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RESEARCH PAPER

Expression of unprocessed glutelin precursor alters polymerization without affecting trafficking and accumulation

Yuhya Wakasa, Lijun Yang, Sakiko Hirose and Fumio Takaiwa*

Transgenic Crop Research and Development Center, National Institute of Agrobiological Sciences, Kannondai 3-1-3, Tsukuba, Ibaraki 305-8604, Japan

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Abstract

Rice glutelin is synthesized as a precursor in the endosperm endoplasmic reticulum and then deposited within the protein storage vacuole protein body-II (PB-II) as an aggregate, with a high degree of polymerized higher-order structure comprising mature acidic and basic subunits after post-translation processing cleavage. In order to investigate the functional role of this processing and its effect on folding assembly, wild-type GluA2 and its mutant cDNA (*mGluA2*), in which the conserved processing site (Asn-Gly) at the junction between the acidic and basic chains was replaced with Ala-Ala, were expressed under the control of the endosperm-specific *GluB1* promoter in the mutant rice a123 line lacking glutelin *GluA1*, *GluA2*, and *GluB4*. The mGluA2 precursor was synthesized and stably targeted to PB-II without processing in the transgenic rice seeds like the wild-type GluA2. Notably, the saline-soluble mGluA2 precursor assembled with the other type of processed glutelin GluB as a trimer in PB-II, although such hetero-assembly with GluB was not detected in the transformant containing the processed GluA. Furthermore, the mGluA2 precursor in the glutelin fraction was deposited in PB-II by forming a quite different complex from the processed mature GluA2 products. These results indicate that post-translational processing of glutelin is not necessary for trafficking and stable accumulation in PB-II, but is required for the formation of the higher-order structure required for stacking in PB-II.

Key words: assembly, glutelin, glutelin mutant, *Oryza sativa* L., processing.

Introduction

Glutelin is the major seed storage protein of rice, comprising ~60–80% of the total amount of the seed storage proteins (Takaiwa *et al.*, 1999). This protein is classified into the GluA, GluB, GluC, and GluD families. Furthermore, the major glutelin components GluA and GluB are made up of three (GluA1, GluA2, GluA3) and four members (GluB1, GluB2, GluB3, GluB4), respectively (Takaiwa *et al.*, 1987, 1991; Mitsukawa *et al.*, 1998; Kawakatsu *et al.*, 2008). Irrespective of its solubility properties, it has been established that rice glutelin is homologous to dicotyledonous 11S globulin, based on similarities in their biogenesis, processing sites joining mature acidic and basic chains, the positions of cysteine residues involved in inter- and intramolecular disulphide bonds and their amino acid sequences (Takaiwa *et al.*, 1999).

11S globulins have a molecular weight (MW) of ~360–400 kDa and consist of six subunits with an MW of ~55–60 kDa. Each subunit in a hexamer is composed of two polypeptide chains that are linked covalently by disulphide bonds. They are initially synthesized and assembled into a trimer in the endoplasmic reticulum (ER) of the endosperm cell, and then are transported into protein storage vacuoles. After post-translational proteolysis at the conserved Asn-Gly site by Asn-specific cysteine protease to release mature acidic and basic polypeptides, they are assembled into hexamers in the protein body (Jung *et al.*, 1998; Gruis *et al.*, 2004).

* To whom correspondence should be addressed. E-mail: takaiwa@nias.affrc.go.jp

Rice glutelin is also synthesized as pre-proglutelin on ER and subsequently transported into the ER lumen through signal peptides, which are ultimately targeted to the protein storage vacuole, called protein body-II (PB-II), by a vesicle-mediated pathway. Proglutelin in PB-II is processed to yield the mature acidic and basic chains like other 11S globulins, but it is notable that they exist as large macromolecular complexes formed by disulphide and hydrophobic interactions of acidic and basic polypeptides (Yamagata *et al.*, 1982; Krishnan and Okita, 1986). The hexameric oligomer structure has not yet been observed for rice glutelins in maturing seeds.

It has been reported that rice glutelin shares approximately a 70% amino acid sequence identity with oat 12S globulin (Shotwell *et al.*, 1990). In spite of the high homology in the sequences, the physical properties are quite different, because oat globulins are deposited in protein bodies as hexameric oligomers with an MW of ~350 kDa, comprising six subunits with an MW of ~55 kDa, and are soluble in saline solution. Little is known about the molecular mechanisms which cause the differences in solubility and the oligomeric structure deposited in protein bodies.

Post-translational cleavage in the biogenesis of 11S globulins is one of the critical steps determining folding and assembly. Post-translational cleavage has been known to be required for assembly of hexameric formation by *in vitro* assay (Jung *et al.*, 1997). Processing occurs at the highly conserved Asn-Gly sites linking mature acidic and basic polypeptides, although there are a few deviations from parts of the pea legumin (Asn-Phe) (March *et al.*, 1988), ginkgo 11S globulin (Asn-Asn) (Arahira and Fukazawa, 1994), and rice glutelin GluC (Asn-Val) (Mitsukawa *et al.*, 1998). It has been reported that asparaginyl endopeptidase (cysteine protease) with specificity toward an Asn residue acts as a vacuolar processing enzyme (VPE) which is involved in post-translational cleavage (Hara-Nishimura *et al.*, 1995; Jung *et al.*, 1998). Knock-out mutants of VPE were isolated in *Arabidopsis* and their involvement in storage protein accumulation (processing) has been reported (Gruis *et al.*, 2004).

