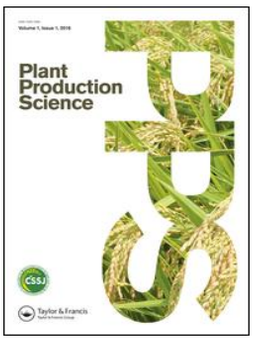

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Glutelin is partially degraded in globulin-less mutants of rice (*Oryza sativa* L.)

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ABSTRACT

Multigenic glutelins and monogenic globulin are major storage proteins accumulating in vacuole-derived protein body (PB-II) of rice (*Oryza sativa* L.) seeds. Because their interplay in PB-II formation was scarcely known, the effect of globulin-less mutation on glutelin accumulation was investigated. In globulin-less mutants, no phenotypic defect was found in seed and plant growth, while PB-II was deformed and apparent glutelin composition was changed, producing new glutelin α polypeptides X1–X5. 2D-PAGE of different combinations of globulin-less and glutelin subunit mutations suggested that the X1/X2, X3, and X4/X5 were derived from glutelin GluB1/GluB2/GluB4, GluA3, and GluA1/GluA2 subunits, respectively. Western blot with glutelin GluB4 subunit-specific and its variable region discriminable antibodies indicated at least in part the new spots X1/X2 are partially degraded products of GluB4 α polypeptides by the removal of 2–39 residues from C-terminus. Time course experiments with maturing seeds indicated the partial degradation of GluB4 occurred earlier (from 7 days after flowering) and higher than that of GluA1/GluA2. Considering the above results together with the fact that globulin accumulates at the periphery of PB-II and its absence produces deformed PB-II, globulin protects glutelins from proteinase digestion and thereby facilitates stable glutelin accumulation.

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Globulin; glutelin; partial degradation; protein body; rice (*Oryza sativa*); seed storage protein

1. Introduction

Rice seeds contain 7–10% protein and are an important source of dietary protein in developing countries in Asia (Khush, 1997). Because rice seed proteins, most of which are storage proteins, are deficit in lysine, an amino acid essential for humans, it is important to improve nutritional quality as well as food processing and health-promoting properties of the rice storage proteins based on the fundamental knowledge regarding structure and function of the proteins and protein bodies.

The predominant proteins (~70%) in rice seeds are glutelins, followed by prolamins (~20%), and globulins (~5%). Contrary to other staple gramineae grains such as maize and wheat, rice accumulates both prolamins and glutelins as major storage proteins (Shewry & Halford, 2002). In rice seeds, prolamins are deposited into ER-derived protein bodies (PB-I) while glutelins and globulin are deposited into vacuole-derived protein bodies (PB-II) (Tanaka et al., 1980; Yamagata & Tanaka, 1986). Okita and Choi (2002) demonstrated that mRNA localization mechanism is responsible for the differential synthesis of prolamins and glutelins on specific ER subdomains and their sorting to different intracellular storage compartments. Prolamin mRNAs are enriched to PB-ER while glutelin mRNAs targeted to the Cis-ER (Choi et al., 2000).

Contrary to the mechanism underlying the sorting of prolamins and glutelins during rice seed development, very little studies have investigated the interrelationship or differences in the synthesis and accumulation of glutelins and globulins. Unlike the multiple gene copies for prolamin and glutelin, globulin is encoded by a single gene (Iida et al., 1998). Transmission electron microscope (TEM) and confocal fluorescence microscopy analyses have demonstrated that globulin accumulates on the exterior surface of PB-II (Krishnan et al., 1992; Kumamaru et al., 2010), where glutelin form a crystalloid with a different angled lattice structure while globulin is present in matrix (Kawagoe et al., 2005). Globulin transport depends on its sorting signals (Crofts et al., 2005; Kawagoe et al., 2005). As is the case with glutelin, globulin travels via the Golgi (Krishnan et al., 1986), while Washida et al. (2012) demonstrated correct globulin RNA targeting to PB-ER is required for efficient transport and arrangement of globulin into PB-II. The function of globulin is unknown since no apparently different phenotype was found in globulin-less mutants so far. However, Ashida et al. (2006) have recently described a gamma-ray-induced globulin-less mutant accumulates

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higher amounts of free amino acids. Analysis of the ultrastructure by TEM showed that the globulin-less mutant had deformed PB-II (Ashida et al., 2011), while a globulin RNAi knock-down transformants had cracked PB-IIs (Kawakatsu et al., 2010). Iida et al. (1993) and Ashida et al. (2006) found a new glutelin band at ~32 kDa in the globulin-less mutant. No new polypeptide was evident, however, in the globulin RNAi knock-down transformants (Kawakatsu et al., 2010).

Glutelin has six major and some minor subunits in the temperate *japonica* subspecies (Katsube-Tanaka et al., 2004). These six subunits have been classified into two subfamilies, GluA and GluB, according to the degree of their relatedness in their nucleotide sequences (Takaiwa & Oono, 1991). Between the two subfamilies, GluB is more nutritious than GluA because it contains more lysine. More than half of the rice glutelin subunits (i.e. GluA1/GluA2/GluA3 and GluB4 subunits out of six major subunits) polymerizes by specific disulfide bonding (Katsube-Tanaka et al., 2004) and hydrophobic interactions to form large macromolecular complexes (Utsumi, 1992), while the other two major subunits GluB1 and GluB2 accumulate as a monomer subunit. However, the difference in the detailed higher order structure and the function between the two glutelin subfamilies is not well known.

