Vacuolar Processing Enzyme plays an Essential Role in the Crystalline Structure of Glutelin in Rice Seed

Kumamaru, Toshihiro
Faculty of Agriculture, Kyushu University

Uemura, Yuji
Faculty of Agriculture, Kyushu University

Inoue, Yoshimi
Faculty of Agriculture, Kyushu University

Takemoto, Yoko
National Institute of Crop Science, National Agriculture and Food Research Organization | Faculty of Agriculture, Kyushu University

他

出版情報：Plant and Cell Physiology. 51 (1), pp.38-46, 2010-01. Oxford University Press (OUP)
バージョン：

Running title: The role of vacuolar processing enzyme in rice seed

Correspondence author

Name: Toshihiro Kumamaru

Address: Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Fukuoka 812-8581, Japan

Telephone number: 81-92-642-3057

Fax number: 81-92-642-3057

e-mail: kumamaru@agr.kyushu-u.ac.jp

Subject areas:

Proteins, enzymes and metabolism

Structure and function of cells

Number of black and white figures: 4

Number of color figures: 2

Number of tables: 1

Title:

Vacuolar processing enzyme plays an essential role in the crystalline structure of glutelin in rice seed

Authors:

Toshihiro Kumamaru[1], Yuji Uemura[1], Yoshimi Inoue[1], Yoko Takemoto[1, 4], Sadar Uddin Siddiqui[1, 5], Masahiro Ogawa[2], Ikuko Hara-Nishimura[3] and Hikaru Satoh[1]

Author's addresses:

[1]Faculty of Agriculture, Kyushu University, Hakozaki 6-10-1, Fukuoka 812-8581, Japan

[2]Faculty of Human Life Science, Yamaguchi Prefectural University, Sakurabatake 3-2-1, Yamaguchi 753-8502, Japan

[3]Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan

Present address:

[4]National Institute of Crop Science, National Agriculture and Food Research Organization, Kannondai 2-1-18, Tsukuba 305-8518, Japan

[5]Plant Genetic Resources Program, National Agricultural Research Center, Islamabad, 45500 Pakistan

Footnote:

The nucleotide sequence reported in this paper has been submitted to DDBJ under accession number AB109637.

Abstract

To identify the function of genes that regulate the processing of proglutelin, we performed an analysis of *glup3* mutants that accumulate excess amounts of proglutelin and lack the vacuolar processing enzyme (VPE). VPE activity in developing seeds from *glup3* lines was reduced remarkably compared to the wild type. DNA sequencing of the *VPE* gene in *glup3* mutants revealed either amino acid substitutions or the appearance of a stop codon within the coding region. Microscopic observations showed that α-globulin and proglutelin were distributed homogeneously within *glup3* protein storage vacuoles (PSVs), and that *glup3* PSVs lacked the crystalline lattice structure typical of wild type PSVs. This suggests that the processing of proglutelin by VPE in rice is essential for proper PSV structure and compartmentalization of storage proteins. Growth retardation in *glup3* seedlings was also observed, indicating that the processing of proglutelin influences early seedling development. These findings indicate that storage of glutelin in its mature form as a crystalline structure in PSVs is required for the rapid use of glutelin as a source of amino acids during early seedling development. In conclusion, VPE plays an important role in the formation of protein crystalline structures in PSV.

Keywords: *Oryza sativa* L., protein storage vacuole, seed, storage protein, vacuolar processing enzyme, crystalline lattice structure

Introduction

During seed development, most seed plants accumulate salt-soluble proteins, 11S and 7S globulins, or alcohol-soluble prolamins in protein bodies (PBs) or protein storage vacuoles (PSVs). However, rice accumulates two kinds of storage proteins, glutelin and prolamin (Krishnan and White 1995, Tanaka et al. 1980). Glutelin, the dominant storage protein in rice, is homologous to leguminous 11S globulins and is accumulated in PSVs (Zhao et al. 1983). Unlike 11S globulins, however, rice glutelins are only soluble in a diluted acid solution. During seed development, rice glutelin polypeptides are initially synthesized on ER membrane as 57-kD proglutelin (Yamagata et al. 1982), which is then transported to the Golgi apparatus and finally to the PSVs (Krishnan et al. 1986, Yamagata and Tanaka 1986), where the proglutelin is cleaved to form acidic and basic subunits (Yamagata et al. 1982).

The PSVs of rice, which is irregularly shaped, accumulate glutelins and a small amount of globulins. The internal structure of the PSV is complex (Beachtel and Juliano 1980, Krishnan et al. 1992), and contains several protein inclusions, which appear to be composed of distinct glutelin aggregates with a crystalline lattice structure (Beachtel and Juliano 1980). Electron microscopy of protein inclusions within PSVs in monocot and dicot seeds suggests that most of the mature 11S globulins assemble to form protein crystalline structures within the PSVs.

In soybean (*Glycine max*), the 11S precursor, proglycinin, is transported into the large central vacuole (Sengupta et al. 1981) where it is proteolytically processed to a mature form. Each mature subunit of glycinin is composed of an acidic and a basic chain that are covalently linked via a single disulfide bond (Staswick et al. 1984). Cleavage of proglycinins is required for the subsequent assembly of proglycinin trimers

into hexamers within the storage vacuoles, suggesting that the cleavage of the protein results in a conformational change that facilitates hexamer formation (Dickinson et al. 1989). This posttranslational processing event is considered an important regulatory mechanism of the vacuolar deposition and assembly of glycinin (Dickinson et al. 1989, Jung et al. 1998, Scott et al. 1992). The cleavage of proglycinin trimers by purified asparaginyl endopeptidase is necessary for their assembly into hexamers *in vitro* (Jung et al. 1998, Nam et al. 1997).

Asparaginyl endopeptidase can be purified from developing leguminous seeds (Abe et al. 1993, Hara-Nishimura 1995, Hara-Nishimura et al. 1991, Muramatsu and Fukazawa 1993, Scott et al. 1992) and is known as vacuolar processing enzyme (VPE). *In vitro* processing assays demonstrated that VPE is an essential processing enzyme that mediates the maturation of several seed proteins in pumpkin and castor beans, including 2S albumin (Hara-Nishimura et al. 1991, Hara-Nishimura et al. 1993), 11S globulins (Hara-Nishimura and Nishimura 1987), and 7S globulin (Yamada et al. 1999). To elucidate the biological function of the enzyme *in vivo*, Gruis et al (2002; 2004) and Shimada et al (2003) generated VPE knockout mutants in *Arabidopsis*. These mutants over-accumulated 12S globulin and 2S albumin precursors in developing seeds. However, the physiological role of VPE in seeds remains unclear because the seeds of the VPE knockout mutants normally germinate and grow, and the proproteins were degraded during germination (Gruis et al. 2002, Shimada et al. 2003).

We have characterized eight independent rice mutants that accumulate large quantities of the 57-kD proglutelin polypeptide. The mutants were named as *endosperm storage protein mutant 2 (esp2)* (Kumamaru et al. 1987, Kumamaru et al. 1988) or *glutelin precursor mutant (glup) 1-7* (Kumamaru et al. 2007, Satoh et al. 1995, Satoh et al. 1994, Satoh et al. 1999, Tian et al. 2001, Ueda et al. 2004a, Ueda et al.

