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OsVPS9A Functions Cooperatively with OsRAB5A to Regulate Post-Golgi Dense Vesicle-Mediated Storage Protein Trafficking to the Protein Storage Vacuole in Rice Endosperm Cells

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ABSTRACT In the rice endosperm cells, glutelins are synthesized on rough endoplasmic reticulum as proglutelins and are sorted to the protein storage vacuoles (PSVs) called protein body IIs (PBIIs), where they are converted to the mature forms. Dense vesicle (DV)-mediated trafficking of proglutelins in rice seeds has been proposed, but the post-Golgi control of this process is largely unknown. Whether DV can fuse directly with PSV is another matter of debate. In this study, we propose a regulatory mechanism underlying DV-mediated, post-Golgi proglutelin trafficking to PBII (PSV). *gpa2*, a loss-of-function mutant of *OsVPS9A*, which encodes a GEF of OsRAB5A, accumulated uncleaved proglutelins. Proglutelins were mis-targeted to the paramural bodies and to the apoplast along the cell wall in the form of DVs, which led to a concomitant reduction in PBII size. Previously reported *gpa1*, mutated in *OsRab5a*, has a similar phenotype, while *gpa1gpa2* double mutant exacerbated the conditions. In addition, OsVPS9A interacted with OsRAB5A *in vitro* and *in vivo*. We concluded that OsVPS9A and OsRAB5A may work together and play a regulatory role in DV-mediated post-Golgi proglutelin trafficking to PBII (PSV). The evidence that DVs might fuse directly to PBII (PSV) to deliver cargos is also presented.

Key words: 57H mutant; dense vesicle; PSV; OsVPS9A; OsRAB5A.

INTRODUCTION

It is generally believed that there are two types of functionally distinct vacuoles in the plant cells: protein storage vacuole (PSV) and lytic vacuole (LV) (Vitale and Raikhel, 1999; Jiang et al., 2000; Park et al., 2004), although they are not necessarily present in the same plant cell at a particular developmental stage (Robinson et al., 2005; Rogers, 2008). Distinct vesicular transport pathways that lead to each type of vacuole have been proposed. Proteins destined to the LV are transported from the endoplasmic reticulum (ER) to the Golgi complex and later to the prevacuolar compartments (PVCs) before reaching LV. This transport pathway is largely similar to the delivery of proteins to the lysosome or vacuole in animal and yeast cells, respectively (Vitale and Raikhel, 1999; Bassham and Raikhel, 2000; Jin et al., 2001; Sohn et al., 2003). In contrast, protein trafficking to the PSV is unique to plants (Muntz, 1998; Park et al., 2004; Vitale and Hinz, 2005). Dependent on a particular storage protein studied, it could be carried in the dense vesicle (DV) or the precursor-accumulating (PAC) vesicles on its way to the PSV (Hohl et al., 1996; Okita and Rogers, 1996).

Rice seeds accumulate large quantities of storage proteins, such as glutelins, prolamins, and α-globulin during

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seed maturation. Glutelins can account for as much as 80% of the total endosperm proteins, and are easily digestible. Therefore, rice glutelins are important protein sources for human consumption. Glutelins are synthesized as 57-kDa glutelin precursors on the rough endoplasmic reticulum (rER) and are sorted to PSVs called PBIIs, where they are proteolytically processed into mature storage proteins, each containing an acidic and a basic subunit (Tanaka et al., 1980; Yamagata et al., 1982a, 1982b). The rice α-globulin is transported with proglutelins together to form the PBII. Prolamins are synthesized and accumulated in ER, distensions of ER eventually transformed into PBIs (Yamagata et al., 1982a). Multiple transport pathways have been reported for proglutelin transport from the ER to PBIIs. Proglutelins are probably transported via DVs budded from the Golgi apparatus (Krishnan et al., 1986) or directly via ER-derived PAC-like compartments in a Golgi-independent manner (Takahashi et al., 2005). However, PAC-like vesicles (Hara-Nishimura et al., 1998) have not been seen in other ultrastructural studies of rice protein body biogenesis (Ogawa et al., 1989; Wang et al., 2009; Shen et al., 2011).

DVs were observed in faba bean (Hohl et al., 1996), pea (Hinz et al., 1999), rice (Krishnan et al., 1986), soybean (Mori et al., 2004), and *Arabidopsis thaliana* (Otegui et al., 2006; Hinz et al., 2007). As carefully defined in pea cotyledon cells, DVs contain an electron-dense core with no coats; they are uniform vesicles of 150 nm in diameter containing major intrinsic membrane protein α-TIP and storage proteins destined for the PSVs (Hohl et al., 1996; Hinz et al., 1999; Hillmer et al., 2001; Wenzel et al., 2005). It has been proposed that vacuolar storage proteins are sorted by physical aggregation starting at the *cis*-Golgi, and the aggregations continue throughout the Golgi stacks until they are released at *trans*-Golgi network (TGN) in the form of DVs (Hillmer et al., 2001; Hinz et al., 2007). In addition to physical aggregation, vacuolar sorting receptors (VSRs) and receptor homology transmembrane–RING H2 motif protein (RMR) have been proposed to be co-receptors for sorting storage proteins into DVs (Otegui et al., 2006; Hinz et al., 2007). En route to the PSVs, the DVs fuse to the intermediate PVC compartments, which further fuse with the developing PSVs to deliver their cargos (Robinson et al., 1998; Jiang et al., 2002; Otegui et al., 2006; Shen et al., 2011). The possibility that DV fuses directly with PSV has not been reported. Similarly, despite the effects made to dissect the mechanisms for sorting storage proteins into DVs at the Golgi apparatus, the regulatory mechanism of post-Golgi trafficking of DVs and/or PVCs to PSV remain largely unknown in plant.

Efforts have been made to dissect the molecular mechanisms of glutelin synthesis, trafficking, and accumulation in rice seeds by screening for mutants with elevated levels of 57-kDa proglutelins (57H mutants). So far, only limited genes have been cloned and characterized using this approach. For example, *esp2* mutant endosperm cells accumulated a new type of protein bodies smaller than PBI and PBII, where proglutelins aggregated with Cys-rich prolamins via disulfide bonds. Protein disulfide isomerase-like 1–1 (PDIL 1–1), a protein functioning as a molecular chaperone for nascent polypeptides in the ER lumen, is responsible for the phenotype of *esp2* mutant (Takemoto et al., 2002; Satoh-Cruz et al., 2010). In *osvpe1/glup3* mutant, proglutelins were not properly cleaved, the typical crystalline lattice structure of wild-type PBIIs was not formed, and *OsVPE1* gene was found to encode a vacuolar processing enzyme that cleaves proglutelins (Wang et al., 2009; Kumamaru et al., 2010). A recent study placed ER-resident *esp2 and* PSV-resident *osvpe1* mutants at the most upstream and downstream of genetic interactions, respectively, among the eight known 57H mutants, namely *esp2* and *Glup1-glup7*. *glup4*, *Glup5*, and *glup6* were suggested to control intermediate steps between *esp2* and *osvpe1* (Ueda et al., 2010). The characterization of *gpa1/glup4* mutant identified OsRAB5A, an ortholog of mammalian RAB5 GTPase. *gpa1* endosperm cells had smaller PBIIs (PSVs); significant amounts of glutelins in the *gpa1* endosperm cells were not delivered to PSV, but instead were mis-targeted and secreted to newly generated subcellular structures, namely 'secretory vesicle-like structures' and 'vesicle filled structures' (also called paramural body, PMB). OsRAB5A was localized to the Golgi and PVC in *Arabidopsis* protoplasts and in DVs in the rice endosperm cells. Therefore, the characterization of *gpa1* mutant provided genetic and cellular evidences for OsRAB5A in the post-Golgi trafficking to the PSV (Wang et al., 2010; Fukuda et al., 2011). However, the nature of 'secretory vesicle-like structures' observed in *gpa1* mutant deserves better characterization (Wang et al., 2010), although they were proposed to be DVs (Fukuda et al., 2011).

In *Arabidopsis*, RHA1/RabF2a and ARA7/RabF2b are conventional types of RAB5 proteins (Ueda et al., 2001). Dominant negative form of ARA7 perturbed embryo development and cell fate decision by inhibiting PIN1 endocytosis, and consequently PIN polarization (Dhonukshe et al., 2008). RAB–GEF is critical in temporal and spatial control of RAB activation to induce downstream events. AtVPS9A is the only GEF for three RAB5 proteins in *Arabidopsis*, and is required for embryogenesis and root development, by orchestra PIN1 endocytosis together with ARA7 (Goh et al., 2007; Dhonukshe et al., 2008). RHA1 and ARA7 are located to the PVC, and might function not only in endocytosis, but also in vacuolar transport pathways (Sohn et al., 2003; Kotzer et al., 2004; Lee et al., 2004; Haas et al., 2007). Evidence that OsRAB5A and OsVPS9A cooperate in the PSV pathway in the rice endosperm cells was recently presented (Fukuda et al., 2013). But the role of DVs and the regulation of DVs in the transport of proglutelins to the PBII (PSV) were not mentioned.