In this paper, we analyzed the gamma-ray-induced globulin-less mutant, combining with some glutelin mutations to obtain fundamental knowledge on the interrelationship and differentiation between globulin and glutelins. This is the first report demonstrating a globulin-less mutation results in glutelin partial degradation with a different degree and process between the two glutelin subfamilies GluA and GluB. The partial degradation seemed to be related to the deformed structure of PB-II and would change the higher order structure of glutelin. The results are fundamentally important because deepened knowledge on the different role of protein body constituents is a prerequisite for designed improvement in the seed protein quality.

2. Materials and methods

2.1. Plant materials

Rice (subspecies *japonica*) of the wild type cultivar Koshihikari and globulin and glutelin mutants were grown using conventional cultivation processes and matured to collect seeds in paddy fields and plastic pots. In the pot experiments, tillers (branches) emerged from a main stem were removed to monitor a developmental stage of the main stem panicle. Developing seeds at 7, 14, 21, 28, and 35 days after flowering were collected and immediately stored in a freezer.

Mutant rice varieties with different combination of globulin and glutelin mutations (K×433, 05TK470, 05TK464, LGC-1, and LGC-Jun) were from a collection of the National Agricultural Research Center for Western Region, Japan (Table 1). The mutant K×433 has a globulin-less recessive mutation (*glb1*) (Iida et al., 1998). The mutant 05TK470 has GluA1-less (*glu3*) and GluA2-less (*glu2*) recessive mutations (Iida et al., 1997). The mutant 05TK464 has *glb1*, *glu3*, and *glu2* mutations. The mutant LGC-1 has a dominant mutation (*Lgc1*) (Iida et al., 1993), which induces RNAi-suppression of GluB subunits including GluB1/GluB2/GluB4. The mutant LGC-Jun has *Lgc1* and *glb1*. *Lgc1* and *glb1* were isolated from ethyleneimine- and gamma-ray-treated populations, respectively (Nishimura et al., 2005) and the other glutelin mutations were identified from ethylmethane sulfonate- or gamma-ray-treated populations (Iida et al., 1997). Pedigree of the above mutants except 05TK470 and 05TK464, which were developed on the way to produce QA28, a quadruple mutant (*Lgc1*, *glu2*, *glu3*, and *glb1*), is shown in the report by Ashida et al. (2006).

2.2. Extraction and preparation of glutelin

The total proteins were extracted with the sample buffer containing 50 mM Tris, pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (w/v) glycerol, and with 5% (v/v) 2-mercaptoethanol (ME) for normal SDS–PAGE analysis or without ME for non-reducing SDS–PAGE. The rice glutelins were extracted with 1% lactic acid containing 1 mM EDTA following the removal of albumins, globulins, and prolamins with a 35 mM KPi (pH 7.6) buffer including 0.4 M NaCl, and 60% n-propanol. Glutelins in the extraction buffer were dissolved in lysis buffer containing 9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, and 5% Bio-Lyte 3–10 (Bio-Rad) for 2D-PAGE analysis.

Table 1. Subunit composition of glutelin and globulin in plant materials used in this study.

	Koshihikari	K×433 (*glb1*)	05TK470 (*glu2*, *glu3*)	05TK464 (*glb1*, *glu2*, *glu3*)	LGC-1 (*Lgc1*)	LGC-Jun (*glb1*, *Lgc1*)
GluB4	+	+	+	+	–	–
GluA2	+	+	–	–	+	+
GluA1	+	+	–	–	+	+
GluA3	+	+	+	+	+	+
GluB2	+	+	+	+	–	–
GluB1	+	+	+	+	–	–
Globulin	+	–	+	–	+	–

Note. The symbols + and – denote presence and absence of glutelin and globulin polypeptides, respectively.

2.3. SDS–PAGE, 2D-PAGE, and western blot analyses

SDS–PAGE was performed according to the procedure by Laemmli (1970) with minor modifications, using 14% (w/v) acrylamide/N,N′-methylene-bisacrylamide gel at a constant voltage of 200 V. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) was performed according to the method by Khan et al. (2008b). Electrophoresis in the first dimension (NEPHGE) was performed at 200 V for 15 min, 300 V for 15 min, 400 V for 15 min, 500 V for 15 min, and 750 V for 5 h. Electrophoresis in second dimension was carried out by higher temperature SDS–PAGE method according to Khan et al. (2008a) at constant voltage of 200 V for 80 min at 45 °C. The immunoblotting was performed according to the method mentioned in our previous report (Khan et al., 2008a). Briefly, the proteins resolved by SDS–PAGE and 2D-PAGE were electrophoretically transferred to nitrocellulose membrane. The membrane was incubated in blocking solution (0.15 M NaCl, 5% (w/v) skimmed milk, 0.05% (v/v) Tween-20 and 0.04 M Sodium-phosphate buffer pH 7.4) with gentle shaking for 1 h at room temperature. Then, the membrane was incubated with primary antibody of anti-glutelin individually or step-by-step (Khan et al., 2008b) at room temperature for 1 h. Following this, the membrane was thoroughly washed three times for 20 min with TBST (0.02 M Tris pH 7.5, 0.9% (w/v) NaCl, 0.05% (v/v) Tween-20) and then incubated with secondary goat anti-mouse or anti-rabbit IgG alkaline phosphatase conjugate (Promega, USA) for 1 h at room temperature. Subsequently, membrane was washed three times for 20 min with TBST. Finally, color development was accomplished with NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl-phosphate). The detection was stopped in deionized water and the membrane was air dried prior to scanning.

