RESEARCH PAPER

Guanine nucleotide exchange factor 2 for Rab5 proteins coordinated with GLUP6/GEF regulates the intracellular transport of the proglutelin from the Golgi apparatus to the protein storage vacuole in rice endosperm

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Abstract

Rice glutelin polypeptides are initially synthesized on the endoplasmic reticulum (ER) membrane as a proglutelin, which are then transported to the protein storage vacuole (PSV) via the Golgi apparatus. Rab5 and its cognate activator guanine nucleotide exchange factor (GEF) are essential for the intracellular transport of proglutelin from the Golgi apparatus to the PSV. Results from previous studies showed that the double recessive type of glup4/rab5a and glup6/gef mutant accumulated much higher amounts of proglutelin than either parent line. The present study demonstrates that the double recessive type of glup4/rab5a and glup6/gef mutant showed not only elevated proglutelin levels and much larger paramural bodies but also reduced the number and size of PSVs, indicating a synergistic mutation effect. These observations led us to the hypothesis that other isoforms of Rab5 and GEF also participate in the intracellular transport of rice glutelin. A database search identified a novel guanine nucleotide exchange factor, Rab5-GEF2. Like GLUP6/GEF, Rab5-GEF2 was capable of activating Rab5a and two other Rab5 isoforms in in vitro GTP/GDP exchange assays. GEF proteins consist of the helical bundle (HB) domain at the N-terminus, Vps9 domain, and a C-terminal region. By the deletion analysis of GEFs, the HB domain was found essential for the activation of Rab5 proteins.

Key words: Guanine nucleotide exchange factor, helical bundle domain, intracellular transport, in vitro GEF assay, Rab5, storage protein.


Introduction

Rice seed storage proteins consist of three types: glutelin, prolamine, and α-globulin, which are soluble in acid and/or alkali, alcohol and salt solutions, respectively. During seed development, glutelin polypeptides are initially synthesized on the endoplasmic reticulum (ER) membrane as a proglutelin (Yamagata et al., 1982). This precursor form is then transported to the protein storage vacuole (PSV) via dense vesicles formed from the Golgi apparatus (Krishnan et al., 1986; Yamagata and Tanaka, 1986), where it is cleaved into acidic and basic subunits. These proteins together with the α-globulins accumulate to form the protein body (PB) type II (Yamagata et al., 1982).

In order to elucidate the mechanism of intracellular transport of the proglutelin from the ER to PSV, we selected and identified mutants, endosperm storage protein2 (esp2) and glutelin precursor (glup) 1–7 (Kumamaru et al., 2007; Ueda et al., 2010) that accumulated abnormal amounts of proglutelin in developing rice endosperm. The glup4 and the glup6 mutants among of them were identified as loss-of-function (null) mutations of the small GTPase Rab5a and its cognate activator, guanine nucleotide exchange factor (GEF), respectively (Fukuda et al., 2011, 2013). Homologous mutations for Rab5a (gpa1: OsRab5A) and GEF (gpa2: OsVPS9a) were isolated and characterized by the Wan laboratory (Liu et al., 2013; Wang et al., 2010).

GLUP6/GEF was demonstrated to activate GLUP4/Rab5a as well as Arabidopsis Rab5 isoforms (Fukuda et al., 2013). GLUP4/Rab5a and GLUP6/GEF are required not only for the intracellular transport of proglutelins from the Golgi to PSV in rice endosperm but also in the maintenance of the general structural organization of the endomembrane system in developing rice seeds (Fukuda et al., 2011). The endosperm in glup4 and glup6 mutants exhibit similar defective phenotypes with elevated proglutelin levels and the novel appearance of paramural bodies (PMBs), with subcellular structures initially formed by abortive endosomal uptake of secreted glutelins, α-globulins, Golgi and post-Golgi components, and subsequently by direct secretion of these constituents into this novel cellular structure (Fukuda et al., 2013).

Rab GTPases are involved in various membrane trafficking events including endosome organization, cytokinesis, trafficking from the Golgi to the plasma membrane and to vacuoles (Woollard and Moore, 2008). The Rab5 members in plant are located not only on the late endosomal compartment, the pre-vacuolar compartment (PVC), but can also be found on the Golgi and the early endosomal trans-Golgi network and network as well (Sohn et al., 2003; Bolte et al., 2004; Kotzer et al., 2004; Lee et al., 2004; Fukuda et al., 2011; Haas et al., 2007; Steinhof and El Kasmi, 2010). In Arabidopsis, three Rab5 orthologues, RHA1/RABF2a, ARA7/RABF2b and ARA6/RABF1, have been identified. ARA7 and RHA1 are conventional Rab5 types, while ARA6 is a plant-specific type conserved in land plants (Ueda et al., 2001; Ebine et al., 2011). Both the conventional type Rab5s, Ara7 and Rha1, and plant-specific Rab5 and Ara6, participate in endosomal trafficking to the vacuole and/or the plasma membrane (Ueda et al., 2001, 2004; Sohn et al., 2003; Kotzer et al., 2004; Lee et al., 2004; Ebine et al., 2011). Although all three Rab5s are located on the multivesicular body/PVC, plant-specific and conventional types are localized on different populations of membrane-bounded compartments in a partially overlapping manner (Ueda et al., 2004; Haas et al., 2007).