2004b). Among them, high resolution mapping analysis of the *glup3* gene revealed that the gene encoding VPE is located within the map region containing the *Glup3* gene (Kumamaru et al. 2002). Recently, it was reported that VPE is a cysteine protease that is crucial for the maturation of rice glutelins in the PSV (Wang et al. 2009). However, it is unknown whether VPE plays a significant role in the proteolytic cleavage and deposition of storage proteins in the PSV in rice.

To clarify whether defective cleavage of glutelin influences the accumulation of the storage protein, we characterized proglutelin-accumulating *glup3* mutants. Here we show that the loss of VPE activity not only resulted in over-accumulation of proglutelin, but also caused alterations in the structure and morphology of the PSVs and in the compartmentalization of storage proteins, suggesting that the activity of VPE almost certainly plays an important role in the formation of glutelin crystalline structures in PSVs.

Results

Proglutelin accumulation in Rice *glup3* mutants

The major storage proteins of rice visible on SDS-PAGE gels consist of the 40-kD acidic and 20-kD basic subunits of glutelin, the 26-kD globulin polypeptide, the 16-kD, 14-kD, 13-kD, and 10-kD polypeptides of prolamin, and small amounts of the 57-kD proglutelin (Fig. 1, see also Tanaka et al. 1980). Figure 1 presents a comparison of the accumulation of 57 kD proglutelin and the processed 40-kD acidic and 20-kD basic subunits in seeds from wild type Taichung65 rice and from the *glup3* mutant EM856. In comparison with the wild-type Taichung65 line, the rice mutant line contains substantially higher levels of 57-kD proglutelin and correspondingly reduced

Fig. 1.

levels of acidic and basic glutelin subunits (Fig. 1A and 1B). Glutelins are encoded by a multigene family that consists of at least six distinct classes (Takaiwa et al. 1999). In wild type plants, glutelin acidic and basic subunits are separated into 11 and 9 bands, respectively, by IEF analysis (Fig. 1C). The *glup3* mutant contains elevated amounts of six proglutelin polypeptide bands in the pI range of 7.8 - 8.2 compared to wild type. Nearly all of the polypeptide bands of the acidic and basic subunits of mature glutelin were reduced uniformly in the *glup3* mutant, indicating that accumulation of 57 kD proglutelin polypeptides occurs at the expense of processed glutelin subunits.

Genomic DNA sequencing of *VPE* in *glup3* mutants

Sequencing analysis of the BAC clone OSJNBa0091D06 revealed that *Glup3* is located on chromosome 4 (Kumamaru et al. 2002). The *VPE* gene reported by Wang et al (2009) was also present on the same BAC clone. In order to verify that *Glup3* encodes VPE, genomic DNA from wild type and *glup3* lines was sequenced. Figure 2 shows the amino acid substitutions in the *VPE* gene in *glup3* lines. A span of DNA from 1900 nucleotides upstream of the ATG initiation codon to 1500 nucleotides downstream of the termination codon of the *VPE* gene in EM856, PAK22 and HO1274 was sequenced. DNA sequencing analysis of the *glup3* allelic genes showed that Gly-333 in the VPE gene in EM856 was replaced by Asp. In PAK22, Gln-90, Glu-317 and Glu-384 were substituted with Arg, a stop codon, and Gly, respectively, and in HO1274, Cys-269 was replaced by Gly. The substitution in HO1247 was same as in the proglutelin accumulating line described by Wang et al (2009). Gly-333, Glu-317 and Cys-269 are conserved in almost all plant VPEs. The 5' and 3' flanking DNA sequences located upstream of the initiation codon and downstream of the termination codon, were the same in both HO1274 and wild type. In PAK22, some differences in

Fig. 2.

sequence were detected in the 5'flanking region compared to wild type. The deduced amino acid substitution of the VPE in all *glup3* lines demonstrate that the *Glup3* gene encodes the VPE.

Expression analysis of *VPE* in *glup3* mutants

To confirm whether expression of the *VPE* gene in *glup3* lines is suppressed, we analyzed its transcript levels in developing seeds of EM856, PAK22, and HO1274 lines by RT-PCR using the specific primer pairs for VPE (Fig. 3). In wild type, the expected size of a product was detected by both primer combinations. As the expression of VPE gene in developing seed peaks at 12 DAF (Wang et al. 2009), the results at 12 DAF were shown. In EM856 and HO1274 seeds, the amplified products were present at nearly the same level as wild type. In contrast, the amplified product from PAK22 seeds was much lower compared to wildtype. Fig. 3.

To further investigate the mechanism of excess proglutelin accumulation, the activity of proteolytic processing in the seeds of *glup3* mutants was measured using the fluorescent substrate, Ac-Ala-Ala-Asn-MCA. VPE activity was not almost detected in the seeds of any of the *glup3* lines (Supplemental Fig. 1). This suggests that in PAK22 seeds, either the presence of a premature stop codon in the ORF or the reduction of promoter activity results in low levels of VPE transcripts, whereas in EM856 and HO1274 seeds, the amino acid substitutions result in a decrease in VPE activity.

Microscopic analysis

The morphology of *glup3* mutants was investigated by electron microscopy. In wild type endosperm, the glutelin-containing PSV is an irregularly shaped structure with a diameter of 3-4 μm (Fig. 4 A and H). In addition to its shape, PSV is Fig. 4.

characterized by the presence of several distinct protein inclusions that differ in electron density, peripheral dark and lighter staining areas. α-globulin antibody is bound to dark-stained area, while the glutelin antibody was distributed to lighter staining area, indicating that the storage proteins were not randomly distributed, but rather stratified within this organelle. The former is crystalloids with characteristic lattice structure, the latter is the amorphous matrix. (Beachtel and Juliano 1980, Kawagoe et al. 2005, Krishnan et al. 1992, Tanaka et al. 1980). Figure 4H shows the distribution of α-globulin and glutelin in wild type PSV. A crystal lattice structure was detected in the crystalloid (Fig.s 4D and 4I), and does not appear in the matrix (Fig. 4G). The distance between the elements of the crystal lattice was measured in several images, averaging approximately 70 Å. PSVs from two *glup3* lines exhibited properties that were very distinct from those seen in wild type endosperm. The PSVs were spherical with uniform electron-dense staining, and the crystalloids and matrix could not be distinguished from one another (Fig. 4B, 4C, 4J and 4L). No crystalline lattices were observed in *glup3* PSVs (Figs. 4E, 4F, 4K and 4M), and immunoelectron microscopy showed that α-globulin and glutelin were uniformly distributed in the PSV (Fig. 4J and 4L).

The spatial distribution of these storage proteins in wild type was also examined by immunofluorescence microscopy (Fig. 5A-5C). α-globulin distributed in the peripheral area, while glutelin distributed in the whole area and both proteins were almost not overlapped in wild type. The uniform distribution of α-globulin in *glup3* strains was confirmed also by immunofluorescence microscopy (Fig. 5D-5I). It seems that the signal strength of α-globulin in *glup3* (Fig.s 5D and 5G) is higher than that of wild type (Fig. 5A). Fig. 5.

