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The Dual Roles of the Golgi Transport 1 (GOT1B): RNA Localization to the Cortical Endoplasmic Reticulum and the Export of Proglutelin and α-Globulin from the Cortical ER to the Golgi

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The rice *glup2* lines are characterized by their abnormally high levels of endosperm 57 kDa proglutelins and of the luminal chaperone binding protein (BiP), features characteristic of a defect within the endoplasmic reticulum (ER). To elucidate the underlying genetic basis, the *glup2* locus was identified by map based cloning. DNA sequencing of the genomes of three *glup2* alleles and wild type demonstrated that the underlying genetic basis was mutations in the Golgi transport 1 (GOT1B) coding sequence. This conclusion was further validated by restoration of normal proglutelin levels in a *glup2* line complemented by a *GOT1B* gene. Microscopic analyses indicated the presence of proglutelin-α-globulin-containing intracisternal granules surrounded by prolamine inclusions within the ER lumen. As assessed by in situ reverse transcriptase polymerase chain reaction (RT-PCR) analysis of developing endosperm sections, prolamine and α-globulin RNAs were found to be mis-targeted from their usual sites on the protein body ER to the cisternal ER, the normal sites of proglutelin synthesis. Our results indicate that GLUP2/GOT1B has a dual role during rice endosperm development. It is required for localization of prolamine and α-globulin RNAs to the protein body ER and for efficient export of proglutelin and α-globulin proteins from the ER to the Golgi apparatus.

Keywords: Endoplasmic reticulum • Golgi transport • Intracellular transport • Proglutelin • Protein body • Storage protein.

Abbreviations: BiP, binding protein; COP, coat protein; CTAB, cetyltrimethylammonium bromide; EM, endosperm mutant; Esp, endosperm storage protein; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; GOT, Golgi transport; Glup, glutelin precursor; MNU, *N*-methyl-*N*-nitrosourea; ORF, open reading frame; PB, protein body; PBS, phosphate-buffered saline; PDIL, protein disulfide isomerase like; PSV, protein storage vacuole; PVC, prevacuolar compartment; RiceXRro, rice expression profile database; Sar, secretion-associated Ras-related protein; Sec, secretory; SNP, single nucleotide polymorphism; TBS, Tris buffer saline; TILLING, targeting induced local lesions in genomes; TIP, tonoplast intrinsic protein; RT, reverse transcript; WAF, week after flowering; WT, wild type.

Introduction

The major seed storage proteins are synthesized on the endoplasmic reticulum (ER) and transported to the protein storage vacuole (PSV) via the Golgi apparatus (Chrispeels 1983). Unlike most other plants, rice seeds synthesize and accumulate abundant amounts of three storage proteins: the acid and alkali soluble glutelins, alcoholic-soluble prolamines and saline-soluble α-globulins. These storage proteins are deposited in two compartments in the endomembrane system. The rice prolamines are deposited as intracisternal granules within the endoplasmic reticulum (ER) lumen to form (PB)-1 (Tanaka et al. 1980, Yamagata and Tanaka 1986, Ogawa et al. 1987), whereas glutelins and α-globulins are transported to the PSV to form PB-II (Yamagata et al. 1982, Krishnan and Okita 1986, Sugimoto et al. 1986). Because PB-Is are bounded by rough ER, the cortical ER is composed of two membrane interconnecting subdomains, protein body ER (PB-ER), which delimits the prolamine intracisternal inclusions, and cisternal ER (Choi et al. 2000, Hamada et al. 2003). These two subdomains can be readily distinguished by the composition of mRNAs and lumenal chaperones. The PB-ER is enriched for prolamine and α-globulin RNAs, while cisternal ER harbors glutelin mRNAs (Choi et al. 2000, Hamada et al. 2003, Li et al. 1993a). The lumenal compartment of PB-I is enriched for binding protein (BiP) (Li et al.

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1993b), while the protein disulfide isomerase like 1-1 (PDIL1-1) is excluded from this lumenal compartment and is observed only in the cisternal ER (Satoh-Cruz et al. 2010).

Proglutelins as well as α-globulins are transported to the PSV via the Golgi apparatus (Yamagata et al. 1982, Krishnan et al. 1986, Sugimoto et al. 1986). At the PSV, proglutelins are proteolytically processed into acid and basic subunits (Kumamaru et al. 2010). Using the levels of proglutelin as a biochemical marker, we identified the *esp2* and *glup1* to *7* mutations that accumulate abnormal elevated amounts of this storage protein (Kumamaru et al. 2007). These mutations are likely in genes that function in the synthesis, transport, processing and packaging of proglutelin. Genetic studies showed that the *esp2* mutation is epistatic to all of the other mutations. While *Glup1*, *glup2* and *glup7* mutations are hypostatic to *esp2* mutation, they are epistatic to *glup4*, *glup5* and *glup6* mutations, which, in turn are epistatic to *glup3* mutation (Ueda et al. 2010).

Several of these mutations have been defined at the gene level. The *esp2* mutant gene codes for a defective PDIL1-1, a chaperone required for the proper folding of proglutelin within the ER lumen (Takemoto et al. 2002, Satoh-Cruz et al. 2010). The *glup4* and *glup6* lines contain mutations in the small GTPase, Rab5a and the guanine nucleotide exchange factor (GEF), the activator of Rab5a, respectively. Both proteins participate in the intracellular transport of proglutelin from the Golgi apparatus to the PSV as well as endosome formation (Fukuda et al. 2011, Fukuda et al. 2013). The *glup3* mutation was shown be an alteration in the gene coding for the vacuolar processing enzyme, which participates in the cleavage of the proglutelin to two subunits within the PSV (Kumamaru et al. 2010). Overall, mutations that affect processes within the ER lumen (*esp2*), from Golgi to the PSV (*glup4* and *glup6*), and at the PSV (*glup3*) have been characterized. However, factors responsible for the intracellular trafficking of storage proteins from the ER to Golgi have not been identified as of yet.

Based on genetic and biochemical evidence, we predict that the *Glup1*, *glup2* and *glup7* mutations affect factors responsible for the export of protein from the ER and their delivery to the Golgi. These mutations are all hypostatic to *esp2* (ER lumen) but epistatic to *glup4* and *glup6* (Golgi to PSV). Moreover, all three mutant plant lines display elevated levels of the molecular chaperon BiP (Ueda et al. 2010), a condition symptomatic of an unfolded protein response within the ER lumen (Okushima et al. 2002). Additional evidence that supports this view is derived from the observations made for the *Arabidopsis mag2* and *mag4* mutant genes. MAG2 and MAG4 are tethering proteins that localize to the ER and cis-Golgi stacks, respectively, which mediate efficient anterograde transport from the ER to Golgi (Li et al. 2006, Takahashi et al. 2010). Mutations in both genes result in the abnormal accumulation of precursors of 2S and 12S storage proteins and elevated levels of BiP. These unprocessed storage proteins accumulate in novel structures, some surrounded by rough ER.

Export of soluble cargo from the ER to the Golgi is dependent on the formation of coat complex II (COPII) vesicles (Barlowe et al. 1994). COPII formation depends on the small GTPase, Sar1p and the coat protein complexes Sec13p/Sec31p and Sec23p/Sec24p (Barlowe et al. 1993). In rice, Sar1 participates in the export of proglutelin and globulin export from the ER (Tian et al. 2013). When Sar1 activity was significantly lowered by suppression of all three Sar1 genes in developing rice seeds, elevated accumulation of proglutelin and novel ER-bounded protein bodies containing both glutelin and α-globulin were observed. Some of these novel protein bodies contained blebs of prolamine. Based on these results, glutelin and α-globulin are transported from the ER to the Golgi by COPII vesicles.

