Comprehensive Expression Profiling of Rice Grain Filling-Related Genes under High Temperature Using DNA Microarray[OA]

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To elucidate the effect of high temperature on grain-filling metabolism, developing rice (*Oryza sativa*) 'Nipponbare' caryopses were exposed to high temperature (33°C/28°C) or control temperature (25°C/20°C) during the milky stage. Comprehensive gene screening by a 22-K DNA microarray and differential hybridization, followed by expression analysis by semiquantitative reverse transcription-PCR, revealed that several starch synthesis-related genes, such as granule-bound starch synthase I (*GBSSI*) and branching enzymes, especially *BEIIb*, and a cytosolic pyruvate orthophosphate dikinase gene were down-regulated by high temperature, whereas those for starch-consuming α-amylases and heat shock proteins were up-regulated. Biochemical analyses of starch showed that the high temperature-ripened grains contained decreased levels of amylose and long chain-enriched amylopectin, which might be attributed to the repressed expression of *GBSSI* and *BEIIb*, respectively. SDS-PAGE and immunoblot analysis of storage proteins revealed decreased accumulation of 13-kD prolamin, which is consistent with the diminished expression of prolamin genes under elevated temperature. Ripening under high temperature resulted in the occurrence of grains with various degrees of chalky appearance and decreased weight. Among them, severely chalky grains contained amylopectin enriched particularly with long chains compared to slightly chalky grains, suggesting that such alterations of amylopectin structure might be involved in grain chalkiness. However, among high temperature-tolerant and sensitive cultivars, alterations of neither amylopectin chain-length distribution nor amylose content were correlated to the degree of grain chalkiness, but rather seemed to be correlated to grain weight decrease, implying different underlying mechanisms for the varietal difference in grain chalkiness. The possible metabolic pathways affected by high temperature and their relevance to grain chalkiness are discussed.

High temperature during the grain-filling stage causes deleterious effects on the yield and quality of crop products (Peng et al., 2004). Temperature above certain growth-optimal temperatures impairs dry matter production, generally decreasing grain size in all major cereal crops, such as rice (*Oryza sativa*), wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), and maize (*Zea mays*). Such small grains result in not only decreased yield but also low milling quality. For *japonica* cultivars of rice, temperatures higher than 26°C render chalky grain appearance as well as reduction of grain weight. Severely chalky brown rice grains are inferior for polishing quality and palatability. The chalky grains ripened under high temperature conditions resulted in lower yield after polishing and less sticky texture after cooking than translucent grains ripened under low temperature (Table I). Because the Japanese market prefers sticky rice, the rice grains with a chalky appearance are easily recognized and only achieve low sale prices, which is one of the recent problems for Japanese rice-producing farmers. To circumvent quantitative and qualitative losses of crop production by forthcoming global warming, studies concerning physiological effects of elevated temperature on grain filling are indispensable.

It has been reported that high temperature at the milky stage of grain filling has the greatest influence on rice grain chalkiness (Tashiro and Wardlaw, 1991a), and the panicle is the most sensitive organ to high temperature (Sato and Inaba, 1973; Morita et al., 2004). There are known to be varietal differences in grain chalkiness among rice cultivars when ripened under a given temperature. The *japonica* varieties 'Koshiibuki' and 'Tentakaku' provide less chalky grains even when they are exposed to high temperature (here defined as high temperature tolerant), whereas 'Hatsuboshi' and 'Sasanishiki' produce severely chalky grains (high temperature sensitive). However, the varietal differences in grain chalkiness are poorly understood at the molecular level. Microscopic observation of the chalky part of high temperature-ripened grains revealed that loosely packed starch granules create air spaces between themselves to reflect light randomly (Tashiro and Wardlaw, 1991a; Zakaria et al., 2002). Thus, to determine the underlying biochemical mechanism for grain chalkiness, the effect of high temperature on starch synthesis in developing caryopses has been investigated so far.

Table I. *Effect of grain-filling temperature on rice polishing yield and palatability*

Cultivar	Treatment[a]	Average Temperature for 0–20 DAH	Grain Weight	Perfect Kernel Ratio[b]	Yield after Polishing[c]	Palatability[d]	Stickiness[d]
		°C	mg	%	%		
'Koshihikari'	NT	26.5	20.7	69.7	90.6	−0.42	−0.21
	ET + house	28.8	20.0	26.8	88.2	−2.46	−1.14
						***	***
'Sasanishiki'	NT	27.7	20.5	56.6	86.3	−0.91	−0.42
	ET + house	28.6	19.3	14.5	81.0	−2.23	−1.00
						***	***

[a]NT, Normal transplanting (on April 26, 2005); ET, early transplanting (on May 19, 2005); house, covered by a plastic-film greenhouse after heading.   [b]The ratio of translucent nonchalky grains measured by a grain-grading machine, ES-1000 (Shizuoka Seiki).   [c]The ratio of the weight of white rice after constant polishing by Ricepal 31 (Yamamoto) to that of brown rice.   [d]Palatability and stickiness of cooked rice determined by 57 tasting panelists comparing to two standard samples, 'Koshihikari' and 'Kochihibiki', for relative value 0 and −2, respectively. ***, Significant at 0.1% level as determined by *t* test.

Starch consists of amylose (linear α-1,4-polyglucan) and amylopectin (α-1,6-branched polyglucans) in rice grains. Although amylose synthesis is exclusively governed by granule-bound starch synthase (GBSS), amylopectin is synthesized via concerted reactions catalyzed by multiple isoforms of enzymes: soluble starch synthase (SS), starch branching enzyme (BE), and starch debranching enzyme (for review, see Nakamura, 2002). In particular, the relative balance of α-1,6-branch formation and the subsequent α-1,4-chain elongation, which are catalyzed by distinct BE and soluble SS isoforms, respectively, are important for determining the amylopectin fine structure. The effect of high temperature on these enzymes was extensively studied in wheat and maize, and activity and/or gene expression for soluble SS was impaired under elevated temperature (Hawker and Jenner, 1993; Singletary et al., 1994; Hurkman et al., 2003). In rice, high temperature resulted in a reduction of activity and gene expression for GBSSI and BEs (Umemoto and Terashima, 2002; Jiang et al., 2003), decrease of amylose content (Asaoka et al., 1989; Umemoto and Terashima, 2002), and increase of long chain of amylopectin (Asaoka et al., 1984; Umemoto et al., 1999).

Prior to the synthesis of amylose and amylopectin, Suc is loaded into endosperm cells by Suc transporters and converted to Glc-6-P via several reaction steps catalyzed by enzymes such as Suc synthase (SuSy) and then into ADP-Glc, the substrate for α-1,4-polyclucan synthesis, by ADP-Glc pyrophosphorylase (AGP). Import of ADP-Glc and Glc-6-P into amyloplasts is conducted by distinct transporters. For each enzyme and transporter involved, several isoforms are known to be differentially regulated. In maize, the activities of AGP and SuSy as well as soluble SS were reduced by high temperature (Singletary et al., 1994; Wilhelm et al., 1999).

To comprehend the effect of high temperature on the wide range of grain-filling metabolic steps simultaneously, proteomic approaches have been employed. The analyses using high temperature-exposed caryopses of rice or wheat revealed the induction of heat shock proteins (HSPs) as well as the repression of several starch metabolism-related enzyme proteins (Majoul

et al., 2003; Lin et al., 2005). HSPs are molecular chaperones that stabilize proteins in their functional conformations and prevent the aggregation of heat-denatured proteins (for review, see Wang et al., 2004). However, these approaches provided information limited to the identification of enzymes or proteins expressed at relatively abundant levels. Because in developing caryopses some proteins such as debranching enzymes expressed at intermediate and low levels have been recently revealed to participate in grain-filling metabolism (Nakamura, 2002) and such low expression level proteins might be barely detectable by proteomic analyses, a more comprehensive expression analysis is required. Current progress on the rice genome project provides cDNA information covering approximately two-thirds of the genome (Kikuchi et al., 2003), and a microarray containing approximately one-half of the genes is available. Considering the minimal time and cost required, DNA microarrays are a powerful tool to comprehend the effect of high temperature on the expression of grain filling-related genes. In this study, we aimed to elucidate the effect of high temperature on metabolism during grain filling at the gene expression level and discussed its relevance to changes in accumulation of storage materials, such as starch and storage proteins, as well as grain chalkiness.

RESULTS

Rice Grains Ripened under High Temperature Showed Chalky Appearance and Low Amylose Content

We intended to identify genes whose transcript levels are responsive to temperature during the grain-filling phase. Because gene expression intensity of field-grown plants is often influenced by numerous stresses from the environment, we raised plants in incubators under controlled illumination and temperature conditions to achieve highly reproducible grain filling. In the system, rice 'Nipponbare' plants exposed to high temperature (33°C/28°C) from 5 to 20 d after flowering (DAF) matured faster than those ripened under a control condition (25°C/20°C). After starting the temperature treatment at 5 DAF, the fresh weight of high

temperature-treated caryopses increased faster than the control plants (Fig. 1A). However, the weight increase completely ceased by 15 DAF, while the weight of the control caryopses continued to increase until at least 20 DAF. At maturity, the grains exposed to high temperature were slightly lighter than the control grains. Weight of high temperature-treated grain was always lower than that of the control grain under the same conditions, although the reduction ratio fluctuated from 88.4% to 99.1% in each set of experiments (93.5% in average, $n = 7$). However, decrease of the weight was significant ($P < 0.02$, $n = 7$). The appearance of high temperature-ripened grains was severely chalky, such that 84% of grains were categorized as immature by an imaging analysis, while the control grains were mostly translucent (Fig. 1B). Furthermore, scanning electron microscopy of transverse sections revealed that endo-

sperm of chalky grains ripened under high temperature contained loosely packed starch granules with large air spaces, while translucent grains ripened under normal temperature were filled with densely packed granules (Fig. 1C).

Amylose content is known to be influenced by ripening temperature. Determined by an iodine colorimetric method, amylose content in the 'Nipponbare' grains ripened in the 33°C/28°C incubator was 17.1%, which was lower than that of the control grains (18.8%; Table II). Total starch contents, the ratio of starch weight to kernel weight, in 25°C/20°C-ripened and 33°C/28°C-ripened grains were 66.8% ± 0.6% and 66.8% ± 1.4% (mean ± SD, $n = 5$), respectively, which are not significantly different. Thus, grains ripened under high temperature with incubators had light weight, chalky appearance, and low amylose content,

