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Identification and Variation of Glutelin α Polypeptides in the Genus *Oryza* Assessed by Two-Dimensional Electrophoresis and Step-by-Step Immunodetection

NADAR KHAN,[†] TOMOYUKI KATSUBE-TANAKA,*[,†] SHUICHI IIDA,[#] TAKESHI YAMAGUCHI,[†] JUNICHI NAKANO,[†] AND HISASHI TSUJIMOTO[†]

The United Graduate School of Agricultural Sciences, Tottori University, Koyama, Tottori 680-8553, Japan, and National Agricultural Research Center for Western Region, Fukuyama, Hiroshima 721-8514, Japan

To obtain fundamental information for nutritional improvement of rice (*Oryza sativa*) seed proteins, the α polypeptides of the major storage protein glutelin varied over the genus *Oryza* were qualitatively and quantitatively characterized with unique methods. The polypeptides were maximally separated by two-dimensional electrophoresis (2D-PAGE) composed of nonequilibrium pH gradient gel electrophoresis (NEPHGE) and higher temperature SDS-PAGE. Then the subunit for each polypeptide spot was identified with the sequential immunodetection called a step-by-step detection method, making use of highly subunit-specific antibodies. The comparative analysis showed considerable variation in the accumulation level of A-type and B-type glutelin subunits and found unknown glutelin subunits that were unable to be identified with the antibodies used. Wild species accumulating a high amount of lysine-rich B-type glutelin subunits and unknown unique subunits were identified as they might play a crucial role in nutritional quality improvement of the cultivated rice.

KEYWORDS: 2D-PAGE; glutelin subunits; subunit-specific antibodies; wild species of rice

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereals and not only a staple food for more than a half of the global population but also a major component of human protein intake, especially in Asian countries (*1*). The demand for rice as a dietary protein is expected to increase dramatically in the future because the world population has been predicted to double by the year 2030 (*2*). Rice seed, like all other cereal crop seeds, is deficient in lysine, which is due to the low content of this amino acid in the storage proteins and also in the free form (*3, 4*).

Recently, the Monsanto group has demonstrated transgenic maize plants accumulating a significant level of free lysine by the inhibition of lysine catabolism as well as deregulation of lysine biosynthesis (*5*). Apart from genetic engineering, however, modification of storage protein composition has been an important research theme since the findings of *opaque2* and *floury2* mutants, because almost all lysine in edible seeds is usually in the form of seed storage proteins. Accordingly, the detailed analysis on the developmental change in the accumulation of different classes of proteins and nonproteinous nitrogen has been conducted (*6*).

Cereal grain proteins have traditionally been classified by Osborne fractionation based on their solubility, that is, albumins in water, globulins in saline, prolamins in alcohol, and glutelins in dilute acid or alkaline solutions. The major endosperm storage proteins of all cereal grains except oats and rice are prolamins (*7*). The most prevalent and dominant part of the storage proteins in rice is glutelin, accounting for about 60% (*8*) to 80% (*9*) of total grain proteins. Rice glutelin is synthesized as a 57 kDa precursor (subunit) on the endoplasmic reticulum (ER), and the precursor is processed proteolytically into a 35 kDa α polypeptide and a 20 kDa β polypeptide within a protein storage vacuole called protein body II (PB II) (*10, 11*). The α and β polypeptides are covalently linked to each other by an intermolecular disulfide bond (*12*).

Rice glutelin is encoded by a small multigene family, which can be classified into two subfamilies, that is, A-type (GluA) and B-type (GluB) glutelin, according to the degree of nucleotide sequence similarity (*9*). The A-type and B-type glutelins of typical *japonica* subspecies of *O. sativa* are composed of six major subunits, GluA1, GluA2, GluA3, GluB1, GluB2, and GluB4, and minor subunits GluB43, GluB22, and GluB42 (*12*). The glutelin α polypeptides are resolved into six major bands from α1 to α6 in decreasing order of molecular weight by SDS-PAGE, and the bands correspond to GluB4, GluA2, GluA1,

* Address correspondence to this author at the Graduate School of Agriculture, Kyoto University, Kitashirakawa, Kyoto 606-8502, Japan (e-mail tanakato@kais.kyoto-u.ac.jp; fax 81-75-753-6065; telephone 81-75-753-6043).

[†] Tottori University.

[#] National Agricultural Research Center for Western Region.