Several GEFs for Rab5, have been reported including the yeast Vps9p, Arabidopsis VPS9a and various animal forms such as Rabex-5, RIN-1, RIN-2, RIN-3, RME-6, Alsin and ALS2CL (Carney et al., 2006; Goh et al., 2007). They all share a highly conserved VPS9 domain (Burd et al., 1996; Horiuchi et al., 1997). The Arabidopsis VPS9a serves as a common activator for the two plant Rab5 types (ARA7, RHA1, and ARA6) (Goh et al., 2007). Mutations in VPS9a result in embryonic lethality and various developmental defects including abnormal root development. Moreover, abnormally enlarged PMBs containing membrane vesicles and accumulation of the 12S globulin precursor were observed in vps9a mutant (Goh et al., 2007; Ebine et al., 2011). These facts suggest that VPS9a is essential for endosomal trafficking and development in Arabidopsis.

In our previous study, analysis of F2 seeds from a cross between glup4 and glup6 lines showed a portion of seeds containing proglutelin levels significantly higher than either parent line (Ueda et al., 2010). This result suggested that pyramiding of both recessive genes mediated a synergistic elevation of proglutelin levels. Rice has several Rab5 homologues expressed during seed development (Fukuda et al., 2011; Liu et al., 2013). Based on these lines of evidence, we hypothesized that the synergistic effect seen with the combined glup4 and glup6 mutations reflected the existence of other Rab5(s) and/or GEF(s) participating in intracellular transport of rice glutelin. In this study the double recessive type of glup4l rab5a and glup6lgef mutation displays much more severe phenotypic alterations in protein trafficking than either parent alone. Moreover, we present evidence that other Rab5 and GEF homologues participate in the trafficking of proglutelin from the Golgi to the PSV.

Materials and methods

Plant materials

The glup6 line EM939 and glup4 lines EM425 and EM956, which were induced by N-methyl-N-nitrosourea (MNU) mutagenesis and which accumulated elevated amounts of proglutelin (Satoh-Cruz et al., 2010b; Ueda et al., 2010), were used in these experiments. The double recessive type of glup4l rab5a and glup6lgef mutant line was isolated from the progenies of the cross between EM939 and EM425 or between EM939 and EM956. All F2 seeds obtained from self-pollinated F1 plants were bisected into embryo and non-embryo portions. The proteins extracted from the non-embryo portion of individual F2 seeds were analysed by SDS-PAGE. Those containing substantially higher levels of proglutelin than either parent line were identified, with the embryo portion of the seed sown in soil and F3 plants cultivated to maturity. By sequencing genomic DNA from the F3 plants, the double recessive type for both genes was confirmed. Developing seeds, 1 to 3 weeks after flowering (WAF), of the double recessive type were analysed as described below.
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**SDS-PAGE and western blot analysis**

Extraction of the proteins from seeds, SDS-PAGE, and western blot analysis was performed as described previously (Ushijima et al., 2011; Fukuda et al., 2013).

**DNA sequence analysis**

DNA sequencing analysis was performed as described previously (Kumamaru et al., 2010). The sequence of total genomic DNAs, extracted by the CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson, 1980) from the leaves of the double recessive type, was determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems Ltd.). DNA sequence analysis was performed using EditView1.0.1 and AutoAssembler 2.1. Comparisons between the double recessive type, two single mutants, and wild type were performed using CLUSTALW of DNA Data Bank of Japan (DDBJ: http://clustalw.ddbj.nig.ac.jp/index.php?lang=en).

**Microscopic analysis**

For immunofluorescence and immunoelectron microscopy studies, the samples were fixed in LR white resin and analysed as described previously (Takemoto et al., 2002). For transmission electron microscopy, samples were embedded in epoxy resin and analysed as described previously (Nagamine et al., 2011).

**Antibodies**

Antibodies against glutelin and α-globulin were raised in mice and rabbits, respectively (Fukuda et al., 2013). Antibodies against the 14 kDa prolamine were raised in rabbits (Nagamine et al., 2011).

**Identification and classification of novel GEFs containing VPS9 domain**

To detect novel GEF isoforms of GLUP6/GEF, the SALADA database was searched for proteins containing the Vps9 domain. Genes encoding for proteins containing the conserved Vps9 domain were annotated by RAP-DB and expression confirmed by RiceXpro. The deduced amino acid sequences corresponding to the GEF genes were aligned using ClustalW of DDBJ. A phylogenetic tree was constructed using the neighbor-joining method as implemented in the MEGA5.0 programme according to the results of ClustalW analysis (Supplementary Fig. S5).

