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Original Article

Proteome analysis of bran proteins in rice (*Oryza sativa L.*)

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Summary

Rice bran has wide potential usage as a source of valuable nutrients, though little is known about its protein composition. Here, we identified rice bran proteins by using proteomic analysis. After extraction of crude protein from the bran of Koshihikari brown rice grains, we separated proteins by 2-D PAGE and analyzed 41 protein spots. N-terminal amino acid sequences were determined for 23 spots, 21 proteins of known function and 2 could not be identified by BLAST searching. For the other 18 spots, the N-terminal amino acids could not be determined. Of these, 4 were identified by using LC-MS/MS. After in-solution digestion of bran samples from Koshihikari and TN-1, we looked for differences in the bran proteins between these cultivars. A total of 83 proteins were detected and classified into 10 categories: storage proteins, carbohydrate metabolic enzymes, proteins related to synthesis and proteolysis, embryo proteins, stress-related proteins, regulatory proteins, antioxidant proteins, redox-related proteins, lipid biosynthesis proteins, and energy-related proteins. In the identified proteins, antioxidant proteins and redox-related proteins, such as peroxiredoxin and glyoxalase, respectively, considered to be unique to bran among seed proteins. Such proteins may play important roles as antioxidants for protecting cells in the embryo and aleurone layer from the stress of desiccation.

Key words：Rice, proteomics, bran, embryo, aleurone, protein sequence, LC-MS/MS

Introduction

Rice is the main food in Japan and many other Asian countries. Recently whole grain (brown rice) has been seen in a new light because of its abundant nutrients, which contribute physiologically and nutritionally to human diets. Rice bran is a byproduct of the milling process and contains valuable components such as protein, fiber, vitamins, minerals, and amino acids (Narai-Kanayama *et al.* 2007, Wang *et al.* 1999). Rice bran is well known to contain vitamin B1, and it also contains various functional components such as gamma-oryzanol, ferulic acid, sterol, wax, ceramide, phytin, and inositol (Taniguchi *et al.* 2012). Rice bran contains 16.8% crude protein (NARO 2009), which include the functional peptides such as α-chymotrypsin- and trypsin-digested peptides from rice bran had significant angiotensin I converting enzyme (ACE) inhibitory activity, an antihypertensive property (Narai-Kanayama *et al.* 2007)

Proteomics is a powerful technique not only for identifying proteins in particular organs and tissues, but also for comprehensively characterizing proteins and for application to plant breeding (Hirano. 2005). Kim *et al.* (2009) reported proteome analysis is useful for improving rice grain quality. Komatsu *et al.* (2004, 2005, and 2006) reported valuable rice proteomic information from which they have constructed a rice proteome database. Researchers conducting proteome analysis of rice seed proteins have examined rice endosperm (Sadimantara *et al*. 1999), white-core-bearing sake-brewing cultivars (Kamara *et al.* 2009), germinated seeds (Sano *et al.* 2012), and developing embryos (Xu *et al.* 2012), but rice bran proteins have not been studied in as much detail. To promote efficient utilization of rice bran as a source of valuable nutrients, several researchers have attempted to conduct proteome analysis (Ferrari *et al.* 2009, Trisiriroj *et al*. 2004, Yano and Kuroda 2006). To

extract bran proteins, some researchers have adopted chemical procedures, *e.g.*, using trichloroacetic acid (Trisiriroj *et al.* 2004) or enzymatic methods, *e.g.*, using phytase, xylanase, or amylase (Tang *et al.* 2003, Wang *et al.* 1999). Ferrari *et al.* (2009) used three different extraction methods on full-fat and defatted rice bran, and they identified a total of 43 proteins in five categories: proteins of signaling/regulation, proteins with enzymatic activity, storage proteins, transfer proteins, and structural proteins. The other articles have reported only a few partially characterized proteins.

Here, we used rice bran obtained from embryo and aleurone layers to identify bran-specific proteins and to classify them into and functional groups. This information may provide increased understanding of the benefits and limitations of rice bran for human health.

Materials and Methods

Plant material

Rice cultivar Koshihikari and TN-1 were field-grown at the university farm of the Faculty of Agriculture, Yamagata University. TN-1 rice is one of the typical *indica* rice cultivar and has been reported to have rice stripe virus resistant (Nemoto et al. 1994). Panicles were sampled at 45 d after flowering and brought into a greenhouse to dry. After 14 d of drying (to about 14% water content), the seeds were harvested and dehulled to obtain brown rice. Then, 5 g of brown rice grains were milled (Peslest; Kett Electric, Tokyo, Japan) for 50 s and the bran was collected.

