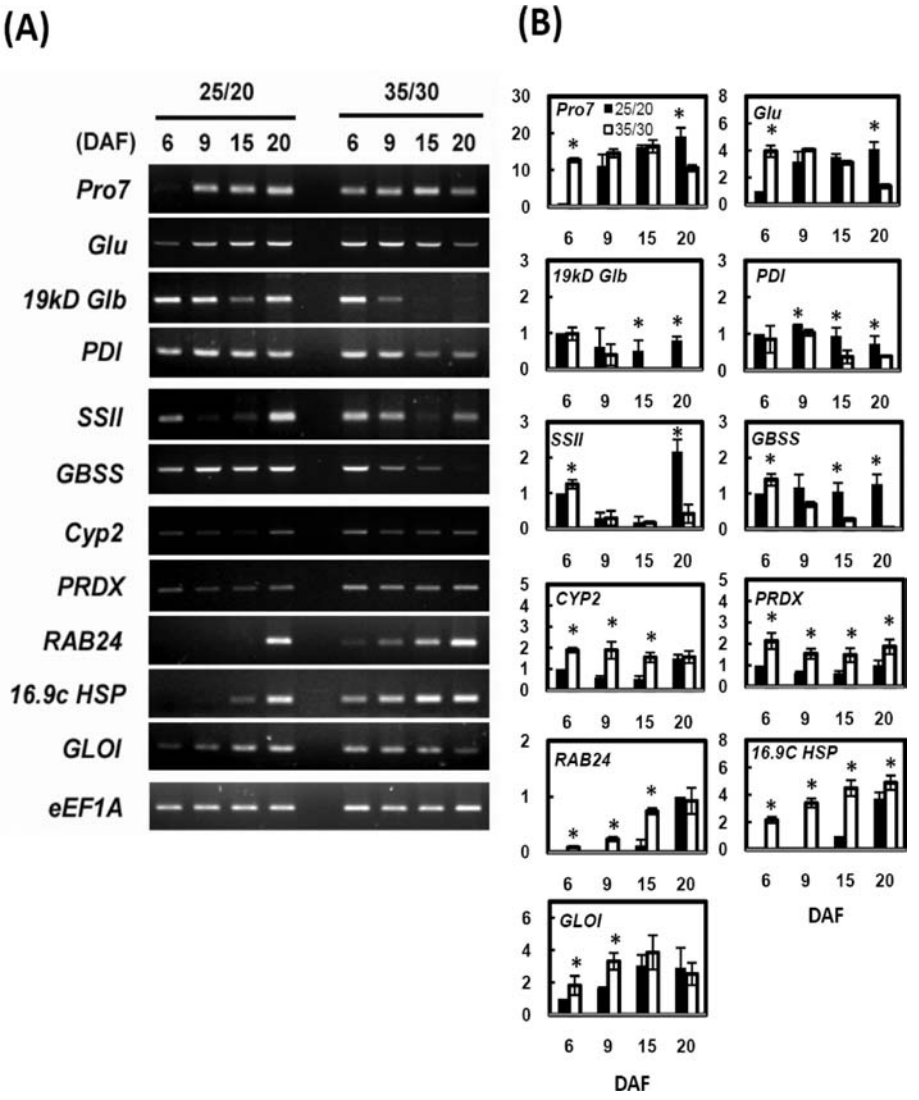

Figure 3. RT-PCR analysis of mRNA expression of genes related to accumulations of starch, storage proteins, and stress responses: (**A**) expression patterns of genes at different days after flowering (DAF) under temperature treatments; (**B**) relative expression of the genes at different days after flowering under temperature treatments. Expression of *eEF1A* at different days after flowering was set to 1 for the calculation of relative expression. Bars indicate standard errors of four biological replications. $*$, $P \leq 0.05$ between temperature treatments.

the relatively consistent amount of crude protein in grain in response to HT. From **Tables 2** and **3**, although HT did not significantly affect the quantity of total grain protein, it did decrease the accumulation of prolamins and globulins in rice grains. In addition, the relatively consistent accumulation of glutelins and decrease in that of prolamin could lead to a higher ratio of glutelin to prolamin in rice grains (**Table 3** and **Figure 5**). The ratio of glutelin to prolamin has been used as an indicator of grain quality for rice wine brewing (*16*). Thus, HT might also degrade grain quality for rice wine brewing.

In general, glutelins contain significantly high amounts of sulfur-containing amino acids, especially cysteine, the amino acid related to disulfide formation (*14*). Reduced relative amounts of prolamins and globulins with HT may result in a higher ratio of glutelins and disulfide bond potential of total storage proteins and then change the physicochemical properties, including gel consistency (**Table 2**) (*18*, *19*).

