The Adaptor Complex AP-4 Regulates Vacuolar Protein Sorting at the trans-Golgi Network by Interacting with VACUOLAR SORTING RECEPTOR1[1]

Kentaro Fuji[2], Makoto Shirakawa[3], Yuki Shimono, Tadashi Kunieda[3], Yoichiro Fukao[4], Yasuko Koumoto, Hideyuki Takahashi[5], Ikuko Hara-Nishimura*, and Tomoo Shimada*

Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606–8502, Japan (K.F., M.S., Y.S., T.K., Y.K., H.T., I.H.-N., T.S.); and Plant Global Educational Project, Nara Institute of Science and Technology, Ikoma 630–0192, Japan (Y.F.)

ORCID ID: 0000-0001-8814-1593 (I.H.-N.).

Adaptor protein (AP) complexes play critical roles in protein sorting among different post-Golgi pathways by recognizing specific cargo protein motifs. Among the five AP complexes (AP-1–AP-5) in plants, AP-4 is one of the most poorly understood; the AP-4 components, AP-4 cargo motifs, and AP-4 functional mechanism are not known. Here, we identify the AP-4 components and show that the AP-4 complex regulates receptor-mediated vacuolar protein sorting by recognizing VACUOLAR SORTING RECEPTOR1 (VSR1), which was originally identified as a sorting receptor for seed storage proteins to target protein storage vacuoles in Arabidopsis (*Arabidopsis thaliana*). From the vacuolar sorting mutant library GREEN FLUORESCENT SEED (GFS), we isolated three *gfs* mutants that accumulate abnormally high levels of VSR1 in seeds and designated them as *gfs4*, *gfs5*, and *gfs6*. Their responsible genes encode three (AP4B, AP4M, and AP4S) of the four subunits of the AP-4 complex, respectively, and an Arabidopsis mutant (*ap4e*) lacking the fourth subunit, AP4E, also had the same phenotype. Mass spectrometry demonstrated that these four proteins form a complex in vivo. The four mutants showed defects in the vacuolar sorting of the major storage protein 12S globulins, indicating a role for the AP-4 complex in vacuolar protein transport. AP4M bound to the tyrosine-based motif of VSR1. AP4M localized at the trans-Golgi network (TGN) subdomain that is distinct from the AP-1-localized TGN subdomain. This study provides a novel function for the AP-4 complex in VSR1-mediated vacuolar protein sorting at the specialized domain of the TGN.

Membrane trafficking in plants shares many fundamental features with those in yeast and animals (Bassham et al., 2008). In general, vacuolar proteins are synthesized on the rough endoplasmic reticulum and then transported to vacuoles via the Golgi apparatus (Xiang et al., 2013; Robinson and Pimpl, 2014). The vacuolar trafficking in plants has been studied by monitoring the transport of reporter proteins to lytic vacuoles in vegetative cells and tissues (Jin et al., 2001; Pimpl et al., 2003; Miao et al., 2008; Niemes et al., 2010). Recently, seed storage proteins became a model cargo for monitoring the transport of endogenous vacuolar proteins in plants (Shimada et al., 2003a; Sanmartín et al., 2007; Isono et al., 2010; Pourcher et al., 2010; Uemura et al., 2012; Shirakawa et al., 2014). During seed maturation, a large amount of storage proteins are synthesized and sorted to specialized vacuoles, the protein storage vacuoles (PSVs). To properly deliver vacuolar proteins, sorting receptors play a critical role in recognizing the vacuole-targeting signal of the proteins. VACUOLAR PROTEIN SORTING10 and Man-6-P receptor function as sorting receptors for vacuolar/lysosomal proteins in the trans-Golgi network (TGN) of yeast and mammals, respectively. The best-characterized sorting receptors in plants are VACUOLAR SORTING RECEPTOR (VSR) family proteins (De Marcos Lousa et al., 2012). VSRs have been shown to function in sorting both storage proteins to PSVs (Shimada et al., 2003a; Fuji et al., 2007) and lytic cargos to lytic vacuoles (Zouhar et al., 2010).