Although post-translational proteolysis is required not only for the synthesis of the mature acidic and basic polypeptides of glutelin, but also for accumulation in protein bodies, there is little experimental evidence showing whether (i) unprocessed mutant glutelin precursor is correctly targeted into PB-II and stably accumulated, (ii) glutelin precursor is stably accumulated in mature seeds, or (iii) it can be assembled together with other glutelins or is self-assembled in mature seeds.

In this study, a GluA2 precursor which cannot be cleaved by VPE was highly expressed under the endosperm-specific *GluB1* promoter in a low-glutelin mutant rice line lacking *GluA1*, *GluA2*, and *GluB4* in order to address the above questions. Mutant glutelin precursor was stably deposited into PB-II in mature transgenic seeds without degradation, and assembled together with other glutelins (e.g. the *GluB* family) in a higher-order molecular form different from the normal mature glutelins.

Materials and methods

Plant materials

Rice (*Oryza sativa* L.) cv. Koshihikari mutant line a123 (Iida *et al.*, 1997), in which *GluA1*, *GluA2*, and *GluB4* are lacking because of mutation, was used for transformation.

Vector construction

Gene cassettes consisted of the endosperm-specific 2.3 kb glutelin B1 (*GluB1*) promoter with 5′ UTR (Accession No. AY427569), the coding region of the glutelin A2 (*GluA2*) gene (Accession No. X05664), or mutated-GluA2 (*mGluA2*), in which the processing site (Asn-Gly) was replaced by Ala-Ala, and 0.65 kb *GluB1* terminator (Accession No. X54314) were introduced into the multiple cloning sites of modified-pBluescript KS+ containing the Gateway recombination sites att L1 and att L2. Then, two gene cassettes were transferred from entry clones to a destination-binary vector (p35:HPT Ag7-GW; Wakasa *et al.*, 2006) by LR clonase II enzyme Mix (Invitrogen, CA, USA). The binary vector constructs are shown in Fig. 1.

Production of transgenic plants

Transgenic rice plants were produced by *Agrobacterium tumefaciens*-mediated transformation. Binary vectors were transferred into *Agrobacterium* strain EHA105 by electro-transformation. Four-week-old calli derived from mature seeds were co-cultured with the transformed *Agrobacterium* for 3 d. The infected calli were successively cultured in DKN selection, DKN regeneration, and MS regeneration media with hygromycin (Wakasa *et al.*, 2007). The regenerated plantlets were grown in a closed greenhouse (Goto *et al.*, 1999).

Total protein extraction and immuno-blot analyses

Mature seeds from independent primary transgenic rice lines were individually ground into fine powder with a multi-bead shocker (YASUI KIKAI, Tokyo, Japan). For total protein extraction, 500 µl of total protein extraction buffer [50 mM TRIS-HCl pH 6.8, 8 M urea, 4% SDS, 20% glycerol, 5% 2-mercaptoethanol (2-MER), 0.01% bromophenol blue] were added to seed powder and vortexed for 1 h at room temperature. The mixture was centrifuged at 13 000 *g* for 20 min at room temperature. Proteins were

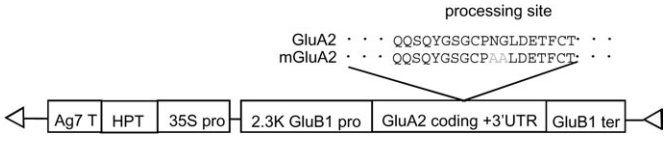

Fig. 1. Binary vector constructs. Ag 7, *Agrobacterium* gene 7 terminator; HPT, hygromycin phosphotransferase coding region; 35S pro, CaMV 35S promoter; 2.3 K GluB1 pro, 2.3 kb rice glutelin B1 promoter; GluA2, wild-type rice glutelin A2 coding region; GluB ter, 0.65 kb glutelin B1 terminator; mGluA2, mutated-glutelin A2 coding region.

subjected to immuno-blot analysis using anti-GluA (GluA1 and GluA2), anti-GluB (GluB1, GluB2, and GluB4), anti-BiP, anti-PDI, and anti-calnexin antibodies.

For quantitative dot blot immuno-blotting analysis, four positive seed extracts per independent transgenic line selected by immuno-blot using anti-GluA antibody were pooled and used for quantification. For the relative comparison of transgene product accumulation, equal amounts of the protein extracts were spotted onto a nitrocellulose membrane (Whatman, Dassel, Germany). The transgene products in each dot were detected immunologically with anti-GluA antibody and quantified with NIH Image J (National Institutes of Health ver. 1.41; Washington, DC, USA).

Sequential protein extraction

Sequential extraction of proteins was performed according to Takaiwa *et al.* (2008). Briefly, globulins were extracted with 500 µl of globulin extraction buffer (0.5 M NaCl, 10 mM TRIS-HCl, pH 6.8) from 25 mg seed powder. After the removal of globulins, the glutelins were extracted from residual proteins with 500 µl of glutelin extraction buffer (1% lactic acid, 1 mM EDTA). Each extraction step was repeated five times and accomplished by sonication for 2 min and centrifugation at 13 000 *g* for 10 min. After the stepwise removal of the globulin and glutelin fractions, prolamins were finally extracted from residual proteins with 500 µl of total protein extraction buffer.

To examine the possible involvement of disulphide bonds for extraction efficiency, the globulin fraction was extracted from 25 mg seed powder with 500 µl of globulin extraction buffer with or without 5% 2-MER. After the acetone precipitation, the globulin pellet was dissolved with 100 µl of total protein extraction buffer. The residual proteins were extracted with 500 µl of total protein extraction buffer. Two microlitres of each sample were used for SDS–PAGE and immuno-blotting analysis.