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Table 1. Species Names of Rice, Their Genome Type, and Accession Numbers Used in This Study

species[a]	genome type[a]	accession no.[b]
O. sativa[c]	AA	
O. nivara	AA	00053927
O. rufipogon	AA	W1549
O. glaberrima	AA	W0025
O. barthii	AA	W1237
O. meridionalis	AA	W1297
O. punctata (2×)	BB	W1514
O. punctata (4×)	BBCC	W1564
O. latifolia	CCDD	W0019
O. alta	CCDD	W0017
O. grandiglumis	CCDD	W1194
O. brachyantha	FF	W0023
O. longiglumis	HHJJ	W1223

[a] The species names and genome type are shown according to refs *20* and *21*. [b] Germplasm of *O. nivara* was from the National Institute of Agrobiological Sciences Genebank, Tsukuba, Japan. The other germplasms were from the National Institute of Genetics (Plant Genetics Laboratory and Experimental Farm), Mishima, Japan. [c] *O. sativa* subspecies *japonica* cv. Koshihikari was used.

GluA3, GluB2, and GluB1, respectively (*12*). In terms of nutritional quality the B type is more nutritious as compared to the A type as the major subunits of the B type contain 20% more lysine on average than the A-type subunits (*9*).

With the realization of many useful genes in wild species of rice, continuous efforts have been made to exploit this genetic pool for crop improvement. There are numerous examples of transfer of genes having resistance to pest and environmental stresses from wild rice species to cultivated rice (*13, 14*). However, research work on wild rice targeted toward nutritional quality improvement of rice seed is limited. Recently, in efforts to find genetic resources with high nutritional value of rice seed, we assessed the diversity of rice glutelin polypeptides in wild species by our unique higher temperature SDS-PAGE method and subunit-specific antibodies and found greater variation in glutelin α polypeptides in wild rice species (*15*). The resolution obtained by one-dimensional gel electrophoresis (1D-PAGE) is not sufficient to detect precise differences in quantity of polypeptides. Obviously for in-depth study of the difference in accumulation level a powerful proteomic approach is needed. The application of proteomic tools such as 2D-PAGE is now a popular technique and is a powerful tool for detecting variation in protein composition, separation, and quantification of protein in a complex mixture (*16–19*). 2D-PAGE coupled with our sequential immunodetection method called "step-by-step detection" enabled us to categorize glutelin α polypeptides of wild rice species into four types of the known subunits and unknown subunits and also allowed us to compare the relative accumulation level of identified subunits. We hope this study will lay the foundations for further research targeted toward nutritional quality improvement of rice seed.

MATERIALS AND METHODS

Plant Materials. Wild rice species of different genome types and two cultivated rice were used as plant materials. The species are listed in **Table 1** with their respective names, genome types, and accession numbers (*20, 21*). Due to the limited number of seeds available for *Oryza minuta* and *Oryza australiensis*, these species were not tested in this study. Germplasms of African cultivated rice *Oryza glaberrima* and all wild species except *Oryza nivara* were provided by the National Institute of Genetics (Plant Genetics Laboratory and Experimental Farm), Mishima, Japan. The seeds of *O. nivara* were obtained from the genetic stocks of the National Institute of Agrobiological Sciences Genebank, Tsukuba, Japan. The plants were sown in pots and grown to maturity at the experimental area of Tottori University, Tottori, Japan. The mature rice seeds were harvested and used for protein extraction.

Glutelin Extraction. Glutelin was extracted stepwise following the method described by Katsube-Tanaka et al. (*12*). One hundred milligrams of rice seeds was dehulled and ground to fine powder by using a mortar and pestle. To defat the samples, the samples were homogenized with 500 μL of cold 100% acetone and shaken at 4 °C for 30 min. Then the samples were centrifuged at 11250*g* at 4 °C for 15 min. After centrifugation, the supernatant was removed. Following this, 1 mL of 35 mM potassium phosphate buffer containing 0.4 M sodium chloride (NaCl) (pH 7.6) was added to the samples to remove albumins and globulins. The samples were mixed by glass rod and vigorously shaken on a shaker at 4 °C for 1 h. Then the samples were centrifuged at 11250*g* at 4 °C for 15 min. The supernatant was discarded after centrifugation. Subsequently, this process was repeated three times. For removal of prolamins from the samples, 1 mL of 60% propanol was added to the samples. Likewise, the samples were mixed by glass rod and vigorously shaken on a shaker at 4 °C and centrifuged at 11250*g* at 4 °C for 15 min. The supernatant was discarded after centrifugation. The pellet was washed with 1 mL of distilled water and centrifuged at 11250*g* at 4 °C. The supernatant was discarded. Finally, 1 mL of 1% (v/v) lactic acid containing 1 mM EDTA was added to the samples, mixed and shaken on a shaker at 4 °C for 1 h to extract glutelin. The samples were centrifuged at 11250*g* at 4 °C for 15 min. The extracted glutelin was either used for subsequent analysis or stored at −20 °C.