To determine whether one of the *GLUP* genes is involved in the transport of rice storage proteins from the ER to Golgi we undertook a study of the *glup2* mutation. Construction of a genetic linkage map of the *GLUP2* gene led to the eventual identification of *GLUP2* as a gene for GOT1B. Microscopic examination showed the presence of novel ER-bound protein bodies consisting of a larger intracisternal inclusion containing proglutelin and α-globulin surrounded by smaller granules of prolamine. Furthermore, prolamine and α-globulin RNAs were found to be mis-directed from the PB-ER to the cisternal ER, a condition that accounts for the abnormal heterotypic interaction of proglutelin and α-globulin proteins in the ER lumen and the close association of prolamine with these storage proteins. Our results indicate that GOT1B plays multiple roles in storage protein expression. In addition to its involvement in mediating export of proglutelin and α-globulin from the ER to Golgi via COPII vesicles, it is also required for the localization of prolamine and α-globulin mRNAs to the PB-ER.

Results

The profile of seed storage proteins in *glup2* allelic lines

The *glup2* mutation, line EM305, has been previously characterized as an accumulator of abnormally high levels of the 57 kDa proglutelin as well as of the luminal chaperone BiP (Ueda et al. 2010). Two additional independent *glup2* lines, EM964 and EM1130, have been subsequently identified. **Fig. 1** shows the protein profiles and grain morphology of wild type (WT) and the three *glup2* allelic lines. Compared to WT, all three EM lines contained large amounts of the 57 kDa proglutelin (**Fig. 1A**). This is also readily evident according to immunoblotting using an antibody raised to the glutelin acidic subunit (**Fig. 1C**) where the signal of the 57 kDa protein in the *glup2* allelic lines was increased substantially in comparison to WT. Interestingly, the amounts of acidic and basic subunits at 40 kDa (arrowhead) and 20 kDa (star), respectively, were relatively unchanged in the *glup2* lines compared with the WT (**Fig. 1B**). This distribution pattern of proglutelin to glutelin subunits differs substantially from the 57 kDa over-accumulating *glup4* and *glup6* mutant lines, which display reduced levels of the glutelin subunits (Fukuda et al. 2011, Fukuda et al. 2013). Unlike the hard vitreous WT grains, those from the *glup2* lines were chalky or floury in appearance and in cross-section (**Fig.1D**), indicating that the *glup2* mutation affects the normal close packaging of starch and storage protein inclusions during the desiccation phase of seed

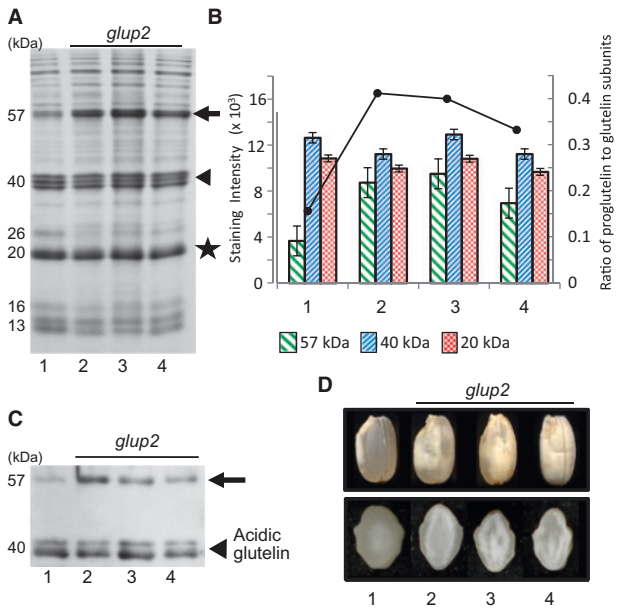

Fig. 1 Storage protein composition and seed morphology of *glup2* lines. (A) Seed protein extracts prepared from wild type (rice variety Taichung65) and various *glup2* rice lines, were resolved by SDS-PAGE. (B) Bar and line graphs indicate the staining intensity level of each polypeptide and the ratio of proglutelin to glutelin subunits, respectively. This value was calculated from densitometry of panel A. (C) Immunoblot analysis of SDS polyacrylamide gel (panel A) using glutelin acidic subunit depicts the elevated amounts of 57 kDa proglutelin in *glup2* mutant lines (lanes 2–4). (D) Whole grains of *glup2* lines were chalky in appearance in contrast to wild type.
Lane 1: Wild type, rice variety, Taichung65; lane 2: EM305; lane 3: EM964; lane 4: EM1130. EM305, EM964 and EM1130 are *glup2* allelic lines. Arrows, arrowheads and star in panels A and C denote 57 kDa proglutelin, 40 kDa acidic and 20 kDa basic glutelin subunits, respectively.

development. No significant differences in plant growth and development were evident for the three *glup2* lines from WT, indicating that the *glup2* mutation affects only seed development.

Construction of genetic linkage map of *GLUP2* gene

As the 57 kDa proglutelins are proteolytically processed to acidic and basic subunits in the PSV (PB-II), the trafficking of proglutelins from the ER to the PSV (via the Golgi) is likely retarded in the *glup2* mutation lines, thereby accounting for the excess accumulation of 57 kDa proglutelin. To identify the causal basis for the elevated proglutelin, a genetic linkage map of the *GLUP2* gene was constructed using seeds from an F2 generation obtained by a cross between the *glup2* line, EM305 and Kasalath (**Fig. 2**). By analyzing proteins from the non-embryo half of the seeds using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), *glup2* homozygous seeds were easily identified. The embryo half of the *glup2* seeds were germinated and grown, whereupon DNA extracted from the leaves of *glup2* homozygous plants was used for the construction of the genetic linkage map of the *GLUP2* gene.

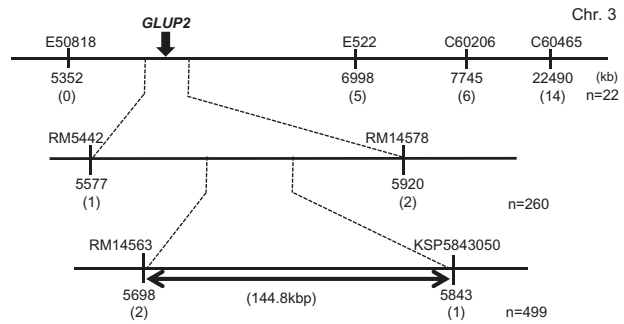

Fig. 2 Delimitation of candidate genomic region of *GLUP2* gene. Genetic linkage map of *GLUP2* gene was constructed by using 499 F2 plants using the SSR and KSP markers on chromosome 3. Molecular markers and inheritance distance (bp) from short arm terminal are indicated. The number in parenthesis indicates the recombination population.

Initial linkage analysis using homozygous *glup2* plants showed that the mutant locus resided between DNA markers, E50818 and E522, located on chromosome 3 (**Fig. 2**). Analysis of 260 plants further revealed that the *GLUP2* gene was located within a 350-kb area bordered by RM5442 and RM14578. The results of the linkage analysis of the area using 499 plants fine mapped the location of the *GLUP2* gene within a 145-kb region between RM14563 and KSP5843050.