Immunogold electron microscopy

The immature seeds (15–20 DAF) were fixed in 4% paraformaldehyde, 0.1% gultaraldehyde buffered at pH 7.2, with 20 mM PIPES over night at 4 °C. After washing in PIPES buffer, the samples were dehydrated in a series of ethanol concentrations and embedded in LR White resin (London Resin, Berkshire, UK). Ultrathin sections were cut with a glass knife using an ultramicrotome (MT2-B; Sorvall, Newtown, CT, USA) and mounted on copper grids. The grids were floated on a drop of PBS containing 3% BSA for 1 h and were incubated for 1 h with anti-GluA diluted 1:3000 with PBS containing 1% BSA. Nonspecifically bound antibodies were removed by washing the grids with washing buffer (PBS containing 0.1% BSA, 0.5 M NaCl, and 0.05% Tween 20) three times for 15 min each. The samples were reacted with 15 nm gold-labelled goat anti-rabbit IgG antibody (Aurion, Wageningen, Netherlands) diluted 1:200 in PBS containing 1% BSA for 1 h. Then the grids were washed three times with washing buffer. The sections were fixed with 1% glutaraldehyde in PBS followed by washing with distilled water twice, then stained with uranyl acetate for 2 h and lead citrate for 7 min. The samples were examined with a transmission electron microscope (H-7100; Hitachi, Tokyo, Japan) running at 75 kV.

Sucrose density gradient (SDG) centrifugation of the globulin fraction

Centrifugation on SDG was carried out using a modified method as described by Takaiwa *et al.* (2008). A globulin fraction was extracted from three mature seeds with 800 µl of exclusive globulin extraction buffer for SDG (35 mM phosphate buffer, pH 7.6, 0.5 M NaCl, proteinase inhibitor). Aliquots of 200 µl were layered onto 10 ml linear 10–30% SDGs on 1 ml of a 65% sucrose cushion containing globulin extraction buffer. After centrifugation at 120 000 *g* for 12 h at 4 °C (Hitachi RPS40T rotor and Himac CP100α ultracentrifuge; Hitachi Koki, Tokyo, Japan), fractions were collected at 0.5 ml/tube from the bottom and precipitated with the same volume of acetone. Precipitate was dissolved in total protein extraction buffer, and was used for SDS-PAGE and immuno-blotting analyses. Globulin fractions extracted from mature soybean seeds were subjected to SDG as an elution marker for trimers and hexamers.

Gel filtration

The glutelin fraction was directly loaded onto a Sephacryl S-300 HR (GE Healthcare, Uppsala, Sweden) column and immediately underwent fraction collection with four drops per tube (~160 µl per tube). To each fraction was added the same volume of 2× sample buffer (100 mM TRIS-HCl pH 6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue) with 10% 2-MER, and was neutralized by 1 M NaOH.

Results and discussion

Production of a transgenic rice plant expressing exogenous GluA2 or mGluA2

Native GluA2 or processing site mutated-GluA2 (mGluA2) under the control of the glutelin *GluB1* promoter (Fig. 1) have been introduced into a low-glutelin mutant rice a123 line. Since the mutant a123 line lacks three glutelins (GluA1, GluA2, and GluB4), exogenous GluA2 and mGluA2 products can be easily detected by using an anti-GluA antibody which recognizes the GluA1 and GluA2 acidic subunits. As shown in Fig. 2B, when anti-GluA antibody was used for immuno-blotting analysis, no signal was detected in non-transgenic a123. By contrast, immunoreactive GluA2 or mGluA2 signals were clearly detected in transgenic a123 seeds. In the highly expressed transgenic rice lines, their products were easily observed as distinct bands in CBB-stained SDS-PAGE gels (Fig. 2A).

In order to examine whether processing from precursor to mature acidic and basic polypeptides is required for trafficking to PB-II or stable accumulation by assembly or aggregation in PB-II, transgenic rice lines that accumulated similar concentrations of exogenous GluA2 or mGluA2 products were selected and then were advanced by self-crossing to obtain homozygous lines. When total proteins were extracted from mature seeds of transgenic rice containing the GluA2 or mGluA2 product and then subjected to immuno-blotting after electrophoresis on SDS-PAGE, the transgene products were detected as a distinct band by CBB staining (Fig. 2A, B). The exogenous GluA2 product was normally processed into acidic and basic subunits in the transgenic rice. The acidic subunit of the GluA2 transgenic rice migrated at the same position as that of the endogenous GluA2 gene product from non-transgenic Koshihikari. Notably, immuno-reactive signals with MWs of 55 kDa and 35 kDa in the wild-type Koshihikari were more intense than in the transgenic with the GluA2 line. This is accounted for by the lack of expression of endogenous immuno-reactive GluA1 and GluA2 in the a123 line used as the host for transformation. In contrast, the transgene product in the mGluA2 transgenic rice seed was only detected as a band of 55 kDa, which corresponded to the size of the uncleaved glutelin precursor. Since the mGluA2 accumulated as proglutelin, Ala-Ala at the mutated processing site was not recognized and cleaved by VPE. Furthermore, it was reported that the VPE had an absolute specificity for Asn on the N-terminal side of 11S globulins but exhibited little specificity for amino acids on the C-terminal side (Jung *et al.*, 1998). In addition, evidence supporting the requirement of an Asn residue at the well-conserved Asn-Gly peptide bond was shown in legumin (Asn-Phe) (March *et al.*, 1988), ginkgo 11S globulin (Asn-Asn) (Arahira and Fukazawa, 1994), and rice glutelin GluC (Asn-Val) (Mitsukawa *et al.*, 1998). Thus, an Asn position may be required for cleavage of proglutelin even in the rice plant in a similar manner observed in other 11S globulins. However, in order to evaluate whether the Asn residue is a crucial one determining the cleavage by VPE, further study using transgenic rice plants containing *mGluA2*, in which the Asn residue only is mutated to Ala, will be required.