2.4. Glutelin subunit-specific antibodies

Glutelin subunit-specific antibodies (polyclonal) reacting against specific peptide sequences of glutelin α polypeptides were used. Five sites (I, II, III, IV-a, and IV-b) from four variable regions within 11S globulin family (I, II, III, and IV) (Adachi et al., 2003; Lawrence et al., 1994) in α polypeptides were compared to produce antibodies. The epitopes are as follows: A1(No.2) in the site II of GluA1; B1(No.4b) in the site IV-b of GluB1; B4(No.3) in the site III of GluB4; B4(No.4a) in the site IV-a of GluB4; B4(No.4b) in the site IV-b of GluB4 (Khan et al., 2008a). Sites IV-a and IV-b are located at the upstream and the downstream of the C-terminus variable region of α polypeptides (also called hyper-variable region), respectively. Anti-A1(No.2) and anti-B1(No.4b) react exclusively with GluA1/GluA2 and GluB1/GluB2, respectively. Anti-B4(No.3), anti-B4(No.4a), and anti-B4(No.4b) react exclusively with GluB4, though the anti-B4(No.4a) gives extra weak signal for a possible partial degradation form of GluB4 as well.

2.5. Microscopic and confocal laser microscopic examinations of rice endosperm

Thin sections for microscopic examination were prepared by cutting the frozen immature seeds of rice mutants (LGC-1 and LGC-Jun) into half and fixed in fixation solution (2% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.3) for about 3 h. Then, the samples were sequentially dehydrated with graded ethanol series (10, 30, 50, 70 and 90%) for 30 min twice. After dehydration, the samples were embedded in L.R. White resin (Sigma–Aldrich, Inc., UK) for about 16 h. For polymerization of samples, the samples were transferred to polymerizing capsule and L.R. White resin was added to the samples. Then, the samples were incubated at 55 °C for about 2–3 days. For obtaining thin sections, the embedded samples were cut with ultramicrotome and glass knives. Then, the thin sections were transferred to glass slide and dried. The thin sections were then stained with 0.25% CBB in 50% (v/v) methanol and 10% (v/v) acetic acid and examined under microscope CX31 (Olympus, Japan).

Sections for confocal laser microscope were prepared from unfixed frozen samples at 7, 14, and 35 DAF using retoratome REM-710 and electrofreeze MC-802A (Yamatokoki, Japan) with Tissue-Tek O.C.T.Compound (Sakura Finetek, Japan). The sections on a slide glass were treated with acetone, PBS, and blocking buffer followed by primary and secondary antibodies. The primary antibodies were 1,000-fold diluted anti-glutelin A1(No.2) antibody of mouse (Khan et al., 2008a) and 2000-fold diluted anti-alpha globulin antibody (prepared against peptide sequence LTGRERFQPMFRRPGALG in rabbit). The secondary antibodies were 500-fold diluted anti-mouse IgG conjugated FITC (green) and 2000-fold diluted anti-rabbit IgG conjugated Cy3 (red). After washing with PBS, the sections were examined with confocal laser microscope FV1200 BX61 (Olympus, Japan).

3. Results

3.1. Plant growth and accumulation of seed storage substances

No apparent phenotypic difference in seed germination, plant growth, and starch accumulation was found between the globulin-accumulating (LGC-1) and non-accumulating (LGC-Jun) mutants (Figure 1(A) and 1(B)). However, a new 32 kDa band, which has been suggested to be glutelin by Iida et al. (1998), was found in the globulin-less mutant