Protein extraction and 2D-PAGE

Proteins were extracted in lysis buffer (O'Farrell 1975) containing 8 mol/L urea, 2% Nonidet P-40, 2% Ampholine (pH 3.5–10; GE Healthcare Biosciences, UK), 5% 2-mercaptoethanol, and 5% polyvinylpyrrolidone-40. The homogenate was centrifuged at 15,000 × *g* for 20 min at 10°C. The supernatant, representing the total extract of bran proteins, was subjected to 2D-PAGE as described by Hirano and Watanabe (1990). The samples were separated in the first dimension by IEF, and in the second dimension by SDS-PAGE using 14% separation gels and 5% stacking gels. The molecular weight of each protein was determined using an Amersham low-molecular-weight calibration kit (GE Healthcare, Buckinghamshire, UK). The pI value of each protein was determined using an Amersham pI Kit (GE Healthcare).

Electroblotting and N-terminal amino acid sequence analysis

Proteins separated by 2D-PAGE were electroblotted in a blotting apparatus (ATTO, Tokyo, Japan) to PVDF membrane (FluoroTrans, Pall Corporation, Port Washington, NY, USA). Electroblotting was performed as described by Hirano and Watanabe (1990). After electroblotting, the PVDF membrane was stained for 5 min with a solution containing 0.1% Coomassie brilliant blue R-250 in 60% methanol, washed with double-distilled water for 5 min, and hung up to dry. The protein spots on the PVDF membrane were excised, set in the upper glass cartridge of the reaction chamber of a protein sequencer (Procise 477A, Applied Biosystems (Life Technologies), Foster City, CA, USA) and analyzed by the pulse-liquid system mode according to the manufacturer's instructions.

Amino acid sequence similarity search

The N-terminal amino acid sequences obtained were used to run searches (web-based NCBI BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the NCBI nonredundant protein database using the protein–protein BLAST algorithm to identify the corresponding proteins.

In-gel enzymatic digestion

Four remaining relatively prominent protein spots for which we could not obtain sequence data because of blocked N-terminal amino acids were analyzed using an LC-MS/MS instrument. The enzyme digestion was performed according to Shevchenko *et al.* (1996) except for destaining. First, four protein spots were cut from 2-D gel and destained in a solution of 30% acetonitrile and 25 mM ammonium bicarbonate. Then the proteins were processed in-gel by reducing alkylation using 10 mM dithiothreitol (DTT) followed by 55 mM iodoacetamide (IAA). In-gel trypsin digestion was performed in a solution of 12.5 ng/μl sequence-grade trypsin (Promega, Madison, WI, USA) and 5 mM ammonium bicarbonate at 37°C for 12 h. After

digestion, the gel cubes were washed twice with 50 mM ammonium bicarbonate. The digested peptides were extracted with 5% formic acid, dried, and stored at –80°C until MS analysis. TOF-MS spectroscopy was performed in the non-labeled native mode on a Xevo Q-TOF MS instrument. Data acquisition and processing were performed using ProteinLynx Global Server (PLGS) Ver. 2.3 software (Waters, Milford, MA, USA). Proteins were identified using PLGS, searching against an *Oryza sativa* protein database downloaded from NCBI using Taxonomy Browser (http://www.ncbi.nlm.nih.gov/guide/taxonomy/). The data included accession number, percent of sequence coverage, number of matched peptides, molecular weight, and pI value.