Growth characteristics of *glup3* seedlings

To clarify the physiological significance of VPE activity, the seed germination and growth rate of *glup3* seedlings were investigated. Although the germination rate of *glup3* mutants was almost the same as wild type, growth retardation of the primary leaf of the seedlings was observed. The length from the base of the primary leaf in the seedling was indicated as the plant height in Fig. 6. Under normal conditions, the length of wild type and *glup3* seedlings was nearly the same up to 9 days post germination. However, the primary leaf length of *glup3* seedlings was significantly lower than that of wild type at 14 days (Fig. 6A). Under high temperature conditions, there was no significant difference in the primary leaf length between *glup3* and wild type seedlings at any time point (Fig. 6B). These results demonstrate that the growth rate of *glup3* seedlings is slower than that of wild type, suggesting that growth retardation of the primary leaf of *glup3* seedlings is due to the accumulation of proglutelin in PSV.

Fig. 6.

Protein content in *glup3* endosperm

In order to elucidate whether the uncleavage of proglutelin by VPE affects the protein content, protein fractions extracted from *glup3* endosperm were measured (Table 1). Proglutelin can be extracted along with mature glutelin by 1% lactic acid (Fig. 1). The content of glutelin fraction in wild type and *glup3* mutants was statistically the same, while the albumin/globulin content in *glup3* was significantly higher than that of WT, indicating that impaired cleavage of proglutelin by VPE in *glup3* mutants does not affect the accumulation of glutelin in PSVs and suggesting that increase of the α-globulin expression may result in the increase of the albumin/globulin content in *glup3*.

Table 1.

Discussion

In rice endosperm, glutelins are synthesized on the ER as a 57-kD proglutelin, which is then translocated into the ER lumen and packaged as processed subunits in the PSV. *glup3* mutants accumulate substantial quantities of proglutelin at the expense of processed acidic and basic subunits. Enzymatic assays showed that *glup3* mutant was devoid of VPE activity, suggesting that this loss-of-function was responsible for the abnormal accumulation of proglutelin. This view was substantiated by the identification of a mutant *VPE* gene in *glup3* lines EM856, PAK22 and HO1274 in subsequent genomic DNA sequencing.

Shimada et al. (2003) suggested that substitution of Gln68 by Lys in βVPE might be the cause of the defective βVPE protein in *Arabidopsis*. This residue is highly conserved in members of the VPE family, suggesting that it is important for protein stability. Similarly, the conserved residues Gly333 and Cys269 were replaced with Asp in EM856 and Gly in HO1274, respectively (Fig. 2). Although the protein expression level is unknown, mRNA was transcribed normally in these *glup3* lines, as judged by RT-PCR (Fig. 3). Cys83, His180 and Cys222 residues, possibly located within active site of VPE (Kinoshita et al. 1995), were conserved in the VPE in EM856 and HO1274. This suggests that Gly333 and Cys269 might be responsible for maintaining protein stability.

A decreased amount of cleaved glutelin was detected in *glup3* endosperm (Fig.1) and levels of VPE activity were almost undetectable (Supplementary Fig. 1). In *Arabidopsis*, the expression of βVPE is seed specific, while α and γVPEs are expressed only in vegetative tissue (Hara-Nishimura et al. 1998). Although seeds from

the *Arabidopsis* βVPE knockout mutant accumulated mature globulin and albumin as well as both precursors, seeds from the α/β/γVPEs knockout mutant accumulated no mature storage proteins, but did accumulate large amounts of both protein precursors (Gruis et al. 2004, Gruis et al. 2002, Shimada et al. 2003). This indicates that vegetative VPEs can compensate for defective βVPE in βVPE knockout mutant seeds and suggests the existence of a VPE homologue that is not coded by the *Glup3* gene in rice. Kato and Minamikawa (1996) identified the cysteine endopeptidases, REP1 and REP2, in late maturing and germinating rice seeds and showed that REP1 was responsible for the degradation of glutelin in germinating seeds. Kato et al. (2003) identified and cloned REP2 from rice seed, showed that it had a gene structure similar to β and γVPEs in *Arabidopsis*, and reported that REP2 might function in the processing of REP1. Since REP2 mRNA is also expressed in the late maturing stage of rice seeds (Kato et al. 2003), REP2 might partially compensate for impaired cleavage of proglutelin in *glup3* mutant seeds.

VPE has a significant effect on PSV morphology and ultrastructure (Figs. 4 and 5). The wild type PSV is irregularly shaped and contains inclusions of varying electron density and ordered structure (crystalline vs. amorphous). The latter two aspects are due to the spatial sequestration of glutelin and globulin. In contrast, the *glup3* PSV was spherical and of uniform electron density, the latter of which was due to a random distribution of glutelin and α-globulin. These drastic changes suggest that the establishment of normal morphology in the PSV requires the cleavage of proglutelin into the mature type by Glup3-VPE.

The seeds in null VPE *Arabidopsis* mutants germinated and grew normally, as their storage protein precursors were readily degraded during germination (Gruis et al. 2002, Shimada et al. 2003). During germination, glutelin acts as a source of amino

acids for early seedling growth. PSVs containing glutelin are disappeared from the central portion by the activation of an internal latent protease at germination. The disappearance of PSVs occurs earlier than that of prolamin-containing PBs (Horikoshi and Morita 1982). Though the glutelin content in *glup3* endosperm was almost the same as in the wild-type (Table 1), growth retardation of the primary leaf in *glup3* seedlings was observed (Fig. 6) and the degradation of proglutelin and mature glutelins in *glup3* was slightly slower compared to that of mature glutelins in wild-type plants (Supplemental Fig. 2). From these results, it is speculated that the mature form with a crystalline structure of glutelin in PSVs may be required for the rapid use of glutelin as a source of amino acids during early seedling development.

Glutelin accumulates in a processed and crystalline form within the PSV (Sugimoto et al. 1986). Cleavage of proglycinins by asparaginyl endopeptidase is required for the assembly of proglycinin trimers into hexamers within the storage vacuoles, suggesting that cleavage results in a conformational change that facilitates hexamer formation (Chrispeels et al. 1982). Moreover, it is likely that hexamer formation enables glutelin to form a larger, more complex crystalline structure and thereby accounts for its spatial isolation from globulin. The size of a glycinin hexamer was reported to be 80-95 Å (Adachi et al. 2003). The average distance between the elements of the crystalline lattice of glutelin was about 70 Å, which was measured and calculated from the images of the PSVs in Fig. 4. Although the size of the glutelin hexamer in PSVs is unknown, the average distance between the elements of a glutelin crystalline lattice and the size of a glutelin hexamer would be similar if it is assumed that the size and structure of glutelin hexamers are similar to glycinin hexamers. Posttranslational processing is indispensable for molecular association of glycinin trimers into hexamers (Adachi et al. 2003), suggesting that hexamer formation is a

prerequisite for crystalline lattice formation of glutelin. The activity of VPE almost certainly plays an important role in the formation of glutelin crystalline structures in PSVs.

Materials and Methods

Plant Materials

Three primary varieties of rice were used in this study: the induced *glup3* mutant line EM856 produced by N-methyl-N-nitrosourea (MNU) treatment of Taichung65; the Chinese rice variety HO1274 in which the high accumulation of substantial amounts of the 57-kD polypeptide have been described (Satoh et al. 1995); and the Pakistan variety PAK22 (Siddiqui et al. 2003). EM856 was used as the representative *glup3* line unless otherwise indicated. The mutant and the parental line, Taichung65, were grown in the field and developing seeds obtained at 5 to 20 days after flowering (DAF) were used in biochemical and electron microscopic studies.

