RESEARCH ARTICLE

GPA5 Encodes a Rab5a Effector Required for Post-Golgi Trafficking of Rice Storage Proteins

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Short title: GPA5 regulates storage protein sorting

One-sentence summary: GPA5 functions as a plant-specific effector of Rab5a required for mediating tethering and membrane fusion of dense vesicles with protein storage vesicles in rice endosperm cells.

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ABSTRACT

Dense vesicles (DVs) are vesicular carriers, unique to plants, that mediate post-Golgi trafficking of storage proteins to protein storage vacuoles (PSVs) in seeds. However, the molecular mechanisms regulating the directional targeting of DVs to PSVs remains elusive. Here, we show that the rice (Oryza sativa) glutelin precursor accumulation5 (gpa5) mutant is defective in directional targeting of DVs to PSVs, resulting in discharge of its cargo proteins into the extracellular space. Molecular cloning revealed that GPA5 encodes a plant-unique phox-homology domain-containing protein homologous to Arabidopsis thaliana ENDOSOMAL RAB EFFECTOR WITH PX-DOMAIN (EREX). We show that GPA5 is a membrane-associated protein capable of forming homodimers and that it is specifically localized to DVs in developing endosperm. Colocalization, biochemical, and genetic evidence demonstrate that GPA5 acts in concert with Rab5a and VPS9a to regulate DV-mediated post-Golgi trafficking to PSVs. Furthermore, we demonstrated that GPA5 physically interacts with a class C core vacuole/endosome tethering (CORVET) complex and a seed plant-specific VAMP727-containing R-soluble NSF attachment protein receptor (SNARE) complex. Collectively, our results suggest that GPA5 functions as a plant-specific effector of Rab5a required for mediating tethering and membrane fusion of DVs with PSVs in rice endosperm.
INTRODUCTION

Plant cells in endosperm and embryonic tissues possess a special type of vacuole: the protein storage vacuoles (PSVs; Jiang et al., 2001; Paris et al., 1996; Shimada et al., 2018). Accumulating morphological evidence has pointed to the general presence of two distinct vesicle-mediated pathways for the trafficking of storage proteins from the endoplasmic reticulum (ER) to PSVs. One is the Golgi-dependent aggregation sorting route via plant-specific dense vesicles (DVvs; 100 to 200 nm in diameter) that are enclosed by a single membrane but devoid of a recognizable protein coat; the other is the direct ER-to-PSV insoluble aggregation sorting route by precursor accumulating (PAC) vesicles (200 to 400 nm in diameter; Herman and Schmidt, 2004; Jolliffe et al., 2005; Robinson et al., 2005; Vitale and Hinz, 2005; Shimada et al., 2018). Although the prevalence of each of the trafficking pathways varies considerably depending on plant species, storage protein compositions, and even developmental stages, the Golgi-dependent pathway is generally deemed to be the prominent route in higher plant seeds (Robinson et al., 2005; Kumamaru et al., 2007).

Rice (Oryza sativa) endosperm has been used as a model system for studying the genetic and molecular mechanisms regulating storage protein trafficking to PSVs because of its agricultural importance and rich resources for molecular genetic studies. Rice has three major types of storage proteins in developing seeds, including glutelins, α-globulin, and prolams (Takemoto et al., 2002). Prolams are directly deposited inside the ER lumen as intracisternal protein granules that eventually bud off from the ER as spherical protein body Is (PBIs), while glutelins are initially synthesized as 57-kDa precursors at the ER, and sequentially pass through the Golgi complex and then bud off from the trans-Golgi network (TGN) in the forms of DVs, which are directionally targeted to PSVs (Krishnan et al., 1986; Kumamaru et al., 2007; Liu et al., 2013; Ren et al., 2014). In PSVs, glutelins are efficiently converted into mature forms by vacuolar processing enzymes and form irregular protein body IIs (PBIIs) together with α-globulin (Krishnan et al., 1992; Wang et al., 2009b; Kumamaru et al., 2010).

Defects in the PSV transport pathways cause a 57-kDa glutelin precursor over-accumulation phenotype (referred to as the 57H defect) in rice dry seeds (Wang et al., 2010). Previous studies have identified a number of 57H mutants (Ueda et al., 2010) and several responsible genes have been cloned and characterized. Among them, ENDOSPERM STORAGE PROTEIN MUTANT 2 (ESP2) encodes a PROTEIN DISULFIDE ISOMERASE-LIKE protein (PDI) and GLUTELIN PRECURSOR MUTANT 3 (GLUP3) encodes a VACUOLAR PROCESSING ENZYME1 (VPE1) and they function in the maturation of glutelin precursors within the ER and in the conversion of glutelins from precursors into mature subunits within the
PSVs, respectively (Takemoto et al., 2002; Kumamaru et al., 2010; Wang et al., 2009b). Moreover, 

GLUP2/GLUTELIN PRECURSOR ACCUMULATION4 (GPA4) encodes GOT1B (for the GOLGI 
TRANSPORT 1B) that functions in ER exit of storage proteins through mediating proper assembly of the 
coat protein complex II (COPII) prebudding complexes (Fukuda et al., 2016; Wang et al., 2016), while 

GLUP4/GPA1 and GLUP6/GPA2 encode a small GTPase Rab5a and its guanine exchange factor (GEF) 
VPS9a, respectively (Wang et al., 2010b; Fukuda et al., 2011; Fukuda et al., 2013; Liu et al., 2013). 
Loss-of-function mutations of GLUP4/GPA1 and GLUP6/GPA2 result in a similar vacuolar protein sorting 
defect: partial mistargeting of DVs to the apoplast space rather than PSVs. Together, these studies have 
elegantly demonstrated that like their mammalian and yeast homologs (Odorizzi et al., 2000; Langemeyer et 
al., 2018), Rab5a and its GEF VPS9a play an essential role in the directional targeting of DVs to PSVs in 
rice endosperm cells. Despite these progresses, the downstream effector proteins of Rab5a responsible for 
tethering and fusion of DVs to the target membrane of PSVs have remained elusive in rice.

In this study, we report the isolation of another rice 57H mutant named glutelin precursor accumulation5 
(gpa5). Cytological evidence demonstrates that the mutation in GPA5 causes fusion of DVs with the plasma 
membrane, thereby discharging their contents into the apoplast space. GPA5 encodes a plant-unique 
phox-homology (PX) domain-containing protein homologous to the previously reported Arabidopsis 
thaliana EREX, EREX-LIKE1 (ERE1), and EREL2 proteins (Sakurai et al., 2016). We show that GPA5 is 
a peripheral membrane protein and that it is specifically localized to mature DVs in developing endosperm. 
We present colocalization, biochemical, and genetic evidence supporting that GPA5 acts as an effector of 
Rab5a likely involved in mediating membrane fusion of DVs with PSVs via interaction with the class C core 
vacuole/endosome tethering (CORVET) complex and VAMP727-containing soluble NSF attachment protein 
receptor (SNARE) complex in rice endosperm. Our results substantiate a model in which the CORVET- and 
VAMP727-dependent storage protein trafficking pathway in rice seeds utilizes the evolutionarily conserved 
Rab5a with its plant-unique downstream effector-GPA5.

RESULTS

The gpa5 Mutant Exhibits a Defect in Storage Protein Transport to the PSV

As part of a continuing effort to understand the molecular mechanisms by which storage proteins are 
transported, we identified another 57H mutant designated gpa5. The gpa5 mutant exhibited defective grain 
development evidenced by white core floury endosperm at maturity (Figures 1A and 1B). Scanning electron
microscopy revealed that \textit{gpa5} endosperm was composed of loosely arranged and round-shaped compound starch granules, in contrast to the tightly packed and polyhedral-shaped compound starch granules in the wild-type seeds (Figure 1C). SDS-PAGE and immunoblotting with storage protein antibodies showed that mature seeds of wild-type plants accumulated large amounts of glutelins mainly in the forms of acidic and basic subunits (Figures 1D and 1E), while \textit{gpa5} mutant seeds abnormally accumulated glutelins in the forms of precursors, accompanied by significantly reduced accumulation of acidic and basic subunits as well as \(\alpha\)-globulin (Figures 1D and 1E). Furthermore, immunoblotting with isoform-specific antibodies verified accumulation of precursors for all glutelin subfamilies including GluA, GluB, GluC, and GluD (Figure 1F). These results suggest that \textit{gpa5} had a defect either in the vacuolar trafficking pathways or in processing of the precursors into mature forms within PBII s. As the latter defect usually causes PBII morphology alteration but has no obvious effect on endosperm development (Wang et al., 2009b; Kumamaru et al., 2010), the floury white endosperm of \textit{gpa5} suggests that it is more likely defective in the vacuolar transport machinery like the previously reported \textit{gpa1}, \textit{gpa2}, and \textit{gpa3} mutants.

A common feature of the previously reported mutants defective in maturation and/or ER exit of glutelins is markedly elevated expression of molecular chaperones, such as BIP1 and PDI1-1, most likely because of a stimulated unfolded protein response (Takemoto et al., 2002; Wang et al., 2016). Immunoblot analysis showed that the accumulation of both BIP1 and PDI1-1 was largely comparable between the wild-type and \textit{gpa5} mutant seeds (Figure 1G), suggesting that maturation and/or ER exit of glutelins is likely unaffected in \textit{gpa5}. In support of this notion, transmission electron microscopy (TEM) examination showed no obvious alterations in the morphologies of ER, PBI, and Golgi in \textit{gpa5} (Supplemental Figure 1). However, unlike the previously reported 57H mutants, \textit{gpa5} exhibited a seedling lethal phenotype (Figure 1H). Together, these results suggest that \textit{gpa5} most likely represents a novel mutant defective in both post-Golgi trafficking of storage proteins to PSVs and vegetative development.