In-solution enzymatic digestion

Bran proteins were extracted from 0.5 g bran of Koshihikari (*japonica*) and Taichung Native 1 (TN-1, *indica*) in lysis buffer (as described above) and precipitated by addition of 50% trichloroacetic acid (final concentration 10%). The resulting mixture was placed at 4°C for 12 h followed by centrifugation at 15,000 × *g* at 4°C for 20 min. The pellets were freeze-dried and stored at –80°C until LC-MS/MS analysis. Quantification of the protein was conducted by the method of Lowry *et al.* (1951). Protein pellets (50 µg) were dissolved in 200 µl of 50 mM ammonium bicarbonate buffer, pH 8.5 with 5 mM DTT and incubated at 60°C for 30 min. Then, 44 µl of 100 mM IAA was added and the mixture was covered with black paper (to keep out light) and incubated at room temperature for 30 min to allow the protein to undergo reducing alkylation. The protein solution was added to 100 µl of 100 ng/µl trypsin (Promega) and incubated at 37°C for 16 h. The reaction was stopped by addition of trifluoroacetic acid (TFA) up to 5% and incubated at 37°C for 30 min. The reaction mixtures were centrifuged at 13,000 × *g* for 10 min, and the supernatants collected. The digested peptides were dried in a vacuum dryer (Labconco, Kansas City, MO, USA). The peptides were stored at –80°C until use for MS spectrometry.

Analysis of tryptic peptides

We used UPLC (ultra-performance liquid chromatography: Waters. Millford. USA) and a Xevo Q-TOF MS system (Waters.Millford. USA). This system can change alternatively between a low-energy scanning mode (MS) and an elevated-energy mode (MS^E) for accurate mass multiplex peptide fragmentation data. This system gave us *m*/*z* of MS^E spectrum, *i.e.*, precursor ions (MS spectra) under low voltage and product ions (MS/MS spectra) produced after collision under high voltage, enabling us to perform differential analysis of protein composition qualitatively and quantitatively (Chakraborty *et al.* 2007, Levin *et al.* 2011). We injected 0.6 µg of tryptic peptides dissolved in 0.1% formic acid and 5% acetonitrile. Peptides were separated by reverse-phase chromatography on a BEH C-18 column (100 × 0.18 mm; Waters) using a 35-min linear gradient from 5% to 50% acetonitrile in 0.1% formic acid at a flow rate of 0.2 ml/min. TOF-MS spectroscopy was performed in the non-labeled native mode on a Xevo Q-TOF MS instrument. We applied three digested protein samples in each cultivar.

Data acquisition and processing were performed using PLGS Ver. 2.3 (Waters). Using the software, we identified proteins and analyzed differences in protein levels between the two cultivars. The mass data were searched against the *Oryza sativa* protein data downloaded to the server and stored in FASTA format from the NCBI using Taxonomy Browser. If at least two out of three samples contained the same proteins, they were assigned a protein identity and listed in the Table. Expression analysis was conducted via normalization of the MS datasets and comparison of the peptide intensities between Koshihikari and TN-1.

Results and Discussion

Determination of sequences of bran proteins separated by 2-D PAGE

Proteins from rice bran were separated by 2-D PAGE into 41 spots; N-terminal amino acid sequences could be determined for 23. Of the 23 protein spots with N-terminal sequences, 21 could be assigned a function by similarity searches at the NCBI BLAST website (Table 1). The percentage of proteins with successfully determined N-terminal amino acid sequences was 56%. The proteins that could not be sequenced were presumed to have blocked

Table 1. Rice bran proteins identified on the bases of N-terminal amino acid sequences and MS/MS analysis

No.	Protein identified	N-terminal sequence	MW(kDa)	pI	Sequence coverage (%)	No.of peptides mached	Accession number
1	Endosperm binding protein	EETKKLGTVIGIDLG	74	4.9			
2	Protein disulfide isomerase	EAAAAEEGGDAAAEA	63	4.5			
3	Capbinding protein	AEGAAAAKAPHKLHR	63	6.5			
4	ATP synthase subunit β	AAAAKEAAPPAPATGK	60	5.0			
5	Enolase		55	5.4	20.6	7	A1YQJ3
6	Globulin 2 precursor	SDDVLQAAFNXRREE	58	6.0			
7	P0648C09.25 (*Oryza sativa japonica*)	EEEEEKGYGGEAMWL	56	6.4			
8	Globulin-like protein		56	6.7	22.3	11	Q8L810
9	Putative embryo-specific protein	ATKAEAGTEDAASKED	46	8.5			
10	P0477B05.10 (*Oryza sativa japonica*)	EEGEAEEDDGNSAGN	46	6.3			
11	Globulin 2 precursor	SDDVLQAAFNXRREE	46	6.7			
12	Globulin-like protein	VLEAAPFAFLQPSTYD	40	6.8			
13	Glyceraldehyde-3-phosphate dehydrogenase	KIKIGINGFGRIGRLVA	38	7.5			
14	Globulin-like protein	MLLNPVSTPGGFEEYF	36	6.4			
15	Glucose and ribitol dehydrogenase		35	5.6	17.7	6	GRDH
16	Glyoxalase I	VYRVGDLDRTIKXYTE	34	5.3			
17	Globulin-1S-like protein	VNITAGSMNAPFYNT	34	5.9			
18	Globulin 2 precursor	SDDVLQAAFNXRREE	33	5.4			
19	Glutelin		33	6.5	11.6	4	Q40689
20	Superoxide dismutase	VLPVALPDLPYDYGALE	32	5.0			
21	Globulin-1S-ike protein	VNITAGSMNAPFYNT	31	5.5			
22	Glyoxalase I	VYRVGDLDRTIKXYTE	20	4.9			
23	Triosephosphate isomerase	KFFVGGNWKCNGTG	29	5.5			
24	1-Cys Peroxiredoxin	PGLTIGDTVPNLELD	29	6.0			
25	1-Cys Peroxiredoxin	GISXDDVQSHKDWIK	28	6.3			
26	1-Cys Peroxiredoxin	HPGDFTPVCTTELA	27	6.7			
27	*Glycine max* allergen protein	FITMEPKTLFVPQYVDS	24	5.8			