Protein Extraction

Rice glutelin and proglutelin were extracted by treatment with 1% lactic acid after pretreatment with 10 mM Tris-HCl (pH 7.5) that contained 0.5 M NaCl for globulin removal. Extraction was accomplished by suspending milled rice in the solvent solution with sonication for 1 min. After centrifugation, the residue was extracted twice more with the solvent.

Protein Measurement

Extracted protein fractions were precipitated according to the method of Kumamaru et al. (1988) and the protein content of each fraction was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin as a standard.

SDS-PAGE and Western Blot Analysis

Storage proteins were extracted from mature seeds in 4% SDS, 4M urea, 5% 2-mercaptoethanol, and 0.125 M Tris-HCl, pH 6.8. SDS-PAGE analysis was conducted on 15% to 25% polyacrylamide concentration gradient gels (Laemmli 1970). After electrophoresis, gels were stained with Coomassie brilliant blue. For western blot analysis, proteins resolved by SDS-PAGE were transferred to nitrocellulose membranes, which were then incubated with indicated antibodies. Antibody-antigen reactions were visualized using a commercial ECL detection kit (GE healthcare, Buckinghamshire, UK).

IEF Electrophoresis

Horizontal IEF slab gels were prepared [6 M urea, 4% acrylamide, 2% NP-40, 2% Ampholine (pH3.5-10.0: pH6.0-8.0: pH8.0-10.5, at a 1:1:1 ratio)] and electrophoresed according to the method described by (Brinegar and Peterson 1982). Glutelins, extracted from rice endosperm as described above, were precipitated by neutralization of the solution with 1N NaOH, and the pellet was dissolved in 8.5 M urea, 2% Nonidet P-40, and 5% 2-mercaptoethanol (O'Farrell 1975) and then subjected to IEF electrophoresis according to the procedure of (Wall et al. 1984). After IEF, the gel was incubated in 15% trichloroacetic acid for 20 min, stained with 0.15% Coomassie brilliant blue R-250 in 50% ethanol and 10% acetic acid, and then destained with 25% ethanol and 10% acetic acid.

Assay of VPE activity

VPE activity was measured according to the method described by Shimada et al. (2003) that uses the fluorescent VPE-specific substrate, Ac-AAN-MCA [Acetyl-glutamate-Seryl-Glutamyl-Asparagine α- (4-Metyl-Coumary-7-Amide); Peptide Institute, Inc., Osaka, Japan]. Crude enzyme extract prepared from developing seeds was incubated with 100 μM Ac-AAN-MCA in an acidic buffer [100mM sodium-acetate (pH 5.5), and 100mM dithiothreitol] for 2h at 20˚C. The fluorescence intensity was measured using a GENios plate reader (TECAN Japan, Tokyo, Japan). The fluorescence was determined at an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

Antibodies

Seed storage proteins were separated by SDS-PAGE and individual bands were excised and solubilized by preparative electrophoresis. Antibodies to glutelin acidic and basic subunits were then raised in rabbit. Antibody to α-globulin polypeptide were raised in mouse.

DNA sequencing analysis

Total genomic DNA from leaves of the three *glup3* lines and wild type was obtained by a cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). DNA sequences were determined using an ABI PRISMR 3100 Genetic Analyzer (Applied Biosystems Ltd.). DNA sequence analysis was performed using EditView1.0.1 and AutoAssembler 2.1. (Applied Biosystems Ltd.). Comparisons between wild type and mutant sequences were done with CLUSTALW

from the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/top-e.html).

RT-PCR Analysis

Total RNA was extracted from developing seeds using an RNA extraction kit (Nippon Gene Co. Ltd., Tokyo, Japan). First strand cDNA was synthesized from total RNA with the T-Primed First Strand Kit (Amersham Bioscience Corp., Piscataway) and the cDNA was used as template. The primers used are as follows:
AL - gaggaggaggaggggaagt; G4R - tcaggaggaggtgatggttc; G5L - gaaccatcacctcctcctga; G6R – tgttgccatttgcaggatta.

Transmission Electron Microscopy

Transverse sections (1-2 mm thick) of developing seeds were fixed for 1h in 1.5% paraformaldehyde and 2.5% glutaraldehyde, buffered at pH 7.2 in PIPES buffer [20 mM piperazine-N, N'-bis (2-ethane sulfuric acid)]. The samples were thoroughly washed with PIPES buffer, post-fixed for 2 h in 1% osmium tetroxide, and dehydrated by a serial ethanol washes. Thin sections were embedded in Spurr's low viscosity resin as described by (Baba et al. 1991), sectioned with an ultramicrotome (Reichert, Depew, NY), then stained sequentially with uranyl acetate and lead citrate solution. Microscopic analyses were carried out with a transmission electron microscope (JEM 200C, Electron Optics Lab. Co., Ltd., Tokyo, Japan).

Immunofluorescence Microscopy

Sections were treated with blocking buffer containing 0.8% BSA, 0.1% gelatin, and 2mM NaN3 in PBS, then incubated in the appropriate antibodies diluted in blocking buffer. Non-specifically bound antibodies were removed by washing the section several

times in PBS. The sections were incubated with secondary antibody [anti-rabbit FITC (Green) or anti-rabbit Rhodamine (Red), Funakoshi Chemical Co., Ltd., Tokyo, Japan] and observed microscopically (AX 80, Olympus, Tokyo, Japan).

Supplemental Material

Supplemental Figure 1

Analysis of VPE activity in developing seeds of *glup3* mutants and wild type at 5 and 10 days after flowering.

Supplemental Figure 2

Developmental changes in the amount of storage proteins in *glup3* and wild type seeds grown in 25˚C and 20˚C conditions during day and night, respectively.

Funding

This work was supported partly by a grant-in-aid for Scientific Research on Priority Areas (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (1213826) and grants from the Bio-oriented Technology Research Advanced Institution (BRAIN), and the Ministry of Agriculture, Forest and Fish of Japan (MP1108).

References

Abe, Y., Shirane, K., Yokosawa, H., Matsushita, H., Mitta, M., Kato, I. and Ishii, S. (1993) Asparaginyl endopeptidase of jack bean seeds. Purification, characterization, and high utility in protein sequence analysis. *J. Biol. Chem.* 268: 3525-3529.

Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B. and Utsumi, S. (2003) Crystal structure of soybean 11S globulin: glycinin A3B4 homohexamer. *Proc. Natl. Acad. Sci. U S A* 100: 7395-7400.

Baba, K., Ogawa, M., Nagano, A., Kuroda, H. and Sumiya, K. (1991) Developmental changes in the bark lectin of Sophora japonica L. *Planta* 183: 462-470.

Beachtel, D.B. and Juliano, B.O. (1980) Formation of protein bodies in the starchy endoserm of rice (*Oryza sativa* L.): a re-investigation. *Ann Bot* 45: 503-509.

Brinegar, A.C. and Peterson, D.M. (1982) Separation and characterization of oat globulin polypeptides. *Arch. Biochem. Biophys.* 219: 71-79.

Chrispeels, M.J., Higgins, T.J. and Spencer, D. (1982) Assembly of storage protein oligomers in the endoplasmic reticulum and processing of the polypeptides in the protein bodies of developing pea cotyledons. *J Cell Biol* 93: 306-313.