Sample Preparation. Four hundred microliters of glutelin extract was mixed with an equal volume of 50% trichloroacetic acid in a test tube. The samples were vortexed and incubated at 4 °C for 30 min followed by centrifugation at 11250*g* at 4 °C for 10 min. After removal of supernatant, the precipitate was resuspended in chilled 100% acetone by vortexing. The samples were incubated at −20 °C for 10 min followed by centrifugation at 11250*g* at 4 °C for 10 min. The supernatant was discarded, and the pellet was air-dried. Finally, the pellet was completely dissolved in 100 μL of lysis buffer containing 9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, and 5% Bio-Lyte (Bio-Rad), pH range from 3 to 10. The samples prepared were used for analysis.

2D-PAGE Analysis. 2D-PAGE was carried out according to the method of Iida et al. (*22*) with some modifications. Gels were prepared in glass tubes (75 × 1 mm) for NEPHGE. The gel components consisted of 9 M urea, 4% (T) acrylamide, 2% Triton X-100, 5% Bio-Lyte, pH 3−10. The lower reservoir was filled with 1 M sodium hydroxide and connected with a cathode, and the upper reservoir was filled with 20 mM phosphoric acid and connected with an anode. After application of samples, 50 μL of overlay buffer (9 M urea, 0.0025% bromphenol blue, 2.5% Bio-Lyte, pH 3−10) was applied to a gel tube. Electrophoresis in the first dimension (NEPHGE) was carried out at 200 V for 15 min, at 300 V for 15 min, at 400 V for 15 min, at 500 V for 15 min, and at 750 V for 5 h. After electrophoresis in the first dimension, the gel was removed from the glass tube. Electrophoresis in the second dimension was carried out by higher temperature SDS-PAGE method according to the method of Khan et al. (*15*). The gel was placed horizontally on a stacking gel made on a 17% (T) acrylamide separation gel. Any trapped bubbles between the NEPHGE gel and the stacking gel were removed gently. Equilibration buffer containing 50 mM Tris, pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (w/v) glycerol, and 5% (v/v) 2-mercaptoethanol was applied on the top of gel. Electrophoresis was conducted at constant voltage of 200 V for 80 min at 45 °C. The slab gel was stained in a staining solution containing 10% methanol, 6.6% acetic acid, and 0.05% Coomassie Brilliant Blue R-250 (CBB) for 1 h and destained in destaining solution (10% methanol, 6.6% acetic acid) for about 1 h until a clear background appeared. Finally, the gel was photographed, and each spot on the gel was quantified by ImageJ 1.38x computer software developed by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD.

Step-by-Step Detection with Antibodies. Proteins from 2D-PAGE gels were partially transferred to nitrocellulose membrane by electroblotting. The membranes were blocked in blocking solution [0.15 M NaCl, 5% (w/v) skimmed milk, 0.05% (v/v) Tween-20, and 40 mM

Table 2. Reactivity of Antibodies against Glutelin Subunits

antibody	glutelin subunit recognized[a]
anti-A1 (no. 2)	GluA1 GluA2
anti-A3 (no. 3)	GluA3
anti-B1 (no. 4b)	GluB1 GluB2
anti-B4 (no.4b)	GluB4

[a] Reactivity is against the glutelin subunits of typical *japonica* subspecies of *O. sativa* (*15*).

sodium phosphate buffer, pH 7.4] for 1 h and incubated with primary antibody at room temperature for 1 h. The membranes were washed (three times for 20 min) with TBST [20 mM Tris, pH 7.5, 0.9% (w/v) NaCl, 0.05% (v/v) Tween-20]. Then the membranes were incubated with secondary antibody (goat anti-rabbit IgG or goat anti-mouse IgG conjugated with alkaline phosphatase) for 1 h at room temperature. After subsequent washing (three times for 20 min) with TBST, color development was accomplished with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

Glutelin subunit-specific antibodies, anti-B4 (no. 4b), anti-A1 (no. 2), anti-B1 (no. 4b), and anti-A3 (no. 3) raised against subunit-specific epitope sequences designed at variable regions of the glutelin α polypeptides for GluB4, GluA1, GluB1, and GluA3, respectively, were previously prepared (*15*) and used in this order unless otherwise mentioned. The specificity of antibodies was evaluated previously and found to be highly subunit specific (*15*). The reactivity of antibodies against the glutelin subunits of typical *japonica* subspecies is presented in **Table 2**. The same blotted membrane was used step-by-step for all four immunodetections (i.e., after detection with one antibody, the same membrane was used for the detection with another antibody following the above-mentioned procedure starting from blocking to color development of membrane). Protein spots remaining in partially electroblotted gels were visualized by CBB staining and compared with signals on membranes to identify subunits but not used for quantification.