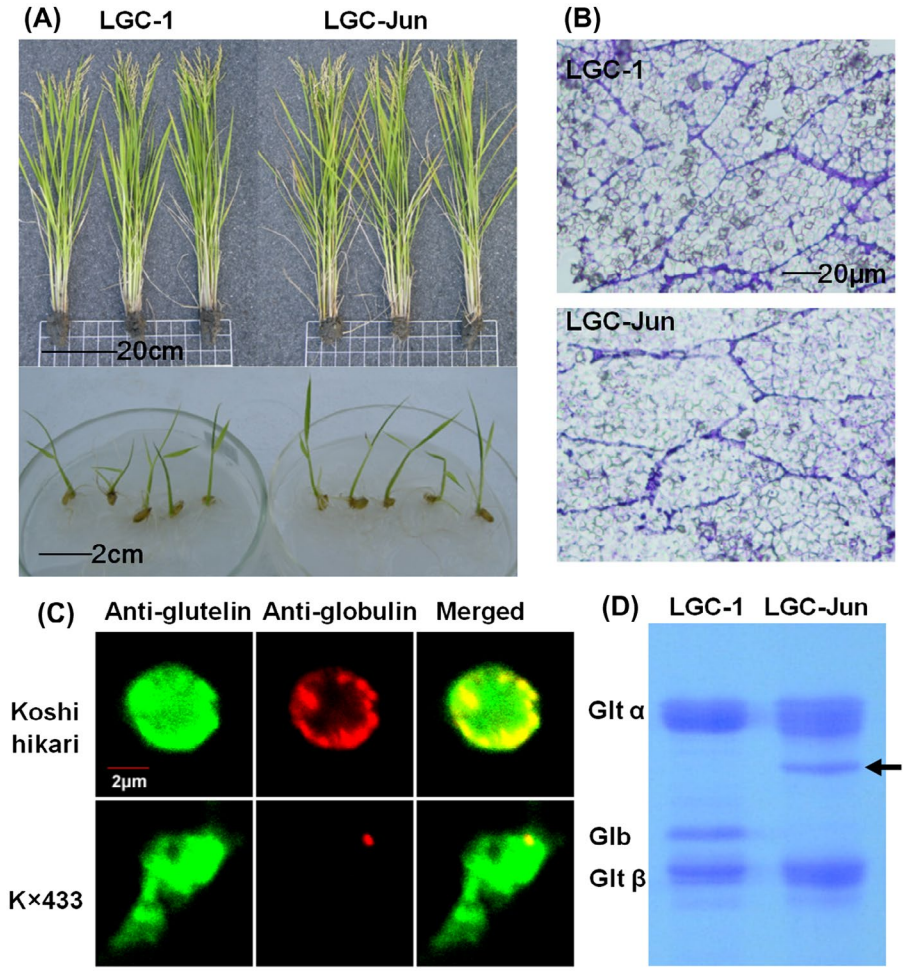

Figure 1. Effects of globulin-less mutation on plant growth and seed storage substances. Plant growth and germination (A), starch accumulation (B) were compared between glutelin GluB knock-down mutants accumulating globulin (LGC-1) or not accumulating globulin (LGC-Jun). Globulin and glutelin localization was examined by immunofluorescent staining of protein body II in the wild type cultivar Koshihikari and the globulin-less mutant K×433 (C). Compositional change of globulin (Glb) and glutelin α (Glt α) and β (Glt β) polypeptides was detected by SDS–PAGE with CBB staining between LGC-1 and LGC-Jun (D). Horizontal arrow indicates the 32 kDa band demonstrated by Iida et al. (1998).

LGC-Jun (Figure 1(D)). Because globulin generally accumulates on the periphery of glutelin-containing protein body II (PB-II) (Figure 1(C)) and deformed PB-II was observed in the globulin-less mutant K×433 (Figure 1(C)), we speculated that globulin is involved in glutelin accumulation.

3.2. Overview of altered glutelin α and β polypeptide structure in globulin-less mutants

Diagonal (non-reducing and reducing) 2D-PAGE was employed to assess the effect of the globulin-less mutation on glutelin structure. The method can distinguish proteins containing an inter-polypeptide disulfide bond like rice glutelins from proteins containing an intra-polypeptide disulfide bond like rice globulin. When total seed proteins were resolved by this 2D-PAGE, glutelins and globulin are almost exclusively displaced to the left and the right side of the central diagonal line, respectively, while proteins lacking a disulfide bond are detected on the diagonal line. In any of the four cultivars/lines examined here, glutelins were horizontally divided into the monomer [(α + β)] and the polymerized [(α + β)n] forms (1st direction) in Figure 2 and then the glutelin subunits were vertically separated into their α and β polypeptides (2nd direction). When the spot distribution patterns between Koshihikari and K×433 and between 05TK470 and 05TK464 were compared, new spots were clearly detected just underneath the α polypeptides of monomer glutelin and faintly detected at much lower position (marked with white arrow heads in Figure 2). Another new spot with a molecular size similar to β polypeptides was also evident between β polypeptides of the monomer glutelin and the diagonal line (marked with white arrow in Figure 2). Because spots migrating to the left side of the central diagonal line are likely to be

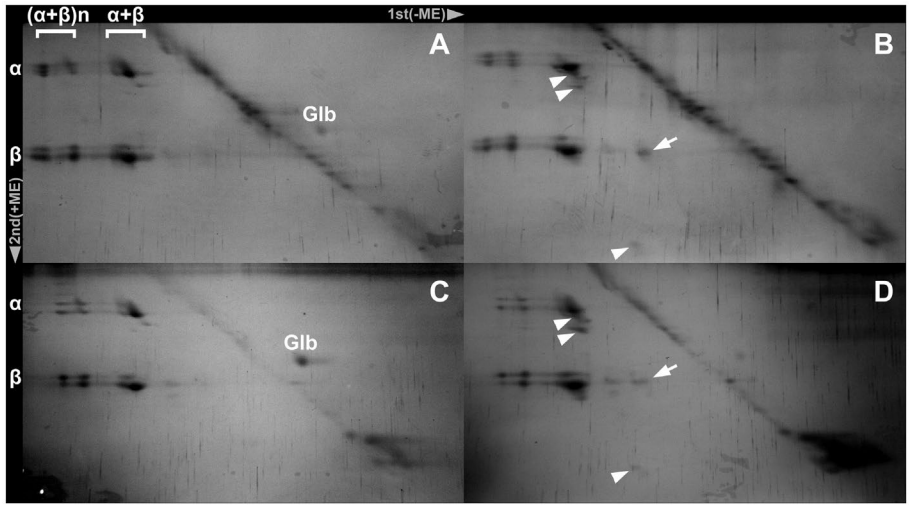

Figure 2. Overall structural change of glutelin in globulin-less mutants assessed by diagonal 2D-PAGE. Total proteins extracted under a non-reducing condition from the wild type Koshihikari (A) and globulin/glutelin mutants K×433 (B), 05TK470 (C), and 05TK464 (D), were electrophoresed under a non-reducing condition [1st (−ME)] followed by a reducing condition [2nd (+ME)]. Proteins containing an inter-polypeptide disulfide bond, i.e. glutelins, were detected at the left side of the diagonal line. Meanwhile, globulin (Glb), which contains an intra-polypeptide disulfide bond was detected at the right side of the diagonal line because of retarded migration under the reducing condition. Polypeptides without a disulfide bond were migrated on the diagonal line. Glutelins were separated into non-polymerized monomer subunits [(α + β)] and polymerized subunits [(α + β)n] in 1st direction, and then separated into each α and β polypeptides in 2nd direction. Possible new α and β polypeptide spots found in globulin-less mutants were indicated by arrow heads and arrows, respectively.

glutelin, the above results suggest that a partial degradation occurred to the α polypeptides, which were covalently linked to intact β polypeptides in the globulin-less mutants. Note that the new α polypeptide spots just under the intact α polypeptides were possibly a monomer glutelin and the ratio of the monomer glutelin to the polymerized glutelin seemed to be increased in the globulin-less mutants (Figure 2).