A gene annotation search of the *GLUP2* gene candidate region within the 145-kb region of chromosome 3 revealed 24 predicted genes (Supplemental Table S1). Among these genes, the SNPs in all *glup2* lines were located within the coding region in only the Os03t0209400 (LOC_Os03g11100.1) gene annotated as Golgi transport 1 (GOT1B). Genomic sequencing of the *GOT1B* gene indicated that all three *glup2* allelic genes contained amino acid substitutions (**Fig. 3**) where a Gly residue was replaced by Asp residue at two positions: at residue 40 in EM964 and EM1130 and at residue 47 in EM305. Using TOPCONS (http://topcons.cbr.su.se), GOT1B is predicted to be a transmembrane protein containing four membrane spanning domains (**Supplementary Fig. S1**). Both Gly residues are highly conserved at positions 40 and 47 and are located in the second transmembrane. Replacement of these conserved Gly residues with a negatively charged Asp residue will likely disrupt the structure of this membrane spanning domain in a hydrophobic lipid environment. A survey of the rice expression profile database (RiceXPro: http://ricexpro.dna.affrc.go.jp) revealed that the GOT1B was constitutively expressed in most tissues examined during all growing stages of plant growth and development except during the day in the leaf sheath (12:00) at the reproductive stage of plant growth (Supplemental Fig. S2).

To validate that the *GOT1B* gene was synonymous to *GLUP2*, complementation tests were performed by transforming a *glup2* plant line with the WT *GOT1B* gene fused to GFP and analyzing the seed protein composition. As shown in Supplemental Fig. S3, all of the transgenic lines of *glup2* expressing WT *GOT1B-GFP* gene as viewed by immunoblot analysis (Supplemental Fig. S3C) showed normal or near-normal proglutelin levels according to SDS-PAGE analysis. Based on the

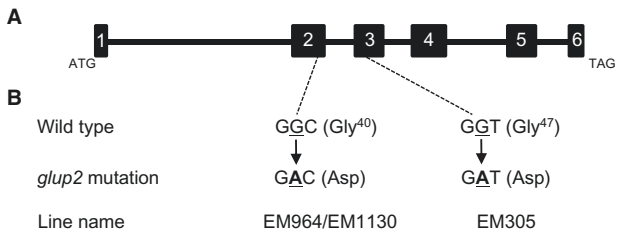

Fig. 3 Mutation site of the *GLUP2* gene in three *glup2* mutant lines. (A) The structure of the *GLUP2* candidate gene, Os03g0209400. ATG and TAG denote the initiation and termination codons, respectively. Black boxes indicate the proximate size and positions of the six exons. The *GLUP2* open reading frame spans 423 bp and codes for 140 amino acids. (B) Mutation sites in *glup2* allelic lines in comparison with wild type, rice variety, Taichung65. In EM305, EM964 and EM1130, a G to A mutation resulted in amino acid change from Gly to Asp.

presence of missense mutations in the three *glup2* allelic lines and restoration of normal 57 kDa proglutelin levels by complementation of the *glup2* mutant line by the WT *GOT1B* gene (Supplemental Fig. S3B and D), we conclude that the *glup2* locus is due to a mutation in the *GOT1B* gene. Since rice has three other GOT1B orthologs (**Supplementary Fig. S1**), the gene is named *GLUP2*/*GOT1B*.

Protein body in *glup2* endosperm

In order to elucidate the role of GLUP2/GOT1B in storage protein accumulation, the morphology of protein bodies in *glup2* mutant lines, EM964 and EM305, was analyzed and compared with the WT. As described in previous studies (Tanaka et al. 1980, Ogawa et al. 1987), the storage proteins accumulate and are packaged into separate protein bodies, PB-I and PB-II. Prolamines are packaged in PB-I, structures bounded by rough ER membranes, while glutelins as well as globulins are deposited in PSV, PB-II (Ogawa et al. 1987). **Figs. 4** and **5** show the immunofluorescence microscopic images of PB-I and PB-II from WT and *glup2* endosperm using antibodies to glutelin, prolamine and α-globulin at 3 weeks after flowering. Microscopic images of PBs from younger endosperm at 1 and 2 weeks after flowering are shown in Supplemental Fig. S4. Compared with WT endosperm (**Fig. 4A to C**, **Fig. 5A to C**, Supplemental Fig. S4A to D), the number and morphologies of PB-I and PB-II were distinct from *glup2* endosperm. Instead of the irregularly shaped PB-IIs seen in the WT, glutelin containing PBs were more spherical in shape and a small proportion were larger in size than those seen in the WT. Moreover, the numbers of small PB-IIs were substantially elevated especially in EM964 (**Fig. 4D G** and **Fig. 5D** and **G**). The deposition of α-globulins in these variant PB-IIs differed as well. Unlike the stratified distribution where α-globulins were located on the peripheral regions of WT PB-II (**Fig. 5C**), α-globulins and glutelins appeared to be homogeneously dispersed in both the large spherical PBs and small irregularly shaped ones.

The morphology of prolamine containing PB-I in *glup2*, especially in EM964, also differs from those seen in the WT. PB-Is appeared normal in shape and size in EM305, although they tended to clump together. In EM964, normal looking PB-Is were

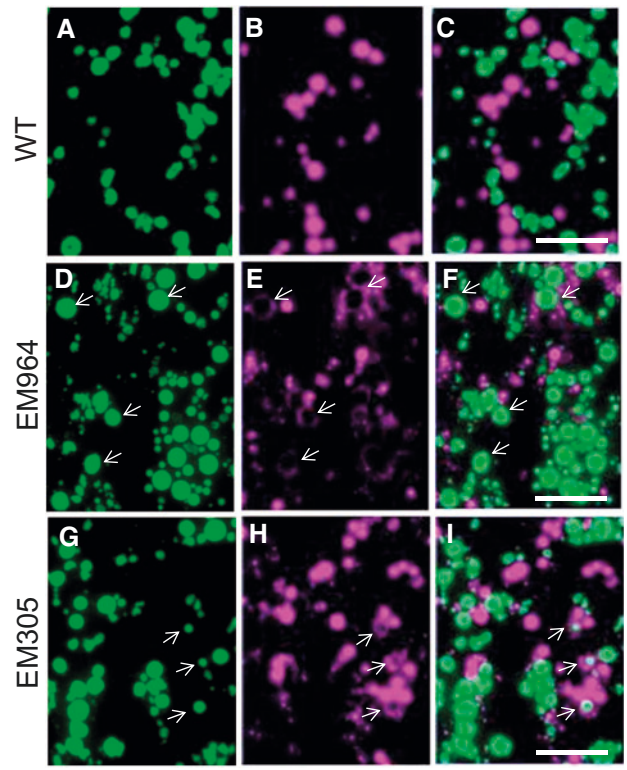

Fig. 4 Immunofluorescence microscopy showing the localization of glutelin and prolamine on the protein bodies in the *glup2* lines, EM305 and EM964 at 3 weeks after flowering. (A to C) WT (Taichung65); (D to F) EM964; (G to I) EM305. Secondary antibodies labeled with FITC (green) and rhodamine (magenta) were used to visualize the binding of glutelin (A, D and G) and prolamine (B, E and H) antibodies, respectively. Panels C, F and I are the merged images of A and B, D and E, and G and H, respectively. Arrows indicate the novel type of spherical protein bodies containing glutelin. Small prolamine PBs were localized to and around the spherical PBs containing proglutelin. Bars = 10 μm.

far fewer in number and were much smaller in size. Instead the majority of the prolamines in EM964 were distributed as ring-like structures as well as small speckles. Such small speckles and ring-like structures were also observed in EM305 but were fewer in number. Interestingly, these prolamine ring-like structures appear to engulf PB-II-like structures when the glutelin and prolamine immunofluorescence images were merged (**Fig. 4F and I**).