Figure 1. Grain filling using plant growth incubators. A, Change in the fresh weight of rice caryopses developing under 33°C/28°C (black circles) or 25°C/20°C (white circles). Values are the mean of at least 50 grains. B, Appearance and weight of dehulled grains. The ratio of perfect (translucent), immature (mainly chalky), damaged, abortive, and colored grains was determined by a grain-grading machine, ES-1000 (Shizuoka Seiki), and is indicated from left to right of the bars in order. Because colored gains were few, the symbol is inside lines. Black and white arrowheads indicate almost translucent and severely chalky grains ripened under 33°C/28°C, respectively. C, Scanning electron micrographs of transverse sections of 25°C/20°C-ripened translucent (left) and 33°C/28°C-ripened chalky grains (right). Top and bottom, Light microscope and scanning electron microscope images, respectively. The areas indicated by boxes were analyzed by scanning electromicroscopy. Bars = 10 μm.
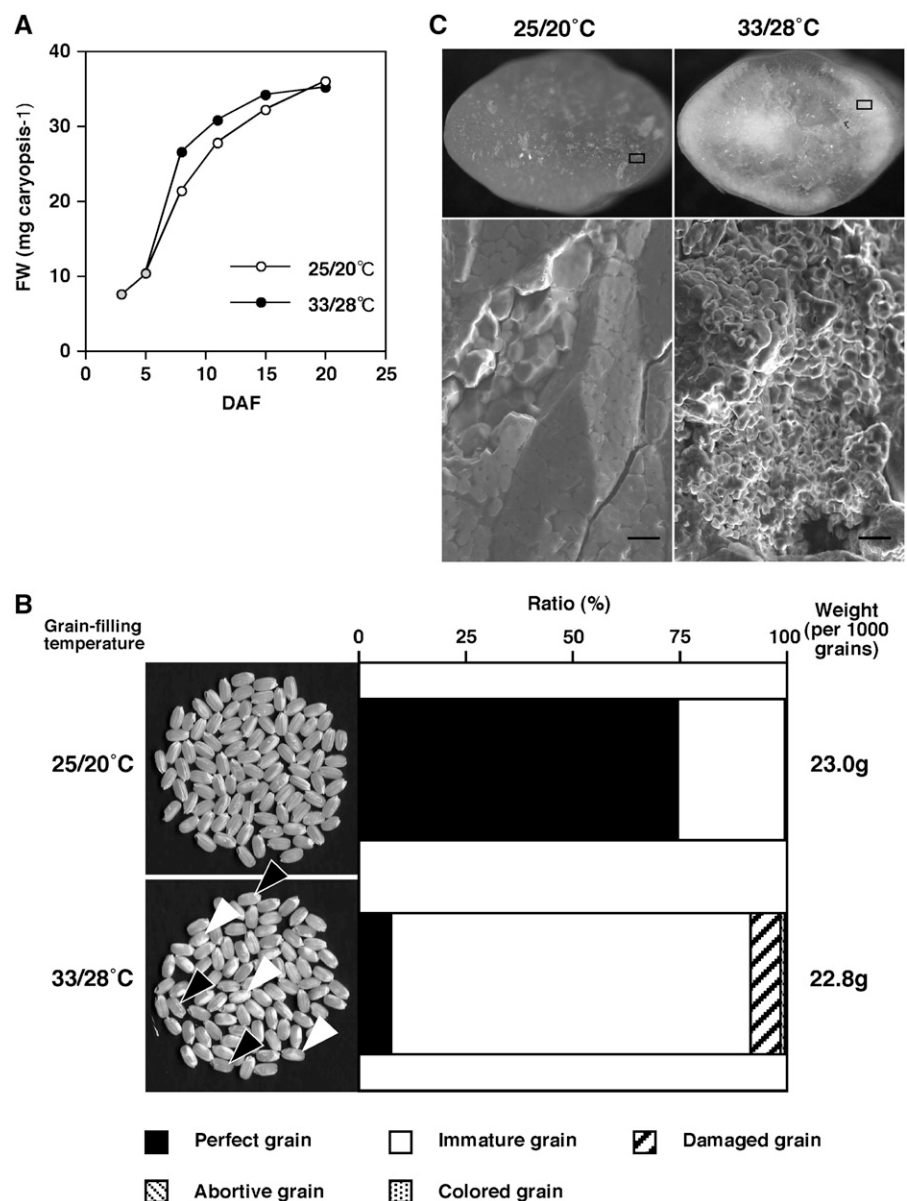
Table II. *Amylose content of rice grains of various cultivars ripened under high (33°C/28°C) and normal (25°C/20°C) temperature*

Cultivar	Amylose Content		Reduction Ratio[b]
	25°C/20°C	33°C/28°C	
	%		%
'Nipponbare'	18.8 ± 0.39[a]	17.1 ± 0.61 ***	91.0
'Koshiibuki'	17.8 ± 0.49	15.1 ± 0.98 ***	84.6
'Tentakaku'	19.7 ± 0.60	15.5 ± 0.80 ***	78.7
'Sasanishiki'	18.4 ± 0.95	16.5 ± 1.13 **	89.6
'Hatsuboshi'	19.8 ± 1.11	15.0 ± 0.48 ***	75.9

[a]Values are means ± SD of five replicates. [b]The ratio of amylose content of 33°C/28°C-treated rice to that of 25°C/20°C-treated rice. ** and ***, Significant at 0.5% and 0.1% level, respectively, as determined by Student's *t* test.

which are common features in field-grown rice grains ripened under high temperature in natural conditions.

High Temperature Affected Expression of Genes Involved in Starch Metabolism, Storage Protein Synthesis, and Stress Responses

To investigate the metabolic alterations caused by high temperature during grain filling, genes whose transcript level was increased or decreased by high temperature were identified from developing caryopses harvested at 10 DAF by: (1) employing the Agilent rice 22-K oligo DNA microarray system; (2) screening of 6×10^4 cDNA clones by differential hybridization; or (3) cloning by PCR with subtracted cDNA libraries. Among 21,938 rice genes on the microarray, 45 genes were up-regulated more than 2-fold by high temperature during grain filling, while 39 were down-regulated to less than one-half the level of the control. In addition, five genes encoding storage proteins and one allergenic protein gene were isolated by differential screening, but none by the subtractive hybridization. Considering their annotation and homology information, all the genes isolated were categorized into carbohydrate-metabolizing enzymes/translocators, storage proteins/related enzymes, HSPs/factor, and other up-regulated or down-regulated genes, as summarized in Table III.

For carbohydrate metabolism, especially starch synthesis, the genes encoding major functional enzymes are predominantly known (Hirose and Terao, 2004; Ohdan et al., 2005), and available genes related to starch metabolism were included in the list of gene expression levels (Table III). For some genes whose full-length cDNA clones were available at the Rice Genome Resource Center, National Institute of Agrobiological Sciences (Tsukuba, Japan), but not included in the Agilent 22-K microarray, their expression levels were briefly checked by dot-blot hybridization. While most of carbohydrate enzymes showed moderate changes at high temperature (the ratio of expression level at high temperature to that of the control deter-

mined by the microarray analysis ranged around 0.80–1.00, 0.89 on average), transcription of *GBSSI* and *BEIIb* was strongly reduced to 35% and 41% of the control, respectively. In contrast, the expression of *Amy3E* was notably induced by high temperature.

Because starch synthesis-related gene isoforms expressed in developing seeds have been identified by previous studies (Hirose and Terao, 2004; Ohdan et al., 2005), expression of the major seed-specific types of genes, including *GBSSI*, *BEIIb*, and *Amy3E*, was investigated for the time course of grain filling by semiquantitative reverse transcription (RT)-PCR analysis using gene-specific primers (Table IV). As shown in Figure 2, the expression of *GBSSI* and *BEIIb*, as well as *AGPS1*, *AGPS2b*, *Susy2*, *GPT1*, and *BT1-2*, was strongly diminished during grain filling at 33°C/28°C. Comparing the sum of densitometrically determined expression intensity values from 8 to 30 DAF to correct for the advancement of grain filling due to temperature variation, the total expression through the ripening period was reduced to 50% and 59% by high temperature for *GBSSI* and *BEIIb*, respectively. The quantification of the expression time course showed that *GBSSI* expression was reduced throughout the ripening phase, and that the transcription of BE genes, especially *BEIIb*, was more impaired than that of soluble SS genes (Fig. 3). In contrast, the expression of α-amylase genes was induced by high temperature and peaked at 15, 15, and 11 DAF for *Amy1A*, *Amy3D*, and *Amy3E*, respectively, and total expression from 8 to 30 DAF for high temperature was 2.43-, 2.29-, and 2.26-fold that for the control, respectively.

Down-regulation of a pyruvate orthophosphate dikinase (PPDK) gene, *PPDKB*, by high temperature treatment was revealed by the microarray analysis (Table III). Rice has two PPDK-encoding genes, *PPDKA* and *PPDKB*, and *PPDKB* has dual promoter sites, producing two distinct transcripts, cytosolic-type *cyPPDKB* and chloroplastic-type *chPPDKB* (Imaizumi et al., 1997). Time-course expression analysis by RT-PCR showed that expression of *cyPPDKB* was repressed by high temperature to 43% of the normal temperature level, while *PPDKA* was slightly induced (Figs. 2 and 3). No transcript for *chPPDKB* was detected in the developing caryopses.

Expression of genes encoding seed-specific storage proteins was differentially affected by rising temperature during grain filling. Expression of the genes for 13-kD prolamin, namely *Pro-7* and *13kD Pro*, increased and peaked at 15 to 20 DAF. However, under 33°C/28°C ripening, the expression was strongly attenuated at 20 DAF, resulting in a reduction of the total 8- to 30-DAF expression level to 57% and 42%, respectively (Fig. 2). In contrast, glutelin genes, most of which were expressed intensively 11 to 15 DAF, were less affected by high temperature. Furthermore, transcription of a gene for protein disulfide isomerase, *PDI*, which is reported to be necessary for precise sorting of storage proteins to protein bodies (Takemoto et al., 2002), was impaired to 68% of the control by high temperature.

Table III. *Expression level of various genes in response to high temperature grain filling*

The values of microarray and dot-blot hybridization are means of the data obtained from independent hybridizations (n = 2 and 4, respectively).

Accession No.	Description	Gene Name[a]	Microarray			Dot-Blot Hybridization			RT-PCR	Identified as
			Fold-Change	25°C/20°C	33°C/28°C	Fold-Change	25°C/20°C	33°C/28°C	Fold-Change[b]	
Starch/carbohydrate-metabolizing enzymes										
AK070431	Rice waxy	GBSSI	0.35	207,723	73,043	0.82	2,836	2,474	0.50	
AK102058	Rice GBSS II precursor	GBSSII	0.61	938	576	0.65	1,247	863		
AK109458	Rice sp. *japonica* soluble SS	SSI	0.97	3,092	2,985				1.25	
AK101978	Rice putative soluble SS II-3	SSIIa	0.77	33,221	25,656				0.87	
AK066446	Rice soluble SS II-2	SSIIb	0.93	2,488	2,315					
AK072339	Rice soluble SS II-1	SSIIc	0.78	758	593					
AK061604	Unknown expressed protein	SSIIIa	0.90	3,711	3,354				0.90	
AK059368	Wheat SS III	SSIIIb	0.95	2,142	2,028					
AK066808	Wheat SS isoform IV; nuclear gene for plastid product	SSIVa	1.11	986	1,097					
AK067577	Wheat SS isoform IV; nuclear gene for plastid product	SSIVb	0.92	5,173	4,771					
AK065121	Rice (*japonica* cultivar group) BE-I	BEI	1.06	12,259	12,946				0.82	
AK108535	Rice BE-3	BEIIb	0.41	127,584	52,108	0.81	2,298	1,970	0.59	
AB093426	Rice (*japonica* cultivar group) isoamylase	ISA1							0.94	
AK101554	Maize isoamylase-type starch debranching enzyme ISO3 (iso3)	ISA3	0.93	307	284					
AB012915	Rice starch debranching enzyme	PUL							0.83	
AK101744	Rice α-amylase, clone pOS103	Amy1A	1.10	280	308				2.43	
AK059671	Rice Amyc2 α-amylase	Amy2A	1.03	380	393					
AK063988	Rice α-amylase, clone pOS137	Amy3A	1.02	727	739					
AK101358	α-Amylase	Amy3C	1.22	206	251					
AK064124	Rice α-amylase, clone pOS137	Amy3D	1.31	128	168				2.29	
AK064300	α-Amylase	Amy3E	3.39	4,367	14,799				2.26	
AK065152	Arabidopsis putative α-amylase (At1g69830/T17F3_14)	Amy4A	0.77	952	737					
AK070300	Arabidopsis At5g45300 putative β-amylase: RAFL16-68-D16	(Bamy)	0.97	9,573	9,321					
AK067249	Arabidopsis putative β-amylase (At3g23920)	(Bamy)	0.93	1,430	1,329					
AK068968	Arabidopsis putative β-amylase (At3g23920)	(Bamy)	1.18	1,365	1,617					
AK100910	Rice (*japonica* cultivar group) AGP large subunit	AGPL1	0.88	4,756	4,165				1.03	
AK071497	Rice AGP large subunit	AGPL2	0.69	71,750	49,582	0.79	1,833	1,522	0.74	
AK069296	Barley AGP large subunit (blpl)	AGPL3	0.48	842	407					
AK121036	*Citrullus lanatus* AGP large subunit 2 (wml2)	AGPL4				1.22	884	1,140		
AK073146	Rice AGP small subunit	AGPS1	0.57	9,680	5,565	0.91	1,124	1,079	0.67	
AK071826	Rice AGP 51-kD subunit	AGPS2a	0.79	69,403	54,531	0.97	2,447	2,502		
AK103906	Rice AGP 51-kD subunit	AGPS2b				1.12	2,257	2,670	0.64	
AK121403	Rice (*japonica* cultivar group) OsCIN1 cell wall invertase	CIN1				1.05	1,217	1,347		
AK072276	Maize cultivar W22 inbred cell wall invertase 2 (Incw2)	CIN2	0.88	4,737	4,152					
AK120779	Rice (*japonica* cultivar group) cell wall invertase 3	CIN3				0.67	1,207	854		
AK069080	*Triticum monococcum* extracellular invertase (exin1)	INV1	1.02	5,862	5,978					
AK099312	Rice (*indica* cultivar group) 'IR64' vacuolar invertase 1	INV2				0.62	1,327	876		
AK072245	*Lolium temulentum* putative soluble acid invertase (inv1:4)	INV3	0.82	1,358	1,111					
AK061240	Rice (*indica* cultivar group) invertase (INV)	(INV)	0.91	2,078	1,898					
AK103334	Rice (*indica* cultivar group) invertase (INV)	(INV)	1.12	289	325					
AK100373	*Lolium temulentum* alkaline/neutral invertase	(INV)	1.01	1,963	1,975					
AK065130	*Allium cepa* invertase	(INV)	1.14	695	794					
AK100334	*Bambusa oldhamii* clone BSUS1b SuSy 2	SuSy1	1.05	2,477	2,590					