RESULTS

Identification of Rice Glutelin Subunits. Glutelin polypeptides of Asian cultivated rice *O. sativa* were resolved by 2D-PAGE, and subsequent subunit identification was accomplished by step-by-step detection method using subunit specific antibodies (**Figure 1**). Glutelin α polypeptides were well separated into nine detectable spots. However, *β* polypeptides were not detected. The possible reason might be that in our 2D-PAGE system we used ampholine ranging from pH 3 to 10, so the *β* polypeptides might have not entered into or run away from the gel (*22*). Anti-B4 (no. 4b) exclusively reacted with one spot of GluB4 subunit. Anti-A1 (no. 2) recognized two major spots, GluA1 and GluA2, and two minor spots. The reactivity of anti-A1 (no. 2) with GluA2 is not surprising because the two subunits share the common epitope (*15*). Therefore, we differentiated GluA1 and GluA2 on the basis of isoelectric point (p*I*) and molecular weight (MW) predicted by their amino acid sequences. The minor spots A1a and A2a are supposed to be post-translationally charge-altered polypeptides derived from GluA1 and GluA2, respectively, because the whole rice genome sequencing of *O. sativa* has revealed no genes other than the three active and one pseudo GluA gene (*9*). Anti-B1 (no. 4b) reacted with both GluB1 and GluB2, revealing the fact that GluB2 has one mismatch in the corresponding amino acid sequence to the epitope of anti-B1 (no. 4b) (*15*). Again, we differentiated GluB1 and GluB2 on the basis of p*I* and MW predicted by their amino acid sequences. Anti-A3 (no. 3) weakly reacted with GluA3. Interestingly, one unknown spot (Unk) was observed, which was not detected by our antibodies (**Figures 1** and **2**).

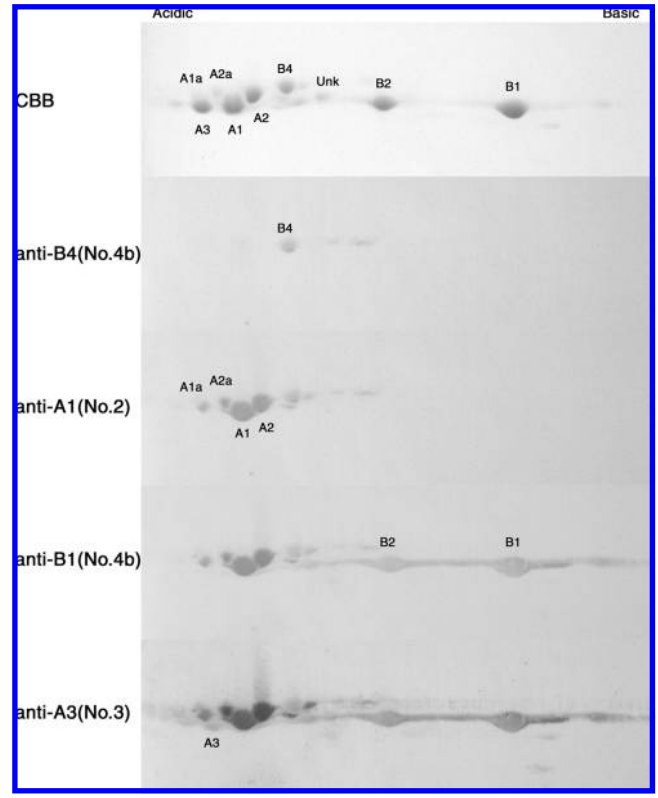

Figure 1. Glutelin α polypeptide subunit identification. Glutelin fraction extracted with 1% lactic acid and 1 mM EDTA from *O. sativa* cv. Koshihikari was resolved by 2D-PAGE. Fractionated glutelin polypeptides were partially electroblotted onto a membrane and detected by sequential use of subunit specific antibodies (step-by-step detection). Untransferred polypeptides were detected on a gel by CBB staining and compared with signals on membranes. Anti-B4 (no. 4b), anti-A1 (no. 2), anti-B1 (no. 4b), and anti-A3 (no. 3) were used in this order for subunit identification. Capital letter and number labels on the spots refer to polypeptides corresponding to glutelin subunits, A1 (GluA1), A2 (GluA2), A3 (GluA3), and B4 (GluB4), whereas the lower case letter (a) denotes possible post-translationally modified polypeptide derived from the corresponding subunit. The spot marked "Unk" shows an unknown spot that has not been identified by the above four antibodies. Gel is oriented with the NEPHGE acid end (+) to the left and the basic end (−) to the right.