Change in Expression Profiles of Storage Proteins with HT. Changes in the expression profiles of glutelins and prolamins were determined by SDS-PAGE and are depicted in **Figure 1**. To determine whether the response of storage protein also appeared in a japonica variety with high eating quality, we added Koshihikari, a well-known premium quality rice cultivar in the international market, to the experiment. The accumulation of glutelins and prolamins was enhanced during the middle filling stage (15 DAA). At maturation, the amounts of glutelins were similar for both the controls and those exposed to HT for all three cultivars, whereas the amount of prolamins was lower in all cultivars (**Figure 1A**). Further densitometry analysis of relative contents confirmed the significant trends of expression patterns revealed on gel (**Figure 1B**).

Figure 2 shows the expression patterns of albumins and globulins in response to HT. SDS-PAGE revealed many polypeptide bands for albumins (water-soluble) and globulins (salt-soluble). The overall amounts of albumins appeared to be similar for both cultivars at 15 DAA and the mature stage. Both cultivars showed high expressions of Alb-1 with HT treatment. The amount of polypeptides was high in the globulin fraction at 15 DAA with HT. At maturation, two polypeptide bands, Glo-1 and Glo-2, were up-regulated and one band, Glo-3, was down-regulated with HT.

The differentially expressed bands in **Figure 2** were further subjected to protein identification by LC-MS/MS as shown in **Table 4**. Three proteins were identified from the Alb-1 band: cyclophilin2, peroxiredoxin, and heat shock protein 16.9C. Glyoxalase

I was identified in the Glo-1 band. A putative RAB24 protein was identified in the Glo-2 band, and globulin 19 kDa was found in the Glo-3 band.

Protein body I (PB-I) and protein body II (PB-II) are found in rice grains and distributed from the outer to inner portion, with a relatively higher number of PB-I and PB-II distributed in the outer endosperm layer than in the inner part (*32*). Prolamins are located in PB-I, and glutelins and globulins are located in PB-II (*33*, *34*). We found prolamins and globulins to be the main storage proteins affected by HT. HT accelerated the accumulation of both proteins during the early filling stage but resulted in a reduced amount at the final mature stage (**Figures 1** and **2**). The lower amounts of prolamins and globulins might change the compositions of PB-I and PB-II and thus affect the structure properties of mature rice grains. In addition, the amount of prolamins has been reported to be negatively related to the digestibility and palatability of rice (*13*, *15*). Thus, the decrease in prolamin and globulin concentraions with HT might also change the physicochemical properties and thus the utilization of rice flour. Further localization approaches are needed to clarify the effects of HT on the distribution of protein bodies, as well as its association with the appearance and physicochemical characters of rice grains. To our knowledge, these results may be the first observation of the effects of HT on the four classes of storage proteins in rice grains. As well, there has been no evidence showing the association of varietal difference in amylose concentration and changes in levels of storage proteins in response to HT (**Tables 2** and **3**).

Effects on Gene Transcription. To understand whether HT also affects the transcription of genes related to storage protein fractions, we performed semiquantitative RT-PCR analysis (**Figure 3**). Candidate genes, especially those identified by SDS-PAGE analysis in the present study, were chosen on the basis of their biochemical relationships with the accumulation of starch and storage proteins, as well as stress responses.

Under the control conditions (25/20 °C), the mRNA level of storage protein-related genes, including *PRO7*, *Glu*, *19kD-Glb*, and *PDI*, was increased gradually from 6 to 20 DAA. The expression of these genes, especially that of *19kD-Glb*, was significantly accelerated with HT until 15 DAA and then decreased thereafter. The mRNA level of *GBSSI* (for starch biosynthesis) increased gradually from 6 to 20 DAA under control conditions, whereas that of *SSIIa* showed a concave binominal pattern from 6 to 20 DAA. In response to HT, the expression of *SSIIa* and *GBSSI* was enhanced only at 6 DAA but showed a lower level at 20 and 15−20 DAA, respectively, than with the control temperature. With regard to stress response-related genes, the expression of *Cyp2* and *PRDX* was significantly increased at 6 DAA and remained high until 20 DAA with HT. The expression of *RAB24* and *16.9C HSP* was enhanced and increased from 6 to 20 DAA with HT. The expression of *GLOI* was increased with HT at 6 DAA and reduced thereafter.