Dickinson, C.D., Hussein, E.H. and Nielsen, N.C. (1989) Role of posttranslational cleavage in glycinin assembly. *Plant Cell* 1: 459-469.

Gruis, D., Schulze, J. and Jung, R. (2004) Storage protein accumulation in the absence of the vacuolar processing enzyme family of cysteine proteases. *Plant Cell* 16: 270-290.

Gruis, D.F., Selinger, D.A., Curran, J.M. and Jung, R. (2002) Redundant proteolytic mechanisms process seed storage proteins in the absence of seed-type members of the vacuolar processing enzyme family of cysteine proteases. *Plant Cell* 14: 2863-2882.

Hara-Nishimura, I. (1995) Vacuolar processing enzyme responsible for maturation of vacuolar proteins. *Seikagaku* 67: 372-377.

Hara-Nishimura, I., Inoue, K. and Nishimura, M. (1991) A unique vacuolar processing enzyme responsible for conversion of several proprotein precursors into the mature forms. *FEBS Lett.* 294: 89-93.

Hara-Nishimura, I., Kinoshita, T., Hiraiwa, N. and Nishimura, M. (1998) Vacuolar processing enzymes in protein-storage vacuoles and lytic vacuoles. *J. Plant Physiol.* 152: 668-674.

Hara-Nishimura, I. and Nishimura, M. (1987) Proglobulin Processing Enzyme in Vacuoles Isolated from Developing Pumpkin Cotyledons. *Plant Physiol.* 85: 440-445.

Hara-Nishimura, I., Takeuchi, Y. and Nishimura, M. (1993) Molecular characterization of a vacuolar processing enzyme related to a putative cysteine proteinase of Schistosoma mansoni. *Plant Cell* 5: 1651-1659.

Horikoshi, M. and Morita, Y. (1982) Changes in ultrastructure and subunit composition of protein body in rice endosperm during germination. *Agric. Biol. Chem.* 46: 269-274.

Jung, R., Scott, M.P., Nam, Y.W., Beaman, T.W., Bassuner, R., Saalbach, I., Muntz, K. and Nielsen, N.C. (1998) The role of proteolysis in the processing and assembly of 11S seed globulins. *Plant Cell* 10: 343-357.

Kato, H. and Minamikawa, T. (1996) Identification and characterization of a rice cysteine endopeptidase that digests glutelin. *Eur J Biochem* 239: 310-316.

Kato, H., Sutoh, K. and Minamikawa, T. (2003) Identification, cDNA cloning and possible roles of seed-specific rice asparaginyl endopeptidase, REP-2. *Planta* 217: 676-685.

Kawagoe, Y., Suzuki, K., Tasaki, M., Yasuda, H., Akagi, K., Katoh, E., Nishizawa, N.K., Ogawa, M. and Takaiwa, F. (2005) The critical role of disulfide bond formation in protein sorting in the endosperm of rice. *Plant Cell* 17: 1141-1153.

Kinoshita, T., Nishimura, M. and Hara-Nishimura, I. (1995) The sequence and expression of the gamma-VPE gene, one member of a family of three genes for vacuolar processing enzymes in Arabidopsis thaliana. *Plant Cell Physiol.* 36: 1555-1562.

Krishnan, H.B., Franceschi, V.R. and Okita, T.W. (1986) Immunochemical studies on

the role of the Golgi complex in protein-body formation in rice seeds. *Planta* 169: 471-480.

Krishnan, H.B. and White, J.A. (1995) Morphometric analysis of rice seed protein bodies (Implication for a significant contribution of prolamine to the total protein content of rice endosperm). *Plant Physiol* 109: 1491-1495.

Krishnan, H.B., White, J.A. and Pueppke, S.G. (1992) Characterization and location of rice (Oryza sativa L.) seed globulins. *Plant Sci.* 81: 1-11.

Kumamaru, T., Ogawa, M., Satoh, H. and Okita, T.W. (2007) Protein body biogenesis in cereal endosperms. In Endosperm – Development and Molecular Biology. Edited by Olsen, O.A. pp. 141-158. Springer-Verlag, Berlin.

Kumamaru, T., Satoh, H., Iwata, N., Omura, T. and Ogawa, M. (1987) Mutants for rice storage proteins. III. Genetic analysis of mutants for storage proteins of protein bodies in the starchy endosperm. *Jpn. J. Get.* 62: 333-339.

Kumamaru, T., Satoh, H., Iwata, N., Omura, T., Ogawa, M. and Tanaka, K. (1988) Mutants for rice storage proteins. 1. Screening of mutants for rice storage proteins of protein bodies in the starchy endosperm. *Theor. Appl. Genet.* 76: 11-16.

Kumamaru, T., Uemura, Y., Takemoto, Y., Ogawa, M. and Satoh, H. (2002) High-resolution mapping of *glup3* gene accumulating high amount of glutelin precursor. *Rice Genet. Newslet.* 19: 62-63.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.

Muramatsu, M. and Fukazawa, C. (1993) A high-order structure of plant storage proprotein allows its second conversion by an asparagine-specific cysteine protease, a novel proteolytic enzyme. *Eur. J. Biochem.* 215: 123-132.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.

Nam, Y.W., Jung, R. and Nielsen, N.C. (1997) Adenosine 5'-triphosphate is required for the assembly of 11S seed proglobulins in vitro. *Plant Physiol.* 115: 1629-1639.

O'Farrell, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250: 4007-4021.

Satoh, H., Kumamaru, T., Ogawa, M., Shiraishi, M., Im, B.G., Son, M.Y. and Takemoto, Y. (1995) Spontaneous 57 kDa mutant in rice. *Rice Genet. Newslet.* 12: 194-196.

Satoh, H., Kumamaru, T., Yoshimura, S. and Ogawa, M. (1994) New 57kDa glutelin genes on chromosome 9 in rice. *Rice Genet. Newslet.* 11: 158-161.

Satoh, H., Li, W.X., Takemoto, Y., Ito, T., Kumamaru, T., Qu, L.Q. and Ogawa, M. (1999) *glup4* controlling a 57H character was located on chromosome 12 in rice. *Rice Genet Newslet* 16: 98-100.

Scott, M.P., Jung, R., Muntz, K. and Nielsen, N.C. (1992) A protease responsible for post-translational cleavage of a conserved Asn-Gly linkage in glycinin, the major seed storage protein of soybean. *Proc. Natl. Acad. Sci. U S A* 89: 658-662.

Sengupta, C., Deluca, V., Bailey, D.S. and Verma, D.P.S. (1981) Post-translational processing of 7S and 11S components of soybean storage proteins. *Plant Mol. Biol.* 1: 19-34.

Shimada, T., Yamada, K., Kataoka, M., Nakaune, S., Koumoto, Y., Kuroyanagi, M., Tabata, S., Kato, T., Shinozaki, K., Seki, M., Kobayashi, M., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (2003) Vacuolar processing enzymes are essential for proper processing of seed storage proteins in Arabidopsis thaliana. *J. Biol. Chem.* 278: 32292-32299.

Siddiqui, S.U., Satoh, H. and Kumamaru, T. (2003) Variation in seed storage proteins of Pakistani rice germplasm. In Advance in Rice Genetics. Edited by Khush, G.S., Brar, D.S. and Hardy, B. pp. 198-200. Intenational Rice Research Institute, Manila.