To uncover the cytological basis of abnormal glutelin precursor accumulation in \textit{gpa5}, we prepared semithin sections of developing endosperm at 9 d after flowering (DAF), followed by Coomassie brilliant blue (CBB) staining. Large amounts of storage proteins were detected in the subaleurone cells of both wild-type and \textit{gpa5} endosperms (Figure 1I). Two types of PBs were readily observed in the wild-type endosperm: round-shaped PBIs and irregularly-shaped PBIIs (Figure 1J). Although both PBIs and PBIIs were observed in \textit{gpa5}, the size of PBIIs was obviously smaller than that of wild-type PBIIs (Figure 1J).
More strikingly, numerous CBB-stained paramural bodies (PMBs) were observed in gpa5 but not in the wild-type endosperm (Figures 1I and 1J).

To further verify the subcellular distribution of storage proteins, double immunofluorescence labeling was performed using specific antibodies against glutelins, α-globulin, and prolamins. In wild-type subaleurone cells, glutelins and α-globulin were deposited in PBIIs with α-globulin at the periphery (Figure 1K), while prolams were sequestered exclusively in PBIs (Supplemental Figure 2). Notably, in gpa5, large amounts of glutelins and α-globulin but not prolams were abnormally accumulated in the PMBs or in granules surrounding the PMBs (Figure 1K; Supplemental Figure 2), suggesting that glutelins and α-globulin are missorted into the PMBs. In line with this notion, the sizes of PBIIs labelled by glutelins were significantly smaller in gpa5 endosperm (Figures 1J to 1L), but the sizes of PBIs labelled by prolams were largely comparable between the wild-type and gpa5 endosperm (Figures 1J and 1M; Supplemental Figure 2). Additionally, some cell wall-like components stained with Calcofluor white were also observed in the PMBs in gpa5 endosperm (Supplemental Figure 2).

The gpa5 Mutation Perturbs the DV-Mediated Post-Golgi Trafficking of Glutelins to PSVs

As DV is the major carrier vesicle for post-Golgi trafficking of storage proteins to the PSVs (Krishnan et al., 1986; Ren et al., 2014), we next examined the effects of the gpa5 mutation on DV biogenesis and subsequent transport at the ultrastructural level using immunogold EM labeling (Figure 2). We observed normal budding of DVs from the TGN in gpa5 endosperm as in the wild-type endosperm (Figures 2A and 2B). Interestingly, many glutelin-positive electron dense granules were observed in the apoplast space in gpa5 but not in the wild-type endosperm (Figures 2C and 2D). As shown in Figures 2E and 2F, these protein granules most likely resulted from membrane fusion of DVs with the plasma membrane. Consistent with the confocal microscopy observation (Figure 1K; Supplemental Figure 2), we detected large amounts of cell wall-like components abnormally accumulated in the enlarged apoplast space (Figure 2G). As endosperm development progressed, numerous DVs continuously appeared to fuse with the plasma membrane and discharge their cargo molecules into the apoplast space, accompanied by abnormal synthesis of cell wall components in the apoplast space and formation of PMBs (Figures 2H to 2J). In support of this notion, we also observed mistargeting of another DV cargo, TIP3 (a specific aquaporin for PSV; Hinz et al., 1999; Ren et al., 2014), to PMBs in the PGlo:TIP3-GFP (GFP-fused TIP3 driven by a globulin promoter) transgenic plants in the gpa5 homozygous background (Supplemental Figure 3). As a result of storage protein
mistargeting to the apoplast space, PBIIs were not efficiently filled by storage proteins in gpa5 endosperm (Figures 2K to 2N) and thus the sizes of PBIIs labelled by TIP3-GFP in gpa5 were also obviously smaller than those in wild-type endosperm (Supplemental Figure 3). The above gpa5 phenotypes (Figure 1) and subcellular defects (Figure 2) are most reminiscent of our previously reported gpa1, gpa2, and gpa3 mutants, suggesting that GPA5 may function in the same DV-mediated post-Golgi trafficking pathway as GPA1, GPA2, and GPA3.

**GPA5 Encodes a Plant-Unique PX Domain-Containing Peripheral Membrane Protein That Can Bind to PI3P and Is Broadly Expressed in Rice**

Using 198 F2 mutant individuals from a cross between a gpa5 heterozygous plant and an indica variety Dular, we delineated GPA5 to a physical region of 60.9-kb on chromosome 6 that harbors eight putative candidate genes via a map-based cloning strategy (Figure 3A). Sequencing analysis revealed a single base deletion within the fourth exon of LOC_Os06g43560, which results in a truncated gpa5 protein harboring the N-terminal 143 amino acid residues of wild-type GPA5 protein (Figure 3B). To test whether the mutation in LOC_Os06g43560 is responsible for the gpa5 mutant phenotypes, we performed a complementation test by introducing a 12.6-kb genomic fragment spanning the entire coding region of LOC_Os06g43560, a 2630-bp upstream regulatory region, and a 2733-bp downstream regulatory sequence into gpa5 homozygous calli. Three positive T2 generation transgenic lines survived and exhibited wild-type phenotypes including grain appearance, storage protein composition, and cell wall deposition pattern (Figures 3C to 3E), demonstrating that LOC_Os06g43560 indeed represents GPA5.

GPA5 encodes a predicted protein of 79.8 kDa that harbors an N-terminal PX domain and a C-terminal coiled-coil (CC) domain (Figure 3F). A BLAST search demonstrated that GPA5 is a single-copy gene in the rice genome. Furthermore, phylogenetic analysis showed that GPA5 represents a plant-unique PX domain-containing protein and its homologs can be widely found in monocots and eudicots (Supplemental Figure 4; Supplemental Dataset 1). Notably, GPA5 is homologous to the Arabidopsis EREX proteins (for ENDOSOMAL RAB EFFECTOR WITH PX-DOMAIN; Supplemental Figure 5; Sakurai et al., 2016).

The PX domain is a phosphoinositide-binding domain that is essential for membrane attachment to organelles of the secretory and endocytic pathways (Teasdale and Collins, 2012). To determine whether the GPA5 protein can bind phospholipid and its binding specificity, we conducted an in vitro lipid binding assay. His-tagged GPA5 recombinant protein could specifically bind to phosphatidylinositol-3-phosphate (PI3P), as
the previously reported EREX protein (Sakurai et al., 2016), although a much weaker binding of His-GPA5 with PI4P was also detected (Figure 3G). Furthermore, deletion of the PX domain but not the CC domain drastically reduced the binding affinity (Figure 3G), corroborating that GPA5 can specifically bind to PI3P through the PX domain.

To analyze the expression pattern and membrane association of GPA5, we raised anti-GPA5-specific polyclonal antibodies using recombinant protein of the N-terminal regions of GPA5 as the antigen. The GPA5 antibodies specifically recognized a ~110-kDa band in the total protein extracts from the wild-type and GPA5 overexpression plants (in the gpa5 homozygous background), and a ~130 kDa GPA5-GFP band in total protein extracts from the GPA5-GFP overexpression plants (in the gpa5 homozygous background; Figures 3H and 3I). Notably, the endogenous GPA5 protein band migrated substantially slower than expected (79.8 kDa). The His-tagged GPA5 recombinant protein and immunoprecipitated endogenous protein using the anti-GPA5 antibodies also migrated slower than expected (Supplemental Figure 6A), suggesting that some inherent features of GPA5 protein (such as hydrophobicity or high-order structure) may render its slower migration on the SDS-PAGE gel. Mass spectrometry assays of the recombinant protein and immunoprecipitated endogenous proteins confirmed the identity of GPA5 (Supplemental Figures 6B and 6C).

We next used the anti-GPA5 antibodies to conduct an immunoblot analysis of total protein extracts from various tissues of the wild-type plants and verified that GPA5 was expressed in all tissues examined, with the lowest accumulation in roots and dry seeds (Figure 3J).

**GPA5 Protein Is Localized to the DVs in Endosperm Subaleurone Cells, and Forms Homodimers**

To investigate the subcellular localization of GPA5, we used the anti-GPA5 antibodies to conduct a subcellular fractions assay and found that GPA5 was localized to both the soluble and membrane fractions (Figure 3K). Treatment of the membrane fractions with high salt or alkali efficiently solubilized GPA5 in contrast to the integral membrane protein Sec12b (with a single transmembrane domain at its C-terminus; Wang et al., 2016), suggesting that GPA5 is a peripheral membrane-associated protein (Figure 3L). We also generated transgenic plants expressing GFP-tagged forms of GPA5 under the control of its native regulatory elements (promoter, intron, and downstream regulatory region; Supplemental Figure 7A). The GFP-tagged full-length GPA5 gene driven by its endogenous regulatory elements rescued the endosperm development and storage protein sorting defects of gpa5 (Supplemental Figures 7B to 7D), demonstrating that the
GPA5-GFP fusion protein retains its authentic function in plants. Confocal microscopy revealed that GPA5-GFP was localized to the cytosol and punctate compartments in the cytoplasm in both root tip cells and developing endosperm subaleurone cells at 9 DAF (Figure 4A). To determine the possible roles of the PX and CC domains on the GPA5 localization pattern, we generated PX or CC domain deletion mutant versions of GPA5 and investigated their localization in their respective transgenic root tip cells. Confocal examination revealed that the GFP fusions of GPA5 lacking either the PX or the CC domain were mainly localized to the cytosol (Supplemental Figure 8), indicating a necessary role of both the PX and CC domains in membrane association of the GPA5 protein.