Variation in Accumulation Level of Glutelin α Polypeptides in AA Genome Species. Glutelin α polypeptides of AA genome species were separated by 2D-PAGE and either quantified by CBB staining or identified by step-by-step detection procedure (detection data not shown). Generally, the overall distribution pattern of spots was similar among AA genome species (**Figure 2**). However, the relative accumulation level of spots displayed marked variation (**Table 3**). In most species one spot for GluA1 was detected, except for *O. rufipogon*, *O. barthii*, and *O. meridionalis*, in which an additional spot (A1a) was also observed. The accumulation level of GluA1 drastically decreased in *O. nivara*, *O. glaberrima*, and *O. meridionalis* and slightly reduced in *O. barthii* as compared to that in *O. sativa*. One spot for GluA2 was detected in most species except *O. glaberrima*, in which an extra significant spot (A2b) at a more basic position was noted. The relative accumulation level of GluA2 was highest in *O. nivara*, in which the quantity of GluA2 was more than double compared with that in *O. sativa*. GluB4 subunit showed one spot in *O.*

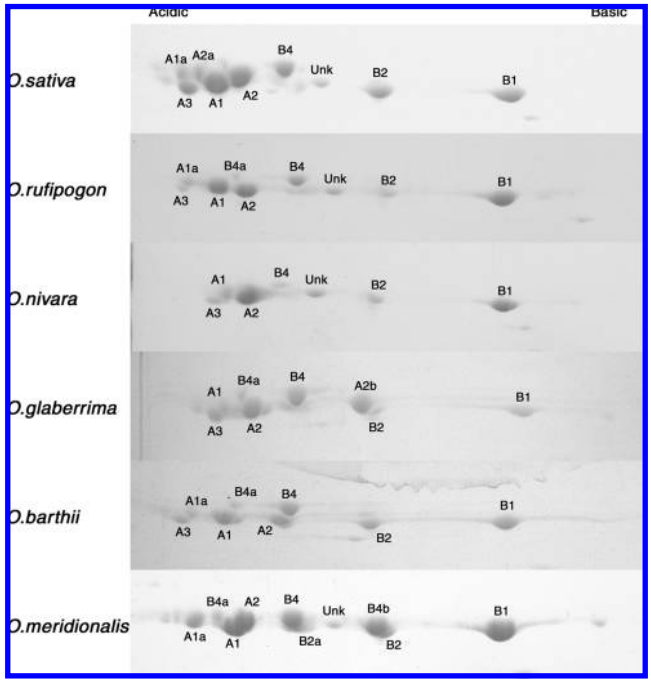

Figure 2. Comparison of accumulation level of glutelin subunits in AA genome wild rice species. Glutelin α polypeptides extracted with 1% lactic acid and 1 mM EDTA from AA genome species of wild rice were separated by 2D-PAGE and detected with CBB. The spots labeled with capital letter and number refer to polypeptides corresponding to glutelin subunits determined by step-by-step detection method in the same way as in **Figure 1**. The lower case letters (a and b) denote other polypeptides related to the corresponding glutelin subunits. The spots marked "Unk" show unknown spots that have not been identified by the four antibodies tested in this study. The names of species are displayed on the left side of the figure. The corresponding subunits were compared with that of *O. sativa* cv. Koshihikari.

nivara and three spots (B4a, B4, B4b) in *O. meridionalis*, whereas in the other species two spots (B4a, B4) were detected. The GluB4 spot (B4) markedly increased in *O. glaberrima* and *O. barthii*; the amount of GluB4 in these species was almost double that in *O. sativa*. It is worth mentioning here that a little increase in GluB4 level was observed in *O. meridionalis*, but the amount of another unique spot (B4b) detected in *O. meridionalis* was higher than the GluB4 spot (B4). In contrast, GluB4 was decreased tremendously in *O. nivara*. In comparison to *O. sativa*, the spot of GluA3 subunit was greatly decreased in most species except *O. glaberrima*, in which the relative amount was unchanged. On the other hand, GluA3 was not detected in *O. meridionalis*. Glutelin subunit GluB1 showed one spot (B1) in all species. Similarly, GluB2 showed one spot (B2) in all species except *O. meridionalis*, in which one minor spot (B2a) was also observed. The amount of GluB1 significantly increased in most species except *O. glaberrima*, and the content of GluB2 greatly decreased in most species except *O. barthii*. *O. rufipogon* accumulated the highest amount of GluB1, in which the quantity of GluB1 was almost double compared to that in *O. sativa*. Some unknown spots (Unk) were also detected, which were not recognized by our antibodies. For example, in *O. rufipogon*, *O. nivara*, and *O. meridionalis*, one unknown spot (Unk) was dominantly detected. Furthermore, the accumulation level of the unknown spot (Unk) was higher in *O. rufipogon* and *O. nivara* compared to that in *O. sativa*.