The accumulation levels of transgene products were investigated for the mature seeds of a total of 40 independent transgenic rice lines selected for the expression of either GluA2 (22 lines) or mGluA2 (18 lines). In this experiment, the immuno-reactivity of anti-GluA antibody with either GluA or mGluA product was very important for estimation of the accumulation levels. As shown in Fig. 2A, B, this antibody exhibited similar immuno-reactivity to the acidic subunit of GluA-2 and precursor of mGluA2 in immuno-blots after SDS-PAGE. A large variation in accumulation levels of GluA2 and mGluA2 products was observed among the individual transgenic lines for each construct. Unexpectedly, the average value of the mGluA2 product from the mature seeds of 18 transgenic rice lines was about double that of the GluA2 product from 22 transformants (Fig. 3). This result indicates that the unprocessed mGluA2 product may accumulate more stably and efficiently than the GluA2 mature polypeptides in transgenic rice seeds. However, further work will be

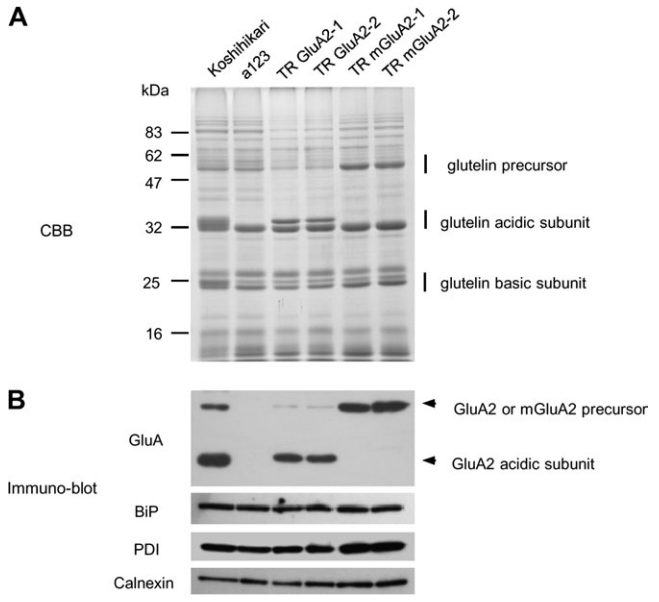

Fig. 2. SDS-PAGE, immuno-blot analyses, and seed phenotype in transgenic rice seed with each construct. Total protein of non-transgenic Koshihikari, non-transgenic a123, transgenic a123 expressing GluA2 (TR GluA2-1 and TR GluA2-2), and transgenic a123 expressing mGluA2 (TR mGluA2-1 and TR mGluA2-2) were subjected to SDS-PAGE gel. (A) Coomassie brilliant blue (CBB) staining; (B) immunodetection with anti-GluA, anti-BiP, anti-PDI, and anti-calnexin.

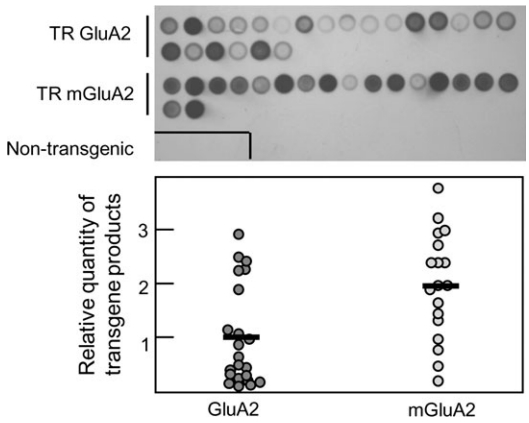

Fig. 3. Relative accumulation levels of transgene products. Comparison of the transgene product levels between GluA2 and mGluA2 are shown. The transgene product in each dot was detected immunologically with anti-GluA antibody (upper panel) and quantified with NIH Image J (National Institutes of Health ver. 1.41, Washington, DC, USA). The signal strength of each dot was plotted in the lower panel. The mean accumulation level of GluA2 was assumed as an arbitrary standard (1.0). Horizontal bars indicate the average of relative accumulation levels in each construct.

required to examine whether this result is applicable to other glutelins.

When the unprocessed mGluA2 precursor was found to be more highly accumulated in transgenic rice seed, the expression levels of three chaperone proteins (BiP, PDI, and calnexin) were investigated in the mature seeds of these transgenic rice plants (Fig. 2B). There was no significant difference in the BiP and calnexin levels between them. It is notable that the PDI level was slightly increased in lines that accumulated the mGluA2 precursor, suggesting that this accumulation may be recognized as a stress factor by the PDI chaperone molecule. This may be related to the evidence that protein folding in the ER is accompanied by the formation of disulphide bonds, which is catalysed by PDI.