**Expression and purification of GST fusion proteins**

GST-conjugates of GLUP6/GEF, Rab5-GEF2, GLUP4/Rab5a, Rab5b, Rab5c, Rab11, and truncated proteins of GEFs were produced in Escherichia coli BL21 (DE3) using the pGEX 4T-1 expression vector (GE Healthcare) and purified according to the procedure described earlier (Goh et al., 2007). The primer sequences for construction of the expression plasmids are shown in Supplementary Table S1 and the experimental procedures are described in the table legend. All Rab proteins were purified in the GDP-bound form in the presence of Mg2+ and without EDTA or GDP. After purification of GST-tagged proteins by chromatography on a Glutathione-Sepharose 4B column (GE Healthcare), the purified proteins were loaded onto a desalting column (GE healthcare) to remove glutathione and then stored frozen at ~80°C. The purification procedure was completed in one day to maximize enzyme activity.

**Guanine nucleotide exchange assay**

The guanine nucleotide exchange assay was performed according to the method described previously (Goh et al., 2007). Each purified GST-Rab protein was preloaded with a 25 M excess of GDP at 25°C for 2 h. The excess GDP was removed by a desalting column. For each assay, 1 μM GST-Rab GDP form was pre-incubated with or without GST-GEFs in GEF assay buffer containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.5 mM MgCl2 for 100 s. The nucleotide exchange reaction was started by the addition of 0.1 mM GMP-PNP. Trp intrinsic fluorescence of Rab proteins was detected at 340 nm by the excitation at 298 nm using a fluorescence spectrophotometer (F-2500, Hitachi). Each experiment was completed within two days as Rab GTPase activity decreased rapidly after thawing.

**Surface plasmon resonance analysis**

Surface plasmon resonance analysis was performed using a BIACORE J system (GE Healthcare UK Ltd.) to analyse protein-to-protein interactions. To monitor the interactions of GLUP4/Rab5a with Rab5-GEF2 and GLU6/GEF, highly purified GLUP4/Rab5a protein was immobilized on a Sensor Chip CM5 according to the manufacturer’s protocol. Various concentrations of purified Rab5-GEF2 and GLUP6/GEF were then applied to the GLUP4/Rab5a immobilized Sensor Chips for 120 s in 10 mM HEPES-NaOH, pH 7.4, 0.15 M NaCl, 0.5 mM MgCl2, 10 mM GDP, and 0.1% Triton X-100 at a flow rate of 30 μl/min. The apparent equilibrium constants (Kd) for GLUP4/Rab5a with Rab5-GEF2 and GLUP6/GEF were calculated from the association and dissociation curves using the BIA evaluation software (GE Healthcare UK Ltd.).

**Accession numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: GLUP4/Rab5a (AK061116, Os12g0631100), Rab5b (AK121527, Os03g0151900), Rab5c (AK067459, Os03g0666500), Rab11 (AK103220, Os08g0525000), GLUP6/GEF (AK070551, Os03g0262900), Rab5-GEF2 (LOC_Os03g62580.1), ΔC-terminal Rab5-GEF2 (AK070821).

**Results**

The double recessive type of glup4/rab5a and glup6/gef mutant accumulates more proglutelin than each single mutant type

We had previously reported that a subset of F2 seeds from a cross between glup4 and glup6 lines showed a more pronounced abnormal accumulation of proglutelin than seeds from the two parents alone (Ueda et al., 2010). In order to determine whether the glup4 and glup6 mutations interacted synergistically to further elevate proglutelin accumulation, plant lines containing both recessive genes were identified by sequencing the GLUP4/Rab5a and GLUP6/GEF genes in F2 plants obtained from a cross between glup4/rab5a lines, EM425 or EM956, with the glup6/gef line, EM939.

The self-pollinated seeds of the double recessive type were then analysed by SDS-PAGE (Fig. 1A). The levels of proglutelin and glutelin subunits in the double recessive type were substantially elevated and reduced, respectively, compared with each parent line (Fig. 1A). This view is readily evident by analysis of the ratio of proglutelin to glutelin subunits where the double recessive type has a much higher ratio than that seen for each parent line (Fig. 1B). In addition, the grain morphology of the double recessive type exhibited a more severe floury grain phenotype than each parent line (Fig 1C). These results suggest that the glup4 and glup6 mutations interact...
In order to elucidate the influence of the combined glup4/rab5a and glup6/gef mutations on intracellular transport of proglutelins, protein bodies in the endosperm of the single and double recessive type were analysed by immunofluorescence microscopy (Fig. 2, Supplementary Fig. S1). In the wild-type endosperm, protein bodies containing prolamines (protein body type I: PB-I) and those containing glutelins and α-globulins (protein storage vacuole: PSV) increased in size and number as the seed develops (Fig. 2A–C, Supplementary Fig. S1A–C). In each mutant type, prolamine containing PB-I were estimated to be similar in size and number to that seen for the wild type (Fig. 2B, E, H, K). In contrast, glutelin-containing PSVs were smaller and fewer in number in each mutant type (Fig. 2E, H, K) compared with the wild type (Fig. 2B). The severity of this condition varied among the mutant types, with glup4/rab5a showing a moderate reduction in size and number of PSVs (Fig. 2D, E) followed by glup6/gef (Fig. 2G, H) while the double recessive type showed the largest reduction in number of PSVs (Fig. 2I, K). Nearly all of the synthesized α-globulin was packaged in paramural bodies (PMBs) in the double recessive type (Fig. 2L).