3.3. Size and charge of the new glutelin polypeptides

Because the new 32 kDa band was extracted in glutelin fraction (Iida et al., 1998), the glutelin fraction of the globulin-less and/or glutelin mutants was analyzed by non-equilibrium pH gradient electrophoresis (NEPHGE) followed by SDS–PAGE. The results demonstrated that several new spots were observed at significant levels (X1 and X2) and at lower levels (X3–X5) in the globulin-less mutant K×433 (Figure 3(B)). Although the NEPHGE does not show the exact pI of the polypeptide, the pIs of X3, X1/X4, and X5 seemed to correspond to the pIs of α polypeptides of GluA1 (pI 6.6), GluB2 (pI 7.9), and GluB1 (pI 8.4), respectively. The pI of X2 was more basic. The molecular size of X1 was slightly smaller than that of the α polypeptide of GluB2. The sizes of X3, X4, and X5 were similar to each other, while that of X2 was slightly smaller. The spots X2–X5 corresponded to the new 32 kDa band.

The new spots were also detected in other globulin-less mutants. However, only X1, X2, and X3 were detected in 05TK464 (Figure 3(D)) and X3, X4, and X5 were observed in LGC-Jun (Figure 3(F)). Because 05TK464 and LGC-Jun lack GluA1/GluA2 and GluB1/GluB2/GluB4, respectively, we hypothesized that X4/X5 are related to GluA1/GluA2 and X1/X2 are related to GluB1/GluB2/GluB4. The new spot X3 was detected in all the globulin-less mutants examined here, suggesting that X3 is related to GluA3. If this interpretation is correct, this size/charge alteration in the α polypeptide of GluA3 is the first clear observation as a mutation incompletely affecting GluA3 accumulation. Note that the presence of minor subunits other than GluA1/GluA2/GluA3 became relatively more evident in LGC-1 and LGC-Jun because the accumulation of GluB subunits was severely suppressed by RNAi (Kusaba et al., 2003).

3.4. Partial degradation of glutelin α polypeptides

Like other 11S globulin family proteins, rice glutelin has a hyper-variable region at the C-terminus of the α polypeptides (Argos et al., 1985). The hyper-variable region is not visible in X-ray crystallographic analysis (Adachi et al., 2003) and thought to be located at the molecular surface and, hence, accessible to proteinase. Therefore, we hypothesized that partial degradation occurred at the C-terminus of α polypeptides, resulting in the formation of

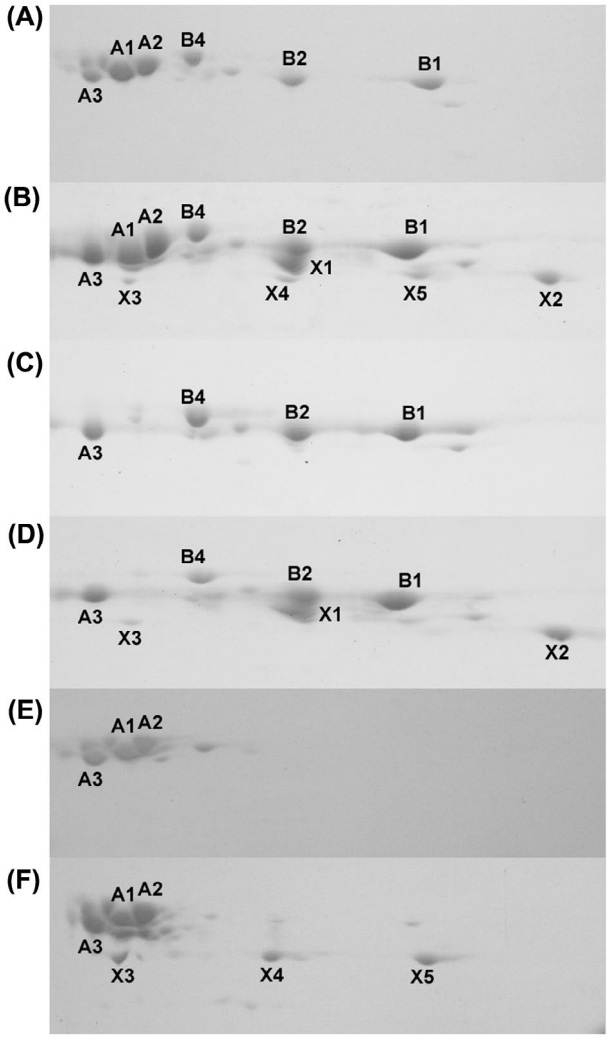

Figure 3. Two-dimensional PAGE of glutelin α polypeptides in globulin/glutelin mutants. Glutelin α polypeptides from the wild type Koshihikari (A) and globulin/glutelin mutants K×433 (B), 05TK470 (C), 05TK464 (D), LGC-1 (E), and LGC-Jun (F) were analyzed by non-equilibrium pH gradient electrophoresis (NEPHGE) followed by SDS–PAGE. The α polypeptides of major six glutelin subunits (GluA1, GluA2, GluA3, GluB1, GluB2, and GluB4) were indicated by A1, A2, A3, B1, B2, and B4, respectively. Newly found glutelin α polypeptide spots in globulin-less mutants, which correspond to spots with the upper two arrowheads in Figure 2B, 2D, were shown by X1, X2, X3, X4, and X5. Acidic, left; basic, right.