Intracellular localization of exogenous GluA2 and mGluA2

The subcellular localization of exogenous GluA2 and mGluA2 in the endosperm cell was examined by immuno-cytochemical electron microscopy using antibody against both of the GluA2 and mGluA2 products (Fig. 4). A large number of immunogold particles were observed in PB-II, but not the other organelles, for both constructs. In contrast, no signal was detected in non-transgenic a123. Furthermore, there is little difference in subcellular structures of the endosperm cell between non-transgenic and transgenic rice expressing the GluA2 and mGluA2 products. Thus, the mGluA2 unprocessed precursor was normally trafficked into PB-II like the normal GluA2 product, indicating that processing from the precursor into mature subunits is not required for trafficking to PB-II.

Different solubility of GluA2 and mGluA2

A sequential extraction experiment was carried out in order to examine the interaction of exogenous GluA2 or mGluA2 products with endogenous glutelins in endosperm cells of a123 mutant line. Rice seed proteins were sequentially extracted with saline solution (globulin fraction), acidic solution (glutelin fraction), and urea and 2-MER solution, in that order.

As shown in Fig. 5, most of the GluA2 or mGluA2 products expressed in seeds of the a123 line could be extracted in the glutelin and residual prolamin fractions. It is interesting to note that a small portion of the mGluA2 product could be extracted in the globulin fraction, which was accompanied by a higher level of endogenous GluB product in the globulin fraction compared with that in the control a123 line. Since the GluB could not be detected in a globulin fraction from Koshihikari (Fig. 5B), recovery in the soluble fraction of the GluB product may be related to high expression of the mGluA2 precursor in mGluA2 transformant or low levels of mature GluA product in a123. By contrast, when GluA products are mainly accumulated as mature subunits in GluA transformant or Koshihikari, the GluB acidic subunits could not be extracted in the globulin fraction. These results suggest that the GluB subunits interact with the GluA precursor in a different manner from disulphide bonds and recovered as hetero-complexes in the saline solution.

As shown in Fig. 6, the GluA2 or mGluA2 product was examined by extraction with the saline solution in the presence or absence of 2-MER. Addition of 2-MER apparently enhanced the extraction efficiency of the GluA2 and mGluA2 products in the globulin fraction, resulting in a yield of 12.2% and 22.8% of total seed protein, respectively (Table 1). This is in contrast with the extraction

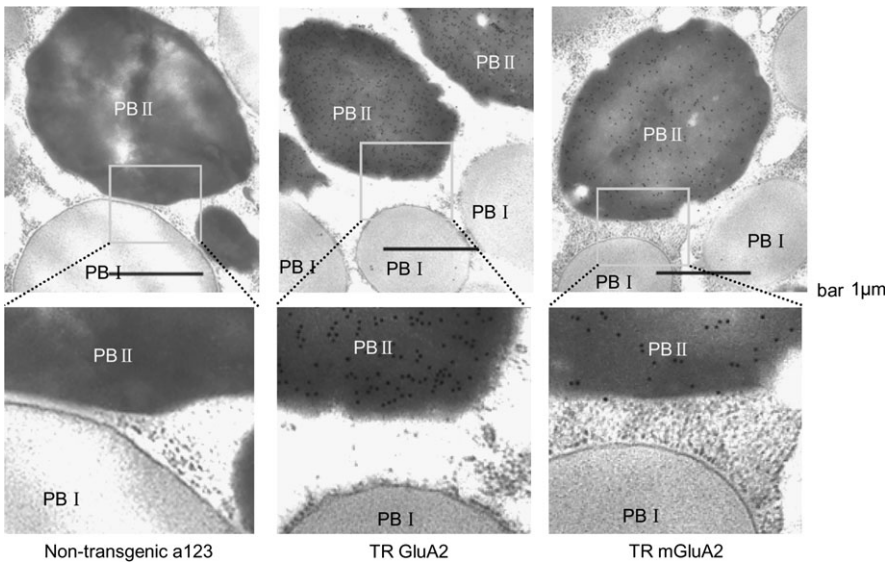

Fig. 4. Immnolocalization of exogenous GluA2 and mGluA2 in rice endosperm cells. The 15 nm gold particles reveal that GluA2 or mGluA2 is localized in PB-II only in transgenic rice seed. Lower panels are enlargements of the areas outlined in white in the upper panels. PB-I, Protein body I; PB-II, protein body II. Scale bar=1 μm.