To determine the possible effect of phosphoinositide binding for the localization pattern of GPA5, we treated transgenic root tip cells expressing the GPA5-GFP transgene driven by its endogenous regulatory elements with wortmannin (a potent inhibitor of phosphoinositide 3-kinase activity). Upon drug treatment, GPA5-GFP fusion protein showed a dispersed pattern in the cytosol (Figure 4B). Together, these results suggest that PI3P binding through the PX domain is also necessary for the membrane association of the GPA5 protein.

As organelle markers have not been well established in rice, we next investigated the nature of the GPA5-labeled punctate compartments in protoplasts prepared from Arabidopsis suspension cells. As shown in Supplemental Figure 9, the GPA5-GFP positive punctate structures were distinct from the localization patterns of the Golgi marker (GmMan1-mRFP; Nebenfuhr et al., 1999) and TGN marker (mRFP-AtSYP61; Sanderfoot et al., 2001) but largely overlapped with the PVC marker (mRFP-AtVSR2; Miao et al., 2006), as confirmed by the correlation analysis using the Pearson-Spearman correlation (PSC) plugin for ImageJ. These results suggest that the GPA5-positive puncta in root cells likely correspond to PVCs.

To further evaluate the intracellular localization of GPA5 in developing endosperm, we performed immuno-gold microscopy of ultrathin sections using primary antibodies against GPA5, which were recognized by secondary antibody-labelled 15 nm gold particles. As shown in Figure 4C, 15 nm gold particles were enriched on the surface of mature DVs, but not on those DVs near the Golgi. More strikingly, double immuno-gold labeling using anti-GPA5 antibodies from rabbit (labelled with 5 nm gold particles) and anti-glutelin antibodies from mouse (labelled with 15 nm gold particles) showed the distribution of 5 nm gold particles on glutelin-containing DVs likely in the fusing process with PBIIs (Figure 4D). Thus, we deduced that GPA5 is localized to DVs and that it may mediate membrane fusion between DVs and PBIIs.
Previous studies have demonstrated that many PX domain-containing proteins such as sorting nexins (SNXs) can form homo- or heterodimers through the linker or other domains in yeast and mammalian cells (Vollert and Uetz, 2004; Xing et al., 2004). In Arabidopsis, SNXs have also been shown to form homo- and heterodimers through the BAR domain (Pourcher et al., 2010). However, it remains unknown whether plant-unique PX domain-containing proteins can form homodimers. To evaluate this possibility, we employed the yeast two-hybrid assay (Y2H) and found that GPA5 can interact with itself (Figure 4E). In addition, a firefly luciferase complementation imaging (LCI) assay confirmed self-dimerization of GPA5 in leaf epidermal cells of *Nicotiana benthamiana* (Figure 4F). Furthermore, an in vivo coimmunoprecipitation assay showed that GPA5-Flag can be coimmunoprecipitated by GPA5-GFP in the total protein extract of *N. benthamiana* (Figure 4G). These results suggest that GPA5 is capable of forming homodimers.

**GPA5 Acts Downstream of Rab5a and VPS9a**

Our previous studies demonstrated that Rab5a and its GEF VPS9a function cooperatively in regulating post-Golgi trafficking of storage proteins to the PSVs in rice endosperm (Wang et al., 2010b; Liu et al., 2013). In addition, earlier studies showed that a subpopulation of Rab5a is localized to DVs in developing endosperm (Fukuda et al., 2011). The colocalization of Rab5a and GPA5 together with their similar function in storage protein sorting suggest that GPA5 may act in the same trafficking pathway as Rab5a in rice endosperm. As live-cell imaging is not yet feasible in developing endosperm, we examined the requirement of GPA5 for the membrane association of Rab5a in root cells of wild-type and *gpa5* homozygous plants expressing GFP-Rab5a. Loss of GPA5 function had no obvious effect on the localization pattern of GFP-Rab5a (Figure 5A). We also examined the effect of Rab5a and VPS9a on the membrane association of GPA5 through transforming GPA5-GFP into the *gpa5*, *rab5a*, and *vps9a* mutant backgrounds. Notably, depletion of either Rab5a or VPS9a caused delocalization of GPA5-GFP from the membrane to the cytosol in the root cells (Figure 5B), suggesting that both Rab5a and VPS9a are required for the membrane association of GPA5.

**GPA5 Physically and Genetically Interacts with Rab5a and VPS9a**

As GPA5 most likely functions downstream of Rab5a in the post-Golgi trafficking to PSVs, we next explored the possible molecular links between GPA5 and four Rab5 members (including two canonical types, Rab5a and Rab5c, and two plant-unique type, Rab5b and Rab5d; Liu et al., 2013) using the Y2H assay. GPA5 specifically interacted with the wild-type and GTP-fixed (Q70L) forms but not GDP-fixed
(S25N) form of Rab5a (Figure 6A). Furthermore, GPA5 showed a similar interaction relationship with Rab5c, but failed to interact with the GTP-fixed forms of Rab5b and Rab5d (Figure 6A; Supplemental Figure 10A). These results suggest that GPA5 may only function as an effector of the canonical Rab5 members. To determine the region in GPA5 that is required for the interaction with Rab5a, we generated various domain deletion variants of GPA5 for the interaction assay with the GTP-fixed form of Rab5a and found that the PX domain of GPA5 is essential for the interaction between GPA5 and Rab5a (Figure 6B; Supplemental Figure 10B). Interestingly, we found a stronger interaction between the CC domain deleted form of GPA5 with Rab5a than the interaction between full-length GPA5 with Rab5a, suggesting that the CC domain of GPA5 may have an inhibitory role on the interaction. In addition, truncations of either N-terminus or the linker between the PX domain and CC domains attenuated the binding activity. Together, these results suggest that GPA5 specifically interacts with Rab5a via the PX domain and its flanking regions.

We next verified the interactions of GPA5 with both wild-type and the GTP-fixed forms of the canonical Rab5a and Rab5c, but not the plant-specific Rab5s, using an in vivo LCI assay (Figure 6C) and an in vivo bimolecular fluorescence complementation (BiFC) assay in *N. benthamiana* leaf epidermal cells (Figure 6D; Supplemental Figures 10C to 10F). Moreover, we also verified direct interaction between GPA5 and Rab5a by performing a coimmunoprecipitation assay with the lysates from developing endosperm at 9 DAF either overexpressing free GFP or expressing GPA5-GFP under the control of its endogenous regulatory elements (in the *gpa5* homozygous background). The immunoprecipitates were subjected to SDS-PAGE, followed by CBB staining (Figure 6E) or immunoblotting with an anti-GFP antibody (Figure 6F). Immunoblot analysis using antibodies against Rab5a showed that Rab5a was indeed coimmunoprecipitated by the anti-GFP antibody (Figure 6F). Furthermore, we analyzed these interacting proteins using mass spectrometry. Notably, we detected all Rab5 members including the canonical Rab5a and Rab5c as well as the plant-specific Rab5b and Rab5d in the GPA5-GFP precipitate but not in the free GFP precipitate, although the abundances of Rab5b and Rab5d appeared obviously lower than those of the canonical Rab5 members (Figure 6G; Supplemental Dataset 2). Therefore, we speculated that the negative interaction between GPA5 and Rab5b/5d in Y2H and BiFC (Figures 6A; Supplemental Figure 10) might be due to a weak or transient interaction between these proteins.

To further investigate the genetic interaction between *Rab5a* or *VPS9a* and *GPA5*, we tried to cross female heterozygous *gpa5* (+/−) with male *rab5a* or *vps9a* plants and found that no *gpa5 rab5a* and *gpa5 vps9a* double homozygous mutants could be established (Supplemental Tables 1 and 2). We speculated that this is
most likely due to a deficiency in the gametophyte of the double mutant and/or embryonic lethality of the double homozygous grains.

As an alternative approach to test the genetic relationship between GPA5 and VPS9a, we overexpressed GPA5-GFP in the wild-type and vps9a backgrounds and found that overexpression of GPA5 significantly aggravated the phenotypic defect of vps9a but not wild-type endosperm in storage protein sorting, which was exemplified by the glutelin precursor accumulation phenotype (Supplemental Figure 11). These results further support the notion that GPA5 may function cooperatively with Rab5a and VPS9a in the DV-mediated post-Golgi trafficking of storage proteins to PSVs.

**GPA5 Is Coimmunoprecipitated with the CORVET Tethering Complex**

Rab5 is a key regulator of endosomal/vacuolar trafficking in eukaryotic cells, which acts in tethering of endosomes to the target membrane through interaction with specific effectors (Stenmark, 2009; Uemura and Ueda, 2014). Previous studies have also shown that CORVET and HOPS, two homologous tethering complexes, sequentially mediate membrane fusion in endosomal/vacuolar trafficking in animal and yeast cells (Balderhaar and Ungermann, 2013). A recent study suggests that CORVET and HOPS complexes might also be involved in the trafficking of distinct cargo proteins through mediating membrane fusion of PVCs and vacuoles in plant cells (Takemoto et al., 2018). The CORVET and HOPS complexes share a core subcomplex comprising VPS11, VPS16, VPS18, and VPS33, but each also contains distinct subunits. CORVET contains VPS3 and VPS8, while HOPS contains VPS39 and VPS41 (Balderhaar and Ungermann, 2013). Our cytological, biochemical, and genetic data suggest that GPA5 acts as a plant-unique effector of Rab5a (Figure 6) and that it likely plays a role in mediating membrane fusion between DVs and PBII s (Figure 4D). Thus, we next examined the potential association between GPA5 with the HOPS and/or CORVET tethering complexes. Interestingly, mass-spectroscopy analysis uncovered all four core subunits and two CORVET-specific subunits (VPS3 and VPS8) but not the HOPS-specific subunits (VPS39 and VPS41) in the GPA5 immunoprecipitate (Figure 7A; Supplemental Dataset 2). Further, immunoblot analysis of the GPA5 immunoprecipitates using antibodies against VPS3, VPS11, VPS18, and VPS39 confirmed that GPA5 is coimmunoprecipitated specifically with the hexameric CORVET complex (Figure 7B; Supplemental Figure 12). Together, these results suggest that GPA5 functions together with the CORVET complex to mediate tethering of DVs to PBII s/PSVs in rice endosperm.