Of 27 protein spots 23 were identified by N-terminal amino acid sequencing followed by BLAST searches. The other 4 spots (No. 5, 8, 15, and 19) were identified by LC-MS/MS (Xevo Q-TOF MS). Data acquisition and processing for the 4 spots were performed using ProteinLynx Global Server (PLGS) Ver. 2.3. Proteins were identified using PLGS, searching against an *Oryza sativa* protein database of NCBI.

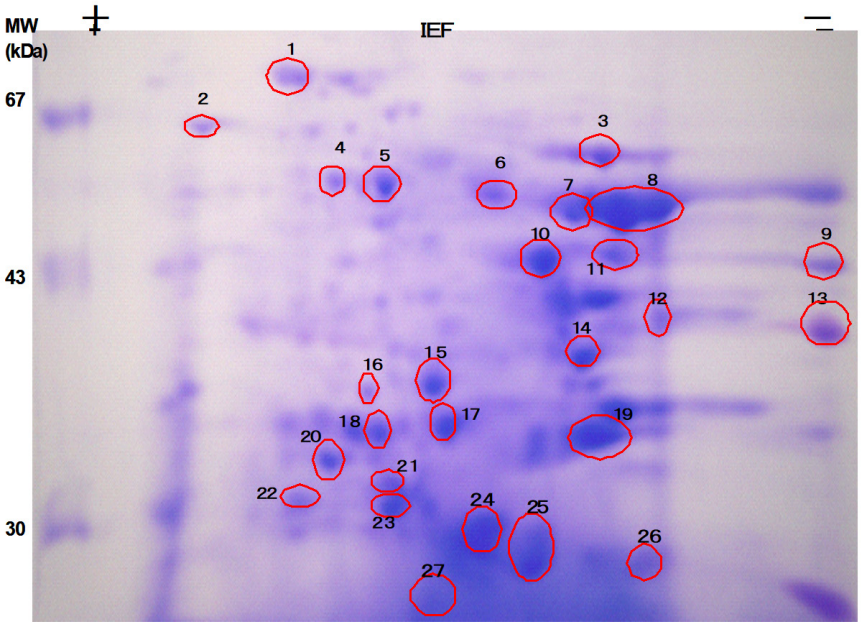

Fig. 1 Two-dimensional gel electrophoresis patterns of proteins in rice bran. The numbers on the 2-D gel correspond to the numbers in Table 1.

N-termini. Of these, four relatively prominent protein spots (Table 1, #5, 8, 15, and 19) could be identified by performing in-gel digestion, LC-MS/MS analysis, and database searching using the ProteinLynx Global Server. Consequently, we identified a total of 27 protein spots resolved from the rice bran sample (Table 1).

Characterization of rice bran proteins identified by 2-D PAGE

Among the identified proteins were some antioxidant proteins that are involved in cell defense. We detected three spots of peroxiredoxin (Table 1. #24, 25, 26) and one of superoxide dismutase (Table 1. # 20), both of which are considered to function as antioxidants. The N-terminal amino acid sequences of the three 1-Cys peroxiredoxins were all different, which may indicate that the number of amino acid residues is different and consequently differentiate the length of signal peptides. We also detected two spots of glyoxalase I, which is related to redox regulation and catalyzes the conversion of glutathione to *S*-lactoyl glutathione. These spots gave the same N-terminal amino acids sequences, though the molecular weights were considerably different.