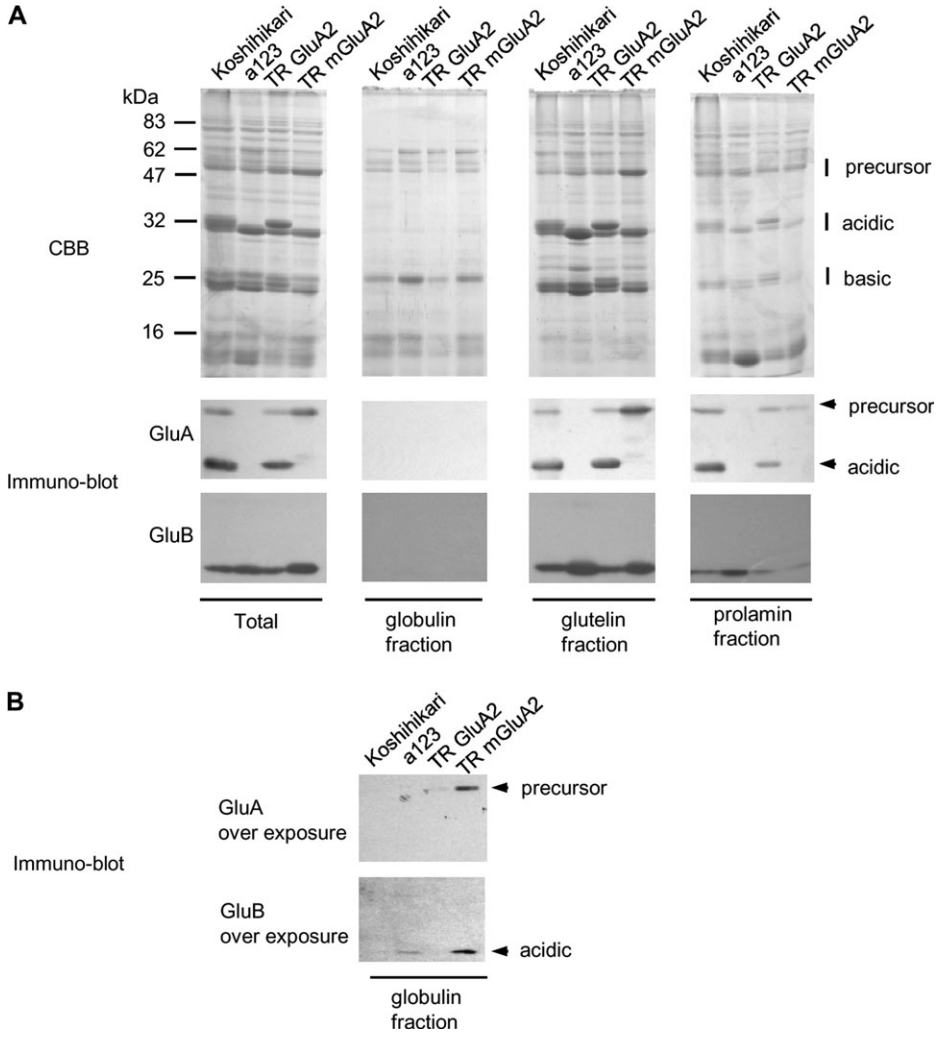

Fig. 5. Sequential extraction of seed proteins from Koshihikari, a123, and transgenic rice with GluA2 and mGluA2. (A) Each extracted protein fraction was subjected to SDS-PAGE and immuno-blot analysis using anti-GluA and anti-GluB antibody. (B) In the globulin fraction, overexposed X-ray film is also shown.

efficiency of 2.5% and 9.6% in the absence of 2-MER. This evidence indicates that disulphide bonds are involved in the interaction between GluA and other glutelins. Notably, the GluA2 precursor could be easily extracted with saline solution, irrespective of 2-MER, whereas a reducing agent was required for the extraction of mature GluB subunits (Fig. 6A). These results indicate that interactions among GluA and GluB products are mainly mediated by disulphide bonds.

Glutelin precursor assembles as a trimer

In order to investigate the potential assembling capacity of saline-soluble glutelins, globulin fractions extracted from transgenic mature seeds were subjected to centrifugation on an SDG. Saline-soluble glutelins from mGluA2- and GluA2-transgenic rice seeds sedimented at a peak of 7S, indicating that some of them were assembled in the form of a trimer (Fig. 7). It has been reported that precursor of leguminous 11S globulin homologous to rice glutelin is assembled as a trimer in ER in the course of its synthesis and then deposited into the protein storage vacuole (Jung *et al.*, 1998). Thus, a higher amount of mGluA2 precursor is predicted to be assembled into trimers in the processing-defective mGluA2 transgenic rice, when compared with the normal GluA2 transgenic rice. Expectedly, higher immunoreactive GluA precursor was detected in the 7S region for the mGluA2 transformant (Fig. 7).

Since the mGluA2 product mutated at the post-translational processing site could not be cleaved by VPE and deposited as a precursor in PB-II (Fig. 3), it is suggested that assembly into a hexamer or another further complicated complex formation is perturbed in PB-II, so that the trimer remains as the assembly state in mature seeds. Furthermore, it is of interest to determine whether mGluA2 precursor can assemble with other glutelins. As shown in Fig. 7, it is notable that saline-soluble glutelin GluB extracted from the mGluA2 transgenic rice seeds could be detected as a trimer by sedimentation analysis on SDG, whereas GluB glutelin could not be detected as a trimer in the GluA2 transformant. This result indicates that a higher-order complex structure is formed by the interaction