Here, we reported, in *gpa2* (*glutelin precursor accumulation 2*) mutant, the amount of 57-kDa proglutelins increased. In the meantime, abnormal structures accumulated in the

endosperm cells, such as the PMBs and vesicles along the cell wall, were the derivatives of proglutelin-containing DVs. Mistargeting of DVs led to the reduction of PBII (PSV) size. The similar phenotypes were observed in *gpa1*, while the condition was accentuated in *gpa1gpa2* double mutant. Second, the mutated gene encoded OsVPS9A, a GEF for RAB5A protein (mutated in *gpa1*), which interacted with OsRAB5A *in vitro* and *in vivo*. Our data indicated that OsVPS9A and OsRAB5A work cooperatively to regulate post-Golgi, DV-mediated transport of storage proteins to PBII (PSV) in developing rice endosperm. In addition, DVs were proposed to fuse directly to the PSV (PBII) to deliver their cargos.

RESULTS

gpa2 Seeds Accumulate Proglutelins and Develop Abnormal Endosperm

As a continuous effect to dissect the glutelin trafficking pathway in rice seed, we isolated another 57H mutant named *gpa2* after the characterization of *gpa1* (Wang et al., 2010). Similarly to *gpa1*, *gpa2* accumulated a higher amount of 57-kDa proglutelins, with a concomitant reduction in the amount of 20-kDa basic and 40-kDa acidic subunits of the mature glutelins (Figure 1A). An antibody against the 40-kDa acidic subunits (Wang et al., 2010) reacted specifically with proglutelins and the acidic subunits confirmed the result of SDS–PAGE (Figure 1B). These results demonstrated that the proper accumulation of mature forms of glutelins is disrupted in *gpa2* seeds.

Notably, *gpa2* mutant endosperms appeared floury (Figure 1D) in contrast to wild-type (Figure 1C). Scanning electron microscopic analysis revealed that the *gpa2* endosperms comprised round and loosely packaged, compound starch granules (Figure 1F and 1H) instead of tightly packaged, crystal-like structures in wild-type (Figure 1E and 1G). Time course analysis demonstrated that *gpa2* seeds weighed less than wild-type throughout the process of grain filling, and this difference became apparent at 6 days after flowering (DAF) a very early stage of seed development (Figure 1I). As a consequence, the dry *gpa2* seeds had 36.2% weight loss (g per 1000 kernels) accompanied by varied contents of starch, amylose, protein,

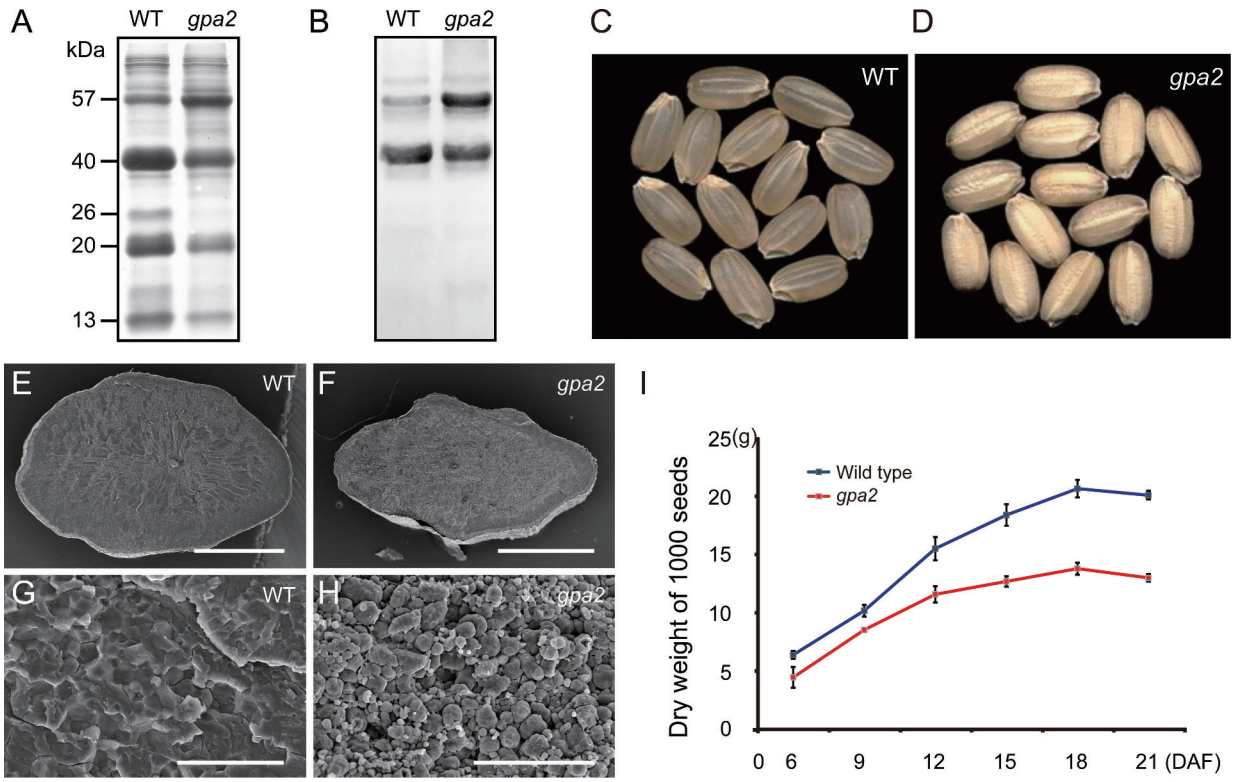

Figure 1. Characterization of the *gpa2* Mutant.
(A) Seed storage proteins revealed by a Coomassie blue-stained SDS–PAGE gel.
(B) Immunoblot analysis of seed storage proteins using antibodies against glutelin acidic subunits.
(C) Wild-type seeds.
(D) *gpa2* seeds.
SEM analysis of the endosperms of wild-type **(E, G)** and *gpa2* **(F, H)**.
(I) Grain-filling process of wild-type and *gpa2* seeds. Scale bars: 1 mm (E, F); 50 μm (G, H).

and lipid when compared to those of wild-type (Supplemental Table 1). In short, *gpa2* mutant was defective in seed development, and the intracellular transport of proglutelins might be disturbed in the *gpa2* endosperm cells.

Reductions of Proglutelin Trafficking to PBII (PSV) and PBII Size Are Accompanied by Novel Structure Accumulation in the *gpa2* Endosperm Cells

Rice seed storage proteins mainly accumulated in the subaleurone layer. Indirect immunofluorescent labeling on semithin sections (1 μm) was adopted to gain a broad view of glutelin deposition in 12-DAF seeds (Figure 2). PBII and PBI revealed respectively by antibodies against glutelin subunits (Figure 2A) and prolamins (Figure 2B) were discrete compartments (Figure 2C) in the wild-type endosperm cells. The PBIIs became smaller and fewer in *gpa2* cells (compare Figure 2A and 2D), and glutelins were seen to accumulate in two categories of abnormal structures at the vicinity of cell wall, one lined along the cell wall (Figure 2D, solid arrowhead) and the other formed large aggregations (Figure 2D, open arrowhead). In the meantime, prolamin deposition appeared normal (Figure 2B, 2E, 2C, and 2F).

In order to quantify the size of PBIIs, an antibody to OsTIP3, a protein highly homologous to known α-TIPs (Jauh et al., 1999; Jiang et al., 2001), was used to label the membrane of PBII. The specificity of the antibody was examined, that it recognized a single band in rice seed crude extracts (Supplemental Figure 1). In the 12-DAF endosperm cells, PBIIs in *gpa2* cells (Figure 2H) looked smaller than that of wild-type (Figure 2G). Quantitative analysis showed that the population of PBIIs with areas smaller than 2 μm^2 was remarkably increased, while that bigger than 4 μm^2 was reduced in *gpa2* mutant (Figure 2I). Quantification with TEM graphs showed that the average area of PBIIs in wild-type and mutant cells is 3.2 and 1.3 μm^2, respectively (Supplemental Figure 2), which agreed with the data shown in Figure 2I. Our results indicated that *gpa2* mutant is defective in the delivery of proglutelins and in the PBII (PSV) formation.