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Accession No.	Description	Gene Name[a]	Microarray			Dot-Blot Hybridization			RT-PCR	Identified as
			Fold-Change	25°C/20°C	33°C/28°C	Fold-Change	25°C/20°C	33°C/28°C	Fold-Change[b]	
AK072074	Rice SuSy	SuSy2	0.77	22,992	17,803	0.86	1,322	1,202	0.58	
AK100306	Wheat SuSy type 2	SuSy3	0.57	32,652	18,686	0.93	1,451	1,422	0.81	
AK099406	Maize SuSy 3	SuSy4	0.70	3,673	2,557	0.83	1,768	1,549		
AK065549	*Citrus unshiu* CitSUSA SuSy	SuSy5	1.01	233	235					
AK063304	Arabidopsis putative SuSy (At4g02280)	SuSy6	0.95	359	341					
AK071525	Maize Suc-phosphatase (spp1)	(SPP)	1.05	282	297					
AK063330	*Aegilops speltoides* Suc-phosphatase	(SPP)	0.90	1,972	1,781					
AK063433	Barley Suc-phosphate synthase	(SPS)	0.88	233	205					
AK069527	Maize Suc-phosphate synthase	(SPS)	1.18	225	265					
AK071732	*Saccharum officinarum* SoSPS2 premature mRNA for Suc-phosphate synthase	(SPS)	0.82	1,085	885					
AK101676	Oncidium 'Goldiana' Suc-phosphate synthase (SPS)	(SPS)	0.99	1,147	1,139					
AK065780	Rice (japonica cultivar group) UGP, UDP-Glc pyrophosphorylase	UGP	0.60	5,831	3,506				0.95	
AK071248	Rice sp. *indica* UDP-Glc pyrophosphorylase (UDPGase)	(UGP)	0.77	467	358					
AK112015	Rice sp. *indica* UDP-Glc pyrophosphorylase (UDPGase)	(UGP)	1.01	500	503					
AK068061	Arabidopsis clone C105270 putative Glc-6-P isomerase (At4g24620)	PGI-a	0.70	6,872	4,792	0.64	810	545	0.84	
AK068236	Rice phospho-Glc isomerase (Pgi-b)	PGI-b	0.74	2,226	1,656	1.06	947	1,061	0.91	
AK100027	Rice (*japonica* cultivar group) Suc transporter	SUT1	1.01	2,198	2,219				0.72	
AK109461	Barley Suc transporter 2 (sut2)	SUT2	1.05	1,318	1,381					
AK071452	Rice (*japonica* cultivar group) OsSUT3 Suc transporter	SUT3	1.30	288	376					
AK065430	*Vitis vinifera* putative Suc transporter (VvSUC12)	SUT4	0.91	512	467					
AK073105	Maize Suc transporter	SUT5	0.99	294	292					
AK060577	Rice GPT	GPT1	0.82	26,786	21,997	0.59	1,135	712	0.66	
AK070124	*Pisum sativum* GPT precursor, nuclear gene encoding plastid protein	GPT2	1.06	1,978	2,094					
AK065618	Rice GPT putative Glc-6-P/phosphate-translocator	(GPT)	0.94	1,274	1,191					
AK062253	Rice GPT putative Glc-6-P/phosphate-translocator	(GPT)	0.93	1,520	1,417					
AK103471	*Solanum tuberosum* brittle1 protein	BT1-1	1.20	820	986	1.47	782	1,219		
AK107368	Maize brittle-1 protein (bt1)	BT1-2	0.89	188,078	166,841	0.92	2,043	1,993	0.70	
AK063766	Rice starch phosphorylase L isozyme	PHOL	0.49	31,965	15,598	0.45	2,211	1,050		
AK103367	Rice starch phosphorylase H isozyme	PHOH	1.02	2,329	2,366					
AK067082	Arabidopsis 4-α-glucanotransferase (At2g40840)	DPE2	0.88	2,678	2,344					
AK065739	Rice sp. *indica* cyPPDKB	PPDKA	1.53	2,107	3,221				1.26	
AK068025	Rice (*japonica* cultivar group) orthophosphate dikinase	PPDKB	0.45	10,499	4,705				0.43	
Storage proteins										
AK108254	Rice 10-kD prolamin	10kD Pro	1.01	37,957	38,427					
AF194115	Rice Prolamin 7	Pro 7				0.72	8,422	6,379	0.57	DS16 (down-regulated)
S39468	Rice 13-kD prolamin	13kD Pro				0.85	7,761	6,967	0.42	DS86 (down-regulated)
AK107785	Rice prolamin, clone: λ RM9	16kD Pro	1.39	143,851	200,016	1.24	4,740	6,212	1.03	
AK107238	Rice preproglutelin	Glu RG21	0.95	292,967	277,529					
AK064478	Rice glutelin, clone: λ RG55	Glu RG55	1.60	131,410	209,827	1.38	5,903	8,618	0.95	
AK107285	Rice glutelin type I (clone pREE 103)	Glu type I	1.15	241,921	277,110	1.17	10,526	13,054	0.78	DS18 (up-regulated)
AK107314	Rice glutelin type II (clone pREE 99)	Glu type II	0.94	182,855	171,010	1.23	10,346	13,473	0.97	DS72 (up-regulated)
AK107343	Rice glutelin	Glu B-1	0.82	242,697	198,004	0.92	9,150	8,924	0.72	DS91 (down-regulated)
AK107271	Rice glutelin 1 (Gt22)	Glu A-3	0.86	250,840	215,299					

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Accession No.	Description	Gene Name[a]	Microarray			Dot-Blot Hybridization			RT-PCR	Identified as
			Fold-Change	25°C/20°C	33°C/28°C	Fold-Change	25°C/20°C	33°C/28°C	Fold-Change[b]	
AK105347	Rice globulin-like protein, clone Ose710	*Glb-like*	2.60	8,580	22,322	2.63	1,231	3,418	0.94	
AK107328	Rice (*japonica* cultivar group) allergenic protein, clone RA14b	*RAG2*	1.66	200,925	333,657	1.55	4,564	7,464	1.20	*DS108* (up-regulated)
AK068268	Rice PDI	*PDI*	0.53	22,285	11,748	0.56	3,818	2,244	0.68	
HSPs										
AK105433	Rice HSP (HSP101)	*HSP101*	1.91	1,091	2,082	1.39	576	846	1.32	
AK063751	Wheat HSP80-2 protein	*HSP82*	2.95	185	546	1.15	889	1,085	3.04	
AK065431	*Spinacia oleracea* heat shock C70 protein	*HSP70*	1.80	8,498	15,267	2.02	1,564	3,333	2.70	
AK063618	Rice HSP 26	*HSP26*	6.72	2,007	13,497	1.40	657	974	2.63	
AK063700	Arabidopsis Columbia endomembrane-localized small HSP AtHSP22.0	*HSP22a*	1.62	3,597	5,825	0.59	683	424	6.73	
AK107883	*Glycine max* low-M_r HSP Hsp22.3 (Gmhsp22.3)	*HSP22b*	2.69	156	419	1.57	1,004	1,665	1.99	
AK071240	Maize an 18-kD HSP	*HSP16.9*	2.34	3,268	7,661	1.76	861	1,605	1.42	
AK106545	Arabidopsis HSF 7 (hsf7)	*HSF*	1.69	1,223	2,069	1.16	824	1,010	4.31	
Up-regulated genes										
AK063493	Barley lipid transfer protein 7a2b		5.26	913	4,804					
AK066733	*Avena fatua* aldose reductase-related protein		5.24	1,080	5,659					
AK073443	*Digitalis lanata* lanatoside 15-*O*-acetylesterase		4.42	1,736	7,680	1.45	955	1,462		
AK103890	Maize ubiquitin/ribosomal protein S27a fusion protein		4.04	285	1,150	1.10	1,542	1,789		
AK064058	Unknown expressed protein		3.43	433	1,486	1.87	623	1,230		
AK064356	Rice class III chitinase homolog (OsChib3H-h), clone: S2321		3.36	972	3,264					
AK102970	Rice pathogenesis-related thaumatin-like protein		3.23	3,185	10,276					
AK107696	Unknown expressed protein		3.20	42,783	136,988					
AK105832	Rice (*japonica* cultivar group) OsENOD93a early nodulin		3.10	5,542	17,179	1.85	793	1,554		
AK069098	Rice Ramy1 (ramy1)		3.10	17,492	54,194	1.86	739	1,455		
AK110620	Arabidopsis putative terminal Flower 1 protein (At1g18100)		3.07	1,001	3,076	1.55	809	1,324		
AK073843	Rice (*japonica* cultivar group) chitinase		3.06	464	1,419	1.57	728	1,212		
AK105267	Arabidopsis unknown protein (At5g01220)		3.05	1,195	3,638	1.84	491	954		
AK062520	Rice lectin-like protein		2.94	786	2,308					
AK059202	Unknown expressed protein		2.89	8,256	23,886					
AK063504	Wheat (cDNA I) EC protein		2.87	15,008	43,048					
AK063126	Rice glucanase (GLU)		2.72	2,581	7,026	0.99	725	758		
AK107301	Arabidopsis purple acid phosphatase (PAP20)		2.66	4,062	10,796	1.50	861	1,364		
AK073083	Rice embryo-specific (Ose705) mRNA		2.60	92,487	240,067					
AK099481	Rice U2 snRNP auxiliary factor, small subunit 35a		2.52	1,060	2,674	1.42	723	1,087		
AK062619	Unknown expressed protein		2.52	998	2,515	1.97	554	1,157		
AK064494	Unknown expressed protein		2.48	10,200	25,337					
AK067257	Rice Bowman-Birk proteinase inhibitor		2.44	47,097	114,783					
AK059772	Rice (*japonica* cultivar group) OsENOD93a early nodulin		2.37	1,909	4,531					
AK061304	Rice sp. *japonica* clone S2148_A putative glutathione *S*-transferase OsGSTU3		2.35	705	1,655	1.02	763	826		
AK070191	Unknown expressed protein		2.21	1,496	3,313					
AK063796	Rice sp. *japonica* clone C63266_1A putative glutathione *S*-transferase OsGSTF5		2.21	756	1,672	1.26	670	892		
AK058507	*Nicotiana tabacum* LIM-domain protein		2.21	2,879	6,362					