Staswick, P.E., Hermodson, M.A. and Nielsen, N.C. (1984) Identification of the cystines which link the acidic and basic components of the glycinin subunits. *J. Biol. Chem.* 259:

13431-13435.

Sugimoto, T., Tanaka, K. and Kasai, Z. (1986) Molecular species in the protein body II (PB-II) of developing rice endosperm. *Agric. Biol. Chem.* 50: 3031-3035.

Takaiwa, F., Ogawa, M. and Okita, T.W. (1999) Rice glutelins. In Seed proteins. Edited by Shewry, P.R. and Casey, R. pp. 401-425. Kluwer Academic Publishers, Dordrecht.

Tanaka, K., Sugimoto, T., Ogawa, M. and Kasai, Z. (1980) Isolation and characterization of two types of protein bodies in the rice endosperm. *Agric. Biol. Chem.* 44: 1633-1639.

Tian, H.D., Kumamaru, T. and Satoh, H. (2001) Gene analysis of 57H mutant gene, *glup6*, in rice. *Rice Genet. Newslet.* 18: 48-50.

Ueda, Y., Satoh, H., Satoh, M., Takemoto, Y., Kumamaru, T. and Ogawa, M. (2004a) Inheritance mode of *Glup5* gene and the genetic relationship with other 57 mutant genes. *Rice Genet Newslet* 21: 49-51.

Ueda, Y., Sugino, A., Takemoto, Y., Satoh, M., Kumamaru, T., Ogawa, M. and Satoh, H. (2004b) A novel 57H mutant gene, *glup7*, located on chromosome 4. *Rice Genet Newslet* 21: 51-54.

Wall, J.S., Fey, D.A. and Paulis, J.W. (1984) Improved two-dimensional electrophoretic separation of zein proteins: application to study of zein inheritance in corn genotypes.

Cereal Chem. 61: 141-146.

Wang, Y., Zhu, S., Liu, S., Jiang, L., Chen, L., Ren, Y., Han, X., Liu, F., Ji, S., Liu, X. and Wan, J. (2009) The vacuolar processing enzyme OsVPE1 is required for efficient glutelin processing in rice. *Plant J* 58: 606-617.

Yamada, K., Shimada, T., Kondo, M., Nishimura, M. and Hara-Nishimura, I. (1999) Multiple functional proteins are produced by cleaving Asn-Gln bonds of a single precursor by vacuolar processing enzyme. *J. Biol. Chem.* 274: 2563-2570.

Yamagata, H., Sugimoto, T., Tanaka, K. and Kasai, Z. (1982) Biosynthesis of storage proteins in developing rice seeds. *Plant Physiol.* 70: 1094-1100.

Yamagata, H. and Tanaka, K. (1986) The site of synthesis and accumulation of storage proteins. *Plant Cell Physiol.* 27: 135-145.

Zhao, W.-M., Gatehouse, J.A. and Boulter, D. (1983) The purification and partial amino acid sequence of a polyepeptide from the glutelin fraction of rice grains; homology to pea legumin. *FEBS Lett.* 162: 96-102.

Figure legends

Figure 1.

Storage protein composition in the endosperm of wild type Taichung65 (WT) or *glup3*

mutant. Storage protein extracts were separated on SDS-PAGE gels that were stained with Coomassie blue (A) or transferred to nitrocellulose membranes and incubated with antibodies to glutelin acidic subunit or glutelin basic subunit (B). Glutelin extracts by 1% lactic acid from *glup3* and wild type endosperms were analyzed by IEF (C). The pIs of the 57 kD polypeptide and glutelin acidic and basic subunits are depicted. The closed arrowhead denotes the 57-kD polypeptide.

Figure 2.

The structure of the *glup3* candidate sequence and amino acid substitutions in three *glup3* mutant lines. ATG and TGA indicate the initiation and termination codons. Numerals under the sequence indicate the position of the exon. EM856, induced *glup3* mutation; PAK22, Pakistan variety; HO1274, Chinese variety.

Figure 3.

Expression analysis of VPE transcripts in wild type and the three *glup3* lines at 12 DAF. AL, G4R, G5L and G6R are VPE gene specific primers. PDI, protein disulfide isomerase; WT, wild type; 1, EM856; 2, PAK22; 3, HO1274.

Figure 4.

Electron microscopy of developing endosperm at 14 DAF of wild type Taichung65 (A, H) and the *glup3* mutants, EM856 (B, J) and HO1274 (C, L). A-F and G-M are samples embedded in epoxy and LR white resins, respectively. D, E, F, K and M are enlarged images of the areas inside the boxes in A, B, C, J and L, respectively. G and I are the enlarged images of areas inside the upper and lower boxes in H. D and I show the lattice structure in a PSV and the red parallel lines indicate the framework of the

crystal lattice. Stars in A-C indicate prolamin-accumulating PBs. In G-M, anti-glutelin and anti-globulin antibodies were labeled with 5nm and 15nm immunogolds particles, respectively. Bar=1 μm in A, B and C, 0.4 μm in H, J and L, 0.04 μm in D, E, F, K and M, 0.02 μm in G and I. St in B indicates the starch granule.

Figure 5.

Immunofluorescence microscopy of the developing endosperm of wild type Taichung65 (A, B, C) and the *glup3* mutants, EM856 (D, E, F) and HO1274 (G, H, I) at 20 DAF. A, D and G were treated with an antibody against globulin, and C, F and I were treated with glutelin antibody. Secondary antibodies labeled by FITC (A, D, G) and Rhodamine (C, F, I) were used. B, E and H are the merged images of A and C, D and F, and G and I, respectively. Bar=5 μm

Figure 6

Analysis of seedling growth rate in *glup3* mutant EM856 and wild type Taichung65. The length from the base of the primary leaf in the seedlings is indicated as plant height. The seeds were grown on agar medium with a diurnal cycle of 14 h of light and 8 h of darkness under two different temperature conditions: A: 25˚C and 20˚C in light and darkness, respectively; B: 35˚C and 30˚C in light and darkness, respectively. Each point represents the mean of 30 seedlings. Error bars indicate the standard deviation. Asterisks indicate significant differences between *glup3* and wild type by Student's *t*-test (***: $P<0.001$).

Supplemental Figure 1

Analysis of VPE activity in developing seeds of *glup3* mutants and wild type at 5 and

10 DAF. The levels of VPE activity at 30 min. after the start of the enzyme assay are shown. 5 and 10 mean the immature seeds at 5 and 10 DAF, respectively. Five grains in each developing stage were assayed. EM856, PAK22, HO1274 are *glup3* mutant lines. Error bars indicate the standard deviation. WT, wild type

Supplemental Figure 2

Developmental changes in the amount of storage proteins in *glup3* and wild type seeds grown in 25˚C and 20˚C conditions during day and night, respectively. A: wild type, B: *glup3* EM856. M: mature seed.