Next, we observed the *gpa2* endosperm cells at the ultrastructural level by TEM. In Nipponbare, the wild-type of *gpa2*, two types of protein bodies were evident in the endosperm cells. Irregularly shaped PBIIs displayed uniform electron density, and were almost full of storage proteins at 12 DAF (Figure 3A). In *gpa2*, however, a small portion of the storage proteins were transported correctly into PBII, occupying only a limited space (Figure 3B). Meanwhile, the details of two categories of abnormal structures noticed in Figure 2 were revealed. In the first category, so-called 'electron-dense secretory granules' (Wang et al., 2010) scattered around the plasma membrane (PM) (Figure 3D, arrowheads); some of them were seen to fuse with the PM and enter the apoplastic region where they gradually dispersed, losing their characteristic spherical appearances (Figure 3C, stars). Further mergence of the dispersed contents formed a stretch of electron-dense stripes (Figure 3D, stars). Subsequently, it resulted in the cell wall with increased electron densities and the cell wall was over two times wider at later stages (wild-type: 296 nm, n = 12; *gpa2*: 658 nm, n = 15; compare Figure 3A with 3E). For the second type of the novel structures, the 'electron-dense secretory granules' fused with the PM and discharged their contents (Figure 3E, stars), which formed so-called paramural bodies (PMBs) (Marchant and Robards, 1968; Wang et al., 2010; Fukuda et al., 2011) (Figure 3E), together with additional cell wall material depositions (glucan, callose, pectin) (Supplemental Figure 3). In contrast, we did not find any above-mentioned novel structures in the wild-type endosperm cells; PBIs had normal appearances in *gpa2* mutant (Figure 3A).

Therefore, two categories of 'electron-dense secretory granules'-derived novel structures appeared in *gpa2* mutant, with a concomitant reduction of proglutelin trafficking to PBIIs (PSVs).

'Electron-Dense Secretory Granules' Are DVs in *gpa2* Endosperm Cells, and DVs Fuse Directly with PSV (PBII) in the Wild-Type Endosperm Cells

Careful EM study showed that yet-fused discrete 'electron-dense secretory granules' are present at three distinct locations within *gpa2* endosperm cells (near Golgi, along the cell wall, and near the PMB), and they share certain features. First, individual 'electron-dense secretory granule' is surrounded by a translucent outer layer of unknown nature (insertions in Figure 4A–4C), which resembles the peripheral layer of DV enriched in a sucrose-binding-protein homolog (SBP) in pea cotyledon (Wenzel et al., 2005). Second, immunolabeling showed that the individual 'electron-dense secretory granules' contain glutelins (Figure 5). Finally, granules newly budded from Golgi apparatus in *gpa2* endosperm cells have an average of 160 nm in diameter (Figure 4B and 4F, n = 38), comparable to DVs of wild-type rice (150 nm in diameter, n = 33) (Figure 4A and 4F), pea, and *Arabidopsis* (Hinz et al., 1999; Wenzel et al., 2005; Hinz et al., 2007). Compared to the newly budded ones, those appeared along the cell wall (Figure 4C) or near the PMBs (Figure 4D) but yet-fused are larger in average (Figure 4F, 189 nm, n = 57), some of them could be around 350 nm, and the variation might be due to pair-wise fusion of vesicles in close contact (Figure 4E). Collectively, 'electron-dense secretory granules' at the three locations are DVs in nature. Re-examination of the 'electron-dense secretory granules' in *gpa1* mutant reached the same conclusion (Supplemental Figure 4; Wang et al., 2010). Therefore, it seemed that DV-mediated transport pathway to the PBII (PSV) was defective, leading to accumulation of intermediate vesicles in *gpa2* and *gpa1* mutants.

It seems that DV mis-targeting contributes directly to PBII (PSV) size reduction in *gpa2* mutant (Figures 2I and 4). Therefore, DV might act as a transport intermediate that fuses directly with the storage vacuole in the wild-type cells.

Indeed, we observed DVs are being fused with PBII (PSV) (Figure 6A–6C). After entering the vacuole, DV loses its membranous structure (Figure 6D), and the membrane-less DV later fuses with the larger aggregation pre-formed inside PBII (Figure 6E). It is worth noting that, during the grain-filling stage, several DVs could simultaneously appear around or attach to the surface of a particular PBII (Figure 6A); subsequent fusion presumably leads to fast PBII formation.

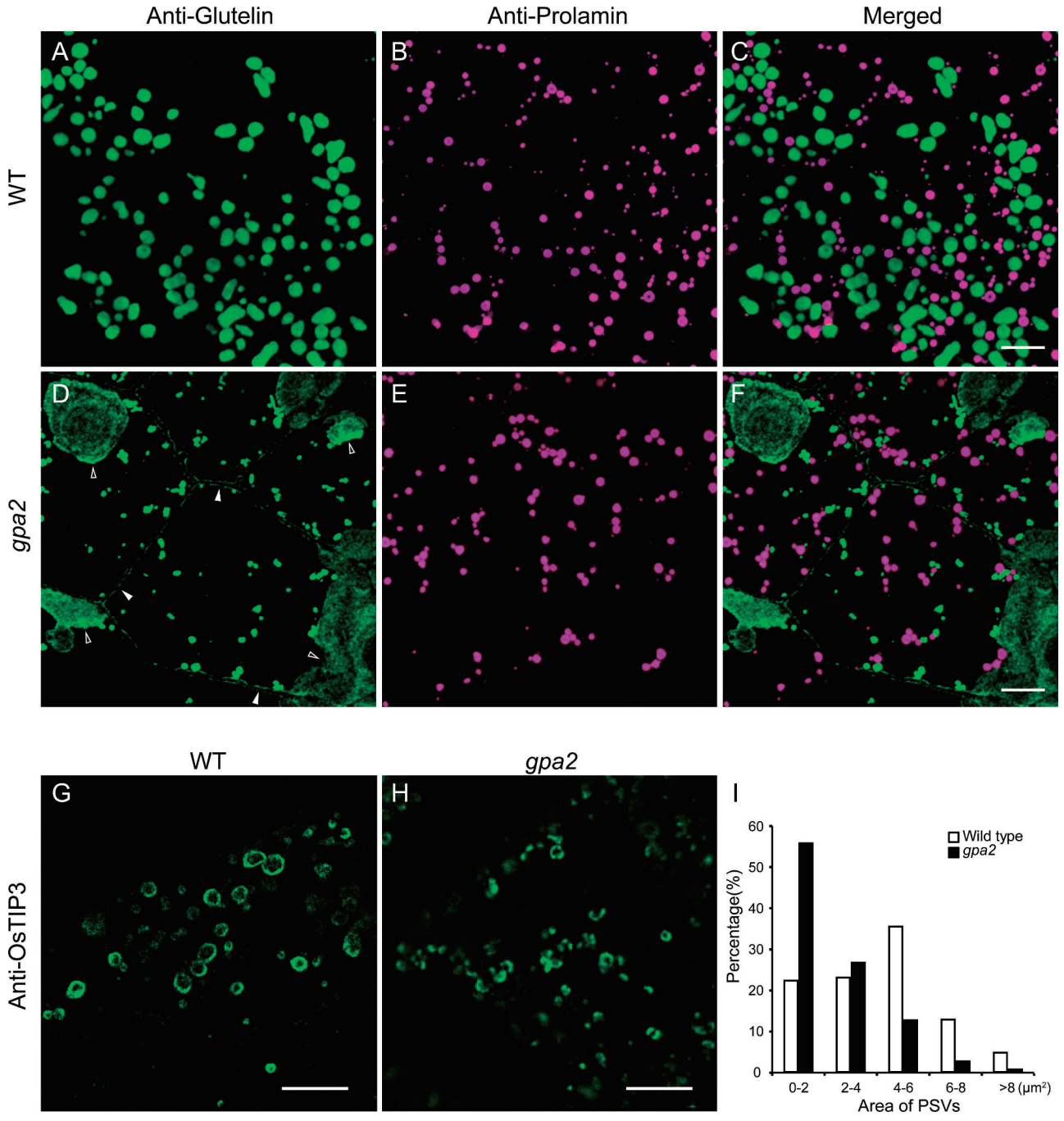

Figure 2. Phenotypic Characterization of *gpa2* by Immunolabeling of Semi-Thin Sections of 12 DAF Seeds.
(A–C) Wild-type.
(D–F) *gpa2* mutant. (A, D) Glutelins were detected by using mouse anti-glutelin antibodies, followed by Alexa-488 conjugated secondary antibodies. Two types of abnormal structures are indicated by arrowheads and open arrowheads, respectively. (B, E) Prolamins were immunolabeled using rabbit anti-prolamin antibodies, followed by Alexa-555 conjugated secondary antibodies. (C, F) Merged images.
(G, H) PBIIs were labeled by rabbit anti-OsTIP3 antibodies, followed by Alexa-488 conjugated secondary antibodies.
(I) Size distribution of PBIIs labeled by anti-OsTIP3 antibodies. Scale bars: 10 μm (A–H).