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Accession No.	Description	Gene Name[a]	Microarray			Dot-Blot Hybridization			RT-PCR	Identified as
			Fold-Change	25°C/20°C	33°C/28°C	Fold-Change	25°C/20°C	33°C/28°C	Fold-Change[b]	
AK108569	Arabidopsis At5g24230 unknown protein, clone: RAFL21-45-L01		2.18	6,823	14,872					
AK108237	*Ananas comosus* polyphenol oxidase		2.13	1,577	3,361					
AK102261	Arabidopsis clone 37751 mRNA		2.12	1,542	3,277					
AK058291	Barley xyloglucan endotransglycosylase-like protein (XEB)		2.11	555	1,170					
AK067373	Unknown expressed protein		2.10	2,319	4,872					
AK099995	Unknown expressed protein		2.10	10,458	21,971					
AK061288	Rice lipid transfer protein LPT IV		2.08	34,166	70,911					
AK107215	Rice (*japonica* cultivar group) allergenic protein, clone RA14c		2.07	61,386	127,317					
AK100844	Rice FK506 binding protein		2.07	2,286	4,735					
AK063691	Rice rab25		2.06	16,210	33,460	1.59	665	1,122		
AK102146	Rice vacuolar H+-pyrophosphatase		2.06	5,505	11,323					
AK062831	Rice (*japonica* cultivar group) thionin Osthi1		2.04	666	1,360					
AK105316	Rice early embryogenesis protein (OSE351)		1.99	135,819	269,660					
AK108345	Maize peroxidase (pox1)		1.96	803	1,571	1.27	676	911		
AK063584	Rice elicitor-responsive gene-3 (ERG3)		1.95	1,660	3,240					
AK069446	Rice (*japonica* cultivar group) catalase		1.92	1,234	2,372	1.10	814	944		
AK063592	Rice hydrophobic LEA-like protein		1.87	5,710	10,657	1.08	773	886		
Down-regulated genes										
AK059459	Unknown expressed protein		0.11	7,257	825					
AK107610	Arabidopsis putative subtilisin Ser protease (At2g05920)		0.22	3,145	697					
AK072149	*Anthoceros formosae* chloroplast rps3, rpl22, ribosomal protein S3, ribosomal protein L22		0.24	1,238	294	0.15	1,002	163		
AK064209	*Lycopersicon esculentum* RNA-directed RNA polymerase		0.24	339	82					
AK066203	Unknown expressed protein		0.25	6,004	1,506	0.42	1,041	466		
AK107401	Arabidopsis Ser carboxypeptidase (At5g36180)		0.27	1,856	497	0.81	1,068	911		
AK061920	Rice EN242		0.32	26,483	8,482	0.99	1,090	1,142		
AK073415	Rice sp. *japonica* putative glutathione *S*-transferase OsGSTZ2		0.32	31,443	10,106					
AK062797	*A. formosae* chloroplast Ycf4 protein		0.36	2,275	828	0.21	808	179		
AK103804	Arabidopsis At4g31120 putative kinase binding protein, clone: RAFL19-91-C03		0.38	1,461	557	0.43	1,239	561		
AK107707	Unknown expressed protein		0.39	348	136	0.62	1,359	892		
AK063467	Arabidopsis Glu dehydrogenase 2 (GDH2)		0.41	2,868	1,172	0.91	984	950		
AK107986	Rice (*japonica* cultivar group) floral organ regulator 1 (FOR1)		0.41	381	157	0.97	1,560	1,608		
AK101511	Unknown expressed protein		0.42	3,610	1,510	0.87	1,666	1,533		
AK103071	Unknown expressed protein		0.42	3,068	1,293	0.85	1,097	984		
AK107597	Unknown expressed protein		0.44	2,846	1,242	0.89	1,447	1,357		
AK064573	Arabidopsis putative signal recognition particle protein (At5g61970)		0.44	1,859	815					
AK101962	Arabidopsis putative pectin methylesterase (At5g09760)		0.45	4,964	2,217	0.81	1,400	1,196		
AK069206	Rice OSK3		0.45	17,820	8,014					
AK059231	Arabidopsis clone 6145 mRNA		0.45	133,348	60,330					
AK058903	Rice acyl carrier protein II		0.46	10,006	4,634					
AK070669	Arabidopsis putative subtilisin Ser protease ARA12 (At5g67360)		0.46	1,531	712					
AK059234	*L. esculentum* ('Rutgers') ribosomal protein S25		0.46	10,164	4,724					
AK058954	Arabidopsis At4g21105 unknown protein, clone: RAFL19-89-M12		0.47	11,409	5,329	0.75	1,661	1,318		
AK108210	Unknown expressed protein		0.47	190,252	89,440					

(*Table continues on following page.*)

Table III. (*Continued from previous page.*)

Accession No.	Description	Gene Name[a]	Microarray			Dot-Blot Hybridization			RT-PCR	Identified as
			Fold-Change	25°C/20°C	33°C/28°C	Fold-Change	25°C/20°C	33°C/28°C	Fold-Change[b]	
AK062052	Maize ribosomal protein L17 (rpl17)		0.47	6,637	3,124					
AK060247	*A. formosae* chloroplast rps7 ribosomal protein S7		0.47	20,809	9,826					
AK072845	Rice putative histone deacetylase HD2		0.48	5,248	2,498	0.71	1,921	1,451		
AK100959	Rice β-expansin (EXPB3)		0.48	12,169	5,866					
AK062048	Wheat Sec61p (sec61)		0.48	6,665	3,216					
AK102932	Arabidopsis putative C3HC4 zinc finger protein (At2g30580/T6B20.7)		0.49	4,118	2,018	0.90	1,239	1,178		
AK059477	Maize chloroplast large ribosomal protein 2 (rpl2)		0.50	9,265	4,610					
AK071514	*Elaeis guineensis* clone opsc112 protein disulfide isomerase		0.50	49,301	24,556	0.86	1,291	1,177		
AK061951	*Lupinus luteus* tRNA-Gln synthetase		0.50	2,429	1,221					
AK072751	*Lolium perenne* nucleoside diphosphate kinase (NDPK)		0.52	30,084	15,533	0.74	2,059	1,602		

[a]Names in parentheses indicate tentative categories, not gene names. [b]The fold-change ratio for RT-PCR analysis is the ratio of cumulative expression levels for 8 to 30 DAF.

HSP or heat shock factor (HSF) genes were also induced by high temperature. While induction ratios ranged from 1.62 to 6.72 in the microarray analysis and from 1.32 to 6.73 in the RT-PCR analysis (Table III), low-M_r HSPs, such as *HSP26* and *HSP22a*, and a putative transcription factor gene (*HSF*) homologous to *RHSF2* were most prominently induced (Fig. 2).

For other responsive genes, many genes encoding pathogenesis-related defense proteins, such as lipid transfer proteins, chitinases, thaumatin, glucanase, proteinase inhibitor, and thionin, were notably up-regulated more than 2-fold by high temperature in the microarray analysis (Table III).

Because the sample lot used for microarray and hybridization screenings is different from that used for RT-PCR analysis, it is difficult to compare the gene expression levels obtained by microarray, dot-blot hybridization, and RT-PCR analyses to each other. However, a similar tendency (correlation coefficient [R] = 0.668 for 67 available gene expression data sets; Table III) was observed for change ratio of gene expression between data from microarray and dot-blot hybridization, for which the same RNA sample set was used. In contrast, data from RT-PCR analysis showed less correlation to microarray data (R = 0.424 for 38 available data sets) and dot-blot hybridization data (R = 0.072 for 29 available data sets), probably because of difference in analyzed time point (cumulation of 8–30 DAF versus 10 DAF) as well as difference in used sample lots.

Grains Ripened under High Temperature Contained Amylopectin with Longer Side Chains

Because the reduction of expression of starch BE genes (*BEI* and *BEIIb*) under high temperature might be accompanied by structural alterations of amylopectin, the distribution of amylopectin side chains by length was elucidated for the 'Nipponbare' rice by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis. As shown in Figure 4, rising temperature during the grain-filling period resulted in a decrease of short side chains of degree of polymerization (DP) 10 to 19 and increase of relatively longer side chains of DP 21 to 32 and DP more than 41.

13-kD Prolamin Had Reduced Accumulation in Grains Ripened under High Temperature

Among various seed storage proteins, the expression of 13-kD prolamin genes was most remarkably decreased under high temperature ripening (Fig. 2). SDS-PAGE analysis of total protein in mature grains also revealed that high temperature-ripened grains contained relatively less 13-kD prolamin, while they accumulated an apparently similar amount of both large and small subunits of glutelin compared with the control grains (Fig. 5). Furthermore, an immunoblot analysis using a 13-kD prolamin-specific antibody showed that approximately 50% to 70% amount of the protein accumulated in high temperature-exposed grains compared to the control.

Among High Temperature-Ripened Grains, the Chalky Grains Contained Amylopectin Enriched with Longer Side Chains Compared to the Translucent Grains

As shown in Figure 1B, grains with various degrees of chalkiness were obtained by ripening under high temperature. To elucidate the differences in the biochemical constituents between chalky and translucent grains both ripened under 33°C/28°C, the severely chalky, milky-white grains (indicated by white arrowheads in Fig. 1B) and less chalky, almost translucent grains (indicated by black arrowheads)

Table IV. *Gene-specific PCR primers and PCR cycles for semiquantitative RT-PCR amplification*

The nucleotide sequences used for designing the PCR primers are shown by their accession number. The designed primer pairs yielded a strong single band for each gene.

Enzyme/Property	Gene Name	Accession No.	Primer Pair	Amplicon Size[a]	No. of Cycles	Synonymous to (Reference)
SS (granule-bound)	GBSSI	AK070431	F: AACGTGGCTGCTCCTTGAA	218	19	Waxy (Okagaki, 1992)
			R: TTGGCAATAAGCCACACACA			
SS (soluble)	SSI	AK109458	F: GGGCCTTCATGGATCAACC	279	22	
			R: CCGCTTCAAGCATCCTCATC			
	SSIIa	AK101978	F: GGCCAAGTACCAATGGTGAA	272	23	SSII-3 (Hirose and Terao,
			R: GCATGATGCATCTGAAACAAAGC			2004; Jiang et al., 2004)
	SSIIIa	AK061604	F: GCCTGCCCTGGACTACATTG	334	23	SSIII-2 (Hirose and Terao,
			R: GCAAACATATGTACACGGTTCTGG			2004; Dian et al., 2005)
BE	BEI	AK065121	F: TGGCCATGGAAGAGTTGGC	191	22	
			R: CAGAAGCAACTGCTCCACC			
	BEIIb	AK108535	F: ATGCTAGAGTTTGACCGC	261	20	ae (Nishi et al., 2001); RBE3
			R: AGTGTGATGGATCCTGCC			(Mizuno et al., 1993)
Debranching enzyme	ISA1	AB093426	F: TGCTCAGCTACTCCTCCATCATC	132	22	Sugary-1 (Kubo et al., 1999)
			R: AGGACCGCACAACTTCAACATA			
	PUL	AB012915	F: ACCTTTCTTCCATGCTGG	202	22	
			R: CAAAGGTCTGAAAGATGGG			
α-Amylase	Amy1A	AK101744	F: GCGCCTGGTGTCAATCAGAA	309	30	
			R: CGGATCGGATACAGCTCGTTG			
	Amy3D	AK119761	F: TGCACGGCAAGGACTACAGC	305	31	
			R: CCAACGGTTACAAACTGCGTGA			
	Amy3E	AK064300	F: GAGCACGCTGGACATCCTCA	309	23	
			R: GCTCGTACACATCTCGCAGCA			
AGP (large subunit)	AGPL1	AK100910	F: ATGCAGTGCAGTGCGTCTTT	183	23	OsAPL3 (Akihiro et al., 2005)
			R: ACTTCACTCGGGGCAGCTTA			
	AGPL2	AK071497	F: CGGGACCGTCATATAAAAGCA	242	19	OsAPL2 (Akihiro et al., 2005)
			R: TCCCATTCCAAAACAAACCA			
AGP (small subunit)	AGPS1	AK073146	F: AGAATGCTCGTATTGGAGAAAATG	258	22	OsAPS1 (Akihiro et al., 2005)
			R: GGCAGCATGGAATAAACCAC			
	AGPS2b	AK103906	F: AACAATCGAAGCGCGAGAAA	186	21	Shrunken (Kawagoe et al.,
			R: GCCTGTAGTTGGCACCCAGA			2005); OsAPS2 (Akihiro
						et al., 2005)
SuSy	SuSy2	AK072074	F: TTCAGCAGGAGAAGCCGTCAGC	150	22	RSus2 (Huang et al., 1996)
			R: CCGGCGTTTATTTGAGGCAAGC			
	SuSy3	AK100306	F: CGGTGAAAAGAATGGGCAATG	180	21	RSus3 (Huang et al., 1996)
			R: CCATGAAAAGGCCAGAGCAT			
UDP-Glc pyrophosphorylase	UGP	AK065780	F: TCCTGGCCCGGTTTAAGTCA	258	23	
			R: TGCCGAATGCACACGACAAT			
Phospho-Glc isomerase	PGI-a	AK068061	F: ATCCAGCACATGGCAGCAAA	324	24	
			R: AAGGGCACGGGATGACAAGA			
	PGI-b	AK068236	F: TGGGGAGTGGAACTGGGAAA	237	26	
			R: CAGAATATGCCGGCTCAACC			
Suc transporter	SUT1	AK100027	F: AGTTCCGGTCGGTCAGCAT	241	26	
			R: ACCGAGGTGGCAACAAAG			
GPT	GPT1	AK060577	F: AGAAGGGATCCAGATGAAGAATG	150	22	
			R: AACAAGAAACGAGCAACATAGACC			
ADP-Glc translocator	BT1-2	AK107368	F: TGATTGTGCATGGGTGTGATG	132	22	
			R: AACAGAGGAAATCGAATCCTACG			
PPDK	PPDKA	AJ004965	F: TTGCAAATGCAGAAACAACCAT	410	26	
			R: TGCTGCTAGCCTTGCAATTG			
	cyPPDKB	D87952	F: GCTCCGGCTCAATGTGCTCGT	165	24	
			R: CTCCGTCGACACCGTGAAC			
	chPPDKB	D87952	F: CCAGGATGCCGTCGGTTTCGA	368	NA[b]	
			R: CTCCGTCGACACCGTGAAC			
Prolamin	Pro 7	AF194115	F: GCGCAGCAGCTACAACTCCA	206	15	DS16 (this work)
			R: TGAGCTTATTTTTAACTTCCGAACCA			
	13kD Pro	S39468	F: GGCCATAGCGCACCAGCTAC	233	15	DS86 (this work)
			R: TGTCACATACGATGATACCTGAGC			