To sort the receptors in the TGN into vacuoles/lysosomes, the adaptor protein (AP) complex binds the cytosolic domain of the receptors. The AP complexes form evolutionarily conserved machinery that

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[2] Present address: RIKEN Innovation Center, Wako 351–0198, Japan.

[3] Present address: Department of Botany, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4.

[4] Present address: Department of Bioinformatics, Ritsumeikan University, Kusatsu 525–8577, Japan.

[5] Present address: Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma 630–0192, Japan.

* Address correspondence to ihnishi@gr.bot.kyoto-u.ac.jp and tshimada@gr.bot.kyoto-u.ac.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Tomoo Shimada (tshimada@gr.bot.kyoto-u.ac.jp).

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mediates the post-Golgi trafficking in eukaryotic cells (Robinson, 2004). There are five types of AP complexes, AP-1 to AP-5. The functions of AP-1, AP-2, and AP-3 have been established. AP-1 appears to be involved in trafficking between the TGN and endosomes (Hirst et al., 2012), AP-2 is involved in clathrin-mediated endocytosis (McMahon and Boucrot, 2011), and AP-3 is involved in protein trafficking from the TGN/endosomes to the vacuole/lysosomes (Dell'Angelica, 2009). However, little is known about AP-4 and AP-5. Mammalian AP-4 may be involved in basolateral sorting in polarized cells and in the transport of specific cargo proteins, such as the amyloid precursor protein APP, from the TGN to endosomes (Burgos et al., 2010). The fifth AP complex, AP-5, was recently identified, and its orthologs are widely conserved in the eukaryotic genomes (Hirst et al., 2011). The AP complexes exist as heterotetrameric proteins that consist of two large subunits (β1-5 and one each of $\gamma/\alpha/\delta/\varepsilon/\zeta$), one medium subunit ($\mu$1-5), and one small subunit (σ1-5). The sorting mechanism is best characterized for the medium (μ) subunit, which is known to recognize the Tyr-based YXXΦ motif (where Φ represents Leu, Ile, Phe, Met, or Val) that is present in the cytosolic domains of cargo proteins (Ohno et al., 1995). Mutations of the YXXΦ motif abolish the interaction with μ and alter the subcellular localization of the cargo proteins.

The genome of Arabidopsis (*Arabidopsis thaliana*) contains all five sets of putative AP genes (Bassham et al., 2008; Hirst et al., 2011). The function of AP-4 in membrane trafficking and its physiological roles in plants are largely unknown. In this study, we identified and characterized the AP-4 complex in Arabidopsis. Mutants lacking the AP-4 subunits exhibited defects in VSR1-mediated vacuolar sorting of storage proteins in seeds. Our results provide new insights into the receptor-mediated vacuolar trafficking in post-Golgi pathways.

RESULTS AND DISCUSSION

Identification of *gfs* Mutants That Accumulate an Increased Level of VSR1

We previously reported a novel screening method, GREEN FLUORESCENT SEED (GFS), to isolate Arabidopsis mutants deficient in vacuolar protein sorting (Fuji et al., 2007). Seven *gfs* mutants, *gfs1*, *gfs2*, *gfs3*, *gfs4*, *gfs5*, *gfs9*, and *gfs10*, exhibited defects in the vacuolar sorting of storage proteins. During further characterization of these *gfs* mutants, we found that *gfs4* and *gfs5* abnormally accumulated a large amount of VSR1 in their dry seeds compared with the wild type (Fig. 1A, lanes 3 and 7). The abnormal accumulation of VSR1 in *gfs4* and *gfs5*, hereafter designated *gfs4-1* and *gfs5-1*, respectively, may be related to the vacuole-sorting defects of these mutants, since VSR1 plays a critical role in the vacuolar sorting of storage proteins (Shimada et al., 2003a). We isolated three additional *gfs* mutants that had increased levels of VSR1 in their dry seeds (Fig. 1A, lanes 4, 8, and 11). Two are allelic to *gfs4-1* and *gfs5-1*, and we designated them *gfs4-2* and *gfs5-2*, respectively (see below). The third one was a previously unidentified mutant, designated *gfs6-1*.