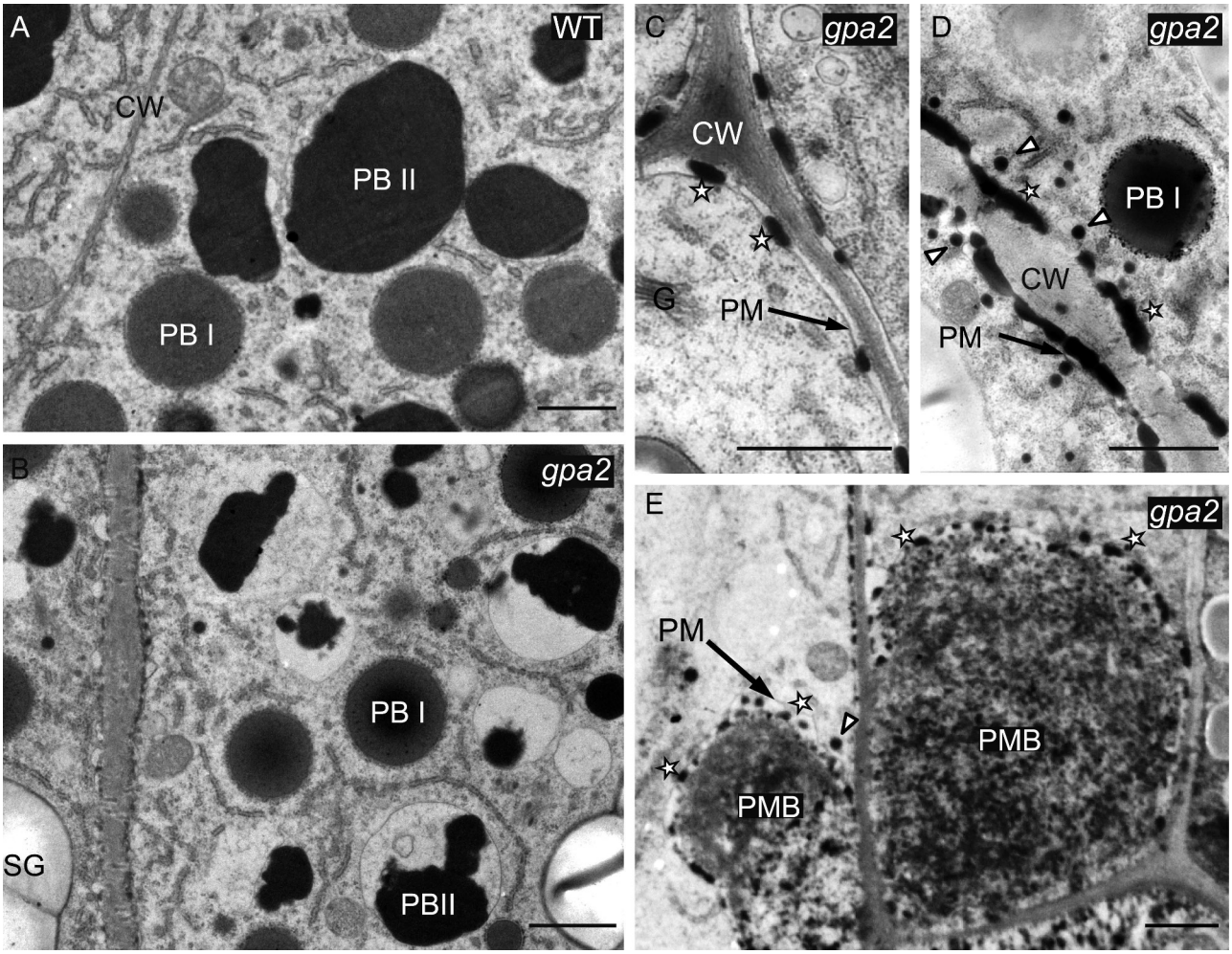

Figure 3. Ultrastructures of 12 DAF Endosperm Cells of *gpa2* and Wild-Type.
(A) Wild-type.
(B–E) *gpa2*. Arrowheads in (D, E) indicate individual yet-fused 'electron-dense secretory granules'. Stars in (C, D, E) indicate abnormal structures in the apoplastic region. DVs just fused with PM (C); Discharged DV contents merged to form electron-dense stripes (D); PMB filled with discharged DVs (E). SG, starch granule; PMB, Paramural body; PM, plasma membrane; G, Golgi apparatus; LB, lipid body. Scale bars: 1 μm.

Disruption of DV-Mediated Proglutelin Transport Leads to Reduced Glutelin Contents in PBII (PSV) of gpa2 Mutant Endosperm Cells

The accumulation of abnormal glutelin-containing structures revealed by the immunofluorescence labeling of anti-glutelin antibodies (Figure 2) implied that glutelins were mis-sorted in *gpa2*. Immunoelectron microscopy was used to gain the detailed information. In the wild-type cells, the glutelins were detected in PBIIs (Figure 5A), the Golgi apparatus, and the DVs newly budded from the Golgi apparatus (Figure 5C). In *gpa2*, glutelins were observed inside individual DVs at various places (Figure 5D and 5E), also in the abnormal structures (Figure 5E and 5F). Thus, glutelins were mis-targeted in *gpa2* mutant, leading to less glutelin filling in PBIIs (Figure 5B). The results conformed with the immunofluorescence data presented above (Figure 2).

Map-Based Cloning of *GPA2*

gpa2 was first identified from a screening of T-DNA insertion lines of the *japonica* variety Nipponbare. Unfortunately, the phenotype was not linked to the T-DNA. To identify the gene using map-based cloning, the mutant was crossed with the *indica* variety 93-11 and 651 F_2 recessive individuals were isolated. The *GPA2* locus was first located between two simple sequence repeats (SSR) markers (RM3467 and RM14764) on chromosome 3, which was further narrowed down to an approximately 42-kb region with more molecular markers (Supplemental Table 1). The 42-kb region was situated on the BAC clone OJ1041F02, which contained eight putative open reading frames (ORFs) (Figure 7A). DNA sequencing revealed a single nucleotide change from Guanine to thymine in the third exon of LOC_Os03g15650. This gene was named *OsVPS9A*, as it contained a conserved Vacuolar Protein Sorting