We found three spots of globulin-like proteins and three spots of globulin 2 precursor. These proteins are classified as storage proteins, though the amino acid sequences were largely different from that of the 19-kDa storage globulin (Shorrosh *et al.* 1992). The N-terminal amino acid sequences of the two spots of globulin 2 precursor were identical and the two spots of globulin-1S-protein also identical, but the molecular weights estimated from 2D-PAGE were different. This might be explained by post-translational processing that removes part of the C-terminal region during grain desiccation.

Several of the proteins were related to carbohydrate metabolism (*e.g.*, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and enolase), though rice seeds are known to contain many proteins related to carbohydrate metabolism (Kamara *et al.* 2009, Sano *et al.* 2012). We detected glutelin, which is the main endosperm storage protein and may represent contamination from the outer layer of rice endosperm. Two protein spots (#3 and #7 in Table 1) were identified as hypothetical proteins on the basis of their N-terminal sequences, which are registered in the rice protein database.

Differential expression of bran proteins between Koshihikari and TN-1

After in-solution digestion of rice bran samples from Koshihikari and TN-1, we attempted identification and differential expression analysis of proteins from the two cultivars by analyzing m/z of MS spectra and MS/MS spectra, and we obtained MS expression data using ProteinLynx Global Server. We detected 83 different bran proteins in the two cultivars, of which 18 (21%) were same as those identified by 2D-PAGE and N-terminal amino acid sequencing, and we compared the levels of these proteins in the two cultivars (Table 2). Each protein was cataloged and classified into one of 10 functional categories (Fig. 2): storage protein (16.9% of the 83 proteins), carbohydrate metabolism (15.7%), protein biosynthesis (15.7%), embryo protein (12.0%), stress-related protein (10.8%), regulation protein (7.2%), antioxidant-related protein (7.2%), redox-related protein (7.2%), lipid biosynthesis (4.8%), and energy-related protein (2.4%).

Several proteins related to carbohydrate metabolism were detected in the two cultivars. These were mainly associated with glycolysis, such as fructose-bisphosphate aldolase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, enolase, and malate dehydrogenase. This class of proteins was relatively abundant in the bran of Koshihikari, which may indicate the presence of active carbohydrate metabolism even in the aleurone and embryo of rice seeds. There were many stress-related proteins, such as 16.9-kDa heat shock protein (HSP), 17.4-kDa HSP, 17.7-kDa HSP, 17.9-kDa HSP, and WSI18 protein. These proteins may be produced under stress conditions, especially under high temperatures, during grain filling and seed desiccation.

In the category of protein synthesis, we detected 40S and 60S ribosomal proteins, DnaK-type molecular chaperonin, elongation factors 1 and 2, and protein disulfide isomerase. We included the 70-kDa HSP in this group because it is well known to have a chaperonin function,

Table 2. Differential expression of bran proteins between cultivars, Koshihikari and TN-1.