the new spots in the globulin-less mutants. In other words, the spots X3 and X4/X5 were derived from the partial degradation of GluA3 and GluA1/GluA2, respectively. And the spots X1/X2 were derived from the partial degradation of GluB1/GluB2/GluB4. In order to confirm the above-mentioned hypothesis, we employed step-by-step immunodetection (Khan et al., 2008b) using GluB4 subunit-specific and its variable region discriminable antibodies for the NEPHGE/SDS–PAGE of 05TK470 and 05TK464 (Figure 4). The analysis showed that anti-B4(No.4b) could react with only the spot B4 and anti-B4(No.4a) could react with the spot X1 and not with the spot X2, while anti-B4(No.3) could react with the spot X2. The above observations suggest that the spots X1 and X2 were at least in part derived from GluB4 and that the spot X1 was shortened and the spot X2 was more shortened at the C-terminus of α polypeptides. The anti-B4(No.4a) and anti-B4(No.4b) were prepared against the epitopes of 32–39 residues and 2–11 residues from the C-terminus of GluB4 α polypeptide, respectively (Khan et al., 2008a), implying that the epitope regions were degraded in the spot X2 and X1, respectively. Because the MW of X3/X4/X5 was similar to that of X2, we speculated that partial degradation occurred at the equivalent region to X2 in X3/X4/X5. Note that it is not excluded that the spots X1 and X2 are also derived from GluB1/GluB2.

3.5. Time course of the partial degradation during seed maturation

It is important to know the timing when the partial degradation occurs in developing seeds. Therefore, we examined the time course of accumulation of the intact and partially degraded polypeptides in Koshihikari and K×433 using anti-A1(No.2) and anti-B4(No.4a). When examined by CBB staining, glutelins accumulated from 7 days after flowering (DAF) and gradually increased and reached their maximum amounts by 21 or 28 DAF (Figure 5(A)). The Western blotting demonstrated that the intact α polypeptides of GluA1, GluA2, and GluB4 (labeled with A1, A2, and B4, respectively in Figure 5(B–C)) were detected from 7 DAF and attained their maximum levels by around 28–35 DAF. Meanwhile, the partial degradation band X1 was detected from 7 DAF and reached its maximum at 21 DAF (Figure 5(C)). The partial degradation band X4/X5 was detected from 14 DAF and reached its maximum at 21 DAF (Figure 5(B)).

It should be noted that the rate of partial degradation estimated by the abundance ratio of partial degradation bands to intact bands seemed to be much higher in GluB4 than in GluA1/GluA2. The accumulation level of the band X1 became equal to or surpassing that of B4, while the abundance of X4/X5 was significantly lower than that of A1/A2 at 35 DAF. Such difference in the partial degradation rate might affect the superficially different timings of the appearance of partial degradation bands between glutelin subunits. Therefore, GluB4 and possibly GluA1/GluA2 seemed to be attacked by proteolytic enzyme from the early stage of seed maturation in the globulin-less mutants.

4. Discussion

4.1. Compensation and degradation in glutelin and globulin mutants

Rice seeds are a staple food for most Asian countries. For developing countries, nutritional improvement in seed

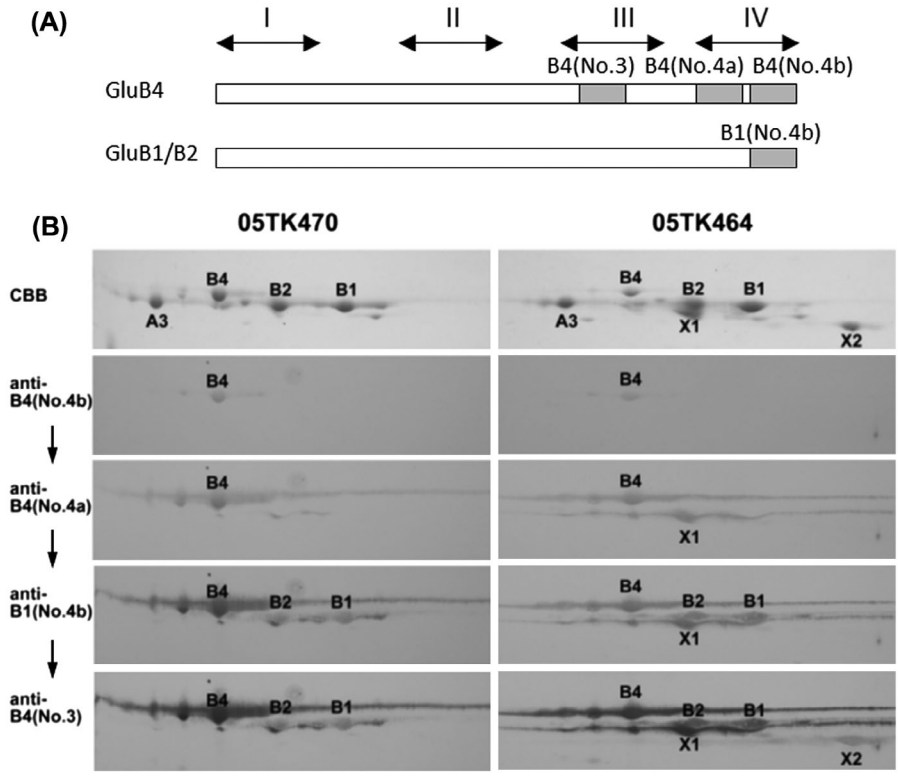

Figure 4. Step-by-step immunodetection of glutelin α polypeptide in globulin/glutelin mutants. (A) Schematic representation of GluB glutelin α polypeptides and epitope sites (shaded box) of antibodies used in this study. Four variable regions, in which the epitope sites were designed, were indicated with horizontal arrows and roman numerals. (B) Glutelin α polypeptides from the globulin/glutelin mutants 05TK470 and 05TK464 were analyzed by non-equilibrium pH gradient electrophoresis (NEPHGE) followed by SDS–PAGE with CBB staining and Western blotting. The anti-B4(No.4b), anti-B4(No.4a), anti-B1(No.4b), and the anti-B4(No.3) were employed 'step-by-step' in the order to overlay signals according to the method of Khan et al. (2008b). A3, B1, B2, and B4 indicate the intact α polypeptides of glutelin subunits GluA3, GluB1, GluB2, and GluB4, respectively. X1 and X2 indicate the partially degraded α polypeptides of glutelin GluB1, GluB2, and/or GluB4. Acidic, left; basic, right.