(*Table continues on following page.*)

Table IV. (*Continued from previous page.*)

Enzyme/Property	Gene Name	Accession No.	Primer Pair	Amplicon Size[a]	No. of Cycles	Synonymous to (Reference)
Glutelin	*16kD Pro*	AK107785	F: TTGCCAGGCTATTTGCACCA	285	15	
			R: CGAACAGCCAAAGACTATTCCAAA			
	Glu RG55	AB016501	F: CCAGCAACAATGCCAACCAG	207	18	
			R: TCAGGCCTTGGAGCCTCAAC			
	Glu type I	AK107285	F: TTCCGTGCTCTCCCAAATGA	230	14	*DS18* (this work)
			R: TGGACAGTACATAGCAGCAAAACA			
	Glu type II	AK107314	F: GGTGCATTCACTCCCCTCCA	225	16	*DS72* (this work)
			R: CATTGGAACGGGAACACAAAAA			
	Glu B-1	AK107343	F: CGCCGTTCTGGAACGTCAAT	316	14	*DS91* (this work)
			R: TTCTTGAGGCTTCGGGCTTG			
Globulin	*Glb-like*	U45322	F: CGAGAACGGCGAGAAGTGGT	214	21	
			R: GCCCTTGCTGAAGCTCGACA			
Allergen	*RAG2*	AK107328	F: GTCGACGACAGCTGGTGCAG	313	16	*DS108* (this work)
			R: TGCTTCCTGACAAATGAAAGCA			
PDI	*PDI*	AK068268	F: GGATGCAACTGCGAACGATG	277	23	
			R: GTCAGGTCCCGTCTCCTCCA			
HSP	*HSP101*	AK105433	F: GAGCTGGCCTACAGGGTGGA	270	24	
			R: TTCAACGGAGACACCCCACA			
	HSP82	AK063751	F: AGCTCGGCCTCAACATCGAC	309	30	
			R: CGATGGCTTAGAAAGATGTACGC			
	HSP70	AK065431	F: GCCCCAAGATCGAGGAGGTC	288	28	
			R: CAAACAATGCGCACATGCAA			
	HSP26	AK063618	F: AGCGCAGCGTGAGCTCCTAC	284	25	
			R: TCATTCGCTCGTTCGCTGAG			
	HSP22a	AK063700	F: CGGTCCTACGGCAGGTTCTG	315	31	
			R: CCGGAACATGTGCTCTGCAA			
	HSP22b	AK107883	F: GAGGTTCTGGAGGCGGTTCA	232	34	
			R: GGTGTCGCCGATCACTATTCA			
	HSP16.9	AK071240	F: CGTGCAAGTACCTGCGGATG	342	27	
			R: TCGCATACGGCATACAGACCA			
Heat shock transcription factor	*HSF*	AK106545	F: TCCAGCTCCAGCCAAACGAT	255	30	
			R: CTTGAACTTTCCGCCGCAAC			

[a]Numbers indicate the size in bp of amplified fragments.   [b]NA, Not amplified.

were selected by hand and measured separately for amylopectin side chain length distribution and amylose content. Although the amylose contents of translucent perfect grains (16.4% ± 0.66%) and severely chalky grains (17.1% ± 0.80%) were not significantly different ($P > 0.10$), the amylopectin extracted from chalky grains consisted of a slightly smaller proportion of short side chains (DP 10–21) and larger proportion of long chains (DP > 23) than that from translucent grains (Fig. 6).

Difference in the Extent of Chalkiness among *japonica* Rice Cultivars Was Apparently Not Related to Amylose Content and Amylopectin Structure

To determine whether the differences in the extent of chalky grain occurrence caused by high temperature among cultivars are attributed to alteration of starch composition or not, the correlation between chalky grain ratio, grain weight, amylose content, and amylopectin side chain length distribution was examined. 'Koshiibuki' and 'Tentakaku', which are high temperature-tolerant cultivars, contained more translucent perfect grains even when exposed to high temperature,

while high temperature-sensitive 'Hatsuboshi' and 'Sasanishiki' contained more chalky grains (Fig. 7A). Decreases in grain weight were slight for 'Koshiibuki' (the ratio of 33°C/28°C-treated to 25°C/20°C-treated; 95.7%) and 'Sasanishiki' (92.9%), but larger for 'Tentakaku' (89.3%) and 'Hatsuboshi' (87.3%). Reductions of amylose content were 84.6%, 78.7%, 89.6%, and 75.9% for 'Koshiibuki', 'Tentakaku', 'Sasanishiki', and 'Hatsuboshi', respectively (Table II). Alteration in length distribution of amylopectin side chains by high temperature is shown in Figure 7B, and the cumulative values of the decrease in the proportion (%) of side chains of DP 10 to 19 and increase in the proportion (%) of side chains of DP 22 to 31, here tentatively designated the elongation index, were 2.82, 4.04, 3.21, and 3.59 for 'Koshiibuki', 'Tentakaku', 'Sasanishiki', and 'Hatsuboshi', respectively. For the expression of the genes described above in response to high temperature, these cultivars showed quantitatively similar induction/repression profiles to those observed for 'Nipponbare' (data not shown). As shown in Table V, the extent of reduction in amylose content and amylopectin side chain elongation index were apparently not related to the reduction of translucent perfect
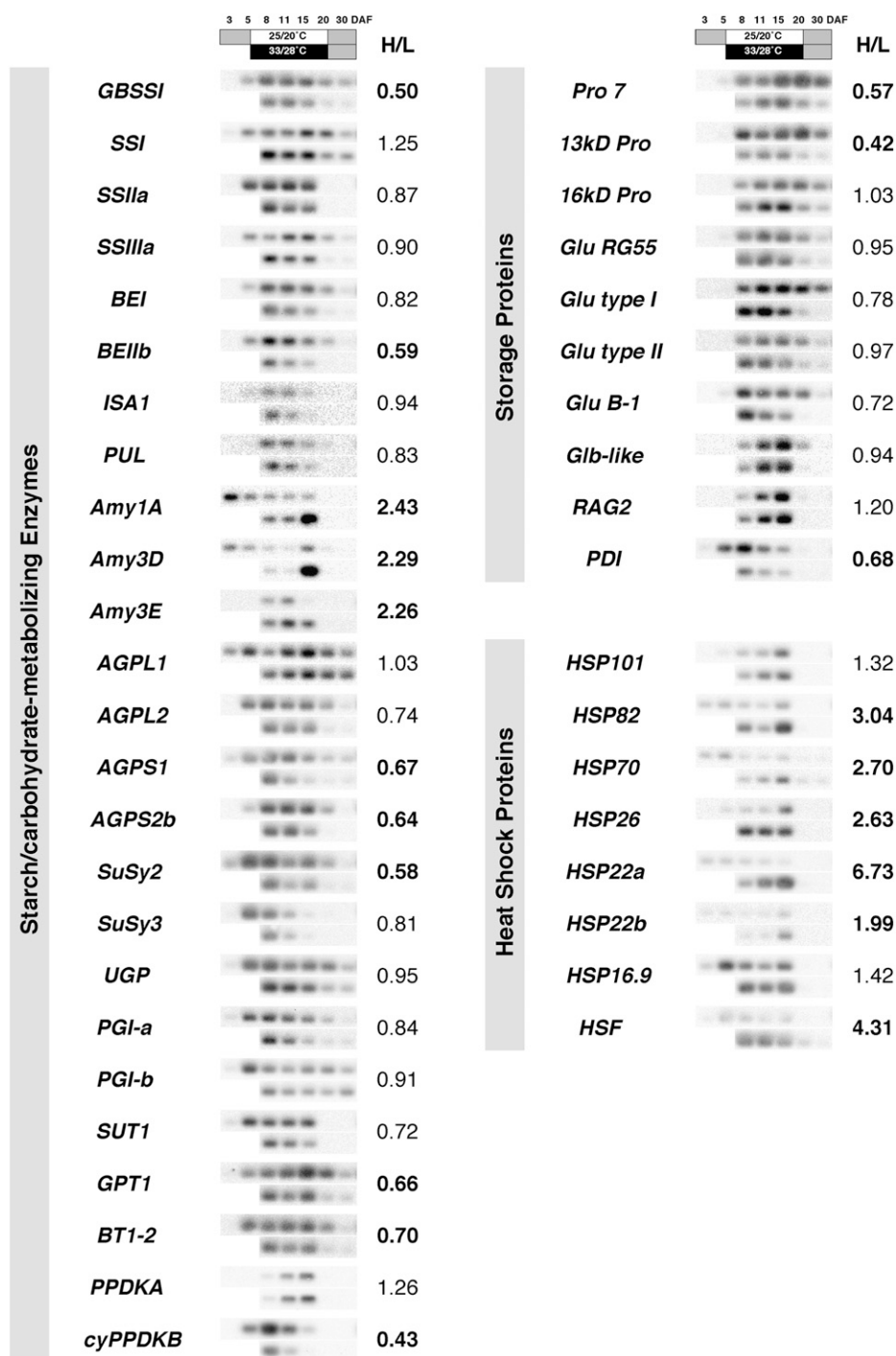
Figure 2. Expression of genes related to starch metabolism, storage protein synthesis, and heat stress response in developing caryopses, as revealed by semiquantitative RT-PCR analysis. The time-course profiles for ripening under 25°C/20°C and 33°C/28°C are shown in the top and bottom rows for each gene, respectively. The developmental stage of the caryopsis is indicated by DAF. The expression levels were quantified densitometrically, and the ratio of accumulation of the transcript levels for 8 to 30 DAF of 33°C/28°C to that of 25°C/20°C (H/L) is indicated for each gene.

grains ($R = 0.061$ and 0.051, respectively) and seemed rather to be correlated to grain weight reduction ($R = 0.795$ and -0.836, respectively).

DISCUSSION

For better understanding of the effect of high temperature on the various metabolic processes of filling grains, it is important to establish the proper condi- tions of growth and high temperature treatment, which reproduce accurately what is observed in high temperature-ripened rice grains under field condi- tions. It is widely accepted in the field-grown rice that chalky grain appearance (Tashiro and Wardlaw, 1991a), decreased grain weight (Tashiro and Wardlaw, 1991b), and low amylose content (Asaoka et al., 1989; Umemoto and Terashima, 2002) are the typical symp- toms caused by the grain filling under high tempera- ture. In this study, we could reproduce all of these
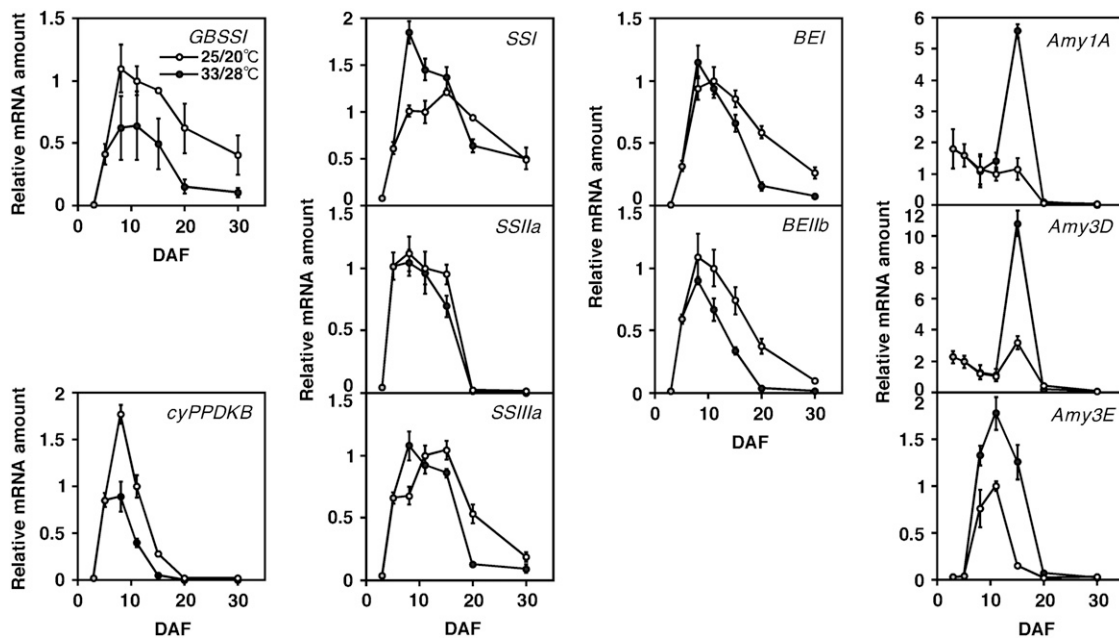
Figure 3. Comparison of expression of genes for SSs, BEs, and α-amylases, and *cyPPDKB*. The expression levels were determined by semiquantitative RT-PCR analysis and densitometry, and the value of 25°C/20°C, 11 DAF was set to "1" for each gene. Results from four independent PCRs are shown with error bars (sd).

three symptoms in the high temperature plot (Fig. 1; Table II) as well as comparable grain weight and maturity to those of field-grown rice in the control plot, which is probably attained by the enhanced illumination and extensive management of plant growth including tiller number.