**GPA5 Is Coimmunoprecipitated with the VAMP727-Containing SNARE Complex**

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After tethering of transport vesicles to the target organelle, SNARE complexes ultimately execute membrane fusion between the transport vesicles and target membranes (Chen and Scheller, 2001; Jurgens, 2004). A functional SNARE complex is composed of four coiled-coil helical bundles, three of which belong to Q-SNARE and the fourth of which is R-SNARE. Two distinct SNARE complexes have been shown to act in the vacuolar trafficking pathways in Arabidopsis. Both complexes share a core subcomplex containing Qa-SYP22, Qb-VTI11, and Qc-SYP5. In addition to these core components, one has R-VAMP71, the other has the seed plant-unique R-VAMP727 (Saito and Ueda, 2009; Uemura and Ueda, 2014). Interestingly, we also detected the presence of VAMP727 together with three types of Q-SNARE components in the GPA5 immunoprecipitates. Although a small amount of VAMP71 homologs was also immunoprecipitated with GPA5-GFP, their abundance was comparable to that in free GFP samples, suggesting that it is most likely due to background contamination (Figure 7C; Supplemental Dataset 2). These results suggest that the VAMP727-containing SNARE complex is also involved in mediating membrane fusion between DVs and PSVs. In support of the above notion, a mass spectrometry assay and immunoblot analysis verified that the major components of the ~55-kDa band in the GPA5 immunoprecipitate (Figure 6E) correspond to glutelin precursors (Figure 7D, Supplemental Dataset 3).
**DISCUSSION**

*gpa5* Is Defective in both DV-mediated Post-Golgi Trafficking of Storage Proteins and Plant Growth and Development.

We have previously characterized *gpa1/rab5a*, *gpa2/vps9a*, and *gpa3* as three independent loss-of-function rice 57H mutants defective in post-Golgi trafficking of storage proteins to PBIIs/PSVs (Wang et al., 2010b; Liu et al., 2013; Ren et al., 2014). A number of Arabidopsis mutants defective in storage protein sorting have also been reported, such as *vssr1*, *vps29*, *vps35*, *kam2*, *vps9a*, *vth12*, *amsh3*, *snx1*, *snx2a*, *ap-4*, *free1/fyve1*, and *mon1/-sand* (Shimada et al., 2003; Shimada et al., 2006; Goh et al., 2007; Tamura et al., 2007; Yamazaki et al., 2008; Isono et al., 2010; Pourcher et al., 2010; Cui et al., 2014; Singh et al., 2014; Gao et al., 2015; Kolb et al., 2015; Fuji et al., 2016). Notably, GPA5 encodes a protein homologous to three previously reported Arabidopsis proteins (EREX, EREL1, and EREL2). The EREX and EREL1 proteins have been reported to redundantly function in the transport of storage proteins in Arabidopsis (Sakurai et al., 2016).

However, the precise role of these proteins in mediating storage protein trafficking in Arabidopsis remains unsubstantiated due to the lack of detailed immunoelectron microscopy examination of the phenotypic defects of storage protein trafficking in their mutants. In this study, we conducted detailed immunofluorescence microscopy and biochemical studies of GPA5. Our results showed that both glutelins and α-globulin were missorted to the PMBs and cell periphery, accompanied by the smaller PBIIs/PSVs in *gpa5* endosperm (Figures 1I to 1M; Supplemental Figures 2 and 3). Further, immunoelectron microscopy demonstrated that DVs were mistargeted to the plasma membrane, and after membrane fusion, DVs release their contents into the apoplast, leading to the formation of PMB structures (Figure 2). Given the similar phenotypic defects with the previously reported *gpa1*, *gpa2*, and *gpa3* mutants, we deduced that GPA5 likely functions together with *GPA1*, *GPA2*, and *GPA3* in the same DV-mediated post-Golgi trafficking pathways in rice endosperm.

It is worth noting that although the *gpa5* mutant shares a similar defect in storage protein trafficking as the previously reported *gpa1*, *gpa2*, and *gpa3* mutants, *gpa5* exhibited a seedling lethal phenotype (Figure 1H), whereas the other earlier reported mutants have normal plant development (Wang et al., 2010b; Liu et al., 2013; Ren et al., 2014). These observations suggest that GPA5 may also play a housekeeping role during vegetative development. Supporting this notion, GPA5 is indeed abundantly expressed in several vegetative tissues examined (Figure 3J). It is notable that the Arabidopsis *erex erel1* double mutants also exhibit a severe growth retardation phenotype at the juvenile stage, but eventually restore to the wild-type phenotype.
The seedling lethal phenotype of \textit{gpa5} might be due to the presence of only a single copy of \textit{GPA5} in the rice genome, whereas Arabidopsis has three homologues (\textit{EREX}, \textit{EREL1}, and \textit{EREL2}). How the GPA5/EREX proteins regulate plant vegetative development will be an interesting avenue for future research.

**PI3P Binding Combined with Protein–Protein Interaction Is Required for the Membrane Association of GPA5**

Phosphoinositides are key coordinators that control vesicle targeting and/or tethering to target membrane in eukaryotic cells (Odorizzi et al., 2000; Di Paolo and De Camilli, 2006; Stenmark, 2009). The effects of phosphoinositides are modulated mainly through their direct interaction with the lipid-binding domains of regulatory proteins essential for membrane trafficking, such as the pleckstrin homology (PH), FYVE, ENTH, and PX motifs (Odorizzi et al., 2000; Hurley and Meyer, 2001; Ellson et al., 2002; Teasdale and Collins, 2012). Particularly, the PX domain has been characterized as an emerging endosomal recruitment module that specifically binds PI3P and also as an essential protein–protein interaction domain in the secretory and endocytic pathways in mammalian cells (Teasdale and Collins, 2012). In this study, we showed that GPA5 is a peripheral membrane-associated protein (Figures 3A to 3F, 3K, and 3L). A phospholipid binding assay showed that the PX domain in GPA5 indeed has a strong affinity to PI3P (Figure 3G). We further found that either PX domain deletion or wortmannin treatment enables delocalization of GPA5 from the membrane to the cytosol in root cells (Figure 4B; Supplemental Figure 8), suggesting that PI3P binding by the PX domain of GPA5 is necessary for membrane association of GPA5. We further showed that GPA5 physically interacts with Rab5a via the PX domain and its flanking sequence (Figures 6A and 6B; Supplemental Figures 10A and 10B). Notably, membrane localization of GPA5 is significantly impaired in the loss-of-function mutants of Rab5a or Rab5a-GEF VPS9a (Figure 5B). The residual membrane localization of GPA5 observed in the \textit{gpa1/rab5a} and \textit{gpa2/vps9a} backgrounds might be due to the existence of homologous genes of \textit{Rab5a} (i.e. \textit{Rab5b}, \textit{Rab5c}, and \textit{Rab5d}) and \textit{VPS9a} (i.e. \textit{VPS9b}) (Wang et al., 2010b; Liu et al., 2013). Strikingly, deletion of the CC motif, known as a domain for protein–protein interaction, also dramatically impaired the membrane localization of GPA5 (Supplemental Figure 8), implying that the CC domain-interacting proteins may also play an important role in regulating the localization/function of GPA5. Consistent with this proposition, the Arabidopsis \textit{FREE1/FYVE1} protein has been reported to be capable of binding to PI3P through its FYVE domain, and its mutation causes a defect in storage protein targeting to PSVs (Gao et al., 2014; Gao et al., 2015; Kolb et al., 2015). A comparable DV-to-PM fusion defect caused by the \textit{gpa5}
mutation was also observed in wortmannin-treated mung bean (*Vigna radiata*) cotyledons (Wang et al., 2009a).

**GPA5 Likely Functions in Mediating Tethering and Fusion of DVs with PSVs**

In eukaryotic cells, Rab GTPases are key coordinators of vesicular trafficking, which serve as the binding platforms for the membrane trafficking machinery involved in the targeting and tethering of transport vesicles to the target compartments (Odorizzi et al., 2000; Langemeyer et al., 2018). In mammalian and yeast cells, CORVET and HOPS act as the tethering complexes in the vacuolar/lysosomal trafficking pathways in a sequential manner: the former controls homotypic fusion of early endosomes, while the later regulates membrane fusion between late endosome and vacuole/lysosome as well as homotypic fusion of vacuolar/lysosomal membranes (Balderhaar and Ungermann, 2013). By contrast, recent studies in Arabidopsis showed that CORVET and HOPS act in distinct vacuolar trafficking pathways involving different sets of SNARE proteins. The CORVET complex works together with the VAMP727-containing SNARE complex and is responsible for transport of SYP22, while the HOPS complex interacts with the VAMP71-containing SNARE complex and is required for transport of 12S globulin and GFP-CT24 to vacuoles (Ebine et al., 2014; Singh et al., 2014; Takemoto et al., 2018; Minamino et al., 2019). These reports suggest that plants employ distinct pathways to transport different cargo proteins.