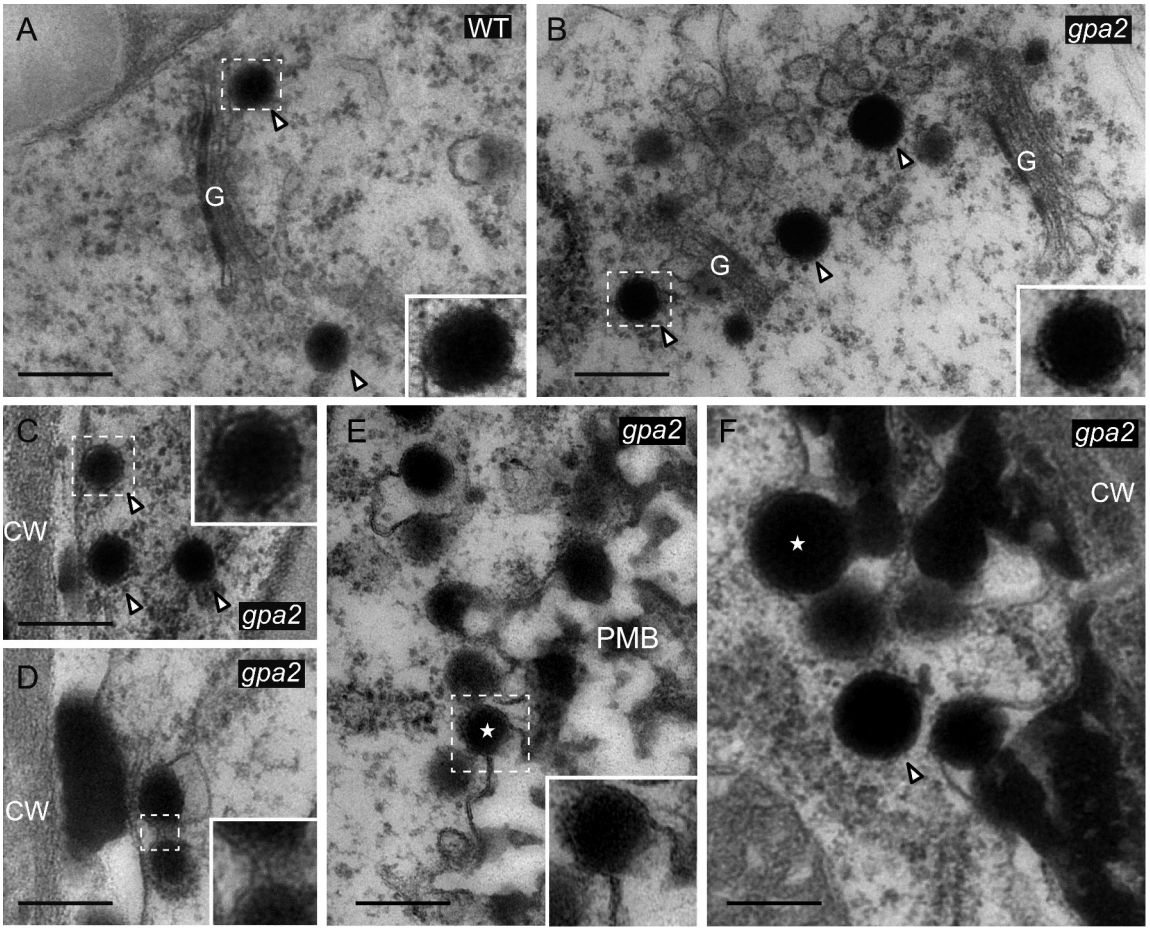

Figure 4. DVs Accumulated in 12 DAF *gpa2* Mutant.
(A) Wild-type.
(B–F) *gpa2* mutant. (B) DVs in the immediate vicinity of the Golgi apparatus. (C) DVs that are going to fuse with the plasma membrane (arrowheads). (D) Two DVs are fusing with each other. (E,F) DVs are fusing with the plasma membrane to form the PMB. Fused aggregations (stars) are present in the apoplastic region. Arrowhead in (F) indicates a yet-fused DV. Scale bars: 250 nm.

9 (VPS9) domain (Figure 7B). Guanine-to-thymine transversion generated a stop codon leading to a premature translational product of 137 amino acids without the VPS9 domain, indicating a correlation between the loss of GEF activity and the mutant phenotypes. Complementation was performed by introducing *p35S:VPS9A-3×FLAG* into *gpa2* mutants. More than 10 transgenic lines restored the translucent appearance of seeds (Figure 7C). One line was chosen randomly for further analysis. In the particular line, VPS9A–FLAG proteins were properly expressed, as demonstrated by anti-VPS9A and anti-FLAG antibodies (Figure 7E). The amount of proglutelins (Figure 7D) and the ultrastructures of the endosperm cells (Figure 7F) were restored to normal. In conclusion, *OsVPS9A* is the gene responsible for *gpa2* mutant phenotypes.

OSVPS9A Might Be a Guanine Nucleotide Exchange Factor of RAB5A GTPase in Rice

Like RAB5s in *Arabidopsis*, OsRAB5s are classified into conventional and plant-specific types (Ebine et al., 2011). OsRAB5A and OsRAB5C belong to the conventional type, while OsRAB5B and OsRAB5D are of plant-specific type (Supplemental Figure 5). Except OsRAB5A, the function of OsRAB5B, OsRAB5C, and OsRAB5D are largely unknown. VPS9 domain-containing proteins catalyze nucleotide exchange on RAB5 GTPases in eukaryotic cells (Carney et al., 2006). The interactions between the OsVPS9A and OsRAB5 family members were examined using yeast two-hybrid assay. OsVPS9A showed strong interactions with the GDP-fixed and the nucleotide-free forms but not with wild-type or GTP-fixed forms of all OsRAB5s, which is consistent with a GEF-like function (Figure 8A). The reason why OsVPS9A did not interact with wild-type OsRAB5s might be due to high GTP:GDP ratio in yeast cells, where wild-type OsRAB5s might be in their GTP-bound forms. OsVPS9A did not interact with other subclasses of RAB GTPases, OsRAB7 and OsRAB11, indicating the specificity of interactions between OsVPS9A and OsRAB5s (Figure 8A). Co-immunoprecipitation (Co-IP) assay demonstrated that OsVPS9A interacted with wild-type and S25N

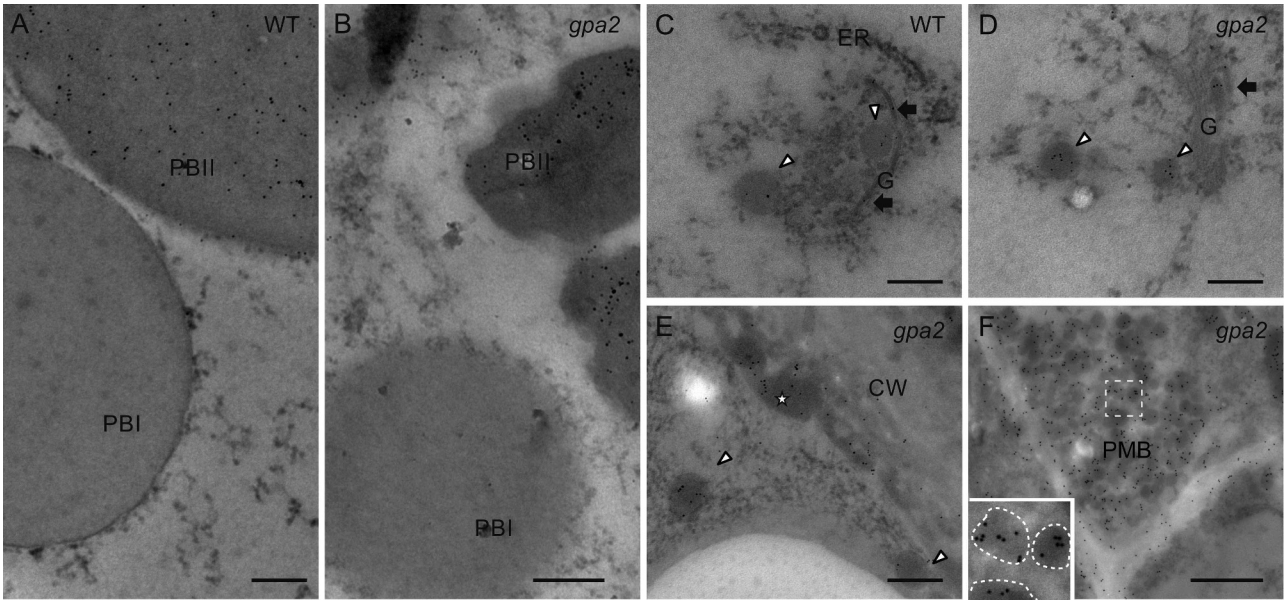

Figure 5. *In Situ* Distribution of Glutelins in the Rice Endosperm Cells Revealed by Immunogold Electron Microscopy.
(A, C) wild-type.
(B, D–F) *gpa2*. (A) Glutelins accumulated exclusively in PBIIs in the wild-type endosperm cells. (B) Size-reduced PBII containing glutelins. (C, D) Glutelins appeared in Golgi and newly budded DVs. Arrows indicate glutelin labeling of Golgi. (E) Glutelins in DVs and apoplastic region (Star). Arrowheads in (C–E) indicate DVs with glutelin labeling. (F) Glutelins in the PMB. Insertion in (F) outlines the fused DVs in PMB that are yet dispersed. Scale bars: (A–E) 200 nm, (F) 1 μm. 6-nm gold particle conjugated secondary antibodies were used in (A–E), while 15-nm ones were used in (F).

forms of OsRAB5A *in vivo*, but not with its Q70L mutant, indicating that OsVPS9A is a potential GEF for OsRAB5A *in vivo* (Figure 8B). There are two *VPS9* genes, *OsVPS9A* and *OsVPS9B*, in rice. While *OsVPS9B* gene was predicted to have different transcripts resulted from alternative splicing, the most abundant one (AK070821) was chosen for the further experiments. The VPS9 domain of this OsVPS9B isoform shares 62% amino acid sequence identity with that of OsVPS9A (Supplemental Figure 6). Co-IP experiment showed that OsRAB5A interacted with this isoform, albeit the interaction is much weaker than with OsVPS9A (Supplemental Figure 7).

gpa1gpa2 Mutant Had More Severe Defects in DV-Mediated Transport of Glutelins from Golgi to PBII (PSV)

To examine the genetic interaction between *OsRAB5A* and *OsVPS9A*, *gpa1gpa2* double mutant was generated. Four independent lines chosen randomly had significantly higher amount of 57-kDa proglutelins than each single mutant, and both the acidic and basic subunits were present at a very low level (Figure 9A). Quantification analysis of the protein band intensities (Figure 9A) clearly showed that the percentage of proglutelins in total amount of glutelin proteins (57 kDa/<57 kDa + 40 kDa + 20 kDa>) in *gpa1gpa2* double mutant was much higher than those of single mutants (Figure 9B). Correspondingly, the size and number of glutelin-containing PBIIs were further reduced, with concomitant increase in glutelin mis-targeting to abnormal structures (Figure 9C–9N). Careful EM dissection reached the same conclusion (Figure 9O–9R), and it was clear that PBIIs were much less filled (Figure 9O). Hence, the *gpa1gpa2* double mutant showed exaggerated defects in glutelin transporting to PBII (PSV) and the formation of PBII (PSV) (Figure 9). These genetic interactions between *osvps9a* and *osrab5a* mutations strongly suggest that OsVPS9A and OsRAB5A function coordinately in the same membrane trafficking pathway.