As described previously (Fukuda et al., 2011, 2013), PMBs were conspicuous in each single mutant (Fig. 2D, G). Many cells contained multiple PMBs and these novel organelles were clustered at the cell’s periphery adjacent to the cell wall. This cell wall relationship is readily evident in the double recessive type as the extracellular space separating individual cells was swollen with glutelin and α-globulin storage proteins (Fig. 2I, K, L). To examine the origin of PMBs in the double mutant, immunofluorescence studies were carried out with sections taken from developing endosperms of the wild type, each single mutant and double recessive type at 1 and 3 weeks after flowering (WAF) (Supplementary Fig. S1). At 1 WAF, glutelin-containing PSVs were beginning to accumulate in the wild type, glup4 and glup6 (Supplementary Fig. S1A, D, G), whereas few PSVs were observed in the double recessive type with nearly all of the glutelins secreted and located at the cell’s boundary or in small PMBs (Supplementary Fig. S1J). This pattern was maintained at 3 WAF where few glutelin-containing PSVs were seen in the double recessive type with nearly all of the glutelins as well as α-globulins either secreted extracellularly or located in the PMBs (Supplementary Fig. S1K, L). Hence, normal transport and packaging of glutelins and α-globulins to PSVs were severely disrupted in the double recessive type than each single mutant.

To obtain additional insight on the trafficking pathways of glutelin and α-globulin to the PSVs and PMBs in the double recessive type, transmission electron microscopy studies were conducted (Figs 3, 4; Supplementary Fig. S2). At 1 WAF, electron-dense granules and the PMBs were detected in the paramural space created by the invagination of the plasma membrane away from the cell wall in the double recessive type (Fig. 3D, E) as well as in each single mutant (Supplementary Fig. S2B, F) but not in the wild type (Fig. 3A). The extracellular electron-dense granules in the double recessive type were labelled with glutelin antibodies (Supplementary Fig. S3B). This observation together with the reduction of glutelin-containing PSVs in the cytoplasm indicates that proglutelin granules, likely in the form of Golgi-derived dense vesicles, were secreted (Fig. 3D). Some proglutelins were normally transported to PSV in the double recessive type (Fig. 3D, F) as well as in each single mutant (Supplementary Fig. S2A, E). In 2 WAF endosperm, the PMBs in the double recessive type were substantially larger than those observed at 1 WAF (Fig. 3G) and from those present in each single mutant (Supplementary Fig. S2C, G). Unlike the condition seen at 1 WAF, electron-dense granules were not readily observed in the cell wall areas suggesting that these granules were taken up by the PMBs. Similar to that seen for the cell wall-associated electron dense granules, those located in the

**Fig. 1.** SDS-PAGE of seed storage proteins in the double recessive type of glup4/rab5a and glup6/gef. (A) CBB staining of the gel. (B) Ratio of proglutelin to sum of glutelin subunits. This value was calculated from densitometry of image A. (C) The floury endosperm of mutant lines. Lane 1, the wild type, Taichung65; lane 2, the wild type, Kinmaze; lane 3, glup4/rab5a, EM425; lane 4, glup4/rab5a, EM956; lane 5, glup6/gef, EM939; lane 6, double recessive type from the crossing of EM425 and EM939; lane 7, double recessive type from the crossing of EM939 and EM956.

**Protein body formation is more severely disrupted in double recessive type of glup4/rab5a and glup6/gef**

Synergistically to elevate proglutelin and, in turn, reduce glutelin subunits as well as mediate a more severe chalky grain phenotype.
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PMB were labelled with glutelin antibody in the double recessive type (Supplementary Fig. S3D, E).

PSVs in glup4 and glup6 endosperms contained a single inclusion similar to those observed in the wild type. Some of the inclusions observed in the single mutant differ from the smaller and spherical type seen in the wild type in having an irregular shape (Fig. 3B, C; Supplementary Fig. S2D, H). By contrast, the PSVs of the double recessive type at 2 WAF were only partially filled and contained multiple small electron-dense granules (Fig. 3H). These small electron-dense granules, labelled with glutelin antibodies (Supplementary Fig. S3C) completely filled up the small irregularly-shaped PSVs of <1 μm at 3 WAF in the double recessive type (Fig. 3I, J arrows; Supplementary Fig. S3F).