Variation in Glutelin α Polypeptides in Non-AA Genome Species. When glutelin fractions of wild rice non-AA genome species were resolved by 2D-PAGE and examined as earlier described (detection data not shown), we observed great variation in the distribution pattern and in the number and accumulation level of spots (**Figure 3**). The distribution pattern of spots is so diverse in non-AA genome species that we are unable to align respective spots for comparison purpose. However, glutelin subunits of most spots were successfully identified. The number and apparent amount of the spot A1 corresponding to GluA1 subunit varied in most species. GluB4 showed two major spots (B4) in *O. latifolia* and *O. grandiglumis* and one major spot in *O. punctata* (2×) and *O. alta*. On the other hand, the GluB4 subunit was absent in *O. brachyantha* and *O. longiglumis*. Glutelin subunit GluB1 exhibited varying numbers of spots with varying intensities and positions in most species. The GluA3 subunit was either absent or not detected properly (confused with A1) in some species. Most of the species also showed the presence of unknown spots (Unk). Two major unknown spots of high intensity were detected in *O. grandiglumis*, *O. brachyantha*, and *O. longiglumis*, whereas, on the other hand, three unknown spots of high intensity were observed in *O. punctata* (4×).

DISCUSSION

Glutelin of Asian cultivated rice is encoded by a small multigene family consisting of subfamilies GluA and GluB. The GluA subfamily is composed of three subunits, GluA1, GluA2 and GluA3, which show 73−96% sequence identity to each other. Major subunits of the GluB subfamily are GluB1, GluB2, and GluB4, the amino acid sequences of which show 80−88% identity to each other, whereas the sequence between GluA and GluB shares 60−65% identity (*9*). Due to this close sequence identity among glutelin subunits it seems rather difficult to discriminate and identify individual subunits by conventional protein-based biochemical means (*12*). Moreover, previous research demonstrated significant diversity in the size and number of glutelin α polypeptides among wild rice species (*15*). Therefore, we analyzed the glutelin fraction of 11 wild species representing various genome types and two cultivated rices by high-resolution 2D-PAGE coupled with higher temperature SDS-PAGE and step-by-step immunodetection. The combined tools proved to be powerful for subunit identification and determining relative amounts of subunits.

Our step-by-step detection approach resulted in the successful subunit identification of multigenic glutelin α polypeptides and proved to be very useful, convenient, and time saving, especially when used for subunit identification of polypeptides separated by 2D-PAGE. 2D-PAGE is labor intensive and time-consuming and also has problems in obtaining reproducible spot distribution. As in step-by-step detection approach, the same membrane is used for subsequent immunoreactions, so one good electroblotted membrane could be enough for subunit identification of polypeptides. However, the current step-by-step detection method needs further improvement because polypeptides having a low amount and/or weak affinity against an antibody are seldom detected due to a decreasing signal to background noise ratio. The optimization for the order of antibody reaction might be useful for overcoming the above-mentioned problem. For example, in this study the GluA3 subunit was sometimes not detected properly because anti-A3 (no. 3) antibody was used at last. Changing the order of antibody application in the detection scheme improved the detection of the GluA3 subunit (data not shown).

Significant differences were found in accumulation levels of glutelin α polypeptides in AA genome species. Similar variation in wild rice species was revealed in our earlier paper

Table 3. Relative Accumulation Level of Glutelin α Polypeptides in AA Genome Species[a]

	polypeptide												
species	A1a	A3 (3.0)	A2a	A1 (2.5)	B4a	A2 (2.5)	B4 (3.2)	B2a	Unk	A2b	B4b	B2 (3.6)	B1 (3.4)	B/A[b]
O. sativa	3.8	9.6	4.9	24.0		20.4	10.7		2.1			10.2	14.3	0.6 a
O. rufipogon	3.4	1.8		23.6	1.4	22.5	9.6		4.9			3.6	29.1	0.9 b
O. nivara		5.0		9.0		45.1	2.5		7.7			6.0	24.7	0.6 a
O. glaberrima		9.0		5.3	4.1	21.9	20.7			23.5		3.9	11.5	0.7 c
O. barthii	2.2	4.2		19.8	1.4	17.0	18.0					13.1	24.1	1.3 d
O. meridionalis	8.0			9.1	2.4	17.3	13.4	4.6	2.2		15.3	4.4	23.4	1.8 e

[a] Relative accumulation level of glutelin α polypeptides within each AA genome species was determined by scanning and processing **Figure 2** with ImageJ computer software. Values are the mean of triplicate determinations, which are presented as percentages. Parenthetical values shown with major polypeptide names represent the lysine content (mol %) of the corresponding subunit in *O. sativa* the typical *japonica* subspecies. [b] B/A represents the ratio of GluB to GluA polypeptides content.Statistical analysis was done according to Tukey's test at $P < 0.05$. Values in the column followed by different letters differ significantly at $P < 0.05$.