The protein bodies in *glup2* mutant were analyzed by immunocytochemistry at the electron microscopy level. In the WT, the spherical PB-Is and irregularly shaped PB-IIs were readily distinguishable (**Fig. 6A, B**). Prolamines were accumulated within the ER as PB-I (**Fig. 6B**), while glutelin and α-globulins are accumulated at specific locations within PB-II (**Fig. 6C**). In EM964, small granules were evident around larger PBs (**Fig. 6D**). Immunocytochemical analysis showed that these small granules contained prolamine, which surrounded the larger glutelin containing PBs (**Fig. 6E**). Collectively, these structures were contained within the ER lumen (**Fig. 6E**). α-Globulin polypeptides were located in the smaller prolamine-containing granules and larger glutelin-containing PBs (**Fig. 6F**). A similar condition was also observed in EM305, where larger PBs were surrounded by

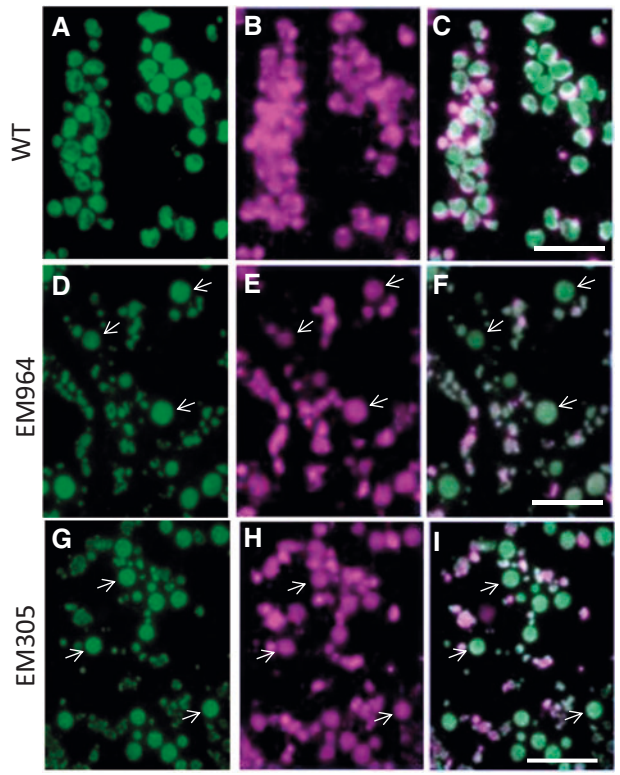

Fig. 5 Immunofluorescence microscopy showing the localization of glutelin and α-globulin in novel protein bodies in the *glup2* lines, EM305 and EM964 at 3 weeks after flowering. (A to C) WT (Taichung65); (D to F) EM964; (G to I) EM305. Secondary antibodies labeled with FITC (green) and rhodamine (magenta) were used to visualize the reaction of glutelin (A, D and G) and α-globulin (B, E and H) antibodies, respectively. C, F and I are the merged images of A and B, D and E, and G and H, respectively. Arrows indicate the novel type of spherical protein bodies containing proglutelin and α-globulin. Bars = 10 μm.

smaller granules (**Fig. 6G**). Immunocytochemical analysis showed that the larger glutelin-containing PBs were surrounded by smaller prolamine-containing granules within the ER lumen (**Fig. 6H**).

These immunocytochemical studies were extended to PBs from 1- and 2-week-old endosperm from EM964. In 1-week-old endosperm, a few particles representing prolamine and α-globulins polypeptides were observed at the periphery of the glutelin PBs, which were surrounded by rough ER (**Fig. 7E and F**). By 2 weeks, these larger glutelin-containing PB structures showed blebs containing prolamine on their periphery (**Fig. 7G**). Although α-globulins particles were readily seen randomly distributed within the larger glutelin-containing PB structure, a few of these blebs appeared to contain α-globulins. As the endosperm matures, these blebs appear to enlarge and eventually are displaced from the glutelin PB-like structures to form the numerous granules surrounding the glutelin PB seen in **Fig. 6E**. Overall, these results suggest that the export of proglutelin and α-globulins from the ER is retarded by the *glup2* mutation and that GLUP2/GOT1B participates in the transport of the proglutelin and α-globulin from the ER to Golgi apparatus.

Intracellular localization of GLUP2/GOT1B

To gain further insight into the role of GLUP2/GOT1B in the intracellular transport of proglutelin and α-globulins, endosperm sections from transgenic rice lines expressing GLUP2/GOT1B tagged with GFP (**Supplementary Fig. S3A**) were subjected to microscopic examination. Immunofluorescence microscopic images of endosperm sections using antibodies to GFP and prolamine are shown in **Fig. 8**. Very strong signals by anti-GFP antibodies were co-localized with the signal by prolamine antibody (**Fig. 8**, arrows), suggesting that GLUP2/GOT1B-GFP is localized on PB-I. In addition to the strong GFP signals associated with PB-I, fainter GFP signals on amorphous structures were also detected (**Fig. 8D, 8F**, arrow heads). As these amorphous structures are located proximal to the PB-ER, it is likely that these structures are cisternal ER. Given the role of GOT1B in COPII vesicle formation and transport, these amorphous structures may represent ER export sites on the cisternal ER.

Prolamine and a-globulin RNAs are mis-localized in *glup2*

In WT endosperm, prolamine and α-globulin polypeptides are synthesized on the PB-ER that surrounds PB-I, while glutelins are synthesized on the cisternal ER (Li et al. 1993a, Choi et al. 2000, Hamada et al. 2003). This segregation of storage protein biosynthesis is due to the location of their respective RNAs, where prolamine and α-globulin RNAs are localized to the PB-ER, while glutelin RNAs are distributed on the cisternal ER. The close association of both prolamine and α-globulin polypeptides with glutelin proteins suggests some disruption in their normal sites of synthesis on the ER membrane complex. Indeed, examination of the distribution of storage protein RNAs by in situ reverse transcriptase polymerase chain reaction (RT-PCR) of developing rice sections from *glup2* indicates that prolamine and α-globulin RNAs are displaced from their normal location on the PB-ER and located on the cisternal ER similar to what is seen with glutelin (**Fig. 9**). The translation of these mis-localized prolamine and α-globulin mRNAs on the cisternal ER, the site of glutelin synthesis, is responsible for the close spatial distribution of these storage proteins within the ER lumen.

Discussion

Glutelin and α-globulin are accumulated in the PSV (PB-II) in developing rice endosperm (Sugimoto et al. 1986, Kumamaru et al. 2010). These storage proteins are synthesized on different subdomains of the cortical ER; α-globulins are translated, like prolamine RNAs, on the PB-ER that delimits the prolamine PBs (PB-I), while glutelins are synthesized on adjoining cisternal ER. These proteins are then exported to the Golgi and then sorted to the PSV where they accumulate within stratified regions to form PB-II.