Although many of the endosperm cells of the double recessive type contained normal PB-I and aberrant PSVs, some cells located close to the aleurone layer showed a large mass of electron-dense material in the cytoplasm at 3 WAF (Fig. 4). Immunocytochemical analysis showed that this electron-dense material contained spherical granules of prolamine (Fig. 4A arrows) embedded within the larger electron-dense matrix, which was labelled with glutelin antibodies (Fig. 4B arrowheads). Moreover, fusion of PB-I and PSVs was observed (Fig. 4C, D). These results indicate that in some cells of the double recessive type, the membrane fusion occurs among PB-I and PSVs.

The more severe biochemical and cellular structural changes observed in the double recessive type compared with the individual parent lines indicate that combining the glup4/rab5a and glup6/gef genes further disrupts the intracellular transport of proglutelins and α-globulins and the development of the protein bodies. The more severe effect seen when the glup4/rab5a and glup6/gef were combined led us to the hypothesis that homologues of GLUP4/Rab5a are also activated by GLUP6/GEF, and conversely that GLUP4/Rab5a and other Rab5 homologues are activated by other GEF proteins. Collectively, these redundant activities participate in the intracellular transport of the proglutelin from Golgi apparatus to the protein storage vacuole. To substantiate this hypothesis, Rab5 and GEF homologues were identified and their properties examined as described below.

GLUP6/GEF activates other Rab5 members

Rice contains four Rab5 GTPases which fall into two subgroups: conventional Rab5 [GLUP4/Rab5a (Os12g0631100) and Rab5c (Os03g0666500)] and plant-specific Rab5 [Rab5b (Os03g0151900) and Rab5d (Os10g0441800)] (Fukuda et al.,
Analysis of the rice expression profile database (RiceXRro: http://ricexpro.dna.affrc.go.jp/) indicates that all four Rab5 GTPase genes are expressed in developing rice endosperm (Supplementary Fig. S4).

In order to elucidate whether the GLUP6/GEF can activate other Rab5s in rice, in vitro guanine nucleotide exchange assays using tryptophan autofluorescence were conducted using GST-tagged recombinant proteins as described previously (Fukuda et al., 2013). In this study, Rab5b and Rab5c were selected as representative of the plant-specific type and conventional type of Rab5 isoforms, respectively. As shown in Fig. 5, Rab5b and Rab5c were activated by GLUP6/GEF in a dose-dependent manner, whereas, in contrast, Rab11 was not activated by GLUP6/GEF. These results indicate that GLUP6/GEF activates not only GLUP4/Rab5a but also at least two other Rab5 isoforms present in developing rice endosperm. Evidence for the genetic and physical interactions of GLUP6/GEF to Rab5 family proteins was also reported by Liu et al. (2013). Overall, the collective evidence suggests that, in addition to GLUP4/Rab5a, other Rab5 isoforms such as Rab5b and Rab5c may participate in glutelin trafficking.

**Fig. 3.** Transmission electron microscopy of the endosperm from the double recessive type of glup4/rab5a and glup6/gef. A−C, wild type; D−J, the double recessive type from the crossing of EM425 and EM939. A−C depict sections of developing wild-type endosperm at 1 WAF, 2 WAF and 3 WAF, respectively. Corresponding sections from the double recessive type are shown in D−F, G and H, and I and J. I, J: gold particles of 15 nm and 5 nm indicate the reaction of glutelin and prolamine antibodies, respectively. The square area in J shows the PSV is indicated as enlarged image in Fig. S3F. Arrows in A, D, F, H, I, and J show the PSVs. CW, cell wall; PMB, paramural body. Bars: 1 μm in A to F; 2 μm in G to J.

**Fig. 4.** Electron dense inclusion of the endosperm from the double recessive type of glup4/rab5a and glup6/gef at 3 WAF. A−D: the double recessive type from the crossing of EM939 and EM956. B and D are the enlarged images of the square areas in A and C, respectively. Gold particles of 15 nm and 5 nm indicate the reaction of prolamine and glutelin antibodies, respectively. The arrows show the electron-dense material contained spherical granules of prolamine. The arrowheads in B and D show the gold particles by the reaction of glutelin antibodies. Bars, 500 nm.
Identification of a GEF homologue

In addition to multiple Rab5 isoforms, homologues of GLUP6/GEF capable of activating Rab5 forms may also be present in developing rice endosperm. A search for VPS9 domain-containing proteins in the SALADA database (Pfam) (http://salad.dnaaffrc.go.jp/CGViewer/en/cgv_search_pf.html) revealed two rice GEF genes, Os03g0262900 and Os03g0842700. The former corresponded to GLUP6/GEF while the latter encoded a novel guanine nucleotide exchange factor for Rab5, which was named Rab5-GEF2 (Supplementary Fig. S5A). Os03g0842700 (or LOC_Os03g62580.1 using the MSU rice genome annotation database) is a functional gene, as a search of the rice annotation project database (RAP-DB: http://rapdb.dnaaffrc.go.jp/) revealed a corresponding cDNA (AK070821). Moreover, inspection of the RiceXPro microarray RNA expression database revealed that AK070821 were expressed in developing seeds (Supplementary Fig. S5B). The expression of this gene in the embryo after 7 DAF (days after flowering) was higher than in the endosperm.