The comprehensive screening using microarray and conventional differential hybridization methods revealed that many genes were responsive at the transcriptional level to elevated temperature during the milky stage of grain filling, which is the most heat-sensitive phase leading to the production of chalky grains. The chalky appearance has been reported to be associated with the development of numerous air spaces between loosely packed starch granules and a change in light refraction (Tashiro and Wardlaw, 1991a; Zakaria et al., 2002). Actually, chalky grains ripened under high temperature in this study were observed to have similar ultra-microstructure (Fig. 1C). The expression level of genes encoding starch- or carbohydrate-metabolizing enzymes and translocators was diminished to 89% of the control on average by exposure to high temperature (Fig. 2). Among them, the transcription of *GBSSI* and genes for BE, which are indispensable for the production of starch components, amylose and amylopectin, respectively, was remarkably repressed throughout the grain-filling phase, whereas genes for α-amylase, a starch-consuming enzyme, were transiently induced by high temperature (Fig. 3). Reduction of amylose content by high temperature (Table II) can be explained by the repression in expression of *GBSSI*, whose encoding protein and activity also has been reported to be down-regulated

(Hirano and Sano, 2000; Umemoto and Terashima, 2002). Decreased expression of BE genes, especially *BEIIb*, with relatively less changed or increased expression of soluble SS genes, might provide amylopectin with fewer but more elongated branches. Amylopectin in high temperature-ripened grains was found to be rich in middle and long side chains compared to the control (Fig. 4), which is consistent with a previous observation using rice plants grown under natural light (Umemoto et al., 1999). Down-regulation of the transcription of *GBSSI* and BE genes as well as the activity of their product enzymes was also reported for *indica* rice (Jiang et al., 2003), suggesting conservation of these responses to high temperature within rice subspecies.

Starch metabolism-related genes reportedly expressed during the milky stage of grain filling, as well as other genes for carbohydrate-metabolizing enzymes or translocators whose strong expression in 10-DAF caryopses was revealed by microarray analysis, were subjected to time-course expression analysis by semiquantitative RT-PCR. The cumulative gene expression levels from 8 to 30 DAF were determined as total potential contribution to de novo starch synthesis. By placing the calculated ratios of the level of high temperature-treated caryopses to that of the control beside each reaction, a metabolic map was tentatively depicted for starch-related metabolism to indicate reaction steps responsive to elevated temperature at the transcriptional level (Fig. 8). This map allowed a global comprehension regarding the effects of high temperature on starch-related metabolism and revealed that several genes other than those described above, such
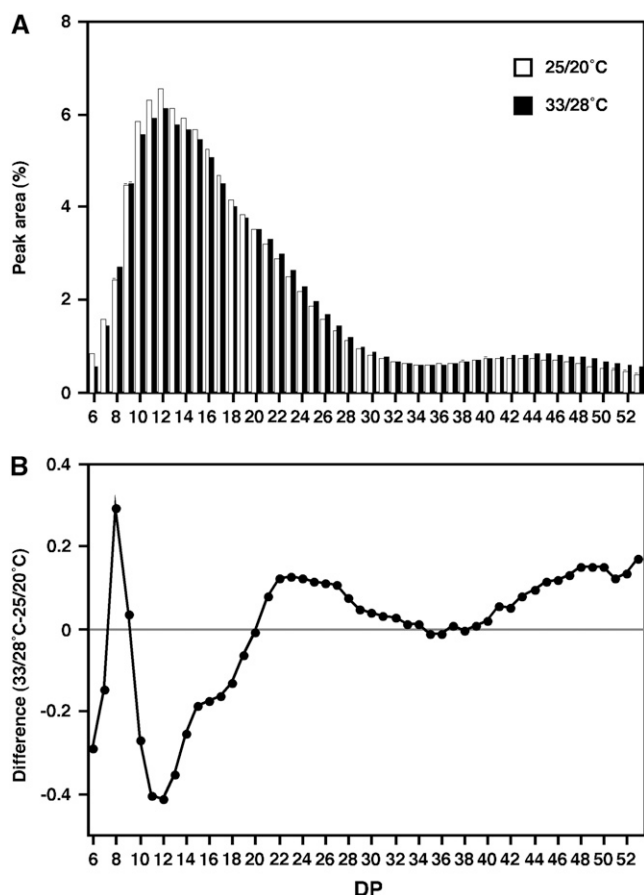
Figure 4. Comparison of the chain-length profile of amylopectin in 'Nipponbare' grain ripened under different temperatures. A, Chain-length profile. Debranched amylopectin extracted from 25°C/20°C-ripened (white bars) or 33°C/28°C-ripened (black bars) grains were analyzed by HPAEC-PAD, and the relative peak area of the chromatogram is shown for the individual DP. The data is the mean ± SD of five independent measurements. B, Difference in the chain-length distribution of amylopectin. The difference in the relative peak area in A between 33°C/28°C-ripened and 25°C/20°C-ripened grains is shown in the DP range of 6 to 53.

as small subunit of AGP (*AGPS1* and *AGPS2b*), ADP-Glc translocator (*BT1-2*), *GPT1*, and *SuSy2*, were down-regulated. Signal peptide-dependent targeting of α-amylases Amy1A, Amy3D, and Amy3E to plastids has been reported in rice seedling leaves and suspension-cultured cells (Chen et al., 2004; Asatsuma et al., 2005). However, whether similar localization occurs in developing caryopses or not remains to be determined.

PPDKB has been recently identified as the causative gene for *floury endosperm-4* (*flo-4*) mutation, whose grains showed a chalky appearance phenotype (Kang et al., 2005). High temperature impaired expression of cytosolic-type transcript of the gene *cyPPDKB* (Figs. 2 and 3), while no transcript for *chPPDKB* was detected in the caryopses, suggesting that decreased expression of *cyPPDKB* is one of the possible factors triggering for grain chalkiness, although the underlying mechanism

of how down-regulation of the enzyme renders floury chalky endosperm is unclear.

Production of storage proteins, the other major constituent of mature rice grains, was also affected by high temperature during ripening. Among them, 13-kD prolamin, one of the major storage proteins, specifically decreased in response to high temperature for both transcripts and protein, while glutelins were less affected (Figs. 2 and 5). A previous proteomic approach had revealed up-regulation of Prolamin 7, a 13-kD prolamin, in developing caryopses harvested 9 DAF (Lin et al., 2005). This discrepancy might be attributed to differences in the analyzed samples. Because grain maturation progresses faster under high temperature (Fig. 1A), comparison of snap-shot images of expression level at a given early stage of grain filling, such as 9 DAF, corresponding to the onset of storage material synthesis, might not be consistent with that of the cumulative expression level throughout the whole ripening period as described in this research. Actually, under the high temperature conditions in our study, *Prolamin 7* transiently showed higher expression level at 8 DAF than the control (Fig. 2).

Genes for various species of HSPs, typical molecular chaperones, were also induced by ripening under high temperature (Fig. 2). Among them, *HSP101* is the orthologous gene of Arabidopsis (*Arabidopsis thaliana*) *HSP101*, whose heterogeneous overexpression in rice plants has been reported to confer thermotolerance to the plant (Katiyar-Agarwal et al., 2003). However, whether rice HSP101 could contribute to physiological
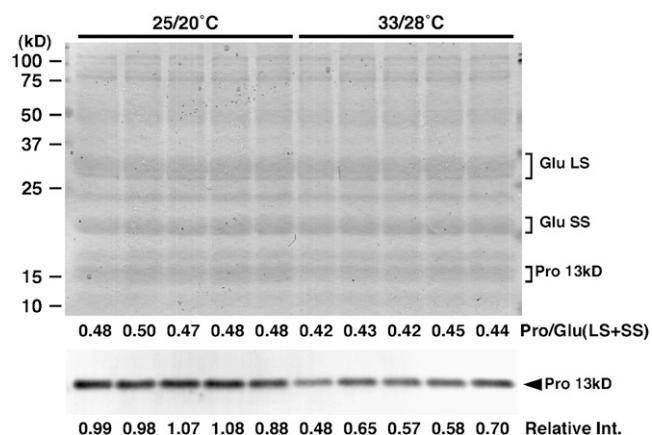
Figure 5. SDS-PAGE and immunoblot analyses of seed storage proteins. Total protein was extracted from mature grains ripened under 25°C/20°C or 33°C/28°C, and five independent extracts for each were separated on a SDS-polyacrylamide (18%) gel, followed by Coomassie Brilliant Blue staining or immunoblot detection with a polyclonal antibody raised against purified 13-kD prolamin. Large and small subunits of glutelin and 13-kD prolamin are indicated on the right side, and their relative amount was quantified densitometrically. For the SDS-PAGE, the ratio of 13-kD prolamin amount to the sum of glutelin large and small subunits is shown at the bottom, and, for the immunoblot, the relative amount of 13-kD prolamin is shown.
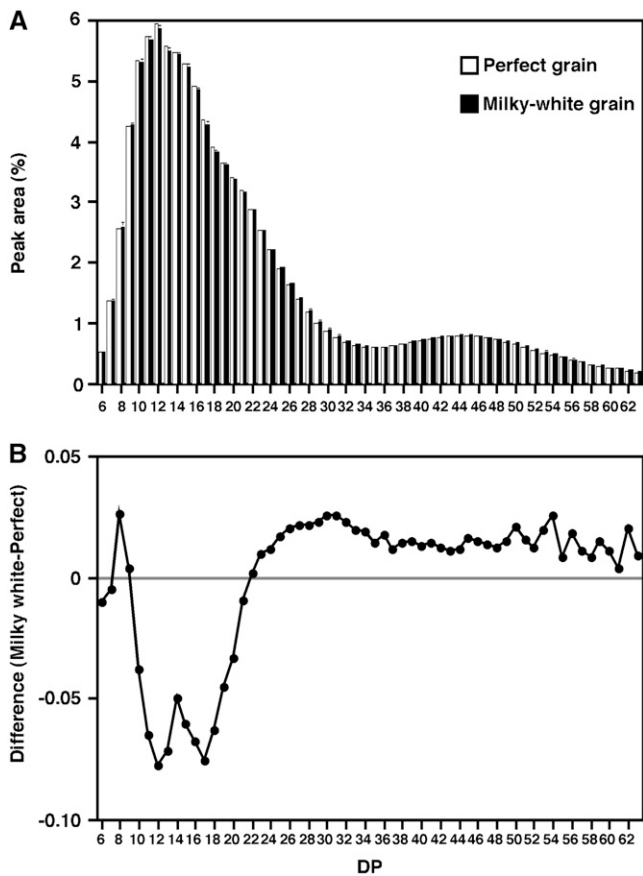
Figure 6. Comparison of the chain-length profile of amylopectin in perfect (translucent) and milky-white (severely chalky) grains ripened under 33°C/28°C. A, Chain-length profile. Debranched amylopectin extracted from 'Nipponbare' perfect (white bars) or milky-white (black bars) grains was analyzed by HPAEC-PAD, and the relative peak area of the chromatogram is shown for the individual DP. The data is the mean ± SD of five independent measurements. B, Difference in the chain-length distribution of amylopectin. The difference in the relative peak area in A between milky-white and perfect grains is shown in the DP range of 6 to 63.

protection of the developing caryopses against elevated temperature or not remains to be determined. In Arabidopsis, heat shock-inducible transcription factors HSF1 and HSF3 govern the expression of arrays of HSPs and regulate thermotolerance (Prändl et al., 1998; Lohmann et al., 2004). Whether *HSF*, a putative HSF strongly induced by high temperature ripening, has a similar function or not remains unclear.