Information is beginning to emerge on the biochemical and cellular processes that are responsible for the sorting of these storage proteins to PB-II. The export of glutelin and

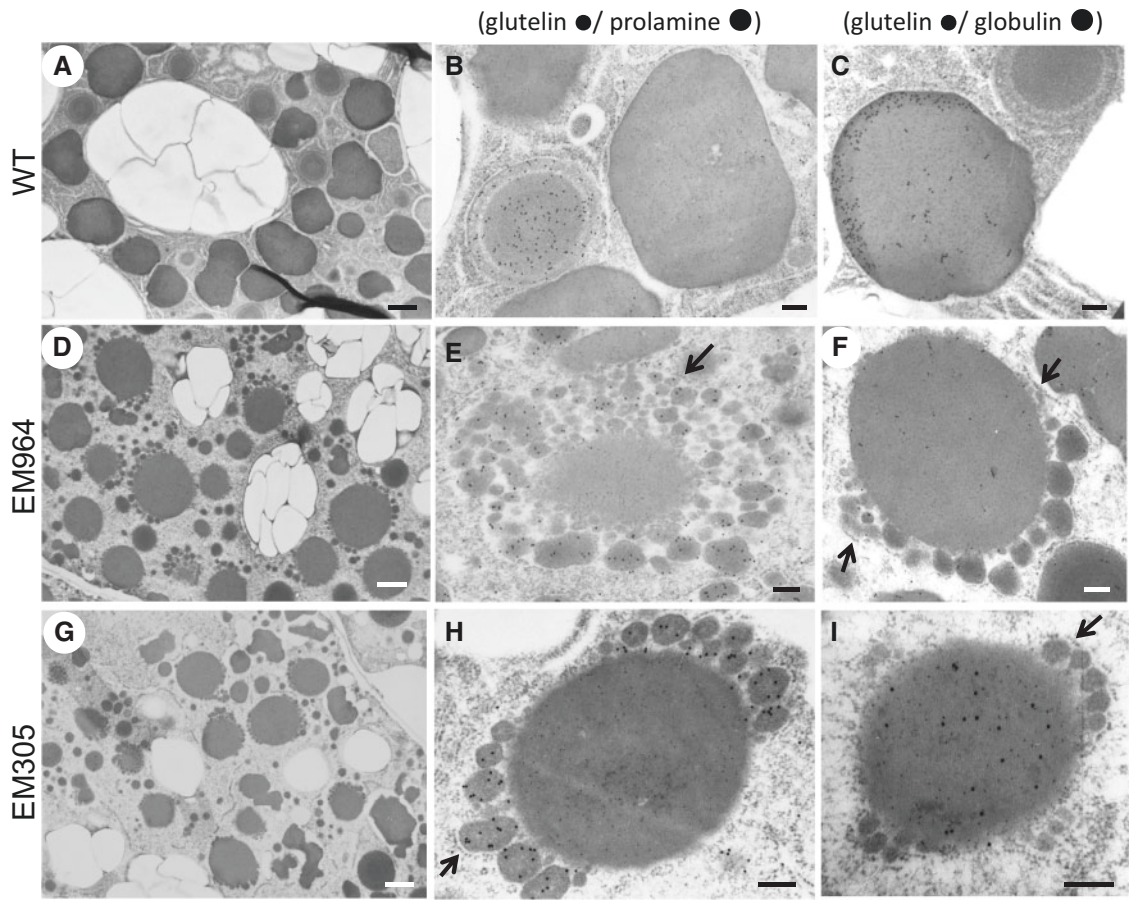

Fig. 6 Immunolocalization of glutelin, prolamine, and α-globulin in protein bodies of *glup2* lines at 3 weeks after flowering. (A to C) WT (Taichung65); (D to F) EM964; (G to I) EM305. B, E and H: Gold particles of 5 nm and 15 nm indicate the reaction of glutelin and prolamine antibodies, respectively. C, F and I: Gold particles of 5 nm and 15 nm indicate the reaction of glutelin and α-globulin antibodies, respectively. Arrows indicate the ER membranes in E, F, H and I surrounding the large intracisternal granules containing glutelin and α-globulin. Bars = 1 μm in A, D and G; 200 nm in B, C, E, F, H and I.

α-globulin from the ER is suppressed by the reduction of Sar1 activity indicating the role of COPII vesicles in the trafficking of these storage proteins to the Golgi (Tian et al. 2013). At the Golgi, these storage proteins are packaged into dense vesicles where they are eventually transported to PB-II, likely via the intermediate prevacuolar compartment (PVC) (Fukuda et al. 2011). These events at the Golgi to the PVC are dependent on the small GTPase, Rab5a, its nucleotide exchange factor (Wang et al. 2010, Fukuda et al. 2011, Fukuda et al. 2013, Liu et al. 2013, Wen et al. 2015), and a plant-specific kelch-repeat protein, which form a protein complex (Ren et al. 2014).

The present study identifies yet another factor involved in the transport of glutelin and α-globulin proteins to PB-II. The *glup2* phenotype, which accumulates elevated amounts of proglutelin, is due to a mutation in the *GOT1B* gene. In yeast, it is reported that Got1p protein participates in vesicular trafficking between the ER and Golgi apparatus (Conchon et al. 1999, Lorente-Rodriguez et al. 2009) and our cytological studies support a similar role in rice. Specifically, GOT1B is involved in COPII vesicle formation for export from the ER (Conchon et al. 1999, Lorente-Rodriguez et al. 2009) as the disruption in COPII-dependent export would account for the observed accumulation of glutelin polypeptides within the ER lumen as intracisternal granules and, thereby, the over-accumulation of proglutelin, which are normally processed at PB-II.

The storage protein profiles exhibited by the *glup2* mutation contrast with those seen for *glup4* and *glup6*, two other mutations that over-accumulate the 57 kDa proglutelin. Unlike the severe reduction in the levels of acidic and basic subunits in *glup4* and *glup6* due to the failure of proglutelin being transported to the PSV, the amounts of these glutelin subunits are not significantly affected in *glup2* (**Fig. 1**). As these glutelin subunits are generated in PB-II via a specific vacuolar protease (Wang et al. 2009, Kumamaru et al. 2010), the normal amounts of these proteins indicate that the bulk of the proglutelins are correctly trafficked to the PSV where they are processed and assembled to form PB-II. Rice codes for four GOT1Bs of which GLUP2/GOT1B (LOC_Os03g11100) is the dominant one expressed during endosperm development (**Supplementary Fig. S5**). Two other GOT1Bs are expressed during endosperm development but at much lower levels than GLUP2/GOT1B. These two other GOT1B isoforms may partially compensate for the loss of GLUP2/GOT1B and allowing export from the