A comparison of the amino acid sequences of AK070821 (obtained from KOME: http://cdna01.dnaaffrc.go.jp/cDNA/), and the predicted primary amino acid sequence of LOC_Os03g62580.1 revealed that AK070821 was not full length and was missing about 80 amino acids at the N-terminus (Supplementary Fig. S6). To generate a functional cDNA corresponding to the LOC_Os03g62580.1 gene, the full-length open reading frame was amplified with gene specific primers (Supplementary Table 1) and single strand cDNA was reverse-transcribed from mRNA extracted from immature seeds of the wild-type plants as a template. Sequence analysis of the single amplified cDNA demonstrated that the Rab5-GEF2 cDNA possessed an ORF of 927 bp which encoded 308 deduced amino acids (Supplementary Fig. S7), which coincided to the deduced amino acid sequence of the 5 exons of LOC_Os03g62580.1. Similar to GLUP6/GEF, the Rab5-GEF2 contained a conserved helical bundle domain (residues 1–101) and a conserved VPS9 domain (residues 102–245).

Rab5-GEF2 activates Rab5s

In order to determine whether Rab5-GEF2 has the potential to activate GLUP4/Rab5a, we tested whether the two proteins physically interacted using surface plasmon resonance (SPR). The \( K_D \) value of Rab5-GEF2 for GLUP4/Rab5a was measured at 1.7 \( \mu \)M, which is close to the \( K_D \) (2.1 \( \mu \)M) observed for GLUP6/GEF. Moreover the overall response over time curves observed for the Rab5-GEF2 experiment was similar to that seen when GLUP6/GEF was used as the Rab5 activator (Fig. 6). This result indicates that Rab5-GEF2 physically interacts with GLUP4/Rab5a just as efficiently as with GLUP6/GEF.

In order to elucidate whether the Rab5-GEF2 can activate Rab5s including GLUP4/Rab5a, in vitro guanine nucleotide exchange assays were conducted. As expected, Rab5-GEF2 activated GLUP4/Rab5a, the plant-specific Rab5b, and Rab5c in a dose-dependent manner, while the control Rab11 was not activated by Rab5-GEF2 (Fig. 7). This result indicates that Rab5-GEF2 serves as a general activator of Rab5 isoforms. These results provide ample evidence that both GLUP6/GEF and Rab5-GEF2 activate multiple Rab5 proteins present in developing rice endosperm.

The role of domains in GEFS for activity

The deduced primary sequence of Rab5-GEF2, 308 amino acids in length, is 172 residues shorter than GLUP6/GEF as it lacks the longer C-terminal region of GLUP6/GEF (Fig. 8A). As Rab5-GEF2 activates Rab proteins, the C-terminal region in GLUP6/GEF is likely not required for activation of the Rab5 proteins. To obtain evidence to support this view, a truncated form of GLUP6/GEF (ΔC-GLUP6/GEF) was constructed, which lacks 162 amino acids at the C-terminal region (Fig. 8A). As shown in Fig. 8B, ΔC-GLUP6/GEF was capable

![Fig. 5.](image) **Fig. 5.** In vitro GEF assay of GLUP6/GEF. The conformational changes of Rab5s and Rab11 (as 1 \( \mu \)M GST fusion proteins) upon GDP/GMP-PNP exchange were measured by monitoring Trp autofluorescence when incubated with 0 \( \mu \)M (blue), 0.25 \( \mu \)M (magenta), 0.5 \( \mu \)M (yellow), and 1 \( \mu \)M (red) GST-GLUP6/GEF. Note that GST-GLUP6/GEF activates Rab5b and Rab5c, but not Rab11.

![Fig. 6.](image) **Fig. 6.** Physical interactions of GLUP4/Rab5a with Rab5-GEF2 and GLUP6/GEF. Purified GLUP4/Rab5a was immobilized on Sensor Chip CM5, and various concentrations (1.5, 3.5, 5, 6.5, and 10.5 \( \mu \)M) of Rab5-GEF2 (A) and GLUP6/GEF (B) were loaded onto the chip for 120 s. Each sensogram showed the actual binding responses obtained by subtraction of the background responses. Surface plasmon resonance analysis result showed that Rab5-GEF2 and GLUP6/GEF exhibited the same patterns of interaction with GLUP4/Rab5a.
of activating GLUP4/Rab5a and Rab5c in a dose-dependent manner, indicating that the C-terminal region is not required for activation of Rab5 proteins.