proteins is desired while for developed countries, low digestible protein content is attracting more and more interests because of increasing number of people suffering nephritic syndrome. To address both problems, a deepened fundamental knowledge on seed storage proteins is required.

In rice storage protein mutants, deletion or suppression of some proteins is assumed to be compensated by other storage proteins. For example, our previous analysis on some glutelin subunit mutants using capillary electrophoresis, a technique suitable for accurate quantification, has demonstrated that mutants Type1, Type2, Type3, and a-123less, in which GluB4, GluA2, GluA1, and their three subunits are deleted, respectively, had apparently no other changes in protein composition and total protein content, suggesting the deletions were compensated evenly by other storage proteins (Katsube-Tanaka et al., 2010). LGC-1, in which glutelin B type subunits are suppressed by RNAi (Kusaba et al., 2003), accumulates increased level of globulin and 13 kDa prolamin instead (Iida et al., 1993) and similar level of total nitrogen (Ashida et al., 2006), indicating functionally redundant and flexible nature of seed storage proteins. Glutelin, globulin, and prolamin knock-down lines produced by Kawakatsu et al. (2010) demonstrated the reduction in one or several storage proteins was compensated by other storage proteins preferentially in terms of sulfur-rich or sulfur-poor characteristics of the proteins. In this study, however, globulin-less mutation was unique to induce partial degradation of glutelin rather than compensation. Although compositional change of protein bodies induced by RNAi was demonstrated to result in irregular structure of PB in maize (Holding, 2014), wheat (Gil-Humanes et al., 2011), and rice (Kawakatsu et al., 2010; Nagamine et al., 2011), partial degradation has not ever been reported. Considering our results together with the fact that globulin accumulates on the surface of PB-II (Krishnan et al., 1992; Kumamaru et al., 2010) and its absence results in structurally deformed PB-II (Ashida et al., 2011), globulin protects glutelins from proteinase digestion and thereby facilitates stable glutelin accumulation. Nonetheless, the globulin-less mutation did not express any other malfunction in germination, plant growth, and

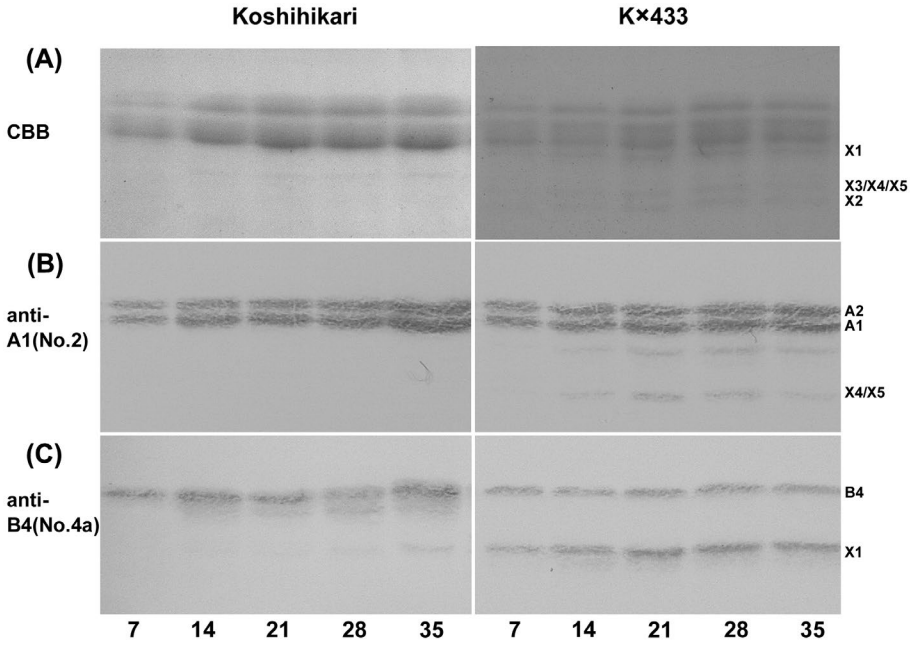

Figure 5. Temporal accumulation of the partially degraded forms of glutelin α polypeptides in developing seeds. Glutelin fractions extracted from the wild type Koshihikari and the globulin-less mutant K×433 at the 7, 14, 21, 28, and 35 day-after-flowering were electrophoresed and detected by CBB staining (A), Western blotting with anti-A1(No.2) (B) and anti-B4(No.4a) (C). The intact α polypeptides of GluA1 (A1), GluA2 (A2), and GluB4 (B4) and partially degraded polypeptides (X1-X5) were indicated at the right side of the figure. The specificity of reactions with anti-A1(No.2) and anti-B4(No.4a) was documented in the text.

storage substances accumulation, resulting in no apparent defect phenotype in conventional cultivation processes. It might be required to examine the performance of the seeds with deformed PB-II under a different condition, for example, more stressful and unfavorable environments.