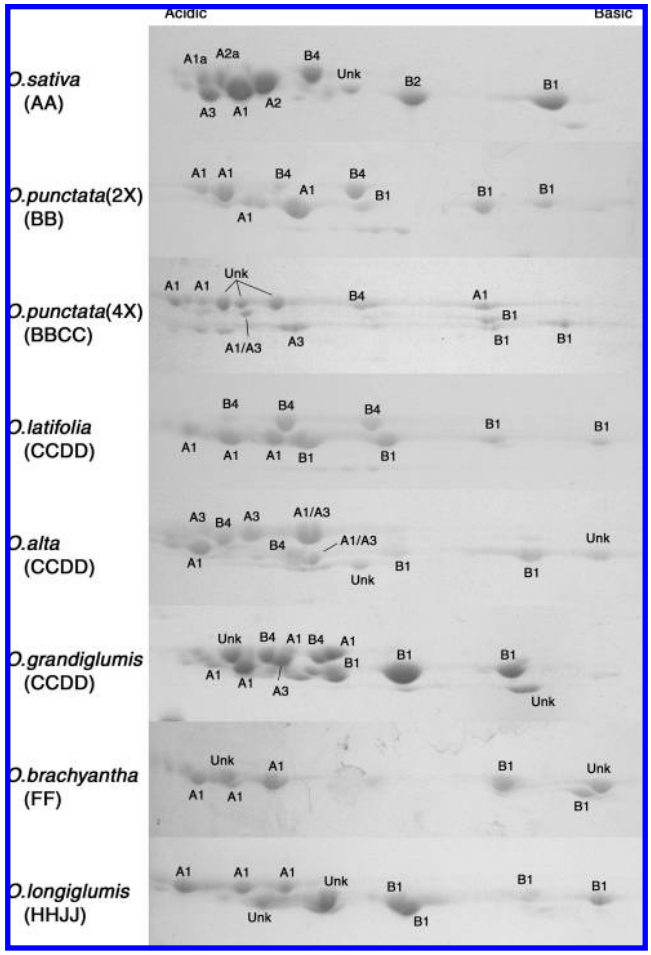

Figure 3. Glutelin subunit variation in non-AA genome species of wild rice. Glutelin α polypeptides extracted with 1% lactic acid and 1 mM EDTA from non-AA genome species of wild rice were separated by 2D-PAGE and detected with CBB staining. The spots labeled with capital letter and number denote polypeptides corresponding to the glutelin subunits determined by step-by-step detection method in the same way with **Figure 1**. The spots marked "Unk" show unknown spots that have not been identified by the four antibodies tested in this study. The label "A1/A3" denotes ambiguity that is A1 or A3. The names of species are presented on the left. The genome type of the rice species is shown under the species name. The corresponding subunits were compared with that of *O. sativa* cv. Koshihikari.

(*15*) when the diversity of glutelin α polypeptides was assessed by 1D-PAGE and the same subunit-specific antibodies. However, the resolution of glutelin α polypeptides achieved by 1D-PAGE is not enough for precise assessment of accumulation level, and there is ambiguity whether differences in immunoreacted band intensity are due to differences in accumulation level or differences in affinity against antibodies. Therefore, we used 2D-PAGE with higher temperature SDS-PAGE in the second direction for better resolution. Our combined approach enabled us to compare the accurate accumulation level of glutelin α polypeptides. Marked variation was observed in accumulation levels of A-type and B-type glutelin polypeptides in AA genome species. It is readily evident from our results that wild species *O. barthii* and *O. meridionalis* accumulate B-type glutelin (especially GluB1 and GluB4) more than *O. sativa*, resulting in high GluB/GluA ratios of 1.3 and 1.8, respectively (**Table 3**). In addition, *O. glaberrima* accumulated more GluB4 than *O. sativa*, even though the two species have low GluB/GluA ratios of 0.7 and 0.6, respectively. These results are consistent with our previous findings (*15*). Furthermore, the current study could differentiate GluB1 and GluB2 and enabled us to know the contribution of individual subunits to the increase in the overall accumulation level of B-type glutelin. For example, it is evident that *O. rufipogon* and *O. nivara* have higher levels of GluB1 than *O. sativa*, although the two wild species have lower amounts of GluB2 and GluB4. The lysine content of B-type glutelin varies among subunits, with GluB2 having the highest lysine content (3.6 mol %) in *O. sativa* (**Table 3**). Therefore, one might assume that the reduction of GluB2 would reduce the overall lysine content in some AA genome wild species. It is supposed that the lysine content of each subunit is conserved within AA genome species including *O. sativa*; however, the overall lysine content in the glutelin fraction of the two wild species is estimated not to fall significantly below that of *O. sativa* by multiplying the relative accumulation value of each polypeptide with the lysine content of the corresponding subunit (data not shown). Accordingly, the seed protein of all AA genome wild species tested in this study seems not less nutritious than that of *O. sativa*. Thus, *O. rufipogon* and *O. nivara* as well as *O. glaberrima*, *O. barthii*, and *O. meridionalis*, the significance of the last three having been pointed out previously, may play a leading role in nutritional quality improvement of cultivated rice.