To elucidate the causative biochemical factors for the chalky appearance of high temperature-ripened grains with regard to starch components, severely chalky grains and almost translucent grains, both of which were harvested from high temperature-exposed 'Nipponbare' plants, were compared for amylose content and amylopectin chain-length distribution. The chalky grains were shown to have amylopectin consisting of more elongated side chains than the translucent grains (Fig. 6), although the amylose content

was not significantly different. The alteration of amylopectin side chain distribution is reminiscent of the *amylose-extender* (*ae*) mutant, which is deficient in *BEIIb* gene. Grains of the *ae* mutant have amylopectin lacking in short side chains of DP 6 to 16 and enriched in long chains of DP more than 19 and with a severely chalky opaque appearance (Nishi et al., 2001). The chalkiness of the mutant was recovered by introducing the wild-type *BEIIb* transgene in an expression level-dependent manner (Tanaka et al., 2004). Considering that the expression of the *BEIIb* gene was repressed under high temperature ripening, one possible explanation for grain chalkiness is the production of *ae*-like amylopectin by down-regulation of *BEIIb* expression.
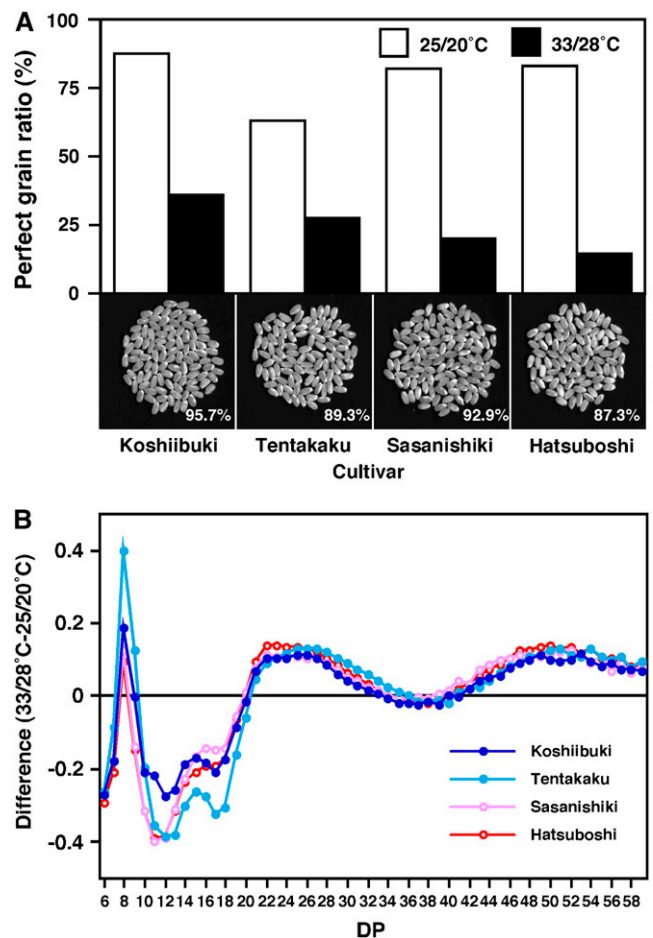
Figure 7. Comparison of high temperature-tolerant ('Koshiibuki' and 'Tentakaku') and -sensitive ('Sasanishiki' and 'Hatsuboshi') cultivars. A, Appearance grade of grains matured under 25°C/20°C (white bar) and 33°C/28°C (black bar). The ratio of perfect (translucent) grain was determined by a grain-grading machine, ES-1000 (Shizuoka Seiki). Appearance of grains ripened under 33°C/28°C is shown in the photograph with the ratio of reduction of grain weight to that of the 25°C/20°C-treated control. B, Difference in the chain-length distribution of amylopectin. For the respective cultivars, the difference in the relative peak area on the HPAEC-PAD chromatogram between 33°C/28°C-ripened and 25°C/20°C-ripened grains is shown in the DP range of 6 to 59.

**Table V.** *Correlation between grain quality, weight, amylose content, and amylopectin structure*

	Cultivar				Correlation Coefficient R to Perfect Grain Reduction Ratio	Correlation Coefficient R to Grain Weight Reduction Ratio
	'Koshiibuki'	'Tentakaku'	'Sasanishiki'	'Hatsuboshi'		
Perfect grain reduction ratio (%)[a]	41.2	43.9	24.4	17.7	–	–
Grain weight reduction ratio (%)[a]	95.7	89.3	92.9	87.3	0.431	–
Amylose content reduction ratio (%)[a]	84.6	78.7	89.6	75.9	0.061	0.795
Amylopectin side chain elongation index[b]	2.82	4.04	3.21	3.59	0.051	−0.836

[a]The ratio of the value of 33°C/28°C-treated rice to that of 25°C/20°C-treated rice. [b]The total value cumulative decrease in the proportion of side chains of DP 10 to 19 and increase in the proportion of side chains of DP 22 to 31 in Figure 7B.

Chalky appearance of the *ae* mutant, whose severity changed in a manner dependent on *Ae* allele dosage or expression level, became prominent when the BEIIb protein amounts were lower than approximately 50% of the wild-type level (Nishi et al., 2001; Tanaka et al., 2004). In this study, ripening under high temperature reduced expression of *BEIIb* to 59% of the control level (Fig. 2). The extent of alteration of amylopectin side chain length distribution by high temperature was almost similar to that reported for the *ae* grains with moderate BEIIb protein levels. Thus, the critical expression level of the changes for the chalky appearance might be estimated around one-half of the level under the normal temperature, and reduced *BEIIb* expression as well as other perturbed metabolic factors would result in grain chalkiness under high temperature.

However, the underlying mechanism of how the *ae*-like amylopectin renders chalky grain appearance is not clear at this time, and the precise relevance between the down-regulation of *BEIIb* and chalky appearance under high temperature remains to be determined. In *Chlamydomonas*, synthesis of amylopectin long chains by GBSSI has been reported (Delrue et al., 1992; Maddelein et al., 1994). Altered expression of *GBSSI* as well as *BEIIb* might be involved in the amylopectin structural modification in high temperature-exposed caryopses.

Furthermore, to clarify the underlying mechanisms for varietal differences in the degree of grain chalkiness, correlations between the extent of grain chalkiness, reduction of amylose content, and degree of amylopectin side chain elongation were investigated
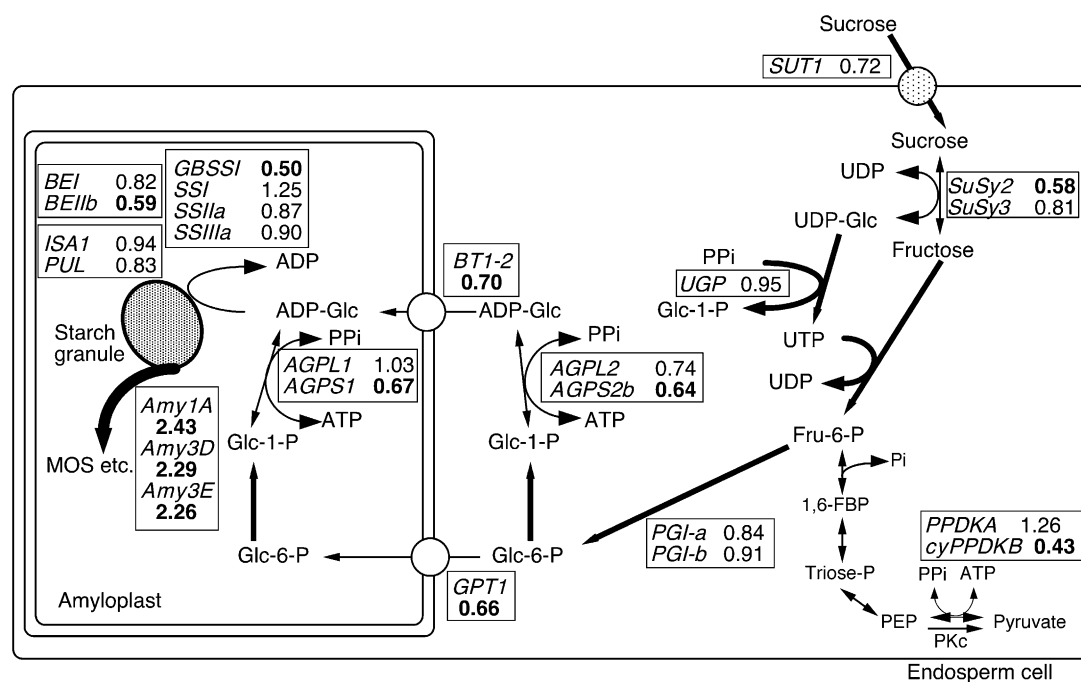
Figure 8. Ratio of cumulative expression level during 8 to 30 DAF of starch metabolism-related genes. The cumulative expression levels during 8 to 30 DAF were determined by semiquantitative RT-PCR and densitometry for grain filling under 25°C/20°C and 33°C/28°C, as described in the legend of Figure 2, and the ratio of cumulative transcript level for 33°C/28°C to 25°C/20°C is shown for the respective genes encoding enzymes/translocators on the starch-metabolizing pathway. The genes induced >1.5-fold and the corresponding reaction step are indicated in bold and with a thick arrow, while the genes repressed to <0.70-fold and the corresponding steps are indicated in bold and with thin arrows. The products of *AGPL1* and *AGPS1* genes have been estimated to be localized in amyloplasts, while those of *AGPL2* and *AGPS2b* in the cytosol (Akihiro et al., 2005; Ohdan et al., 2005). The pathways described in the text are solely depicted. MOS, Maltooligosaccharide; PKc, cytosolic pyruvate kinase.

using cultivars ranging from high temperature toler-ant ('Koshiibuki' and 'Tentakaku') to high temperature sensitive ('Sasanishiki' and 'Hatsuboshi'). However, the reduction of amylose content and amylopectin side chain elongation by high temperature was not corre-lated to the grain chalkiness, but rather correlated to grain weight reduction (Table V), suggesting that the difference in the extent of grain chalkiness between high temperature-tolerant and -sensitive cultivars might be attributed to neither the reduction of amylose content nor the alteration in amylopectin structure. However, these biochemical alterations of starch com-ponents, amylose and amylopectin, were shown to be conserved among *japonica* rice cultivars.

In conclusion, we elucidated the effect of elevated temperature on grain-filling metabolism at the gene expression level and revealed down-regulation of sev-eral genes for starch or storage protein synthesis (such as *GBSSI*, *BEIIb*, and prolamin) and up-regulation of genes for starch-consuming α-amylases and HSPs. Especially, down-regulation of 13-kD prolamin and *cyPPDKB* (*flo-4*) and up-regulation of α-amylase genes were, to our knowledge, newly identified in this study, which is probably attained by excellent reproducibility of the incubator system as well as high sensitivity of microarray analysis. However, the activity of several starch-metabolizing enzymes, including BEIIb, has been recently reported to be interdependently con-trolled by posttranslational modifications such as pro-tein phosphorylation and redox modulation (Tetlow et al., 2004). Actually, expression of several redox reaction-related genes, such as PDIs (AK068268 and AK071514) and glutathione *S*-transferases (AK061304, AK063796, and AK073415), was changed by high temperature in the microarray analysis (Table III). Analyses of such posttranslational regulation are re-quired to understand the effect on starch metabolism. To clarify the precise mechanism for chalky grain occurrence, further research such as gene functional analyses using transgenic plants is necessary along with the determination of key enzyme activities and quantification of their metabolite levels. According to recent studies, ectopic overexpression of α-amylase genes resulted in grains with decreased weight and chalky appearance even ripened under ambient tem-perature (Asatsuma et al., 2006), and antisense sup-pression of Suc transporter gene *SUT1* decreased the grain weight in the transgenic rice plant (Scofield et al., 2002). Expression of α-amylase genes and *SUT1* was up-regulated and down-regulated by high tempera-ture ripening, respectively (Fig. 2), suggesting that these alterations in gene expression might contribute to grain weight reduction and/or grain chalkiness. The grain-filling system using incubators offers a suitable experimental model for the evaluation of transgenic plants with highly reproducible grain fill-ing achieved because restricted time and space are available for growing transgenic plants in many lab-oratories. The analyses using transgenic plants are currently in progress.