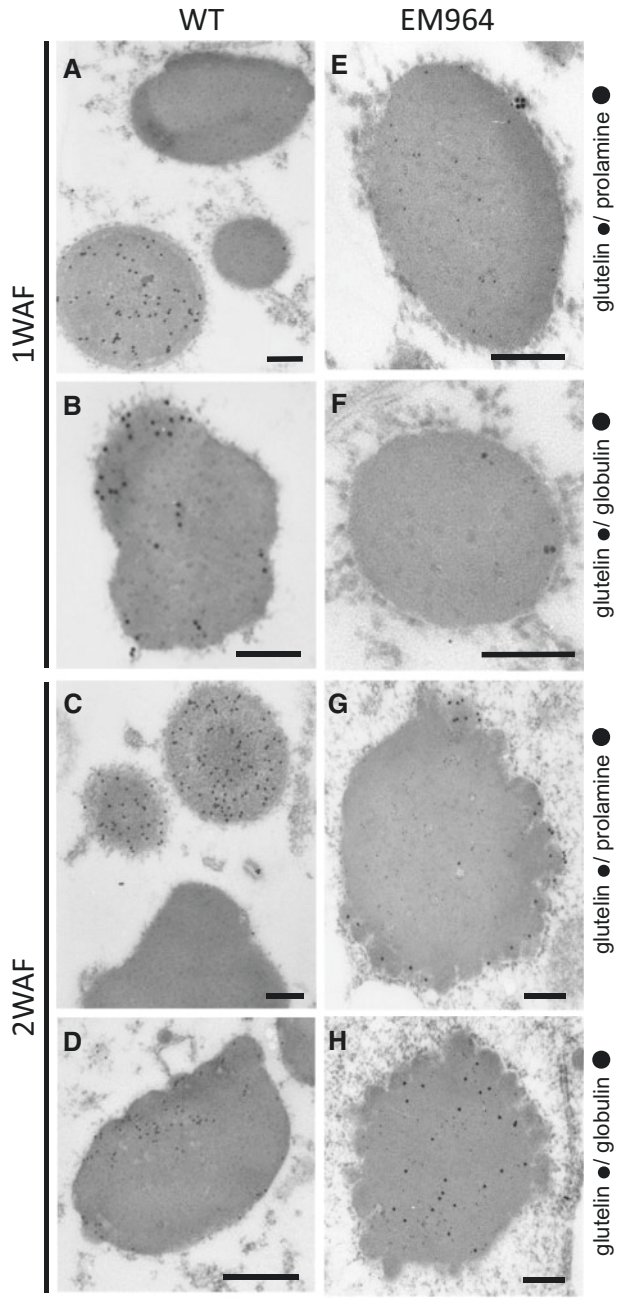

Fig. 7 Immunolocalization of glutelin, prolamine and α-globulin on protein bodies in the developing *glup2*, EM964 endosperm at 1 and 2 weeks after flowering. (A to D) WT (Taichung65); (E to H) EM964. A, B, E and F: 1 week after flowering (WAF); C, D, G and H: 2 WAF. A, E, C and G: Gold particles of 5 nm and 15 nm indicate the reaction of glutelin and prolamine antibodies, respectively. B, F, D and H: Gold particles of 5 nm and 15 nm indicate the reaction of glutelin and α-globulin antibodies, respectively. Bars = 200 nm.

ER especially during early seed development when storage protein synthesis is beginning to accelerate. In addition to GOT1B, the structurally related SFT2 (**Supplementary Fig. S1**) may also serve to partially compensate for the reduction in GLUP2/GOT1B. The yeast Got1p was initially identified as a mutation that showed synthetic lethality with *sft2* (Conchon et al. 1999). SFT2, like GOT1B, has four transmembrane domains and may help mediate formation and transport of COPII vesicles in the absence of GOT1B.

In addition to its role in exporting glutelin and α-globulin from the ER, GOT1B is also required for the faithful localization of prolamine and α-globulin RNAs to the PB-ER. In the WT, both prolamine and α-globulin RNAs are transported and targeted to the PB-ER where they are translated (Li et al. 1993a, Choi et al. 2000, Hamada et al. 2003). While prolamine accumulates within the ER lumen, α-globulins are rapidly exported to the Golgi and then transported to the PB-II (Washida et al. 2012). The transport and localization of these RNAs to the PB-ER is dependent on zipcodes, mRNA sequences that serve as cis-determinants (Choi et al. 2000, Crofts et al. 2004, Crofts et al. 2005, Washida et al. 2012) that recognize specific trans-acting RNA binding proteins (Wang et al. 2008, Crofts et al. 2010, Doroshenk et al. 2012, Yang et al. 2014). Transport and localization of glutelin RNAs to the cisternal ER are also dependent on specific zipcode sequences and trans-factors (Washida et al. 2009, Doroshenk et al. 2014, Tian and Okita 2014). In addition to these two regulated RNA transport pathways, a third default pathway to the cisternal ER is also evident.

The segregated synthesis of α-globulin on the PB-ER and glutelins on the cisternal ER enables these proteins to properly fold to form a protein conformation competent for ER export. When synthesized in the same ER location via mistargeting of α-globulin RNAs to the cisternal ER, they apparently interact, as these storage proteins are uniformly distributed within PB-II instead of being asymmetrically distributed (Washida et al. 2012). In addition, instead of folding in a compact polypeptide, newly synthesized α-globulins at the cisternal ER may interact with each other via inter-chain disulfide bond formation, an interaction that may impede their transport to the Golgi as evident by the presence of numerous α-globulin-containing speckles in the cytoplasm (Washida et al. 2012).

In *glup2* endosperm, prolamine and α-globulin RNAs are mis-targeted to the cisternal ER where they are translated and accumulated as intracisternal granules. This mistargeting of these RNAs accounts for the location of these storage proteins on the periphery of the glutelin intracisternal granules (**Figs. 4–7**). In the WT, immunofluorescence studies showed that GOT1B is dispersed throughout the cortical ER but apparently enriched on the PB-ER (**Fig. 8**). Its location on the PB-ER membranes infers a possible role as part of a receptor complex or as a membrane tether for anchoring of transported prolamine and α-globulin RNAs targeted to the PB-ER membrane in addition to its role in exporting α-globulin polypeptides from the ER to the Golgi.

The involvement of GLUP2/GOT1B in the targeting of prolamine and α-globulin RNAs to the PB-ER provides further evidence that RNA localization during rice endosperm development is mediated by trafficking of membrane vesicles. Additional evidence for the involvement of membrane trafficking is the observed mis-localization of glutelin RNAs to the PB-ER in *glup4* (Doroshenk et al. 2010) and *glup6* (Crofts et al. 2004) mutant lines. The *GLUP4* locus contains DNA sequences for the

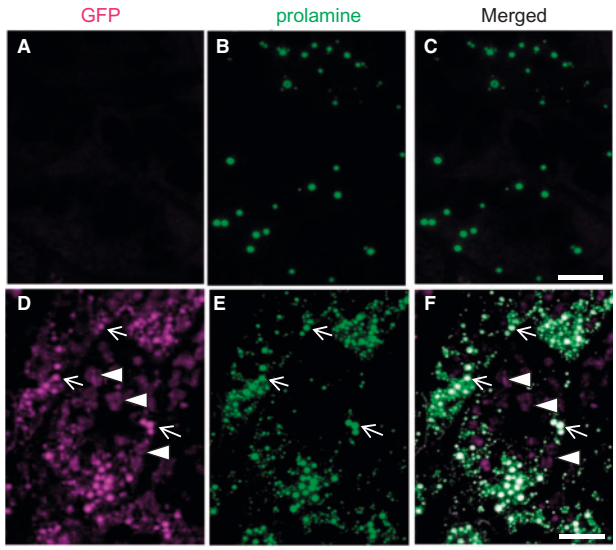

Fig. 8 Immunofluorescence microscopy showing the localization of the GOT1B-GFP in 2-week-old developing seeds. (A to C) WT (Taichung65); (D to F) Transformant of *glup2* line, EM964, with *GOT1B-GFP* gene construct. Secondary antibodies labeled with rhodamine (magenta) and FITC (green) were used to visualize the reaction of GFP and prolamine antibodies in A and D, and B and E, respectively. C and F are the merged images of A and B, and D and E, respectively. Arrowheads in D and F denote the amorphous structures, which are likely cisternal ER due to their close spatial relationship with PB-ER. Bars = 10 μm.