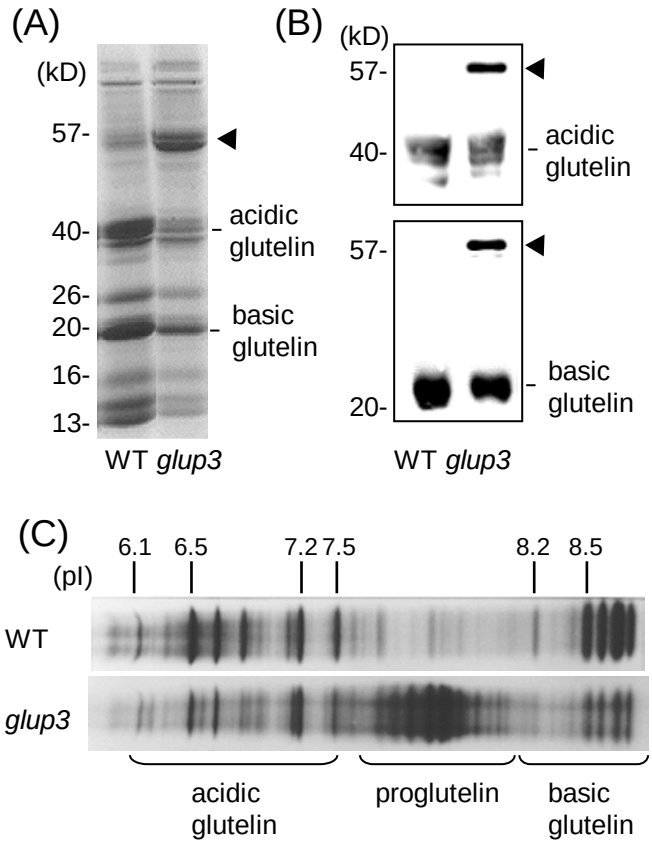


Figure 1

Kumamaru et al

glup3 line	amino acid substitution		
EM856		Gly333Asp	
PAK22	Gln90Arg	Glu317stop	Glu384Gly
HO1274	Cys269Gly		