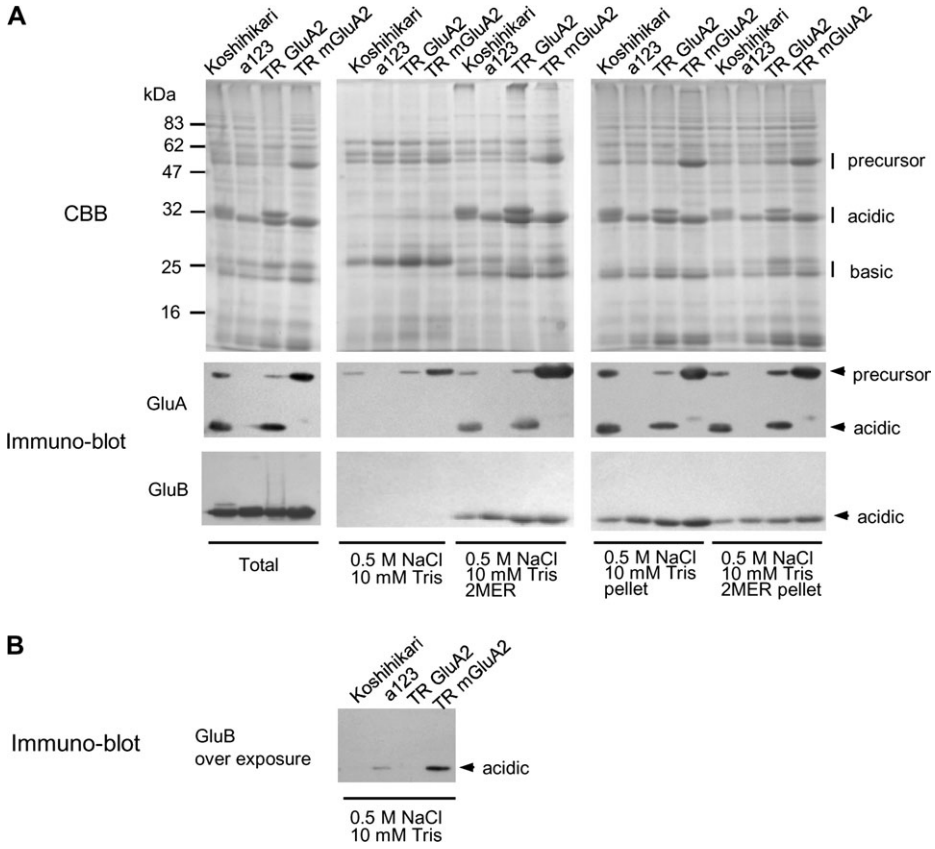

Fig. 6. SDS-PAGE and immuno-blot analysis of transgene products extracted by total protein extraction buffer after pre-extraction by globulin extraction buffer with or without 5% 2-mecaptoethanol. The globulin extraction buffer-extracted fraction (supernatant) was 5-fold concentrated by acetone precipitation compared with the pellet fraction. (A) Each extracted protein fraction was subjected to SDS-PAGE and immuno-blot analysis using anti-GluA and anti-GluB antibodies. (B) In the globulin fraction, in order to detect the matured GluB signal, overexposed X-ray film is also shown.

Table 1. Approximate percentages of soluble GluA2 or mGluA2 by extraction using salt buffer in transgenic rice seed

Signal strengths of the immno-blot in the second and third panels in Fig. 6A were quantified with NIH ImageJ (National Institutes of Health, Washington, DC, USA).

Sample	MER–	MER+
GluA2	2.5%	12.2%
mGluA2	9.6%	22.8%

between the processed GluA and GluB mature subunits. That is, the processing from precursor to acidic and basic polypeptides by VPE is required for the formation of the high-order structure in PB-II. It was also suggested that GluA precursor hetero-assembled with GluB precursor as a trimer in the ER lumen and then is transported into PB-II in the process of biogenesis. Furthermore, it should be noted the GluB glutelin that hetero-assembled with the mGluA2 precursor as a trimer was detected as mature subunits rather than the precursor. This evidence suggests that processing by VPE may be performed in the state of the trimeric structure. Taken together, after glutelins constituting the trimeric structure in their assembly process are completely processed to the mature form consisting of acidic and basic polypeptides, the higher-order complex structure is generated and stacked in PB-II.

Jung *et al.* (1997) reported that post-translational processing into mature subunits is required for hexamer assembly in legumin B, one of the 11S globulins in the field bean. A noncleavable legumin B mutant accumulated as a trimer in transgenic tobacco seeds, whereas wild-type legumin B accumulated as a hexamer. Gruis *et al.* (2004) have reported that the precursor of the legumin-like globulin drastically increased in the knock-out line of VPE genes in *Arabidopsis* seeds. When protein extracts from wild-type and mutant seed were separated by SDG, legumin-like proteins from wild-type seeds were found as two peaks consisting of 2S and 12S (hexamer). By contrast, the same proteins from mutant seeds were found as three peaks of 2S, 5.5S, and 9S (trimer), but 12S (hexamer) was not detected. In rice glutelin, although there have been no experimental data indicating whether glutelin exists in the trimer or hexamer form in seeds, the present results do provide clear evidence that a rice glutelin is generated through a trimer in the assembly process. The trimer form of glutelin is required for transport into PB-II, in a manner similar to the leguminous 11S globulins. Homo- and