Previous studies have shown that in rice endosperm, Rab5a and its GEF-VPS9a act as two key regulators for post-Golgi trafficking of storage proteins to the PBIIs/PSVs, and that their loss-of-function mutations all cause mistargeting of DVs to the apoplast space (Wang et al., 2010b; Fukuda et al., 2011; 2013; Liu et al., 2013). In addition, GPA3, a plant-unique regulator, has also been shown to be required for proper targeting of DVs to PBIIs/PSVs in rice endosperm (Ren et al., 2014). In this study, we showed that GPA5 encodes another plant-unique regulator required for proper targeting of DVs to PBIIs/PSVs in rice endosperm. Our ultrastructural studies of immunolabeled ultrathin sections showed that GPA5 is specifically localized to mature DVs in rice endosperm subaleurone cells (Figures 4C and 4D), which is distinct from Rab5a and GPA3 that are dually localized to both the Golgi/TGN and DVs (Fukuda et al., 2011; Ren et al., 2014). We also observed that GPA5 is localized to DVs appearing to be in the fusing process with PBIIs/PSVs (Figure 4D), a finding consistent with the earlier observations that DVs may directly fuse with PBIIs/PSVs in rice and soybean (*Glycine max*) seeds (Krishnan et al., 1986; Herman and Larkins, 1999; Liu et al., 2013; Ren et al., 2014). Together, these observations suggest that GPA5 likely acts together with Rab5a and GPA3 in regulating the tethering and fusion processes of DVs with PBIIs/PSVs in rice endosperm. This notion is
further supported by the verified physical interaction between GPA5 with Rab5a in vitro and in vivo (Figure 6; Supplemental Figure 10). Strikingly, our coimmunoprecipitation combined with mass spectrum assays showed that GPA5 is specifically associated with the CORVET (but not HOPS complex) and VAMP727-containing SNARE complex (but not VAMP71-containing SNARE complex) in developing rice endosperm (Figure 7). Based on these observations, we propose a model for GPA5 in mediating post-Golgi trafficking of storage proteins to PBIIs/PSVs in rice endosperm (Figure 8). After DV maturation from the TGN where GPA3 first recruits GPA2/VPS9a to activate GPA1/Rab5a, the activated GPA1/Rab5a recruits its effector GPA5 onto DVs, which then interacts with the CORVET and VAMP727-containing SNARE complexes to execute DV-to-PSV direct fusion (Figure 8). Our findings indicate that Rab5a and GPA5 regulation of tethering and fusion of glutelin-containing DVs with PBIIs/PSVs is specifically mediated by the CORVET and VAMP727-containing SNARE complexes, similar to the transport route of SYP22 but different from that of storage proteins (12S globulin) in Arabidopsis (Minamino et al., 2019). Altogether, accumulating evidence suggests that plants use both evolutionarily conserved machinery (such as Rab5, CORVET, and SNARE) and plant-unique factors (such as GPA3 and GPA5) to mediate storage protein transport.

It is worth mentioning that morphology-based evidence suggests that in some dicots, such as Arabidopsis and pea, storage proteins are exited from the Golgi in DVs, which then fuse to prevacuolar compartments (PVCs)/multivesicular bodies (MVBs) before being delivered to the PSVs (Robison et al., 1998; Otegui et al., 2006). However, glutelin-containing PVCs/MVBs with typical internal vesicles have rarely been observed in rice endosperm so far (Krishnan et al., 1986; Wang et al., 2010; Fukuda et al., 2011, 2013; Liu et al., 2013; Ren et al., 2014; Wang et al., 2016), suggesting that PVCs/MVBs plays a minor role, if any, in glutelin trafficking to the PSVs in rice endosperm. Consistent with this notion, we found in this study that the GPA5-labelled structures are completely filled with electron dense storage protein aggregates (Figures 4C and 4D). The presence of numerous glutelin precursors but not mature subunits in the GPA5 immunoprecipitate also indicates the vesicle nature of GPA5-labeling structures (Figures 6E and 7D). Similar secretory vesicles containing 2S albumin precursors were also isolated by the subcellular fractionation experiments using developing *Brassica napus* embryos (Otegui et al., 2006). Nevertheless, we cannot rule out the possibility that GPA5-labeled DVs may represent a distinct type of PVCs, such as the previously reported storage PVC (sPVC) in rice endosperm (Shen et al., 2011). Further studies are required to clarify these issues.
It should be noted that a recent whole-cell electron tomography analysis of Arabidopsis root cells indicated that small vacuoles are mainly derived from homotypic fusion of MVBs (Cui et al., 2019). Although homotypic fusion of DVs has not been observed in developing rice endosperm, our results cannot rule out the possibility that GPA5 may also mediate DV-DV homotypic fusion by interacting with the CORVET and VAMP727-containing SNARE complexes. Future research should be directed to elucidate the detailed mechanisms of how GPA5 interacts with the CORVET and VAMP727-containing SNARE complexes to mediate membrane fusion in rice endosperm.

METHODS

Plant Materials and Growth Conditions

The gpa5 mutant described in this study was derived from a pool of 60Co-irradiated lines of the japonica rice (Oryza sativa) variety Kitaake. As the gpa5 (-/-) homozygous mutant is seedling lethal, a gpa5 (+/-) heterozygous plant was backcrossed to the wild type Kitaake three times to remove other background mutation sites. The gpa5 (-/-) homozygous hulled seeds were surface sterilized and grown in a culture box on 1/2 Murashige and Skoog (MS) plus 0.7% agar and 1% sucrose at 28°C. The gpa5 (+/-) heterozygous plants were separately crossed with gpa1 and gpa2 single mutant to generate corresponding F2 populations, followed by genotyping with the mutation site specific primer pairs. Unless indicated otherwise, rice plants were grown in paddy fields during the normal growing seasons or in a greenhouse at the Chinese Academy of Agricultural Sciences, in Beijing.

Antibodies

Partial cDNAs of GPA5 (LOC_Os06g43560; a.a. 1–350), VPS3 (LOC_Os05g01360; a.a. 582–936), VPS11 (LOC_Os04g31390; a.a. 661–947), VPS18 (LOC_Os08g08060; a.a. 785–1000), and VPS39 (LOC_Os03g50740; a.a. 1–295) were separately cloned into the pET32 vector and transformed into the E. coli strain Rosetta (DE3) for recombinant protein expression, followed by expression and purification using Ni-NTA His-bind Resin (Millipore). These recombinant proteins were injected into rabbits to produce polyclonal antibodies, which were affinity-purified using CnBr-activated Sepharose 4B (Sigma-Aldrich, C9142) at ABclonal Biotechnology Co. (https://www.abclonal.com.cn/). Anti-Glutelin acidic subunits, anti-glutelin basic subunits, anti-α-globulin, anti-GluA, anti-GluB, anti-GluC, anti-GluD, anti-BIP1,
anti-PDI1-1, anti-Sec12b, and anti-Rab5a antibodies were described previously (Wang et al., 2010b; Ren et al., 2014; Wang et al., 2016). Anti-His (Sigma-Aldrich, H1029, dilution 1:2000), anti-GFP (Roche, 11814460001, dilution 1:3000), anti-Flag (Sigma-Aldrich, F1804, dilution 1:3000), anti-EF-1α (Agrisera, AS10 934, dilution 1:3000), and anti-UGPase (Agrisera, AS05 086, dilution 1:3000) antibodies are commercially available.

**Seed protein extraction, SDS-PAGE, and immunoblot analyses**

Total seed protein extraction and SDS-PAGE analysis were performed as described previously (Ren et al., 2014; Wang et al., 2016). Briefly, hulled rice mature seeds were ground into flour, followed by resuspension in a lysis buffer containing 4% (w/v) SDS, 4 M urea, 5% (v/v) β-mercaptoethanol, and 125 mM Tris-HCl, pH 6.8. SDS-PAGE analysis was performed on a 12.5% (v/v) uniform gel, followed by CBB staining or immunoblot analyses. Antibody-antigen reactions were detected with the ECL detection reagent (Thermo Scientific), followed by visualization with the ECL detection system (LI-COR, Odyssey-Fc).

All immunoblot assays were repeated independently at least three times with similar results, and representative results are shown. Quantification of immunoblots was conducted using ImageJ software (http://rsb.info.nih.gov/ij/) as described previously (Lin et al., 2015).

**Microscopy observation**

Scanning electron microscopy of rice mature brown grains was described previously (Ren et al., 2014). Briefly, brown rice was transversely cut by a razor blade, followed by gold sputtering and observed by a scanning electron microscope (Hitachi S-3000N, Tokyo, Japan).

Thick sections (60 μm thickness) were prepared from freshly harvested developing grains (9 DAF), as described previously (Ren et al., 2014). Briefly, developing grains were dehulled and cross-sectioned with a Leica VT1200S vibratome in ice-cold MTSB buffer (50 mM PIPS-KOH pH 6.9, 10 mM EGTA, 10 mM MgSO₄, 1% [v/v] DMSO and 0.1% [v/v] Triton X-100), followed by fixation in the MTSB buffer containing 4% paraformaldehyde. Unspecific cell wall staining with Calcofluor white (Sigma-Aldrich, 18909) was conducted according to a previously described protocol (Ren et al., 2014).

Semithin sections (0.4 μm thickness) of developing grains (9 DAF) were prepared as described previously (Wang et al., 2010b). CBB staining and indirect immunofluorescence staining were performed according to a recent report (Ren et al., 2014). In brief, sections were firstly treated with the blocking buffer containing 3%...
BSA, and then reacted with combinations of primary antibody combination diluted in a blocking buffer containing 1% BSA, followed by washing three times with TBST. Sections were further incubated with Alexa fluor 488 and 555 conjugated secondary antibody combination (Invitrogen) and then washed three times with TBST before confocal imaging using a LSM880 scanning confocal microscope (Carl Zeiss). Sections were incubated with primary antibodies raised against glutelin acidic subunits (1:1000), 13 kDa prolamin (1:500) and α-globulin (1:500) as well as secondary antibodies (1:500).