The accumulation level, distribution pattern, and number of spots greatly varied in non-AA genome species, which reflects the divergence of glutelin subunits in those species. Similarly, major variation in subunit composition of wild soybean (*Glycine soja*) and cultivated soybean (*Glycine max*) glycinin (which belongs to the same 11S globulin family as rice glutelin) has been reported and suggested to be useful in seed quality improvement (*23, 24*). Abe et al. (*25*), Jahan et al. (*26*), and Bhowmik et al. (*27*) observed variation in glutelin α polypeptides among Asian cultivated rice seeds

Table 4. Relative Accumulation of B-Type and Unknown Subunits in Non-AA Genome Species[a]

	B/A[b]	Unk/(A+B)[c]
O. punctata (2×)	0.6 a	0 a
O. punctata (4×)	0.8 b	0.6 b
O. latifolia	1.7 c	0 a
O. alta	0.5 a	0.1 c
O. grandiglumis	2.4 d	0.2 d
O. brachyantha	0.6 a	0.4 e
O. longiglumis	1.3 e	0.6 b

[a] Relative accumulation level of glutelin α polypeptides in non-AA genome species was determined in the same way as **Table 3** but was based on **Figure 3**. Statistical analysis was done according to Tukey's test at $P < 0.05$. Values in a column followed by different letters differ significantly at $P < 0.05$. [b] B/A represents the ratio of GluB to GluA polypeptides content. [c] Unk/(A+B) represents the ratio of unknown to GluA and GluB polypeptides content.

by 2D-PAGE. However, information about individual subunit accumulation levels was limited. The current study revealed that the accumulation level of B-type glutelin in *O. punctata* (4×), *O. latifolia*, *O. grandiglumis*, and *O. longiglumis* increased as confirmed by the higher GluB/GluA ratios (0.8, 1.7, 2.4, and 1.3, respectively) compared that of *O. sativa* (**Table 4**). These results suggest the usefulness of these species for nutritional quality improvement of cultivated rice. Likely, the potential importance of unknown spots in some species, particularly in *O. punctata* (4×), *O. brachyantha*, and *O. longiglumis*, which have major unknown spots and high unknown to known (GluA and GluB) polypeptide ratios (0.6, 0.4, and 0.6, respectively) (**Table 4**), cannot be ignored. Accordingly, these unknown spots need to be identified and characterized, which might have significant implications for the nutritional quality improvement of rice seed.

The almost similar distribution of 2D-PAGE spots among AA genome species may be explained by the same chromosome constitution of these species. In contrast, the three CCDD genome species have more divergent spot distribution patterns, which implies that the variation of glutelin genes in these species is species-specific rather than genome type-specific. These results support the findings based on genomic DNA hybridization and RFLP analysis that the three CCDD genome species are independent and separate species (*28*) and contradict the hypothesis based on molecular, cytological, and morphological data that these are populations of a single complex species (*29, 30*). It is likely, although *O. punctata* (4×) possessing a BBCC chromosome commonly shares BB and CC chromosomes with BB and CCDD genome species, respectively, but it is difficult to find common and comparable spots among these species. Thus, for further nutritional quality improvement research the three CCDD genome species should be assessed and considered independently. The same is true for BB and BBCC species in this study.

ABBREVIATIONS USED

2D-PAGE, two-dimensional electrophoresis; NEPHGE, nonequilibrium pH gradient gel electrophoresis; GluA, A type glutelin; GluB, B type glutelin; 1D-PAGE, one-dimensional gel electrophoresis; CBB, Coomassie Brilliant Blue R-250; p*I*, isoelectric point; MW, molecular weight.

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