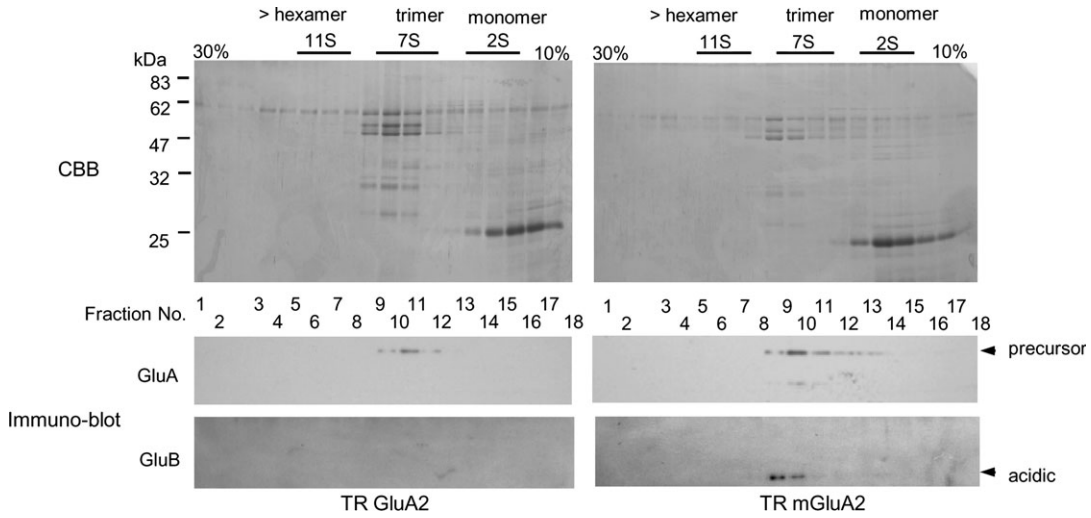

Fig. 7. Assembly of saline-soluble GluA2 and mGluA2. Globulin fractions derived from transgenic rice seeds were subjected to SDG centrifugation. Each fraction was analysed by SDS-PAGE and immuno-blot using anti-GluA and anti-GluB antibodies. The left panels show the transgenic rice with GluA2 (TR GluA2), the right panels mGluA2 (TR mGluA2).

heterotrimer composed of GluA and/or GluB could be extracted in saline solution, whereas 2-MER is required for extraction of higher complexes, indicating that trimer formation as hetero-complexes may be mediated by specific molecular interactions such as hydrophobic interactions.

Unprocessed mGluA2 forms unique complexes with other glutelins in PB-II

In order to investigate the assembly state of the newly synthesized mGluA2 and GluA2 products in the major saline-insoluble glutelin fraction, the glutelin fraction was extracted from transgenic a123 mature seeds containing mGluA2 or GluA2 products with lactic buffer, and was subsequently subjected to gel filtration analysis (Fig. 8). Processed GluA2s were assembled in a high-order polymerized structure with a peak in fractions 51–54. GluBs were also assembled in a high-order structure, but GluB had a slightly lower order complex than GluA2. By contrast, unprocessed mGluA2 precursor was distributed widely in many fractions and the peak of interacting GluB shifted to higher molecular weights (a change from fraction 54 to fraction 47), indicating that polymerization of the glutelin mGluA2 precursor is quite different with that of the mature GluA2 molecule. The unprocessed mGluA2 precursor may form higher molecular complexes than the mature GluA2 molecule. Alternatively, since the mGluA2 precursor may not fold well, the complex containing mGluA2 may have a more loosely compacted structure than GluA2. Furthermore, the peak of interacting GluB shifted to higher molecular weights, clearly suggesting that parts of GluA and GluB were assembled into a hetero-complex.

The polymerization of glutelins is reported to be different between the GluA and GluB subfamilies (Katsube-Tanaka *et al.*, 2004). The number of free cysteine (Cys) residues, besides the four highly conserved Cys residues involved in

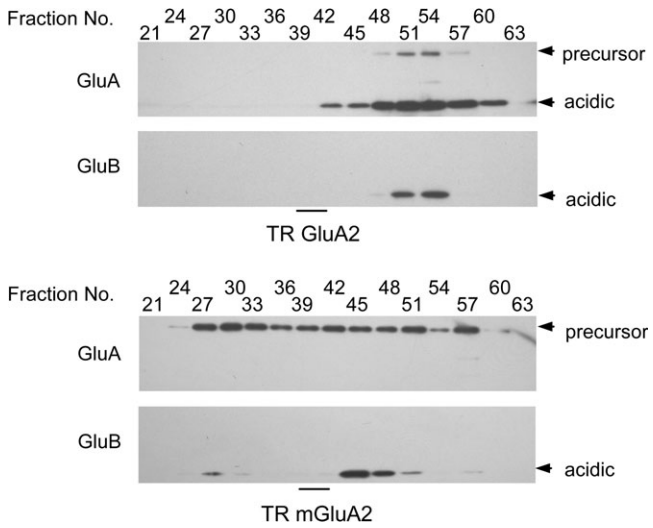

Fig. 8. Gel filtration analysis in saline-insoluble glutelin in a123 and transgenic rice with GluA2 or mGluA2. The numbers are the numbers of fractions collected. Each fraction was analysed by immuno-blot analysis using anti-GluA and anti-GluB antibody. The bars under the panels show the blue dextran (2000 kDa)-through fraction.

intra-molecule disulphide bonds, is different between them, which is responsible for the difference in the polymerized higher-order structure formed through inter-molecule disulphide bonding with other glutelins. GluAs usually have four free-Cys residues in a molecule, whereas GluBs have only one free-Cys residue (Katsube-Tanaka *et al.*, 2004). Unprocessed glutelin precursor (mGluA2) is not involved in the inter-molecule interaction like mature glutelin (GluA2), because free Cys residues may not be exposed to its molecular surface and instead are buried in the molecule. This may explain the difference in polymerization

between them. Further work will be required to determine the relationship between free Cys residues and glutelin polymerization.

Taken together, in rice glutelin, cleavage of the precursor into mature subunits by VPE is necessary for the formation of the compacted high-order structure observed in the processed mature glutelins, because unprocessed mGluA2 precursor resulted in widely distributed unstable polymerized aggregates relative to the endogenously processed GluA2. On the other hand, the processing of glutelin precursor into acidic and basic polypeptides is not responsible for the trafficking of glutelins from the ER to PB-II, or for stable accumulation.

In this study, transgenic a123 expressing glutelin with a mutation at the processing site proved to be useful for studying the glutelin sorting and assembly system in rice seeds. The results obtained using transgenic rice plants clearly address the functional role of post-translational processing from the glutelin precursor to the mature acidic and basic polypeptides during rice seed development.

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