	Cultivars		Sequence coverage (%)	No. of peptides matched	Accession number
	Koshihikari	TN-1			
Storage protein					
19-kDa globulin		○	24.2	6	GL19
α-globulin		○	25.3	5	Q0PH05
Cupin family protein	□	□	35.6	15	Q75GX9
Globulin 2		○	51.5	22	O65043
Globulin-like protein		○	32.3	13	Q8L810
Glutelin C	□	□	8.5	5	Q6T726
Glutelin		○	37.3	12	Q0JJ36
Glutelin type-A1	□	□	32.3	13	GLUA1
Glutelin type-A2	□	□	33.1	11	GLUA2
Glutelin type-A3	□	□	15.3	6	GLUA3
Glutelin type-B1	□	□	27.6	12	GLUB1
Glutelin type-B2	□	□	29.3	12	GLUB2
Glutelin type-B4	□	□	23.2	10	GLUB4
Glutelin type-B5	□	□	22.4	10	GLUB5
Carbohydrate metabolism					
Cytosolic glyceraldehyde-3-phosphate dehydrogenase	○		57.5	13	Q8H6A7
Cytosolic pyruvateorthophosphate dikinase	◎		5.8	4	O82032
Enolase	○		59.6	17	A1YQJ3
Enolase 2		○	35.7	10	Q10P35
Fructose-bisphosphate aldolase	□	□	61.3	21	Q5N725
Glucose and ribitol dehydrogenase	□	□	40.3	10	GRDH
Glyceraldehyde-3-phosphate dehydrogenase	□	□	50.4	14	Q6K5G8
Granule-bound starch synthase		◎	32.2	11	C8CBL4
Malate dehydrogenase		○	37.6	6	A1YQK1
Phosphoglycerate kinase	◎		34.4	11	Q655T1
Pyruvate decarboxylase isozyme 2	◎		11.9	5	PDC2
Triosephosphate isomerase	□	□	58.9	11	TPIS
UDP-glucose pyrophosphorylase	◎		9.4	4	A3QQQ3
Protein synthesis and proteolysis					
40S ribosomal protein S12	○		45.7	4	Q8H2J8
60S ribosomal protein P1	◎		65.0	1	RLA1
70-kDa heat shock protein	□	□	29.6	14	Q10NA1
DnaK-type molecular chaperone Bip	□	□	18.4	7	Q6Z7B0
DnaK-type molecular chaperone hsp 70		○	25.6	12	Q53NM9
Elongation factor 1-α	◎		20.6	6	Q10QZ5
Elongation factor 2	◎		5.7	3	Q6H4L2
Heat shock cognate 70-kDa protein	□	□	29.6	14	Q10NA1
Heat shock cognate 70-kDa protein 2	□	□	23.9	11	Q84TA1
Peptidyl-proyl *cis-trans* isomerase	□	□	56.4	11	Q6ZH98
Protein disulfide isomerase	◎		25.5	11	Q9MB13
Polyubiquitin	○		31.6	4	A0PGH5
Polyubiquitin containing 7 ubiquitin monomers		◎	30.9	3	A6N128
Embryo proteins					
Differentiation embryo protein 31		○	20.2	9	Q2L8Y1
Embryo abundant protein 1	□	□	41.1	4	EMP1
Embryo-specific protein	□	□	30.7	5	Q2R223
Endosperm luminal binding protein	○		18.1	10	O24182
Germin-like protein 8-2	◎		16.3	7	GL82
Late-embryogenesis-abundant protein 1	□	□	34.9	10	LEA1
Late embryogenesis abundant protein 3	○		41.5	6	LEA3
Putative embryonic cell protein		◎	15.0	2	Q94GQ5
Putative group 3 LEA protein		○	30.0	6	B1NEV8
seed maturation protein		◎	18.0	2	Q10CY6
Stress-related proteins					
16.9-kDa class 1 heat shock protein 1		○	50.7	5	HS16A
16.9-kDa class 1 heat shock protein 2		○	45.3	5	HS16B
16.9-kDa heat shock protein A	□	□	41.9	7	E5KIK8
16.9-kDa heat shock protein B	○		42.1	4	E5KIK9
17.4-kDa heat shock protein	◎		31.7	4	E5O3J9
17.7-kDa class 1 heat shock protein	◎		20.8	3	HS177
17.9-kDa class 1 heat shock protein		○	38.5	5	H517A
Stress-inducible membrane pore protein	◎		14.5	3	Q9FP64
WSI18 protein induced by water stress	□	□	45.3	9	Q40709
Regulatory proteins					
Allergenic protein fragment	□	□	51.3	3	Q40720
α-amylase inhibitor	□	□	65.2	8	E6Y8T1