4.2. Molecular assembly and stable accumulation – soybean glycinin and rice glutelin

Soybean glycinin, the most major storage protein and a member of 11S globulin family like rice glutelin, has a hexameric structure in protein bodies, that is a common characteristic for most 11S globulins (Utsumi, 1992) and is likely to be necessary for stable and efficient accumulation. Crystallographic analysis suggested the posttranslational processing at the junction between the acidic polypeptide and the basic polypeptide of proglycinin is crucial for the molecular assembly to the hexamer from a trimer (Adachi et al., 2003; Dickinson et al., 1989). On the other hand, the higher order structure of rice glutelin may not be a prerequisite for stable accumulation because rice glutelin accumulates as both polymerized and non-polymerized forms in wild type (Katsube-Tanaka et al., 2004). However, the partial degradation at the C-terminus of α polypeptides seems to inhibit or prevent polymerization (Figure 2) because the C-terminus region contains a specific cysteine residue which is responsible for the polymerization in wild type (Katsube-Tanaka et al., 2004). Thus, the imperfect partial degradation might affect the proper PB-II formation and the stable accumulation of glutelin through the altered ratio of the polymerized to the non-polymerized form.

Another possibility is that the polymerization interferes with the partial degradation and partly contributes to the stable accumulation of glutelins. Actually the partial degradation rate of GluA subunits, which are all able to polymerize due to the cystein residue at the C-terminus of α polypeptides, was low. Though, the GluB4 subunit, which is exceptionally able to polymerize among the major GluB subunits, was severely suffered by the partial degradation. Thus, it would be interesting to tell the difference in the partial degradation rate between the polymerization-competent GluB4 and -incompetent GluB1/GluB2 subunits. Note that posttranslational cleavage of precursor into α and β polypeptides at the C-terminus of the α polypeptide by vacuolar processing enzyme (Kumamaru et al., 2010) seemed to be carried out regardless of the occurrence of the partial degradation (Figure 2). Thus, future works should be also addressed to see whether the partial degradation occurs or not on unprocessed precursor forms of glutelin.

4.3. Distinct function of rice globulin and glutelin A and B subfamilies

Kawakatsu et al. (2010) demonstrated a cracked PB-II structure in a globulin knock-down transformant, which

would be caused by the similar reason for the deformed PB-II structure observed by Ashida et al. (2011). However, Kawakatsu et al. (2010) did not mention the partial degradation of glutelin. Their globulin knock-down transformant simultaneously has glutelin GluB knock-down. The partial degradation rate was demonstrated to be much slower in GluA subunits than in GluB subunits in this study. That may be the reason why the partial degradation could not be observed in their knock-down transformant. Ashida et al. (2006) has also reported the increased free amino acid content in the globulin-less (*glb1*) mutant (1.3–1.5-fold higher than wild type in major amino acids) while the free amino acid content of the globulin and GluB knock-down transformant was not statistically different from that of wild type (Kawakatsu et al., 2010). Although a free amino acid content is much less than a total amino acid content by double-digits in rice seeds, the above results are consistent with the difference in the partial degradation between the two reports.

Another explanation might be possible by the different temporal and spatial expression between glutelin GluB subunits and globulin. Furtado et al. (2008) demonstrated the globulin promoter directs the expression from an earlier developing stage and at a higher level, especially in the central region of endosperm than the GluB1 promoter. Even though globulin was not detected by the immunoblotting in the globulin knock-down transformant (Kawakatsu et al., 2010), the use of the GluB1 promoter to simultaneously downregulate both GluB subunits and globulin in the study of Kawakatsu et al. (2010) might cause leaky expression of globulin. If the above-mentioned hypothesis is true, even undetectable level of globulin might be enough to suppress the partial degradation of glutelin.

It is worthwhile to mention that a low level of α polypeptide-like spot was detected at the lower right of the intact α polypeptide of the monomer glutelin subunit in Koshihikari and 05TK470 as well as in K×433 and 05TK464 in Figure 2. The possibly equivalent spot was also observed at the lower right of the B1 spot in Figure 3 in the above-mentioned four cultivars, suggesting that partial degradation slightly occurs even in globulin-containing cultivars. Meanwhile, it is interesting to know whether the enhanced accumulation of globulin in LGC-1 (Iida et al., 1993) inhibits the partial degradation or not. However, the lower right spot of the B1 spot in Figure 3 was detected neither in LGC-1 nor LGC-Jun (Figure 3), indicating that the reason for the no apparent degradation in LGC-1 would be the different partial degradation rate between GluA and GluB subunits. In other words, the lower right spot of the B1 spot in Figure 3 would be derived from a GluB-related subunit and so far we cannot tell the effect of the enhanced accumulation of globulin on partial degradation.

Note that the globulin-less mutant examined in this study has ~62.8 k bp deletion (Morita et al., 2009). The deleted region was supposed to contain 10 genes (Morita et al., 2009) coding such as serine/threonine protein kinase domain containing protein, heme peroxidases family protein, UDP-glucuronosyl/UDP-glucosyl transferase family protein, and so on including hypothetical conserved genes and the globulin gene. It would be necessary to consider the possibility that such genes deleted simultaneously with globulin gene might be responsible to the partial degradation of glutelin.

Why is the partial degradation rate different between GluA and GluB subunits? Why does the partial degradation occur at two steps (X1 and X2) in GluB subunits? The cause is probably the different structure between GluA and GluB subunits (Katsube et al., 1999) but so far we cannot tell exact difference. Further research including identification of the exact cleavage position and the responsible proteinase is required.

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