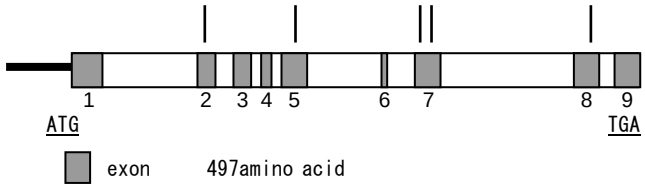


Figure 2

Kumamaru et al

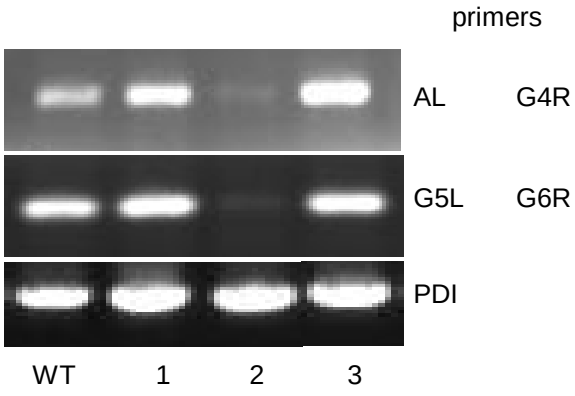


Figure 3

Kumamaru et al

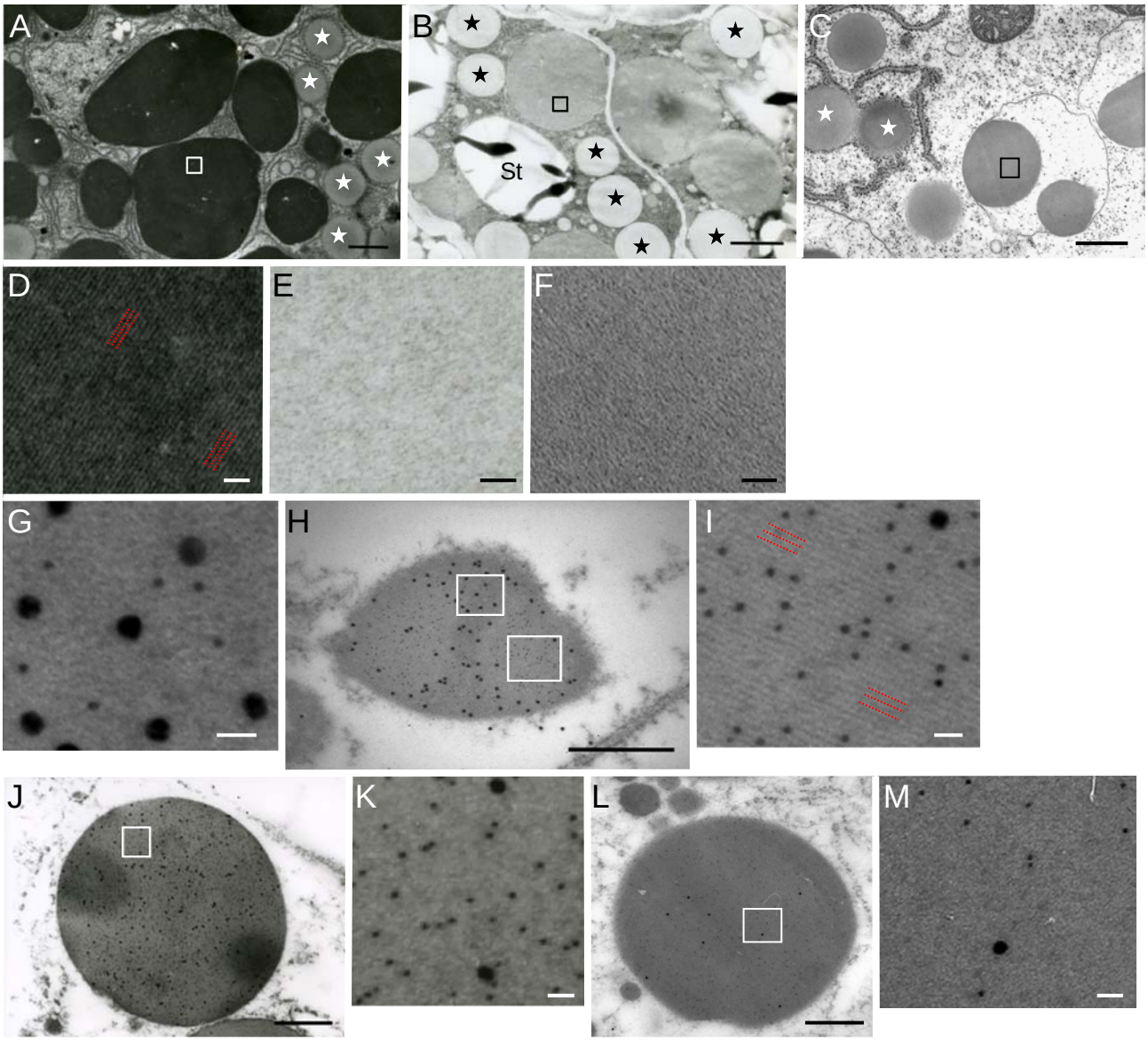


Figure 4

Kumamaru et al

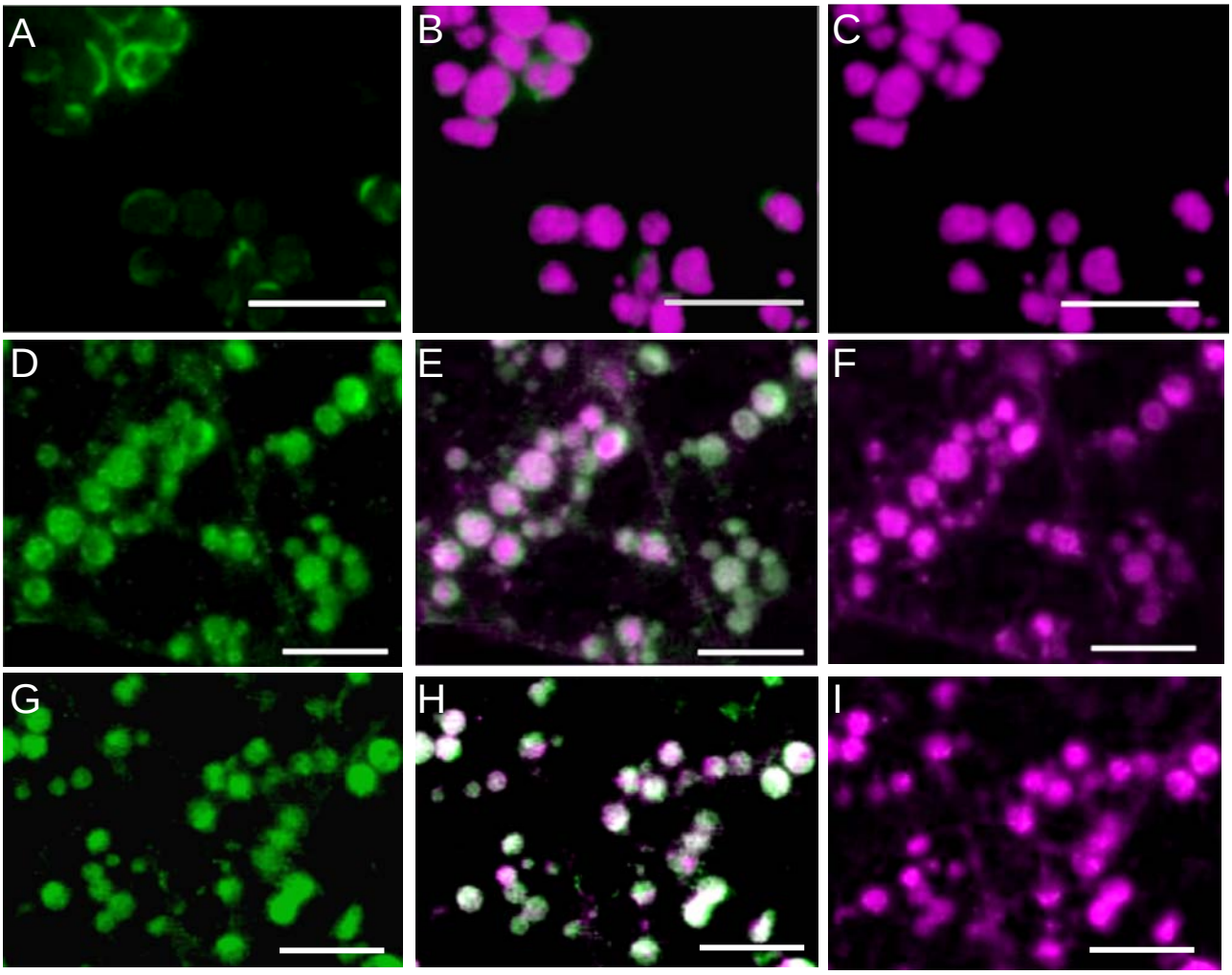
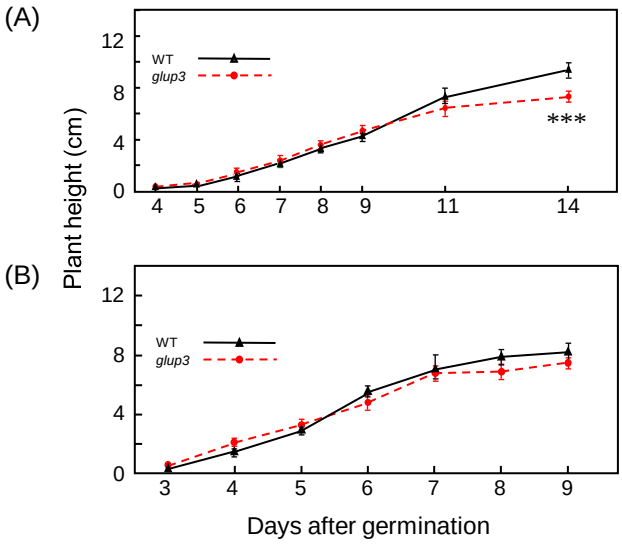


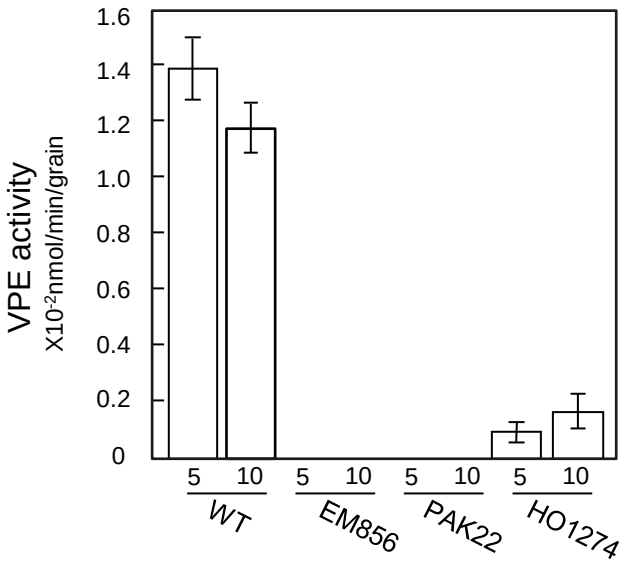
Figure 5

Kumamaru et al



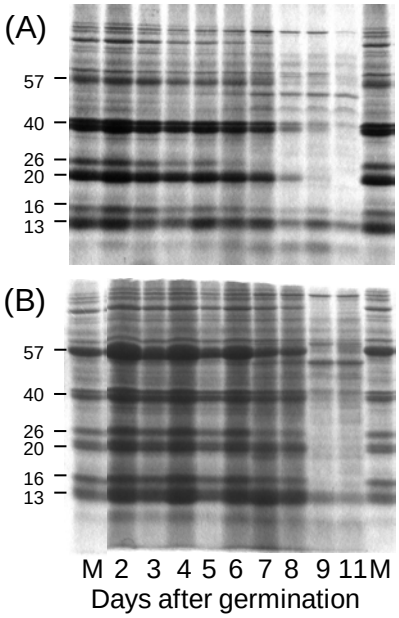
Kumamaru et al

Figure 6



Supplemental Figure 1

Kumamaru et al



Supplemental Figure 2

Kumamaru et al

Table 1. Protein content in *glup3* and wild type endosperms.

	Protein content (mg/100mg polished grain)	
Protein fraction	WT	EM856
Albumin/Globulin	1.372±0.219	2.018 ±0.074***
Glutelin	3.535±0.205	3.495±0.047

Asterisks indicate significant differences between *glup3* and wild type by Student's *t* -test (***: $P < 0.001$).

Values indicate the mean ± the standard deviation