α-amylase/subtilisin inhibitor	○		38.0	5	IAAS
Bowman-Birk type bran trypsin inhibitor	□	□	46.5	11	IBBR
Seed allergenic protein RAG2		◎	18.1	3	B7SDG9
Seed allergenic protein RA5	□	□	23.1	3	RA05
Antioxidant proteins					
1-Cys peroxiredoxin	○		45.0	10	REHYA
Peroxiredoxin		○	68.6	13	B7ERQ1
Peroxiredoxin 5		○	60.3	17	A6MZV8
Peroxiredoxin 2C	○		56.8	5	PRK2C
Peroxiredoxin 2E-1	○		47.4	6	PR2E1
Superoxide dismutase	□	□	22.5	8	Q0DJ64
Redox-related proteins					
Alcohol dehydrogenase 1	◎		20.1	5	ADH1
Glutaredoxin C6	○		44.6	4	GRXC6
Glyoxalase		○	23.4	6	Q0J7H9
Glyoxalase family protein	□	□	38.4	6	Q10N99
Lactoylglutathione lyase		○	18.9	5	LGUL
Thioredoxin H1	○		19.7	2	TPXH1
Oil-related proteins					
Lipoprotein-like		◎	8.7	3	Q94J20
Non-specific lipid-transfer protein 1	○		44.0	4	B7SDG3
Oleosin	○		33.1	6	B7F9V8
Oleosin 18 kDa		◎	36.6	6	OLEO2
Energy-related proteins					
ATP synthase subunit α	○		8.5	5	ATPAM
ATP synthase subunit β	□	□	33.9	12	Q5N7P8

Proteins were identified using the software, ProteinLynx Global server (PLGS) Ver. 2.3 (Waters) embedded ion-accounting algorithm and a search of an *Oryza sativa* database. When at least two out of three samples contained the same proteins, they were assigned a protein identity and listed in the table. The ion detection, peptide clustering, and parametric normalizations were performed in PLGS with a method of Expression$^{E.}$. The detected proteins were compared quantitatively based on the ion intensity of the minimum three peptides in each protein. We conducted two-by-two comparison of each protein between the two cultivars, Koshihikari and TN-1.Sequence coverage percentage, number of peptide matched and accession number for each protein were listed from the data of Koshihikari, adopted higher values of the two in the coverrage percentage and the number of peptides.
◎: protein level more than twise than in the other cultivar, ○: protein level 1.5 to 2 times than other cultivar, □: same amount of protein in both cultivars.

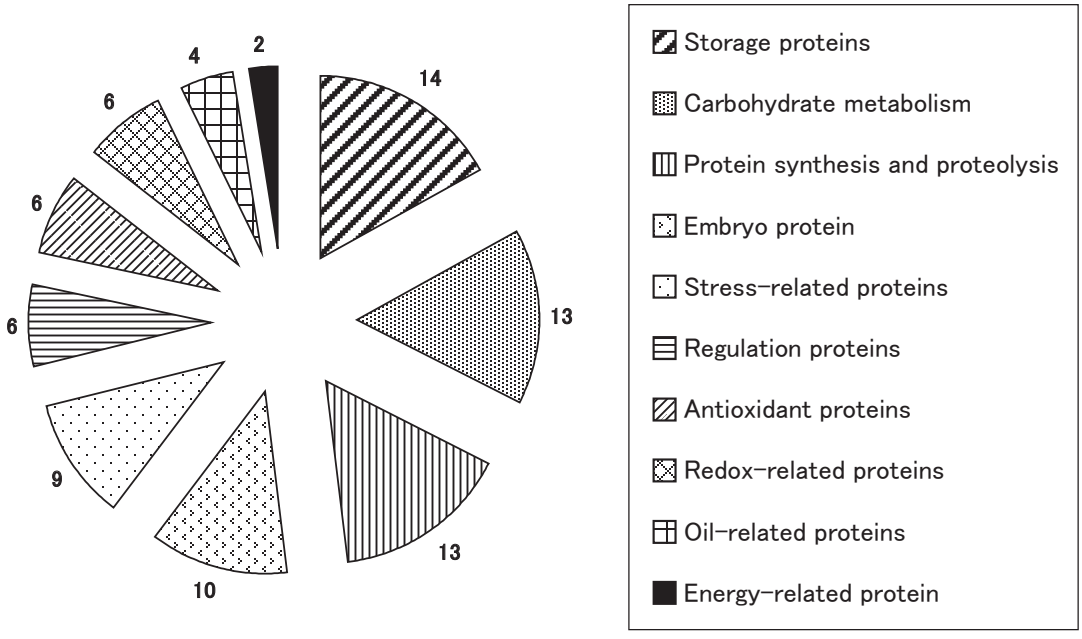

Fig. 2 Classification of identified proteins according to their function of rice bran in Koshihikari and TN-1.