DISCUSSION

gpa2 Is a 57H Mutant in Rice with Altered Endomembrane Organization in Endosperm Cells

Screening of 57H mutants provided us with possibilities to reveal the complex network of proglutelin trafficking and processing in rice seeds. Studies on several 57H mutants showed that the defects in folding of proglutelins in rER (*esp2*, Takemoto et al., 2002), in delivery of proglutelins to PBII (*gpa1/glup4*, Wang et al., 2010; Fukuda et al., 2011), and in proper cleavage of proglutelins inside PBII (*osvpe1*, Wang et al., 2009) could result in 57H proglutelin accumulation. In this study, we reported the isolation and functional study of a 57H mutant *gpa2* where proglutelins trafficking is disturbed, leading to their partial secretion into the apoplast. In *gpa2* endosperm cells, proglutelins were partially mis-targeted in the forms of DVs which fused with PM rather than with PBII (PSV); disperse of DVs in the apoplastic region resulted in abnormal structures, less and smaller PBII (PSV)s. Examination

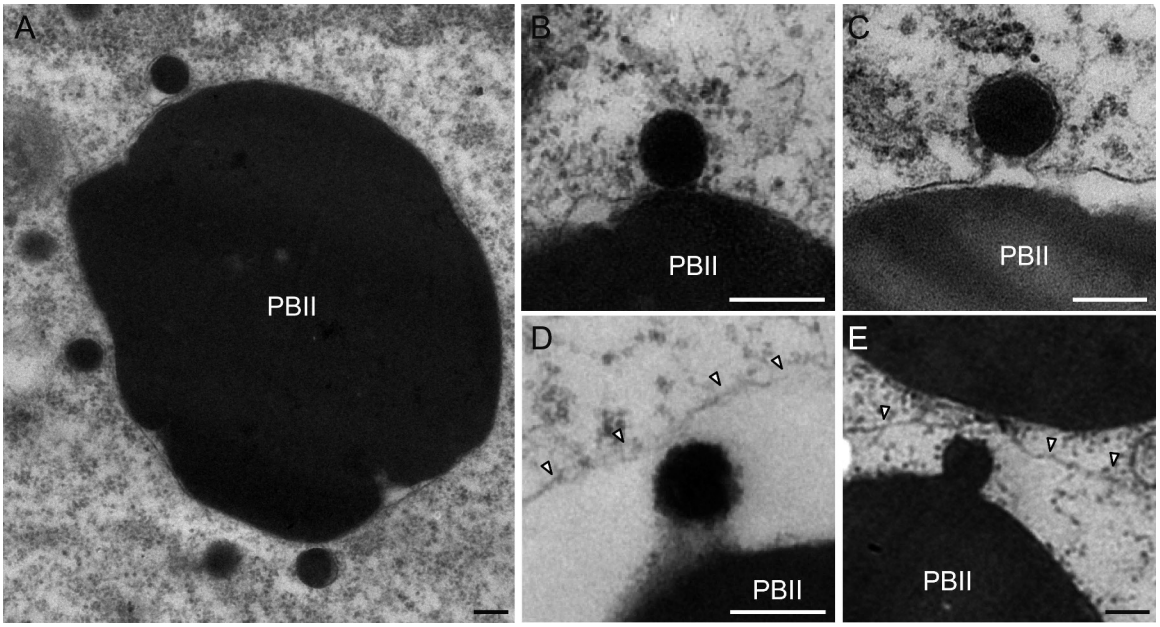

Figure 6. Electron Micrographs of the Wild-Type Endosperm Cells at 12 DAF Showing Progressive Stages of the Formation of DV-Derived PBIIs (PSVs).
(A) DVs scattered around PBII (PSV) before fusion.
(B–E) Gallery of fusion processes. (B) A DV in close contact with PBII before fusion. (C) A DV was fusing with the PBII. (D) A DV fused with the tonoplast and entered the vacuole. (E) A DV was fusing with the extant PBII aggregations. Open arrowheads in (D, E) indicate tonoplast. Scale bars: 200 nm.

of *gpa1* mutants suggested that previously described 'secretory vesicle-like structures' are actually DVs, and 'vesicle filled structures' are PMBs filled with DVs that have been fused with plasma membrane. Therefore, *gpa1* and *gpa2* mutants have overall similar phenotypes with disrupted endomembrane organization. The mutated gene of *gpa2* turned out to be *OsVPS9A* (Figure 7), the potential GEF for *OsRAB5A* (Figure 8), further suggesting a genetic interaction between these two genes in regulating the proglutelin targeting to PBII (PSV).

OsVPS9A and OsRAB5A Interact Biochemically and Genetically to Regulate Post-Golgi DV to PBII (PSV) Trafficking

It seems that proglutelins are sorted correctly into DVs, and DVs containing proglutelins bud off normally from Golgi in *gpa1*, *gpa2*, and *gpa1/gpa2* mutants. But the targeting and/or fusion of DVs are disturbed subsequently, leading to their partial secretion in the apoplast. OsVPS9A physically interacts with the dominant negative, but not the constitutive active, form of OsRAB5A, which agreed with the property of OsVPS9A as a GEF for OsRAB5A (Hama et al., 1999). This interaction and that OsVPS9A could interact with all OsRAB5s tested in yeast two-hybrid assay provided a plausible explanation for why *gpa2* mutant had an overall similar, yet more severe, phenotype than *gpa1*. *gpa1gpa2* double mutant showed additive effects, and it might be due to the existence of OsVPS9B, which also interacts with OsRAB5A (Supplemental Figure 7).

How exactly OsVPS9A and OsRAB5A regulate the process is an important question to ask. The specificity of membrane fusion is ensured by key regulators such as the best-characterized RAB5 proteins (Horazdovsky et al., 1994; Grosshans et al., 2006). In yeast and *Arabidopsis* RAB5 homologs VPS21p, ARA7 and RHA1 are required for the sorting of vacuolar proteins (Horazdovsky et al., 1994; Kotzer et al., 2004) and are found in a prevacuolar, late endosomal compartment (Gerrard et al., 2000; Lee et al., 2004). RHA1 protein was suggested to function in the vacuolar pathway by regulating the fusion between PVCs, PVC with the central vacuole, or vesicles derived from PVC with the central vacuole through the studies of *rha1*-dominant negative mutant in a protoplast-based system (Sohn et al., 2003). It has been demonstrated that a SNARE complex SYP22/VTI11/SYP51/VAMP727 plays a crucial role in PSV biogenesis and the transport of storage proteins by regulating membrane fusion of the PVC with the vacuole during *Arabidopsis* seed development (Ebine et al., 2008). Recently, *rha1syp22-1* double mutant was shown to accumulate 12S proglobulins, as was observed in *vps9a-2* mutant as well (Ebine et al., 2011). These data suggested that VPS9A and RHA1 might cooperatively regulate SYP22 containing SNARE complex formation in the PVC to PSV pathway, but this hypothesis awaits detailed subcellular and biochemical characterizations. This proposed regulation might not be direct, as most evidence showed that RAB might not directly regulate the SNARE function (McBride et al., 1999; Grosshans et al., 2006). Additional players such as tethering complexes HOPS and CORVET might be involved, as suggested by studies in yeast (Peplowska et al., 2007). Homologs of HOPS