In general, the mRNA level of most analyzed genes was increased along with grain filling under the control temperature. With exposure to HT, the expression was accelerated during the early filling stage but decreased after 15 DAA. The expression patterns for the genes related to biosynthesis of starch (*SSIIa* and *GBSSI*) or storage proteins (*PRO7*, *Glu*, *19kD-Glu*, and *PDI*) under HT were similar to those we previously found with our proteomic approach (*23*). A decrease in the expression of genes responsible for the accumulation of both starch and storage proteins can also explain the lower dry weight of grain after exposure to HT (*11*, *23*).

The products of *GLOI*, *RAB24*, and *Cyp2* were identified to be glyoxalase I, peroxiredoxin, and cyclophilin2, respectively

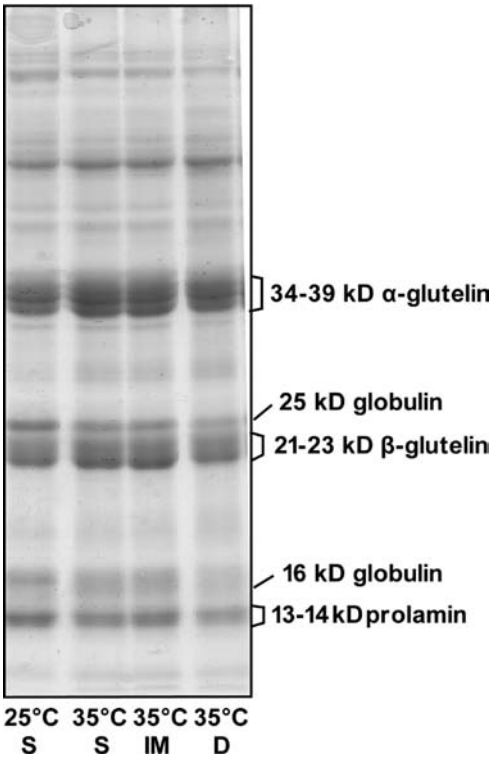

Figure 4. SDS-PAGE analysis of total storage proteins of flour from kernels with different appearances. S, sound kernel; IM, immature kernel; D, dead kernel. Proteins were extracted with 30 mg of brown rice flour, and 20 μL of the protein extract was loaded in each lane. Classes of proteins and their molecular masses in kilodaltons are indicated at the right side of the gel.

(**Table 4**). These genes are documented to be related to the stress responses of plants (*35*−*37*), but we found no identification in rice caryopsis or records of responses to HT treatment in the literature. In our results here, the enhancement or stimulation of both the translational and transcriptional expression of these genes with HT (**Figures 2** and **3**) suggests an internal stressful physiological condition within rice caryopsis as we describe in the next section. The roles of these three genes within rice caryopsis in response to HT are worthy of further study.

Weight and pH Value of Grains with Different Appearances. HT increases the rate of grains with defective appearances (*2*, *3*). Advanced analytical instruments can automatically separate brown rice grains into three classes: sound, immature, and dead. Immature grains have various types of chalky appearance, such as white core, white belly, and white base. Dead grains have a wholly opaque appearance. Under suitable temperature, most brown rice grains are sound. Our results showed that all three types of brown rice grains experiencing HT had significantly lower weight than the sound grains in the control (**Figure 5**). To determine whether the physiological status differed in grains with different appearances, the pH values were further determined (**Figure 5**). Grains grown under HT had significantly lower pH values than did grains grown under the control temperature. Dead grains induced by HT had the lowest pH value. The inner portion of endosperm was suggested to have a lower pH value than the outer layers in barley (*26*). Furthermore, the lower pH might be the result of hypoxia respiration in the inner portion of the endosperm (*38*). The lower pH value found in rice grains grown under HT, especially dead type grains, may be indicative of a hypoxic physiological status induced by HT. Stimulation of the expression of peroxidation responsive genes, RAB 24 (*RAB24*), peroxiredoxin (*Cyp2* and *PRDX*), and glyoxalase I (*GLOI*),