i.e., aiding the folding of targeted proteins. We also detected proteolysis-related proteins such as polyubiquitin that are considered to have important roles in protein degradation. We found ten embryo proteins, such as embryo-specific protein and late-embryogenesis-abundant (LEA) protein, of which the former is thought to regulate germination controlled by glutaredoxin and/or thioredoxin (Yano and Kuroda, 2006), and some kind of LEA proteins have been reported cryo-protective activity after harvesting (Momma, 2003). There were ten stress related proteins which consisted mainly low molecular weight heat shock proteins (HSPs), such as 16.9-kDa HSPs, 16.9-kDa class 1 HSPs 17.4-kDa HSP, 17.7-kDa class 1, 17.9-kDa HSPs and WSI18 protein, which may contribute cell defense in grain filling and desiccation after harvesting. We found several types of regulatory proteins. These were mainly protein inhibitors such as α-amylase inhibitor, α-amylase/subtilisin inhibitor, Brown-Brick type bran trypsin inhibitor, and seed allergenic proteins. The seed allergenic protein RAG2 were more abundant in TN-1 than in Koshihikari.

Among the bran proteins there were a few distinctive proteins: several types of peroxiredoxin, one glutaredoxin, and one superoxide dismutase, all of which are antioxidant proteins. Such proteins are considered to reduce the peroxide proteins and may regulate seed germination (Ferrari *et al.* 2009). In addition to the antioxidant proteins, other distinctive bran proteins included several involved in redox regulation, such as glyoxalase I, lactoylglutathione lyase and thioredoxin. These proteins belong to the glyoxalase family and are related to glutathione biosynthesis and metabolism. We also found ATP synthase subunits α and β, which are part of ATP synthase located on the mitochondrial membrane. Rice bran is about 21% bran oil (NARO 2009); thus, it was not surprising that we found several oil-related proteins such as oleosins and a lipid transfer protein in the bran. Oleosins function in lipid biosynthesis, and lipid transfer protein bind various lipids and transfer of the lipids across membrane, which is also known as a kind of food allergens.

We found many kinds of storage proteins such as glutelin types A1, A2, A3, B1, B2, B4, B5, and C, α-globulin, and 19-kDa globulin, especially in TN-1. These are considered to be contamination of the bran samples with flour from the outer layer of the endosperm. We could not detect prolamin in either Koshihikari or TN-1, though some authors have reported detecting prolamin in rice bran (Ferrari *et al.* 2009, Trisiriroj *et al.* 2004). The reason we could not detect prolamin is related to the lysis buffer we used for protein extraction, because prolamin that is alcohol desolved protein would not be extracted in such a mild lysis buffer.

In this study, we have identified several proteins that are specific to bran among seed proteins, including antioxidant proteins such as peroxiredoxin and superoxide dismutase and redox-regulating proteins such as glyoxalase I which are not found in endoaperm protein (Sadimantara et al. 1999). We speculate that these proteins provide protection under stress during grain filling and desiccation.

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イネにおける米糠タンパク質のプロテオミクス

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米糠は、その栄養価の高さから、広く利用されることが期待されるが、その構成タンパク質に関してはほとんど知られていない。本研究では、プロテオミクスの手法で含有する米糠タンパク質を同定した。コシヒカリ米糠タンパク質の二次元電気泳動法により、41のタンパク質スポットが分離でき、そのうち23のスポットのN末端アミノ酸配列が決定できた。そのうちの21スポットはBlast検索によりタンパク質が同定できたが、2スポットは機能未知であった。他の18スポットのN末端アミノ酸配列データは得られなかった。そのうちの4スポットについて、トリプシンによるゲル内消化後のLC-MS/MS分析により同定した。次に、イネ日本型のコシヒカリとインド型のTN-1の米糠から抽出した、それぞれ混合タンパク質の液相内トリプシン消化により、ペプチド混合物を得てLC-MS/MS分析を行った。両品種に共通する83種のタンパク質が検出され、それらを10のカテゴリーに分類した：タンパク質の種類の多い順に、貯蔵タンパク質、炭水化物代謝、タンパク質合成と分解、胚特異的、ストレス誘導、調節、抗酸化、酸化還元、脂質合成などである。これらの同定したタンパク質のうち、抗酸化作用のある、パーオキシレドキシンや、酸化還元に関係したグリオキサラーゼなどは登熟期および収穫後の乾燥などによるストレス下で、種子の胚や米粉層細胞などで、細胞を防御する役割を果たしていることが推察される。

キーワード：イネ，プロテオミクス，米糠，胚，アリューロン，アミノ酸配列，LC-MS/MS