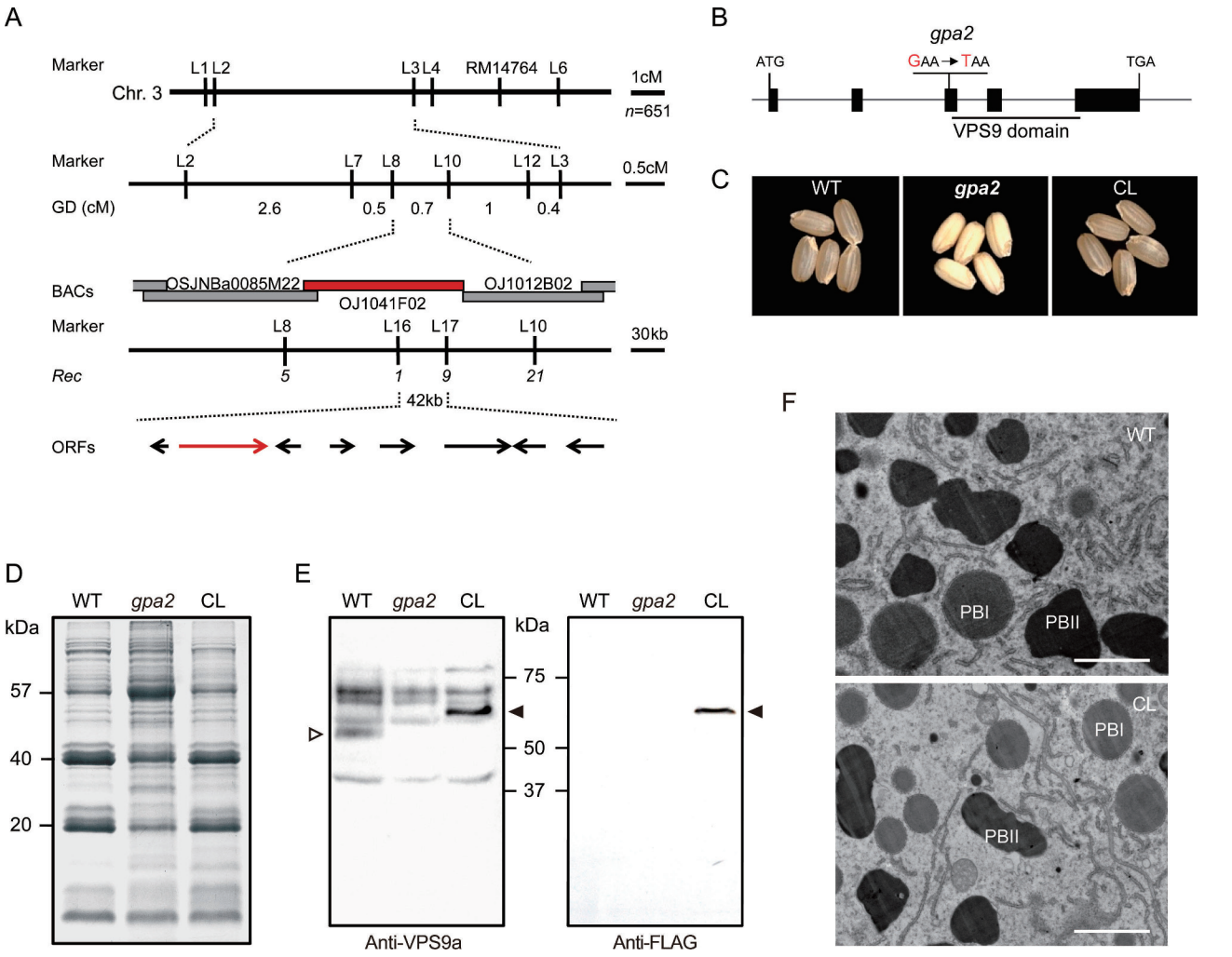

Figure 7. *OsVPS9A* Is the Gene Responsible for *gpa2* Mutant Phenotypes.
(A) High-resolution mapping of mutant gene in *gpa2*. The markers and the number of recombinants are indicated above or below the map, respectively. GD, genomic distance; Rec, recombinants. Eight ORFs were predicted in the mapping region.
(B) A single nucleotide transversion in *OsVPS9A* led to a stop codon before the VPS9 domain.
(C) The complemented line (CL) restored normal seed appearances.
(D) The amount of 57-kDa proglutelins in the CL was comparable to that of wild-type.
(E) VPS9A-3×FLAG fusion proteins were properly expressed in the CLs, black arrowheads indicate the band of OsVPS9A-3×FLAG fusion protein, while open arrowheads indicate the band of the endogenous OsVPS9A protein.
(F) The ultrastructures of endosperm cells in the CL are back to normal. Scale bar: 2 μm.

and CORVET are present in plant (Rojo et al., 2001) and their function in storage protein trafficking await to be explored. In short, the specificity of membrane fusion between DVs or DV with PBII (PSV) might be ensured by OsVPS9A and OsRAB5A proteins in rice endosperm cells.

DVs May Fuse Directly to PBII (PSV) in Rice Endosperm Cells during Seed Development

The DVs were suggested to fuse with the intermediate PVC compartments, which further fuse with the developing PSVs to deliver their cargos (Robinson et al., 1998; Jiang et al., 2002; Otegui et al., 2006; Shen et al., 2011). In this study, DVs were observed to fuse with PBII (PSV) directly, and the discharged DV contents joined the extant crystalloids. Correspondingly, in *gpa1*, *gpa2*, and *gpa1gpa2* mutants, the targeting and/or fusion of DVs with PBII were defective to various extents, leading to less glutelin filling and smaller PBII (PSV). Combining data from wild-type and mutant studies, we showed that DVs could fuse directly with PBII (PSV) in rice endosperm cells.

In summary, our data demonstrated that a post-Golgi, DV-mediated proglutelin transport pathway to PBII (PSV) in the rice endosperm cells, which is regulated by the cooperation of OsVPS9A and OsRAB5A. In addition, DVs were suggested to fuse with PBII (PSV) directly in rice seeds. Whether the DV-mediated direct trafficking of storage proteins to PSV exists in other plants deserves further analysis.

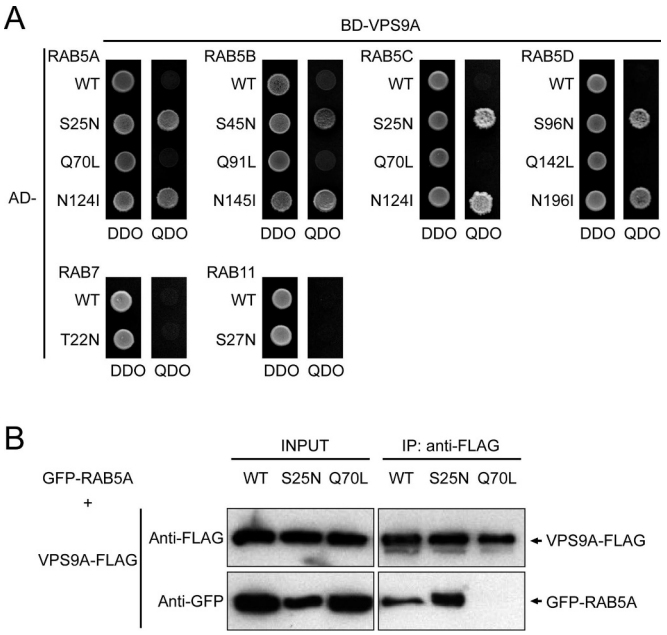

Figure 8. Interactions between OsVPS9 and OsRAB5 Family Members.
(A) OsVPS9A and OsRABs were fused to the DNA binding domain (BD) and activation domain (AD) of GAL4 transcription factor, respectively. The transformants were grown on DDO (SD/-Trp/-Leu) and QDO (SD/-Trp/-Leu/-His/-Ade) plates.
(B) VPS9A could interact with wild-type and S25N form of RAB5A in a Co-IP assay.

METHODS

Plant Materials and Commercial Antibodies

The *gpa2* mutant was identified from mutant lines of the *japonica* variety Nipponbare. The *gpa1* mutant was isolated from a ^{60}Co-irradiated mutant pool of the *indica* cultivar N22 (Wang et al., 2010). *gpa1gpa2* double mutant plants were isolated from the F_2 population by genotyping with primers T5390-F/T5390-R and Q4041-F/Q4041-R (Supplemental Table 2). All plants were grown in the field during the natural growing season in Nanjing, China. *gpa2* complementation lines were established by introducing a construct bearing *p35S:VPS9A-3×FLAG* into the mutant. Individual plasmid was transformed into the *Agrobacterium tumefaciens* strain *EHA105* and then introduced into calli of various backgrounds for regeneration. Monoclonal antibodies against callose (1,3-β-glucan), (1,3;1,4)-β-glucan, and pectin (JIM7) were purchased from Biosupplies Australia and University of Georgia.

Measurement of Starch, Lipid, and Total Protein Contents

Fresh harvested rice grains were baked in an oven for 5 d, and ground into fine flour with a miller before being measured. Amylose, lipid, and total protein contents were subsequently measured according to the previous reports (Han et al., 2012).

Scanning Electron Microscopy

Transverse sections of dry seeds from *gpa2* and wild-type plants were sputter-coated with gold palladium, and then were observed with a Hitachi S-3400N scanning electron microscopy.