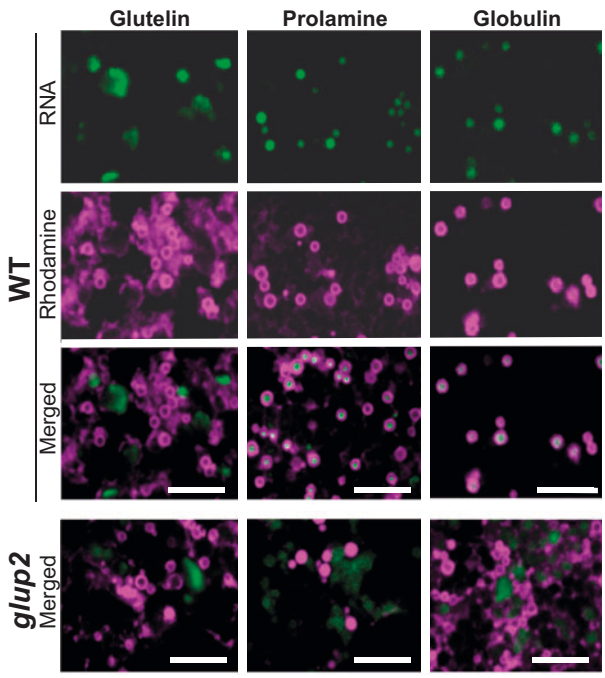

Fig. 9 Distribution of storage protein RNAs in wild type and *glup2*, EM305. The relationship of RNA (green) and prolamine PBs (stained with rhodamine) are shown together with a merged image. Note coincident localization of prolamine and α-globulin RNAs to PB-ER, whereas glutelin RNAs are distributed to the cisternal ER. In *glup2*, prolamine and α-globulin RNAs are mis-localized to the cisternal ER. WT: wild type, rice variety, Kita-ake. Bars = 10 μm.

Rab5a gene, while *GLUP6* is the nucleotide exchange factor for Rab5. The loss of GLUP4/Rab5a or GLUP6/GEF activities reroutes the normal transport of glutelin and α-globulin proteins from the PSV to the extracellular space. These secreted storage proteins subsequently accumulate into paramural bodies (PMBs) formed initially by the aborted endosomal uptake of these storage proteins followed by the direct secretion of proteins associated with the major endomembrane compartments (Fukuda et al. 2011, Fukuda et al. 2013, Wen et al. 2015). In addition to the disruption of glutelin and α-globulin transport to PSV, glutelin RNAs are mis-localized to the PB-ER and secreted to the PMBs while localization of prolamine and α-globulin RNAs is unaffected. In contrast as described in this study, prolamine and α-globulin RNAs are mis-localized in *glup2*, while glutelin RNAs are unaffected. Overall, these altered RNA localization patterns exhibited by the *glup2*, *glup4* and *glup6* rice lines indicate that these mutations directly affect specific membrane trafficking processes for these RNAs and are not due to a secondary, indirect consequence of these mutations.

GLUP4/Rab5a and GLUP6/GEF are required for normal transport of cargo from the Golgi to the PSV (Fukuda et al. 2011, Fukuda et al. 2013), while GLUP2/GOT1B is involved in ER to Golgi transport. Hence, the targeting of prolamine/globulin RNAs and glutelin RNAs possibly exploit activities involved in different membrane trafficking pathways (ER to Golgi for prolamine and α-globulin RNAs and Golgi to PSV for glutelin RNAs). Further studies are required to identify other membrane trafficking factors that may be involved in RNA localization.

RNA localization is well established in governing gene expression in other organisms (Marchand et al. 2012, Blower 2013, Weis et al. 2013). Although there are relatively fewer examples of RNA localization in plants (Okita et al. 1994, Crofts et al. 2004, Tian and Okita 2014) it is likely an essential process in all plant cells. As the bulk of the protein synthesis occurs at the cell's cortex, a region rich in cytoskeletal elements and rough ER, RNAs as well as ribosomes, must be exported from their site of synthesis, the nucleus, and then transported and targeted to the cortical region. While not all RNAs have to be targeted to specific ER subdomains like the storage protein RNAs, their localization to restricted sites on the ER would facilitate the subsequent intracellular localization of the coded protein product, assist in the assembly of heterotypic protein complexes and avoid the formation of deleterious protein interactions (Okita et al. 1994, Okita and Choi 2002, Crofts et al. 2004, Crofts et al. 2005, Washida et al. 2012).

Although not as conspicuous as in developing rice endosperm cells, Rab5, GEF and GOT1 activities are likely required for RNA and protein localization processes in other rice cell types. In fulfilling their role of storing carbon and nitrogen reserves, rice endosperm cells exhibit high protein synthetic capacity. While GLUP4/Rab5a, GLUP6/GEF and GLUP2/GOT1B are the major activities during rice endosperm development, other isoforms of these proteins are present that can complement, albeit only partially, the functions of these mutated proteins (Fukuda et al. 2011, Fukuda et al. 2013, Wen et al. 2015). Hence,

in other cells, which exhibit protein synthetic activity much lower than that evident for developing endosperm cells, these other isoforms may more fully complement the loss of these major endosperm activities, thereby allowing normal plant growth and development to proceed.

Based on our results, GLUP2/GOT1B serves two roles in storage protein biosynthesis. It is required for faithful targeting of prolamine and globulin mRNAs to the PB-ER and for the export of proglutelin and α-globulins from the ER to the Golgi (**Fig. 10**). In the absence of GLUP2/GOT1B, the major GOT1B isoform expressed during endosperm development, some proglutelins are retained in the ER lumen to form intracisternal granules. At the same time, mis-targeting of prolamine and α-globulin RNAs to the cisternal-ER results in the synthesis of these storage proteins close to glutelin synthesis enabling prolamines to assemble as small granules surrounding the larger proglutelin-α-globulin inclusions. While a portion of the proglutelin and α-globulin are retained in the ER, the bulk are exported from the ER to the Golgi where they are packaged in PB-II as a more homogeneous protein inclusion instead of as independent inclusions.

Materials and Methods

Plant materials

The rice (*O. sativa* L.) *glup2* mutant lines, EM 305, EM964 and EM1130, induced by *N*-methyl-*N*-nitrosourea (MNU) mutagenesis (Satoh et al. 2010), were initially identified by their accumulation of substantial amounts of the 57 kDa proglutelin precursor (Ueda et al. 2010). The original varieties of EM305, and EM964 and EM1130 are Kinmaze and Taichung65, respectively. As protein composition of rice varieties, Kinmaze and Taichung65 is the same (Wen et al. 2015), Taichung65 was used as the WT. Rice plants were grown in the field or glass house (transgenic lines) at Kyushu University and developing seeds were harvested and isolated for biochemical and microscopic analyses. In EM305 and EM964, *glup2* homozygous plants obtained from the cross between *glup2* lines and the WT were used in microscopic analyses.

SDS-PAGE and Western blot analysis

Proteins were extracted from seeds using 0.125 M Tris-HCl, 4% SDS, 4 M urea and 5% β-mercaptoethanol, pH6.8 (Ushijima et al. 2011). The proteins were resolved by SDS-PAGE, transferred electrically to nitrocellulose membranes and then incubated for 1 h with primary antibody raised in mouse against 40 kDa glutelin acidic subunits (1/5000 dilution) in Tris buffer saline (TBS, 10 mM Tris-HCl, 0.15 M NaCl, and 5% skim milk, pH7.5). The blot was washed three times with TBS containing 0.05% Tween 20 (TBST) and then incubated with TBS containing 5% skim milk and secondary antibody, goat IgG against mouse IgG conjugated HRP (Bio-Rad Laboratories, Inc. Berkeley, CA) (1/2,500 dilution). The blot was washed three times with TBST and incubated with ECL detection kit (GE healthcare, Hatfield, UK). The blot was then exposed to X-ray film for visualization of the bound primary antibody. The staining intensity of the SDS-PAGE bands was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Construction of genetic linkage map

The *glup2* line, EM305, was crossed with the *indica* rice cultivar, Kasalath. The F2 seeds were cut in half and total proteins extracted from the non-embryo half seed were subjected to SDS-PAGE. Seeds containing abnormally high amounts of 57 kDa polypeptide as analyzed by SDS-PAGE were judged as homozygous for *glup2*. Homozygous *glup2* plants were cultivated from the embryo containing half seeds and genomic DNA was isolated from the seedlings. A genetic linkage map of the *GLUP2* gene was constructed using DNA markers of chromosome 3.