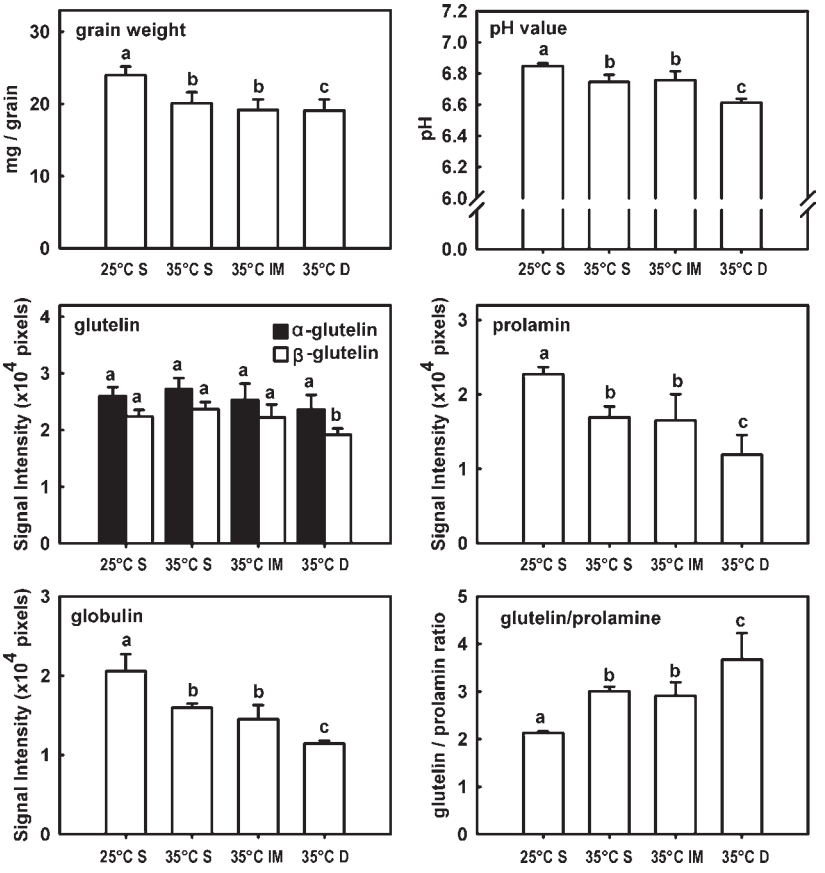

Figure 5. Weight, pH value, and concentration of storage proteins for kernels with different appearances. Densitometry analysis represents results from four biological repeats. Bars indicate standard errors of the mean. S, sound kernel; IM, immature kernel; D, dead kernel. Different letters indicate significant difference at $P \leq 0.05$.

at both transcription (**Figure 3**) and translation levels (**Figure 2**) might also reflect the physiological responses to HT. We conducted further experiments to test the hypothesis. In addition, the change of pH value in rice grains exposed to HT has not yet been documented. The lower pH might decrease the activities of enzymes involved in the biosynthesis of starch and storage proteins (*39*, *40*) and result in lower amounts of starch and storage proteins in grains grown under HT.

Accumulation of Storage Proteins in Grains with Different Appearances. HT is known to induce a defective grain appearance (*2*). The proportions of sound, immature, and dead type grains of TNG 67 were 94.5, 4.5, and 2.7%, respectively, under the control temperature, and 20.8, 45.5, and 33.2%, respectively, under HT. For cultivar TN 1, the proportions were 96.5, 2.3, and 1.3%, respectively, under the control temperature and 28.7, 40.5, and 29.5%, respectively, under HT. The accumulation patterns of storage proteins by classes of sound, immature, and dead type grains are in **Figures 4** (electrophoresis gel pattern) and **5** (amount). In comparison with sound grains grown under control temperature, significantly lower amounts of prolamin and globulin were found in immature and dead grains induced under HT. Low amounts of prolamin were found in all types of grains (even sound grains) grown under HT. A similar expression pattern of storage proteins was found in TN 1 cultivar. These results further confirmed that the accumulation of prolamins and globulins was more vulnerable to HT than that of the other storage proteins. In addition, we searched promoter regions of prolamin and globulin genes and found several putative stress responsive elements, including HSE (heat stress responsiveness), ARE (anaerobic induction), ABRE (abscisic acid responsiveness), and MBS (MYB binding), which suggests possible regulatory relationships between HT and the accumulation of prolamins and globulins.

Chalky appearance is one of the major phenomena of immature or dead grains induced by HT (*2*, *3*, *22*). Cultivars suitable for brewing Japanese sake wine are known for the chalkiness in the center portion of their grains (*41*). As well, a significantly lower concentration of prolamins and globulins has been found in the center chalky region of grains of these sake cultivars (*17*). Our findings of reduced amount of prolamins in dead and immature grains imply an association of prolamins and chalky structure in rice grains. A further localization approach may benefit the understanding of the roles of prolamins or other storage proteins in the formation of chalkiness under HT (*32*). In addition, experiments with more varieties are needed to clarify the relationship among storage proteins, physiological fluctuation, and genotypic variation of grain quality in response to HT.

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