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa* sp. *japonica*) 'Nipponbare', 'Koshiibuki', 'Tentakaku', 'Sasanishiki', and 'Hatsuboshi' were grown at 27°C/22°C (12-h-day/12-h-night cycles) until flowering in a plant incubator (model FLI-301NH; Eyela) equipped with a sodium lamp, which allows continuous illumination at an intensity of 560 μmol photons m^{-2} s^{-1}. The relative humidity, which is an important factor for vigorous growth and grain filling, was approximately 50% to 70%, corresponding to 5 to 16 hPa of vapor pressure deficit. Six plants were grown in a plastic container (15 × 10 × 6 cm) filled with 600 mL of rice nursery culture soil (containing 0.15 g each of nitrogen, phosphate, and potassium), and each plant was restricted to the main culm by the removal of tillers as they developed. The number of days from sowing to heading was 62 d for 'Nipponbare' and 75 d for other cultivars. Approximately 15 to 20 d before heading, 3 g of a fertilizer (containing 0.18 g nitrogen, 0.24 g phosphate, 0.18 g potassium, and 0.06 g magnesium) was supplied per container. Each spikelet was marked on the flowering day for subsequent sampling. Five DAF, the plants were transferred to either a 33°C/28°C or 25°C/20°C chamber for high temperature or control treatment, respectively, and then the temperature was maintained at 25°C/20°C from 20 DAF to maturity. During the course of grain filling, developing caryopses were detached from the ear, weighed, immediately frozen in liquid nitrogen, and stored at −80°C until use. On 40 and 45 DAF, caryopses attached on the upper half of ears were dehulled, weighed, and photographed, and appearance quality was determined by a rice grain image analyzer (model ES-1000; Shizuoka Seiki), which can distin-guish between perfect, immature (mainly chalky), damaged (cracked), abor-tive, and colored grains. The heat stress treatment experiment of 'Nipponbare' was repeated twice: (1) For each of the high temperature-treated and control plots, approximately 1,000 developing caryopses from 40 plants were sampled at 10 DAF and used in bulk for microarray analysis and hybridization screenings; and (2) 50 to 200 caryopses from five plants were sampled at various developmental stages and used for gene expression analysis by RT-PCR. For scanning electron microscopy, grains were cut transversely with a razor blade. The fractured surfaces were viewed with a Keyence VE-7800 scanning electron microscope at an accelerating voltage of 12 kV. For amylose content measurement and HPAEC-PAD analysis of amylopectin, 10 dehulled brown rice grains were polished to remove the embryo and pericarp by a test mill Pearlest (Kett) and crushed with a hammer; the resulting powder was used.

Oligo DNA Microarray Analysis and Dot-Blot Hybridization Analysis

A rice 22-K custom oligo DNA microarray kit (Agilent Technologies) that contains 21,938 oligonucleotides synthesized based on the sequence data of the rice full-length cDNA project (Kikuchi et al., 2003) was used. Total RNA was extracted from developing caryopses that were harvested from both 25°C/20°C- and 33°C/28°C-treated plants 10 d after heading using the method of Chang et al. (1993), but with the polyvinylpyrrolidone and spermidine omitted from the extraction buffer. The yield and RNA purity were determined spectrophotometrically. Integrity was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA (200 ng) was labeled with Cy3 or Cy5 using an Agilent Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies). Fluorescently labeled targets were hybridized to Agilent rice 22-K custom oligo DNA microarrays. Hybridization and wash processes were performed according to the manufacturer's instruc-tions, and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software (Agilent Tech-nologies) was employed for the image analysis and data extraction processes.

For several genes responsive to high temperature grain filling, the change of gene expression level was further confirmed by dot-blot hybridization. Ten nanograms of plasmid clone containing the full-length cDNA, which was obtained from the Rice Genome Resource Center, National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan), was denatured by boiling and blotted onto a nylon membrane (positively charged; Roche Diagnostics) using a dot blotter (model AE-6190; ATTO). Polyadenylated [poly(A$^+$)] RNA was isolated from 25°C/20°C- or 33°C/28°C-treated caryopses using Oligo-tex-dT30 Super (Roche Diagnostics) and used for the synthesis of digoxigenin-labeled single-strand cDNA probes. RT was conducted at 42°C for 1 h in a 20-μL labeling reaction mixture containing 1 μg of poly(A$^+$) RNA, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol, 0.5 mM each of

dNTP except for 0.325 mM dTTP, 0.025 μg/μL oligo(dT)$_{12\text{-}18}$ primer, 0.175 mM digoxigenin-dUTP, and 200 units of SuperScript II reverse transcriptase (Invitrogen). Hybridization was performed in high SDS buffer, and the blot was finally washed in 0.1$\times$ SSC and 0.1% SDS at 65°C. Detection was conducted using CDP-*Star* (Roche Diagnostics) according to the manufacturer's instructions. The signal intensities were determined with a Fluor-S Max imaging system and Quantity-One analysis software (Bio-Rad) and corrected by subtracting the local background.

Screening of High Temperature Grain Filling-Responsive Genes by Differential Hybridization and Subtractive Hybridization

For differential hybridization, poly(A$^+$) RNA was purified from caryopses that were harvested from both 25°C/20°C- and 33°C/28°C-treated plants 10 d after heading. Complementary DNA was synthesized from each RNA and inserted into the Uni-ZAP XR vector (Stratagene) according to the manufacturer's instructions. Approximately 3×10^4 unamplified recombinant phages from each library were differentially screened with radioactively labeled single-strand cDNA probes synthesized from poly(A$^+$) RNA of caryopses harvested from either 33°C/28°C-treated plants (plus probe) or 25°C/20°C-treated plants (minus probe). A 25-μL labeling reaction mixture contained 1 μg of poly(A$^+$) RNA, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 4 mM dithiothreitol, 0.8 mM each of dNTP except for 4.8 μM dCTP, 0.02 μg/μL oligo(dT)$_{12\text{-}18}$ primer, 200 μCi of [α-^{32}P]dCTP, and 200 units of SuperScript III reverse transcriptase (Invitrogen). After incubation at 42°C for 1 h, unincorporated nucleotides were separated by a Sephadex G-50 spin column (Roche Diagnostics), and labeled probe DNA was used for hybridization. A total of 24 plates (12 for the 25°C/20°C-treatment library and 12 for the 33°C/28°C-treatment library), each containing approximately 2.5×10^3 clones, were used to prepare pairs of replica nylon membranes (Hybond-N; GE Healthcare Bio-Science), one of which was hybridized with the plus probe and the other with the minus probe. Hybridization was performed at 65°C for 16 h in 6$\times$ SSC containing 0.1% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and 100 μg/mL salmon sperm DNA. Filters were washed twice in 2$\times$ SSC and 0.1% SDS at 25°C and then five times in 0.1$\times$ SSC and 0.1% SDS at 65°C. The filters were exposed to XAR film (Kodak) with an intensifying screen at -80°C for 20 h. Among 6×10^4 clones screened, 20 hybridized differentially to the two probes. These cDNA clones were excised with helper phage and recircularized to generate subclones in the pBluescript SK$-$ phagemid vector according to the manufacturer's instructions (Stratagene). Sequencing analysis revealed that they encoded six distinct genes. The clones containing the longest cDNA fragments, designated as *DS16*, *DS18*, *DS72*, *DS86*, *DS91*, and *DS108*, were selected for further analysis.

PCR-select subtractive hybridization was conducted using the same set of cDNAs prepared from 25°C/20°C-treated and 33°C/28°C-treated caryopses according to the manufacturer's instructions (BD Biosciences).

Semiquantitative RT-PCR Analysis

Total RNA was prepared from developing caryopses as described above. The isolated RNA (5 μg) was reverse-transcribed (SuperScript II; Invitrogen) using an oligo(dT)$_{12\text{-}18}$ primer. An aliquot of the first-strand cDNA mixture corresponding to 6.25 ng of total RNA was used as a template. The semiquantitative PCR (10 μL total volume) was done using 0.25 units of Taq polymerase (ExTaq; TaKaRa). Thermocycling time and temperature were as follows: 98°C for 2 min, followed by an appropriate number of cycles of 97°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final extension period of 72°C for 2 min. The numbers of PCR cycles in the linear range of DNA amplification for each gene and designed gene-specific primers are listed in Table IV. Amplified bands were cloned and sequenced to confirm that they were fragments of the intended gene. The amplified DNA fragments were separated on a 2.0% (w/v) agarose gel, transferred to a nylon membrane (Hybond-N+; GE Healthcare Bio-Science), hybridized with specific cDNA probes amplified from the corresponding cDNA clone, and visualized by the AlkPhos Direct Labeling and Detection system (GE Healthcare Bio-Science) following the manufacturer's instructions. The signal intensities were determined with a Fluor-S Max imaging system and Quantity-One analysis software (Bio-Rad). For the *GBSSI*, *SSI*, *SSIIa*, *SSIIIa*, *BEI*, *BEIIb*, *Amy1A*, *Amy3D*, *Amy3E*, and *cyPPDKB* reactions, the experiment was repeated four times using the same cDNA sources, and the average mean and SD values of relative transcript abundance were calculated.

Determination of Amylose Content by Iodine Colorimetric Analysis

Apparent amylose content was measured by an iodine colorimetric method (Juliano, 1971). Twenty milligrams of polished rice powder was gelatinized by treatment with 0.1 mL of 95% ethanol and 0.9 mL of 1 M NaOH and stood for 10 min in boiling water. After the addition of 5 mL of distilled water, the solution was homogenized, and filled up to 10 mL with distilled water. An aliquot (1 mL) of the solution was taken and added by 0.2 mL of 1 M acetic acid, 0.2 mL of 0.2% (w/v) I$_2$, 2% (w/v) KI, and 8.6 mL of distilled water. After incubation at 27°C for 20 min, A_{620} was measured using a model DU7400 spectrophotometer (Beckman Coulter). Apparent amylose content was estimated by the method of Juliano (1971) from the base calibration line, which was obtained from the absorbance values by changing the ratio of potato amylose (Sigma, Type III) and waxy rice powder in the iodine solution.

Determination of the Distribution of α-1,4-Glucan Chain Length of α-Polysaccharides by HPAEC-PAD

Five milligrams of polished rice powder was suspended in 5 mL of methanol and boiled for 10 min. The homogenate was centrifuged at 8,000g for 3 min. The precipitated polyglucan was washed twice with 5 mL of distilled water, resuspended in a further 5 mL of distilled water, and then boiled for 60 min. An aliquot (1 mL) of the gelatinized polyglucan sample was added to 50 μL of 600 mM sodium acetate buffer, pH 4.6, and 10 μL of 2% (w/v) NaN$_3$, and hydrolyzed by adding 5 μL of *Pseudomonas amyloderamosa* isoamylase (295 units, Hayashibara Biochemical Laboratories). After incubation at 40°C for 24 h, the hydroxyl groups of the debranched glucans were reduced with 3 mg of sodium borohydride under alkaline conditions for 20 h by the method of Nagamine and Komae (1996). The precipitate was dried in vacuo at room temperature. The reduced isoamylolysate sample was dissolved in 100 μL of 1 M NaOH for 60 min and diluted with 900 μL of distilled water. A 25-μL aliquot of the preparation was injected into a BioLC (model DX-500, Dionex) equipped with a pulsed amperometric detector and a CarboPac PA-1 column (4 mm $\times$ 25 cm). Size fractionation of α-1,4-glucans was performed with a linear gradient of sodium acetate (0–450 mM) in 0.1 M NaOH at a flow rate of 1 mL min^{-1}.

Determination of Starch Content

Ten milligrams of polished rice powder was washed twice with 80% (v/v) ethanol at 80°C. After centrifugation at 10,000g for 5 min, the pellet was resuspended in distilled water, boiled for 16 h, digested with amyloglucosidase (Roche Diagnostics) for 20 min at 55°C, and the resultant Glc determined by an enzymatic method (Nakamura and Miyachi, 1982).

Protein Analysis by SDS-PAGE and Immunoblotting

Ten grains of dehulled brown rice were crushed with a hammer and homogenized in 4 mL of 62.5 mM Tris-HCl, pH6.8, 4% (w/v) SDS, 5% 2-mercaptoethanol, and 8 M urea. After centrifugation at 8,000g for 10 min, the supernatant was boiled for 10 min and an 8-μL aliquot of protein was separated on an 18% T, 0.45% C SDS-polyacrylamide gel, where T and C denote the percentage of both acrylamide and bisacrylamide, and the percentage of bisacrylamide relative to the total concentration, respectively. Proteins were stained with Coomassie Brilliant Blue R-250 and quantified densitometrically using Quantity-One analysis software (Bio-Rad).

For immunoblotting, a 0.01-μL aliquot of protein was separated on the same gel and transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad) by semidry electroblotting. The blot was incubated with rabbit antiserum that contained polyclonal antibodies raised against purified rice 13-kD prolamin (Furukawa et al., 2003; provided by Dr. T. Masumura, Kyoto Prefectural University). Immunoreactive proteins were detected by incubation with alkaline phosphatase-conjugated antibodies against rabbit IgG and visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. The protein bands were quantified using Quantity-One analysis software (Bio-Rad).

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