For immunogold electronic microscopy, the developing grains were high-pressure frozen/freeze-substituted (HPF/FS), followed by ultrasection and immunogold labeling as described previously (Wang et al., 2010a; Ren et al., 2014). Briefly, developing grains of wild type, *gpa5* and complemented plants were cryofixed by high pressure freezing (Leica EMPACT2) and freeze-substituted with 0.2% (w/v) uranyl acetate in acetone at –85°C for 24 h, followed by a series of gradient dehydration. After dehydration, the samples were embedded in LOWICRYL HM20 resin by ultraviolet irradiation in Leica AFS2. Ultrathin sections (70 nm thickness) were prepared with a Leica EM UC7 microtome. Immunogold labelling on HM20 sections was performed with primary antibodies at 50 μg/mL and 5 nm or 15 nm gold-conjugated secondary antibodies at 1:50 dilution (Abcam). After post-staining with aqueous uranyl acetate/lead citrate, the samples were examined using a Hitachi H7700 transmission electron microscope.

**Map-based cloning**

The *gpa5* (+/-) heterozygous plant was crossed with the *indica* variety Dular to generate a mapping population. F2 seeds were individually harvested from the F1 plants. Of them, populations with an aberrant endosperm phenotype segregation were selected to map the *gpa5* locus. To simultaneously perform phenotyping and genotyping of a single seed, F2 seeds with floury endosperm were cut into two halves. The embryoless half was ground into flour, followed by protein extraction and SDS-PAGE analysis for phenotyping. The remaining half (with embryo) defective in glutelin precursor accumulation was further subjected to DNA extraction for mapping. Molecular markers used for fine mapping are shown in Supplemental Table 3.

**Phylogenetic analysis**
Homologous proteins of GPA5 were identified by searching the NCBI database (www.ncbi.nlm.nih.gov/protein) using GPA5 sequence as query. Full-length protein sequences were aligned using the Geneious (v4.8.5), followed by phylogenetic tree construction using Geneious Tree builder module with the neighbor-joining method (Drummond et al., 2010). Bootstrap replication (1000 replications) was used for statistical support of the nodes in the phylogenetic tree. Alignments used to generate the phylogeny presented in Supplemental Figure 4 are listed in Supplemental Data Set 1.

**Vector construction and rice transformation**

For the complementation test, a 12.6-kb wild-type genomic fragment spanning the regulatory elements including a 2630-bp promoter and a 2733-bp downstream regulatory region was cloned into the pCAMBIA2300 vector (at the EcoRI and SmaI sites) to generate the pGPA5:gGPA5 construct. To generate the GFP-tagged GPA5 transgenic plants, GFP with linker peptides were inserted 30-bp upstream of the stop codon of GPA5 to construct the GPA5-GFP fusion under the control of its own regulatory elements including promoter, intron and terminator, followed by cloning into the pCAMBIA2300 vector to produce the pGPA5:gGPA5-GFP construct using the strategy of fluorescent tagging of full-length proteins (Tian et al., 2004; Supplemental Figure 7A).

To generate the GPA5 overexpression construct, the full-length coding sequence of GPA5 was amplified and cloned into the pCUbi1390 vector. For subcellular localization analyses of GPA5 and its variants, the coding sequence of GPA5 and PX or CC domain deleted GPA5 (i.e., GPA5△PX and GPA5△CC, respectively) was separately amplified and cloned into the modified pCAMBIA1305 containing GFP tag driven by the maize (Zea mays) UBIQUITIN promoter. For localization of the TIP3 protein, the coding sequence of TIP3 was cloned into modified pCAMBIA1305 containing a GFP tag driven by a 980-bp α-globulin promoter (Ren et al., 2014). Unless indicated otherwise, all constructs were generated using an infusion cloning kit (Clontech). Constructed binary vectors were individually introduced into the Agrobacterium tumefaciens strain EHA105, and the resulting lines were used to infect calli from Kitaake, or gpa1/Q4041, or gpa2/vps9a, or gpa5.

**Subcellular localization in Arabidopsis protoplasts**

For subcellular localization of GPA5 in protoplasts isolated from Arabidopsis suspension cells, the coding sequence of GPA5 was cloned into pAN580 with a GFP tag driven by the d35S promoter. Protoplasts were transformed according to a previous report (Miao and Jiang, 2007). At least 10 independent protoplasts were
analyzed with the PSC plugin for ImageJ to quantify the colocalization of GPA5-GFP and each marker (French et al., 2008).

**Recombinant protein preparations and in vitro lipid binding assay**

For the in vitro lipid binding assay, the coding sequences of full-length GPA5 and various domain deletion variants were separately inserted into the pET28a vector to generate the His-GPA5, His-GPA5△PX and His-GPA5△CC constructs. Recombinant proteins were expressed in *Escherichia coli* BL21 Rosetta strains (TransGen) upon induction with 0.4 mM isopropyl b-D-1-thiogalactopyranoside at 28°C overnight, followed by purification using Ni-NTA His-bind Resin (Millipore). Lipid binding with PIP strips (Echelon Biosciences) was conducted following the manufacturer’s instructions. Briefly, the PIP strips were incubated with the blocking buffer and then reacted with 5 μg/mL purified proteins in a blocking buffer containing 0.1% Tween 20. After washing three times with PBST, the PIP strip was detected with anti-His antibody (Sigma-Aldrich, H1029 dilution 1: 2000).

**Subcellular fractionation**

Developing wild-type endosperm samples (9 DAF) were used for subcellular fractionation as described previously (Wang et al., 2016). Briefly, 9-day-old endosperm samples were homogenized in ice-cold buffer A (100 mM HEPES-KOH, pH 7.5, 0.3 M sucrose, 5 mM EGTA, 5 mM MgCl₂, and 1×Complete Protease Inhibitor Cocktail). The homogenate was filtered through cheesecloth and then centrifuged at 2000g for 20 min at 4°C to remove large cellular debris. The supernatant was further centrifuged at 100,000g for 1 h at 4°C; the supernatant and pellet were assigned as S100 and P100 fractions for immunoblot analysis, respectively.

The P100 fraction was further resuspended in 150 μL of each solution of buffer A, high salt buffer (buffer A supplemented with 1 M NaCl), alkaline buffer (buffer A supplemented with 0.1 M Na₂CO₃, pH 11), Triton X-100 buffer (buffer A supplemented with 1% [v/v]Triton X-100), and SDS buffer (buffer A supplemented with 1% [v/v] SDS). After incubation for 20 min on ice, these resuspension solutions were centrifuged at 100,000g for 1 h at 4°C to obtain the S100 and P100 fractions for immunoblot analysis.

**Yeast two-hybrid assays**
Yeast two-hybrid assays were conducted using the MatchMaker GAL4 Two-Hybrid System (Clontech) following the manufacturer’s instructions. The coding sequences of genes of interest were cloned into the pGADT7 or pGBK7 vectors (Clontech) and various combinations of plasmids were cotransformed into the yeast AH109 strain. Positive transformants were selected on synthetic drop-out (SD) medium lacking Trp and Leu (DDO), while the screening of interactions was performed on SD medium lacking Trp, Leu, His and Ade (QDO). The experiments were performed at least three times independently with similar results.

Firefly luciferase complementation imaging assays

For the interaction assay between GPA5 and Rab5a or Rab5c, the coding sequence of GPA5 was fused upstream of nLUC in the pCAMBIA-nLUC vector, while the coding sequences of Rab5a and Rab5c were fused downstream of cLUC in the pCAMBIA-cLUC vector. For the interaction assay between GPA5 and itself, the coding sequence of GPA5 was also fused downstream of cLUC in the pCAMBIA-cLUC vector. These constructs were transformed into the EHA105 strain, and various combinations of EHA105 strains were infiltrated into N. benthamiana leaves. After 2 to 3 days, the relative LUC activity was measured as described previously (Chen et al., 2008). Each data point contains three replicates.

BiFC assays

The coding sequences of GPA5 and Rab5 homologs were cloned into pYN1, pYC1 or p2YN vectors (kind gift of Joh A. Lindbo, OARDC, The Ohio State University, Wooster, OH) to generate the YN-GPA5, YC-Rab5a/c (containing a Flag tag), YC-GPA5 and Rab5b/d-YN (containing a Flag tag) constructs, respectively. These constructed vectors were then transformed into the Agrobacterium tumefaciens EHA105 strain, and various combinations of EHA105 strains were used to infiltrate N. benthamiana leaves, as described previously (Waadt and Kudla, 2008). After 24 to 48 h, protoplasts were isolated from the infiltrated plant leaves (Ren et al., 2014), and fluorescence signals were captured using a Zeiss LSM880 laser scanning confocal microscope.

Co-IP assays in N. benthamiana

The coding sequence of GPA5 was separately cloned into the pCAMBIA1305-GFP and pCAMBIA1300-221-Flag vectors (Ren et al., 2014) to generate GPA5-GFP and GPA5-Flag fusions, followed by transformation into the EHA105 strain and infiltration into N. benthamiana leaves. After 2 to 3 days, total leaf lysates were prepared in an ice-cold IP buffer (50 mM Tris-MES pH 7.5, 1 mM MgCl2, 0.5
M sucrose, 10 mM EDTA, 5 mM DTT, 0.1% [v/v] Nonidet P-40 and 1×Complete Protease Inhibitor Cocktail) and were incubated with GFP-Trap magnetic beads (ChromoTek) for 2 h at 4°C with shaking. After washing with IP buffer five times, the IP samples were eluted in a reducing buffer, followed by SDS-PAGE and immunoblot analyses using anti-GFP (Roche, 11814460001, dilution 1:3000) and anti-Flag (Sigma-Aldrich, F1804, dilution 1:3000) antibodies.