The mammalian Rab5 GEF harbours a helical bundle (HB) domain consisting of conserved ~100 amino acids at the N-terminal end of the Vps9 domain, which stabilizes the Vps9 domain (Delprato et al., 2004). In order to evaluate the influence of the HB domain on GEF activity for rice Rab5 GEFs, truncated ΔHB forms for GLUP6/GEF and Rab5-GEF2 were constructed, lacking 90 amino acids of the N-termini (Fig. 8A). When tested in the in vitro GEF assay, the activity of ΔHB/Rab5-GEF2 and ΔHB/ΔC-GLUP6/GEF to all tested Rab5 proteins was greatly reduced (Fig. 8C, D). Liu et al. (2013) similarly reported that a truncated Rab5-GEF2 protein lacking the N-terminal 90 amino acids exhibited weak physical interaction with Rab5 proteins. Overall, the HB domain in GEF proteins is indispensable for the activation of the Rab5 family.

Discussion

Regulation for biosynthesis, intracellular transport, and accumulation of glutelin

The study of the glut mutant lines, which accumulate abnormally high amounts of proglutelin, have identified the function of several factors that participate in the biosynthesis, intracellular transport, maturation and packaging of glutelins into PSVs. Further gene-gene interaction analysis between proglutelin accumulating mutations (Ueda et al., 2010) agrees well with the results obtained by biochemical and histochemical analyses of the mutations. Within the ER, protein disulfide isomerase-like (PDIL) 1-1 is essential for the segregation of proglutelin and prolamine polypeptides within the ER lumen and the maturation of proglutelin, especially when its rate of synthesis significantly exceeds its export from the ER (Takemoto et al., 2002; Satoh-Cruz et al., 2010a). GLUP4/Rab5a and its cognate GDP/GTP nucleotide exchange factor, GLUP6/GEF, participate in the intracellular transport of the proglutelin from the Golgi apparatus to the PSV (Fukuda et al., 2011, 2013). The vacuolar processing enzyme, which cleaves proglutelin to two subunits within the PSV, plays an important role in the formation of the glutelin-containing crystalline structures observed in PSV micrographs (Kumamaru et al., 2010). Additionally, Sar1, a small GTPase, acting as a molecular switch to regulate the assembly of coat protein complex II (COPII), plays a role in the transport of proglutelin and α-globulin from the ER to the Golgi apparatus (Tian et al., 2013).

Loss of GLUP4/Rab5a or GLUP6/GEF activities result in the abnormal accumulation of proglutelin and the appearance of PMBs (Fukuda et al., 2011, 2013). As demonstrated earlier by Liu et al. (2013), the combined loss of both Rab5 and GEF magnified these phenotypic changes. We also demonstrated in the present study that rice contains multiple Rab5 and GEF isoforms that are capable of biochemically interacting with each other (Figs 5, 7). These results provide the causal basis for the apparent synergistic interaction between the glut4/rab5a and glut6/gef mutations resulting in a larger proportion of proglutelin accumulation and more prominent PMBs than that seen in either parent alone. Overall, the available evidence suggests that multiple Rab5 and GEF isoforms participate in the intracellular transport of the proglutelin from the Golgi apparatus to the PSV.

Mutations in the GPA3 gene also result in developing rice seeds having the same phenotypes (i.e. proglutelin accumulation and PMB formation) as glut4 and glut6 (Ren et al., 2014). GPA3 gene codes for a kelch-repeat protein, which interacts with OsVPS9A (GLUP6/GEF) and participates in anterograde post-Golgi trafficking of proglutelin. Double recessive types of kelch-repeat protein and OsRAB5A or OsVPS9A exhibit the same synergistic genetic interactions as Rab5 and GEF indicating that they are involved in the same trafficking events (Ren et al., 2014). In addition to these mutant lines, a dominant Glup5 was identified and genetically classified to the same class as glut4 and glut6 (Ueda et al., 2010). Although the GLUP5 gene has yet to be identified, the available evidence indicates that additional unknown factors participate in the intracellular transport of the proglutelin from the Golgi apparatus.

Rice developed a different Rab5-GEF system from Arabidopsis

Several GEFs for Rab5 had been documented including the yeast Vps9p, the mammalian Rabex-5, RIN-1, RIN-2,
The function of GEF/Rab5 system in intracellular transport of glutelin in rice

RIN-3, RME-6, Alsin, and ALS2CL (Carney et al., 2006) and the Arabidopsis VPS9a and VPS9b isoforms (Goh et al., 2007; Vernoud et al., 2003). The Arabidopsis VPS9a is capable of activating the three Rab5 family proteins and is the major GEF activity, whereas VPS9b is expressed at very low levels in vegetative tissues when viewed by RT-PCR analysis or by analysis of the public expression database. This dominant role of VPS9a is also supported by the severe growth phenotypes exhibited by T-DNA insertion mutants of VPS9a. These lines of evidence point to VPS9a as representing the major GEF activity for Rab5 in Arabidopsis (Goh et al., 2007). Based on neighbor-joining tree analysis, GLUP6/GEF is the rice orthologue of VPS9a. The corresponding counterparts of Rab5-GEF2 and VPS9b, however, do not exist in Arabidopsis and rice, respectively (Supplementary Fig. S5A).