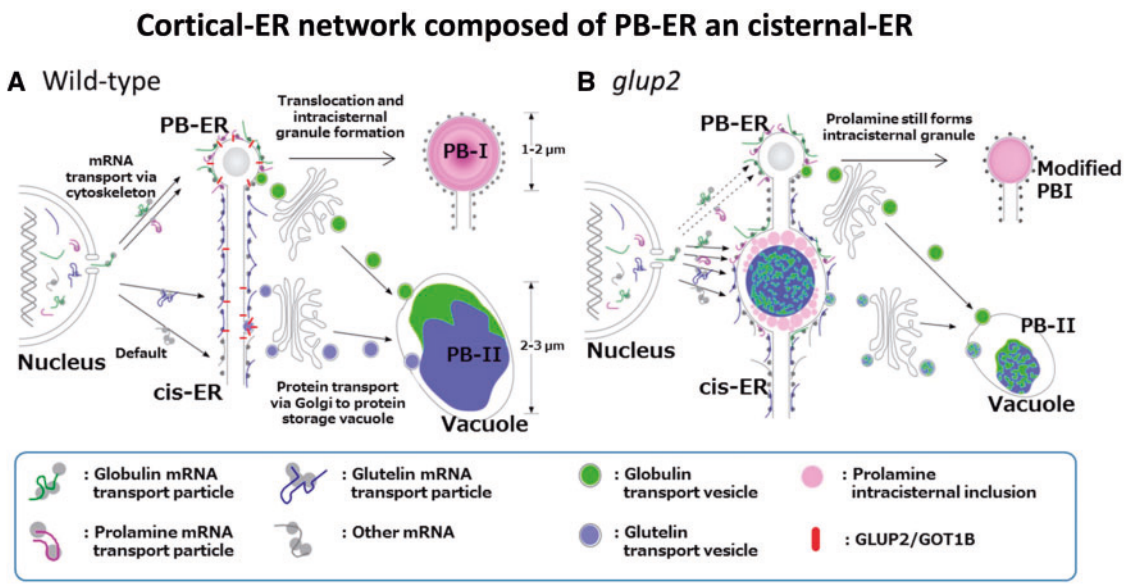

Fig. 10 Model of the role of GLUP2/GOT1B in storage protein RNA localization and storage protein export from the ER. (A) GLUP2/GOT1B is proposed to serve two roles in storage protein biosynthesis. First, it is required for faithful targeting of prolamine and α-globulin RNAs to the PB-ER that bound the prolamine intracisternal inclusions; and second, it is required for efficient export of proglutelin and α-globulin from the ER to the Golgi. (B) The loss of GLUP2/GOT1B, the major GOT1B isoform expressed during rice endosperm development, results in the mis-localization of prolamine and α-globulin RNAs from the PB-ER to cisternal ER, the site of synthesis of proglutelin, and in retarding the export of proglutelin and α-globulin, which enables these proteins to form an intracisternal inclusion surrounded by prolamine granules. The bulk of the proglutelin and α-globulin are exported together as a homogeneous mixture from the ER where they are delivered to PB-II via the Golgi. The structure of these PB-IIs does not display the typical stratification of crystalline glutelin surrounded by amorphous α-globulin inclusions. Many prolamine PBs are also modified in *glup2* as they do not display the typical concentric electron dense rings and alternating electron-lucent and electron-dense layers seen in WT (**Fig. 6C**).

The SNPs in the genes predicted within *GLUP2* candidate region were detected by the TILLING methods described previously (Suzuki et al. 2008).

DNA sequencing analysis

DNA sequencing analysis was performed as described previously (Kumamaru et al. 2010). Total genomic DNAs were extracted from the leaves of the *glup2* lines and WT using the CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson 1980). Sequencing of genomic DNA was performed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Ltd Waltham, MA). DNA sequence analysis was performed using EditView1.0.1 and AutoAssembler 2.1. Comparisons between the WT and mutant sequences were performed using CLUSTALW of DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/top-e.html).

Plasmid construction and rice transformation

A full-length cDNA clone for GOT1B (AK059829) was obtained from the National Institute of Agrobiological Sciences (Tsukuba, Japan). The GOT1B open reading frame (ORF) region was amplified by PCR with primers 5′-ATA AGATCTGCTCAACAATATGGTTTC-3′ and 5′-TATCTCGAGCCCACTGGAAC CCGTTT-3′ using a full-length cDNA clone (AK059829) as a template. The PCR product was then inserted upstream of the spGFP gene (Kawagoe et al. 2005), generating GOT1B-spGFP. The two fusion genes were expressed under the control of the rice *β-TIP* gene promoter (Onda et al. 2009). The binary vector containing the GOT1B-spGFP was constructed by the Gateway system (Invitrogen Carlsbad, CA) as described previously (Onda et al. 2009). Transformation of rice was performed as described previously (Kawagoe et al. 2005).

Microscopic analysis

For immunofluorescence and immunoelectron microscopic analysis, the samples were fixed as described previously (Takemoto et al. 2002).

Immunofluorescence microscopic analysis. Fixed rice seed sections were treated with blocking buffer containing 0.8% BSA, 0.1% gelatin in phosphate-buffered saline (PBS) and then incubated in the appropriate antibodies diluted in blocking buffer. Non-specifically bound antibodies were removed by washing the section five times with PBS. The sections were treated with blocking buffer, followed by incubating with secondary antibodies, goat IgG against mouse IgG, FITC conjugate (green) and goat IgG against rabbit IgG, rhodamine conjugate (Red) (Molecular probes, Thermo Fisher Scientific Inc. Eugene, Or), and then observed microscopically (BZ-9000; Keyence, Osaka, Japan). Samples were treated with antibodies raised against glutelin basic subunit (1:5,000), 14 kDa prolamine (1:1,000) or α-globulin (1:5,000). Red fluorescent images were converted to magenta with Adobe Photoshop (San Jose, CA, USA).

Transmission immunoelectron microscopic analysis. Ultrathin sections (90–100 nm) were cut with a diamond knife and placed on 200 mesh gilder grids. The grids were treated with blocking buffer (TBS, 10 mM Tris, 500 mM NaCl, 0.3% Tween, pH7.2) containing 1% BSA for 60 min. The grids were then incubated overnight with the appropriate antibodies diluted with blocking buffer. Unreacted antibodies were removed by washing the section four times in a drop of blocking buffer. The grids were then incubated for 60 min with secondary antibodies, protein A-gold conjugate 5 nm or 15 nm (BBI Solutions Cardiff, UK), diluted with blocking buffer as described above, then sequentially stained with 0.25% $KMnO_4$ and 1% uranylacetate. The sections were then observed by TEM (JEM-1220) at 80 kV.

Antibodies

Seed storage proteins were separated by SDS-PAGE and individual bands were excised and eluted from the gel by preparative electrophoresis. Antibody against glutelin acidic subunit was raised in mouse and antibodies against α-globulin and 14 kDa prolamine were raised in rabbit. The specificity of the antibodies raised against α-globulin and 14 kDa prolamine was confirmed by immunoblot analysis as described previously (Nagamine et al. 2011, Fukuda et al. 2013). Mouse and rabbit antibodies against GFP for immunofluorescence microscopy and immunoblot blot analysis were purchased from Abcam plc (Cambridge, UK) and Medical & Biological Laboratories Co., Ltd, (Nagoya, JP) respectively.

In situ RT-PCR

The distribution of mRNAs of storage proteins in rice endosperm was performed as described previously (Washida et al. 2012).

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