**Immunoprecipitation and mass spectrometry**

Nine-day-old developing transgenic endosperm (~10 g fresh weight) expressing free GFP or GPA5-GFP were separately homogenized in ice-cold IP buffer (50 mM Tris-MES pH 7.5, 1 mM MgCl₂, 0.5 M sucrose, 10 mM EDTA, 5 mM DTT, 0.1% [v/v] Nonidet P-40, and 1×Complete Protease Inhibitor Cocktail) and centrifuged at 20,000g for 10 min at 4°C to remove the cell debris. The supernatants were further subjected to immunoprecipitation using the μ-MACS GFP-tagged protein isolation kit (Miltenyi Biotec) following the manufacturer’s instructions with minor modification in the column washing step, in which wash buffer 1 was replaced with the IP buffer containing 0.2% [v/v] Nonidet P-40.

For mass spectrometry analysis, the immunoprecipitated proteins were resolved by SDS-PAGE and stained with Coomassie brilliant blue (CBB). The lanes were cut from the polyacrylamide gel, followed by dehydration and digestion with trypsin and subjected to LC-MS/MS analysis using a Thermo Scientific Q Exactive Mass Spectrometer. Data were analyzed using the Mascot server (version 2.3) in house (http://www.matrixscience.com/) and compared with proteins registered in TIGR (http://blast.jcvi.org/euk-blast/data/blastp-Genes_in_TIGR_Rice_Pseudomolecules_PEP-74962-1559646622.html).

**Statistical analysis**

For statistical analysis, two-tailed Student’s $t$ test was used to analyze the significance between two noted samples (**$P<0.01$**). Detailed descriptions of statistical analyses are presented in Supplemental Dataset 4.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: GPA5 (Os06g0643000), Rab5a (Os12g0631100), Rab5b (Os03g0151900), Rab5c (Os03g0666500), Rab5d (Os10g0441800), VPS3 (Os05g0104100), VPS11 (Os04g0382700), VPS18 (Os08g0178100), VPS39 (Os03g0715500), and TIP3 (Os10g0492600).
**Supplemental Data**

**Supplemental Figure 1.** Comparisons of ER, PBI, and Golgi Morphology in Developing Wild-Type and *gpa5* Subaleurone Cells.

**Supplemental Figure 2.** Immunofluorescence Microscopy of Glutelins and Prolamins in Developing Subaleurone Cells.

**Supplemental Figure 3.** The *gpa5* Mutation Affects the Trafficking of TIP3 to PBIIs in Endosperm Subaleurone Cells.

**Supplemental Figure 4.** Phylogenetic Tree of the GPA5 Protein and Its Homologs in Land Plants.

**Supplemental Figure 5.** Amino Acid Sequence Alignments of GPA5 and Its Three Arabidopsis Homologs.

**Supplemental Figure 6.** Mass Spectrometry Identification of the GPA5 Protein.

**Supplemental Figure 7.** Expression of GFP-Tagged GPA5 Rescues the *gpa5* Mutant.

**Supplemental Figure 8.** The PX and CC domains Are Necessary for the Membrane Association of GPA5 in Transgenic Rice Root Tip Cells.

**Supplemental Figure 9.** Subcellular Localization of GPA5-GFP in Arabidopsis Suspension Cells.

**Supplemental Figure 10.** Y2H and BiFC Assays of GPA5 and Rab5s in *N. benthamiana*.

**Supplemental Figure 11.** Overexpression of GPA5 in the *vps9a* Background Aggravates the Defects in the Storage Protein Trafficking.

**Supplemental Figure 12.** Evaluation of the Specificity of Polyclonal Antibodies Developed in This Work.

**Supplemental Table 1.** Segregation Analysis of Progenies with a Glutelin Sorting Defect from *gpa5*/*rab5a*−/− Double Heterozygous F1 Plants.

**Supplemental Table 2.** Segregation Analysis of Progenies with a Glutelin Sorting Defect from *gpa5*/*vps9a*−/− Double Heterozygous F1 Plants.

**Supplemental Table 3.** List of Primer Pairs Used in This Study.

**Supplemental Dataset 1.** Alignments Used to Generate the Phylogeny Presented in Supplemental Figure 4.

**Supplemental Dataset 2.** Detailed Mass Spectrometry Data of Proteins Coimmunoprecipitated with Free GFP or GPA5-GFP.

**Supplemental Dataset 3.** Detailed Mass Spectrometry Data of a 55-kDa Protein Band Coimmunoprecipitated with GPA5-GFP.

**Supplemental Dataset 4.** Results of Statistical Analyses.

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**AUTHOR CONTRIBUTIONS**

J.W., H.W. and Y.R. designed the research. Y.R. screened the mutant material and cloned the gene. Y.R. performed immunofluorescence, HPF-FS, and immunogold labeling experiments. Y.R., Y.F.W., Z.W., and L.G. performed the Y2H assay. Y.F.W., L.G., Z.W., and R.J. constructed some vectors. Y.R., Y.H.W., T.P., and Y.L.W. performed all other experiments. F.W., M.W., L.G., J.C.W., G.W., X.B., B.Z., P.Z., Y.Z., C.L., X.Z., Z.C., Q.L., S.Z., Z.Z., J.W., C.W., and L.Q. provided technological assistance. J.W., H.W. and Y.R. analyzed the data and wrote the paper. Y.R., Y.H.W., T.P., and Y.L.W. contributed equally to this work.

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**FIGURE LEGENDS**

**Figure 1.** The *gpa5* Mutant Has a Defect in Storage Protein Transport to the PSV.

(A) Comparison of representative wild-type (WT) and *gpa5* dry seeds. Scale bars, 1 mm.

(B) Transverse sections of representative wild-type and *gpa5* dry seeds. Scale bars, 1 mm.

(C) Scanning electron microscopy images of transverse sections of wild-type and *gpa5* dry seeds. Scale bars, 10 μm.

(D) Total seed protein profile of wild-type and *gpa5* dry seeds on an SDS gel stained with Coomassie brilliant blue (CBB). Note the over-accumulation of unprocessed glutelin precursors in *gpa5*. pGT, unprocessed glutelin precursors; αGT, mature glutelin acidic subunits; αGlb, α-globulin; βGT, mature glutelin basic subunits; Pro, prolamins.

(E) Immunoblot analysis of storage proteins with anti-glutelin mature acidic subunits, anti-basic subunits, and anti-α-globulin antibodies. Arrow and arrowhead indicate the unprocessed glutelin precursors and mature glutelin subunits (black for acidic subunits, red for basic subunits), respectively.

(F) and (G) Immunoblot analysis of dry seeds with anti-glutelin subfamily-specific (GluA, GluB, Gluc, and GluD; [F]) and anti-molecular chaperone (BIP1 and PDI1-1; [G]) antibodies. EF-1α was used as a loading control in (E) to (G).

(H) The *gpa5* mutant exhibits a lethal phenotype at the seedling stage. Images of wild-type and *gpa5* seedlings grown for 5 d on 1/2 MS medium. Scale bar, 1 cm.

(I) Light microscopy of sections stained with CBB from developing wild-type and *gpa5* grains. Asterisks indicate protein aggregates (PMB structures) in *gpa5*. Endosperm is divided into three types of cell by pink line segments. Al, aleurone layers; Sl, subaleurone layers; En, starchy endosperm cells. Scale bars, 50 μm.

(J) Magnified images of the subaleurone cells. Black and red arrowheads indicate the round-shaped prolamin-containing PBIs and irregularly-shaped glutelin and α-globulin-containing PBIIs, respectively. Asterisks indicate the PMB structures. Scale bars, 10 μm.

(K) Immunofluorescence microscopy of glutelins and α-globulin in wild-type and *gpa5* developing subaleurone cells. Secondary antibodies labeled with Alexa fluor 488 (green) and Alexa fluor 555 (red) were used to detect antigens recognized by the polyclonal anti-glutelin antibodies from rabbit and monoclonal anti-α-globulin antibodies from mouse, respectively. Cell wall components (blue) were visualized by staining with Calcofluor white (a non-specific dye for β-glucan). Arrows indicate PBIs, while arrowheads indicate the protein granules located along the cell periphery and PMB structures (asterisks). Scale bars, 10 μm.

(L) and (M) Measurement of the diameters of PBIIs (L) and PBIs (M). Values are means ± SD. **P < 0.01 by Student’s t-test (n > 400).
Figure 2. Immunogold Labeling of Glutelins Depicting the Post-Golgi Sorting Defects of Storage Proteins in the gpa5 Mutant.
Ultrathin sections were prepared from HPF/FS-fixed samples of wild-type or gpa5 developing subaleurone cells, followed by immunogold labelling using the monoclonal anti-glutelin antibodies from mouse. G, the Golgi; CW, cell wall; AP, apoplast space; PMB, paramural body. Bars in (A) to (F) and (K) to (L) = 200 nm; bars in (G), (J), (M), and (N) = 500 nm; bars in (H) and (I) = 1 μm.
(A) and (B) Electron micrographs show that DVs can bud off from the Golgi in the wild type and gpa5.
(C) and (D) Overview of the cell periphery in the wild type and gpa5.
(E) to (J) Electron micrographs showing the biogenesis of the PMB structure in gpa5. (K) to (N), Electron micrographs showing the filling status of PBIIs in the wild type (K) and gpa5 (L) to (N).