Mammalian cells utilize several GEF isoforms to activate Rab5 at different steps of the endocytic pathway. RME6 participates in formation of clathrin-coated vesicles for endocytosis at the plasma membrane (Sato et al., 2005). Rabex-5 complexed with the adaptor protein Rabaptin-5 is essential for homotypic fusion between early endosomes (Horiuchi et al., 1997; McBride et al., 1999; Lippe et al., 2001) while RIN1 stimulates Rab5-dependent endosome fusion and epidermal growth factor receptor-mediated endocytosis (Tall et al., 2001). Compared with animals and Arabidopsis, rice appears to have developed a more generalized Rab5-GEF system in having redundant GEFs and Rab5 activities.

Fig. 8. *In vitro* GEF assay of truncated GEFs. The conformational changes of Rab5a and Rab11 (as 1 μM GST fusion proteins) upon GDP/GMP-PNP exchange were measured by monitoring Trp autofluorescence when incubated with 0 μM (blue), 0.25 μM (magenta), 0.5μM (yellow), and 1 μM (red) GST-truncate GEFs. (A) Schematic structure of GEF proteins. VPS9a: Rab5 specific GEF in Arabidopsis (Goh et al., 2007). Δhelical bundle of Rab5-GEF2: deletion of helical bundle domain sequence, 90 amino acids from Rab5-GEF2. Δhelical bundle of GLUP6/GEF: deletion of C-terminal sequence, 162 amino acids from GLUP6/GEF. (B) *In vitro* GEF assay of Δc-terminal of GLUP6/GEF. Note that GST-Δc-terminal of GLUP6/GEF activates GLUP4/Rab5a and Rab5c. (C) *In vitro* GEF assay of Δhelical bundle, Δc-terminal of GLUP6/GEF. Note that GST-Δhelical bundle, Δc-terminal of GLUP6/GEF cannot activate GLUP4/Rab5a, Rab5b, and Rab5c. (D) *In vitro* GEF assay of Δhelical bundle of Rab5-GEF2. Note that GST-Δhelical bundle of Rab5-GEF2 cannot activate GLUP4/Rab5a, Rab5b, and Rab5c.
participate in the transport of proglutelins from the Golgi apparatus to the PSV.

**Helical bundle domain of GEF is essential for the physical interaction with Rab5 family**

In addition to the conserved VPS9 domain, many Rab5 GEFs share a second conserved helical bundle (HB) domain (Burd et al., 1996; Horiuchi et al., 1997). This HB domain, originally identified in Rabex-5 3-D structure, stabilizes the Vps9 domain (Delprato et al., 2004). Deletion of the HB domain in Rab5-GEF2 and GLUP6/GEF resulted in loss of ability to activate Rab5 members (Fig. 8C, D), although the truncated Rab5-GEF2 lacking the HB domain weakly interacted with GLUP4/Rab5a (Liu et al., 2013). The truncated GLUP6/GEF containing the deleted HB domain and C-terminal region was also unable to activate Rab5a, although deletion of the C-terminal region in GLUP6/GEF had no significant effect on the activation of Rab5s (Fig. 8). The HB domain is also conserved in VPS9a and Rabex-5 (Uejima et al., 2010) with the sequence similarity in the HB domain of Rab5-GEF2 to GLUP6/GEF, VPS9a, VPS9b, and Rabex-5 being 59.1%, 65.3%, 64.4%, and 40.9%, respectively (Supplementary Fig. S8). These facts suggest that the HB domain is essential for the physical interaction of GEFs with Rab5 proteins.

**Supplementary data**

Supplementary data are available at JXB online.

Supplementary Fig. S1. Immunofluorescence microscopy of the endosperm in the double recessive type of *glu4*/rab5a and *glup6*/gef.

Supplementary Fig. S2. Transmission electron microscopy of endosperm in the *glu4*/rab5a and *glup6*/gef.

Supplementary Fig. S3. Immuno-electron microscopy of endosperm from the double recessive type of *glu4*/rab5a and *glup6*/gef.

Supplementary Fig. S4. Expression analysis of Rab5 homologues in rice.

Supplementary Fig. S5. Analysis of GEF genes for Rab5 in rice.

Supplementary Fig. S6. Alignment of amino acid sequence of transcripts from Os03g0842700.

Supplementary Fig. S7. cDNA and the deduced amino acid sequences corresponding to Os03g0842700.

Supplementary Fig. S8. Alignment of helical bundle domain from different GEFs.

Supplementary Table 1. List of PCR primers.

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