Transmission Electron Microscopy Studies

For conventional ultrathin sectioning, transverse sections (approximately 1 mm in thickness) of developing endosperm were fixed in 0.1 M phosphate buffer (pH 7.2) with 2% (v/v) glutaraldehyde and 0.5% (w/v) paraformaldehyde for 12 h, and then further fixed in PBS containing 2% OsO_4 (pH 7.2). After dehydration in an ethanol series, samples were embedded in LR White resin (London Resin, Berkshire, UK), followed by sectioning using a ultramicrotome (Power TomeXL; RMC, Arizona, USA). Ultrathin sections (50–70 nm) were double stained with 2% (w/v) uranyl acetate and 2.5% (w/v) lead citrate aqueous solution before observation. For glutelin immunogold labeling analysis, fresh endosperm samples were fixed in 0.5% (v/v) glutaraldehyde and 2.5% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The remaining procedures were performed as described above without OsO_4 fixation. After labeling, the grids were stained with 2% (w/v) uranyl acetate. Immunolabeling was done using standard procedures as described previously (Wang et al., 2010). Sections were examined with a Hitachi H-7650 transmission electron microscope.

Fluorescence Microscopic Observation

For immunofluorescence analysis, endosperm sections (approximately 1 mm in thickness) were fixed overnight at 4°C in 2% (v/v) paraformaldehyde, 2% (v/v) glutaraldehyde, and 250 mm sucrose buffered to pH 7.2 with 50 mM PIPES. After being washed three times and dehydration in an ethanol series, the samples were embedded in LR White resin (London Resin). Semi-thin sections (1 μm) were blocked in TBST (10 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20, pH 7.4) containing 3% BSA for 1 h, and then incubated with the primary antibodies for 1 h in TBST containing 1% BSA. The sections were incubated with the Alexa Fluor 488 or Alexa Fluor 555 conjugated secondary antibodies (Invitrogen) for 1 h. Washed samples were observed using a Zeiss LSM710 laser scanning confocal microscope.

For α-TIP labeling, fresh harvested mid-developing rice seeds (12 DAF) were submerged in MTSB (50 mM PIPES–KOH, 10 mM EGTA, 10 mM $MgSO_4$, 1% DMSO, 0.1% Triton X-100, pH 6.9), and 60-μm sections were prepared using a vibratome (VF-300, Leica, Germany). Sections were fixed for 30 min in MTSB containing 4% paraformaldehyde, washed three times with MTSB, and followed by immunolabeling as described above.

Map-Based Cloning of the *gpa2* Gene

To map the *gpa2* locus, the *gpa2* mutant (*japonica*) was crossed with an *indica* variety 93-11 to generate a mapping

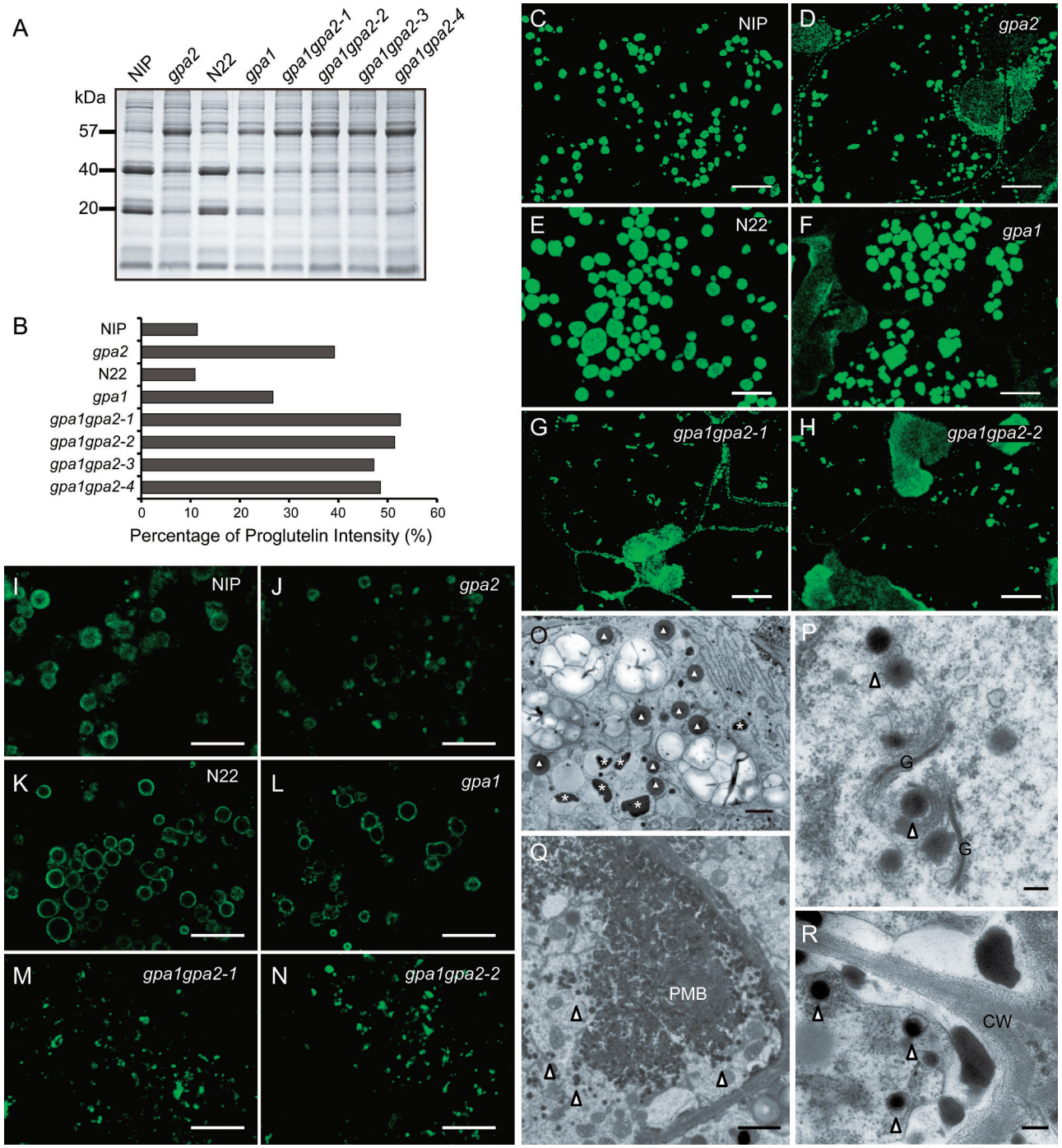

Figure 9. *gpa1gpa2* Double Mutant Showed Additive Effects in 12-DAF Seeds.
(A) Storage protein profiling of the indicated mutants on a SDS–PAGE gel.
(B) Quantification of the band intensity ratio of 57-kDa/(57 kDa+40 kDa+20 kDa) in different wild-type and mutant seed samples.
(C–H) Glutelins were detected by immunolabeling using mouse anti-glutelin antibodies, followed by Alexa-488 conjugated secondary antibodies.
(I–N) PBIIs were labeled by rabbit anti-OsTIP3 antibodies, followed by Alexa-488 conjugated secondary antibodies.
(O–R) TEM of *gpa1gpa2* endosperm cells. Stars and triangles in (O) indicate PBIIs and PBIs, respectively. Open arrowheads in (P–R) indicate DVs. Scale bars: 10 μm (C–N), 1 μm (O, Q), 200 nm (P, R).

population. In the F_2 population, total proteins were extracted from half of an individual rice seed and resolved by SDS–PAGE gel to monitor the accumulation of the 57-kDa band. Meanwhile, the other half of the identified mutant seeds with intact embryos was allowed to grow until seedling stage for DNA extraction. 651 mutant individuals were isolated to fine map the *gpa2* locus. The primer pairs are listed in Supplemental Table 2.

Antibody Generation

Polyclonal antibodies against OsVPS9A were produced in rabbit using purified His-OsVPS9A protein as an antigen. Polyclonal antibodies against OsTIP3, 13-kDa prolamins, and glutelins were raised in rabbits as described elsewhere (Takahashi et al., 2004; Kawakatsu et al., 2010; Wang et al., 2010). Monoclonal antibody against the glutelin acidic subunits was raised in mouse.

Co-IP Assay

Coding sequences of *OsVPA9A* and *OsVPS9B* were introduced into the binary vector pCAMBIA1300-221-Flag, while the wild-type and mutant versions of *OsRAB5* were subcloned into the binary vector pEGAD. Individual construct was transformed into *Agrobacterium tumefaciens* EHA105. Various combinations of *Agrobacteria* were used to transfect tobacco leaves, which were harvested 48 h post infiltration and used for IP as described elsewhere (Liu et al., 2010).

Yeast Two-Hybrid Assay

The cDNAs for wild-type and mutant versions of *RABs* were cloned into pGADT7, while that of *VPS9A* was cloned into pGBKT7. Various combinations of plasmids were co-transformed into AH109 strain and tested according to the manufacturer's instructions (Clontech).

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the accession numbers: OsVPS9A (ABF95105); OsVPS9B (BAF13772); OsRAB5A (BAF30359); OsRAB5B (BAF10902); OsRAB5C (BAF12760); OsRAB5D (BAF26606); OsRAB7(BAF05974); OsRAB11(BAF24204); OsTIP3 (BAF26847).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

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