Figure 3. Map-Based Cloning of GPA5 and Characterization of GPA5 Protein.
(A) Fine mapping of the GPA5 locus. The numbers and arrows indicate the recombinant plants and candidate genes, respectively.
(B) Gene structure and the mutation site of GPA5. The boxes and lines indicate exons and introns, respectively.
(C) to (E) A 12.6-kb wild-type genomic segment of GPA5 rescues the grain appearance (C), the storage protein processing defect (D), and the endomembrane trafficking defects (E) of gpa5. L1 to L3 indicate three independent T2 transgenic lines. Arrow indicates the unprocessed glutelin precursors. Asterisks denote the PMB structures in gpa5. Al, aleurone cells; Sl, subaleurone. Scale bars, 1 mm in (C), 20 μm in (E).
(F) Schematic domain structure of the GPA5 protein.
(G) GPA5 lipid binding assay. Purified His-tagged full-length GPA5, truncated GPA5△PX, and GPA5△CC proteins were separately subjected to in vitro lipid binding, followed by immunodetection with anti-His antibodies.
(H) GPA5 antibodies specifically detect the endogenous GPA5 protein and the GPA5-GFP fusion protein. Arrow indicates the GPA5-GFP fusion protein, while arrowhead indicates GPA5.
(I) EF-1α was used as a loading control in (H).
(J) Protein accumulation profiles of GPA5 in various tissues as indicated. EF-1α was used as a loading control.
(K) and (L) GPA5 is a peripheral membrane-associated protein. Total protein extract from one-week-old seedlings was ultracentrifuged at 100,000 g for 1 h to obtain pellet (P100) and supernatant (S100), followed by immunoblot analysis with anti-GPA5 and specific antibodies for the ER membrane marker anti-Sec12b and the cytosol marker anti-UGPase (K). P100 fraction was suspended in various buffers as indicated. TX100 indicates Triton X-100. These suspensions were ultracentrifuged to obtain pellet (P) and supernatant (S), followed by immunoblot analysis with anti-GPA5 and anti-Sec12b antibodies (L).

Figure 4. GPA5 Is Localized to DVs in Developing Endosperm Subaleurone Cells and Can Form a Homo-Complex in Planta.
(A) Confocal microscopy images show that GPA5-GFP is localized to the cytosol and to punctate compartments in the cytoplasm in root tip cells and subaleurone cells of the complemented transgenic plants. Scale bars, 10 μm.
(B) One-week-old GPA5-GFP transgenic roots were incubated with DMSO or 33 μM wortmannin for 120 min, followed by confocal imaging. Scale bars, 10 μm.
(C) and (D) Immunogold localization of GPA5-GFP in developing subaleurone cells. Ultrathin sections prepared from HPF/FS samples of pGPA5:gGPA5-GFP T2 transgenic subaleurone cells, followed by single immunogold labeling using the polyclonal anti-GPA5 antibodies from rabbit in combination with 15-nm
gold-coupled secondary antibodies (C) and double immunogold labelling using the monoclonal anti-Glutelin antibodies from mouse in combination with 15-nm gold-coupled secondary antibodies and polyclonal anti-GPA5 antibodies from rabbit in combination with 5-nm gold-coupled secondary antibodies (D). The gold particles (for glutelin) are found inside Golgi and DVs, whereas the gold particles (arrows, for GPA5) are found on the surface of DVs away from the Golgi. Scale bars, 200 nm.

(E) Y2H assay shows that GPA5 interacts with itself. DDO, SD/-Trp/-Leu; QDO, SD/-Trp/-Leu/-His/-Ade.

(F) Firefly luciferase complementation imaging assay shows that GPA5 interacts with itself when transgenically expressed in N. benthamiana leaf cells. Colored scale bar indicates the luminescence intensity in counts per second (cps). cLUC, C terminus of LUC; nLUC, N terminus of LUC.

(G) Coimmunoprecipitation assay shows that GPA5-Flag can be coimmunoprecipitated in the total leaf extract of N. benthamiana with GFP-Trap.

Figure 5. Rab5a and VPS9a Regulate the Membrane Association of GPA5.
(A) Subcellular localization of GFP-Rab5a fusion protein in root tip cells of wild-type and gpa5 plants. GFP-Rab5a was firstly transformed into wild-type and gpa5 (+/-) heterozygous plants. The gpa5 homozygous seeds from the F2 population were identified by genotyping and germinated on 1/2 MS medium, followed by confocal microscopy observation. Scale bars, 10 μm.

(B) Subcellular localization of GPA5-GFP fusion protein in gpa5, rab5a, and vps9a mutant backgrounds. Scale bars, 10 μm.

Figure 6. GPA5 Physically Interacts with Rab5s.
(A) Y2H assay showing the interactions between GPA5 and Rab5 members. DDO, SD/-Trp/-Leu; QDO, SD/-Trp/-Leu/-His/-Ade.

(B) Y2H assay showing the interactions between the constitutively active form of Rab5a and the indicated deletions of GPA5.

(C) Firefly luciferase complementation imaging assay showing that GPA5 specifically interacts with wild-type and the constitutively active variants of Rab5a and 5c in N. benthamiana leaf cells. Colored scale bar indicates the luminescence intensity in counts per second (cps). cLUC, C terminus of LUC; nLUC, N terminus of LUC.

(D) BiFC assay shows that GPA5 specifically interacts with the wild-type and constitutively active variant of Rab5a and constitutively active form of Rab5c. Scale bar, 20 μm.

(E) CBB-stained SDS-PAGE gel. Developing rice endosperm (9 DAF) lysates from transgenic plants expressing free GFP or GPA5-GFP driven by its own promoter were subjected to immunoprecipitation using anti-GFP antibody. Precipitated proteins with free GFP or GPA5-GFP were loaded. pGT, unprocessed glutelin precursors.

(F) Coimmunoprecipitation analysis for interaction between GPA5 and Rab5a. Precipitates from (E) were used for immunoblot analysis using anti-GFP and anti-Rab5a antibodies.

(G) Summary of Rab5 proteins that coprecipitated with GPA5-GFP and identified by mass spectrometry in three independent experiments. Scores were calculated by Mascot. Free GFP did not precipitate any member of the Rab5 family.

Figure 7. GPA5 Is Associated with the CORVET and VAMP727-Containing SNARE Complexes in vivo.
Developing rice endosperm (9 DAF) lysates prepared from transgenic plants expressing free GFP or GPA5-GFP driven by its own promoter were subjected to immunoprecipitation using anti-GFP antibody. Precipitated proteins with free GFP or GPA5-GFP were loaded. pGT, unprocessed glutelin precursors.

(A) Summary of CORVET and HOPS subunits that coprecipitated with GPA5 proteins and identified by mass spectrometry in three independent experiments. Protein scores were calculated by Mascot. Free GFP
did not precipitate any member of CORVET or HOPS complex.

(B) Immunoblot analysis of the immunoprecipitate samples with anti-VPS3, anti-VPS11, anti-VPS18, and anti-VPS39 antibodies.

(C) Summary of SNARE subunits that coprecipitated with GPA5 proteins and identified by mass spectrometry in three independent experiments. Protein scores were calculated by Mascot. Free GFP only precipitates a few VAMP71 members.

(D) Immunoblot analysis of the immunoprecipitate samples with anti-glutelin acidic subunits. Arrows indicate unprocessed glutelin precursors, while arrowheads indicate mature glutelin acidic subunits.

Figure 8. A Working Model Depicting How GPA5 Functions in Post-Golgi Trafficking of Glutelins in Developing Rice Endosperm Subaleurone Cells.

GPA2/VPS9a may be recruited to the TGN and DV by GPA3 and then it functions as the GEF of GPA1/Rab5a. Activated GPA1/Rab5a recruits its effector GPA5 onto DVs, which then interact with CORVET and VAMP727-containing SNARE complexes to execute DV-to-PBII fusion.
A

**UBI:GFP-Rab5a**

|     | WT | gpa5 |
|-----|----|------|
| UBI:GFP-Rab5a |    |      |

B

**UBI:GPA5-GFP**

|     | gpa5 | rab5a | vps9a |
|-----|------|-------|-------|
| UBI:GPA5-GFP |    |       |       |
### A

| Accession         | Assigned name | Protein score 1# | Protein score 2# | Protein score 3# |
|-------------------|---------------|------------------|------------------|------------------|
| LOC_Os05g01360    | VPS3          | 1812             | 1115             | 1241             |
| LOC_Os02g03220    | VPS8          | 2537             | 1784             | 1822             |
| LOC_Os04g31390    | VPS11         | 935              | 649              | 1304             |
| LOC_Os01g47650    | VPS16         | 1571             | 1701             | 1460             |
| LOC_Os08g08060    | VPS18         | 1049             | 1321             | 1311             |
| LOC_Os04g14654    | VPS33         | 463              | 413              | 225              |
| LOC_Os03g50740    | VPS39         | -                | -                | -                |
| LOC_Os04g11880    | VPS41         | -                | -                | -                |

### B

- Anti-VPS3
- Anti-VPS11
- Anti-VPS18
- Anti-VPS39

### C

| Accession         | Assigned name | Protein score 1# | Protein score 2# | Protein score 3# |
|-------------------|---------------|------------------|------------------|------------------|
| LOC_Os08g44430    | VAMP727       | 537              | 208              | 1349             |
| LOC_Os01g15110    | SYP22         | 154              | 244              | 190              |
| LOC_Os01g51120    | VTI11         | 302              | 357              | 491              |
| LOC_Os01g37980    | VTI11         | 103              | 141              | 111              |
| LOC_Os08g17600    | SYP5          | -                | 37               | 101              |
| LOC_Os02g02720    | SYP5          | 129              | 104              | 177              |
| LOC_Os06g07780    | VAMP71        | -                | 16               | 34               |
| LOC_Os02g55990    | VAMP71        | 44               | -                | -                |

### D

- Anti-Acidic
- Anti-Acidic Overexposure

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