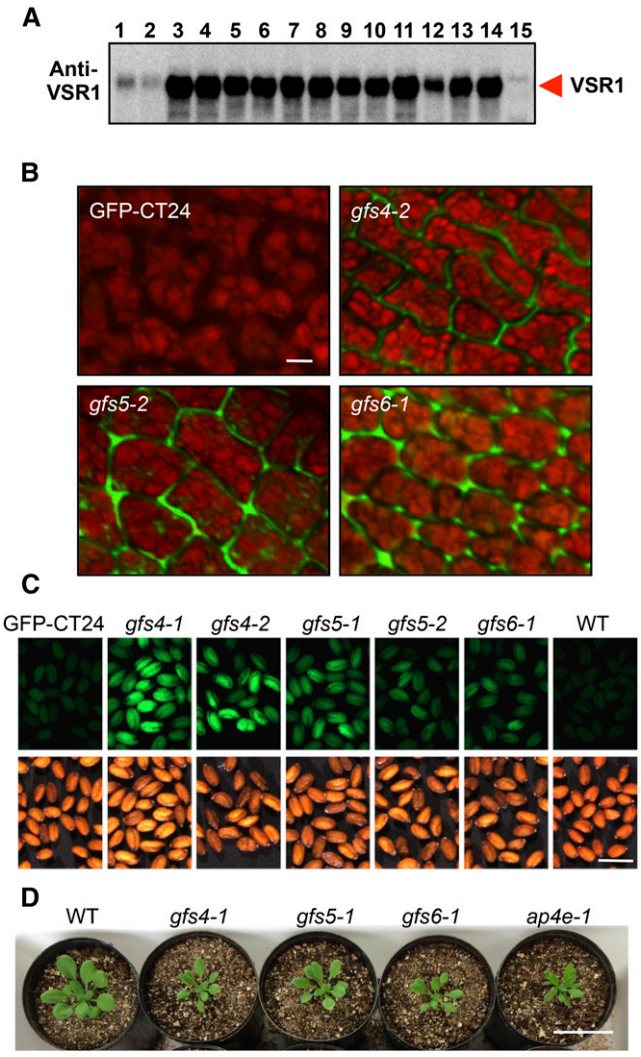

Figure 1. Isolation of Arabidopsis *gfs* mutants that have increased levels of VSR1 in their dry seeds. A, Immunoblot of dry seeds using an anti-VSR1 antibody. Dry seeds of *gfs4-1* to *gfs4-4* (lanes 3–6), *gfs5-1* to *gfs5-4* (lanes 7–10), *gfs6-1* and *gfs6-2* (lanes 11 and 12), and *ap4e-1* and *ap4e-2* (lanes 13 and 14) accumulated higher VSR1 levels than those of the wild type (lane 1) and the parental line GFP-CT24 (lane 2). *vsr1-2* was used as a negative control (lane 15). B, Confocal images of seed cells of *gfs4-2*, *gfs5-2*, *gfs6-1*, and the parental line GFP-CT24. GFP fluorescence was detected outside the cells of these mutants, indicating the missorting of GFP-CT24. The autofluorescence of protein storage vacuoles is shown in red. Bar = 5 μm. C, The Arabidopsis *gfs* seeds have strong GFP fluorescence. Seeds of the wild type (WT), the parental line GFP-CT24, and the *gfs4-1*, *gfs4-2*, *gfs5-1*, *gfs5-2*, and *gfs6-1* mutants were inspected with a fluorescence microscope (top) and a binocular microscope (bottom). Bar = 1 mm. D, Images of wild-type, *gfs4-1*, *gfs5-1*, *gfs6-1*, and *ap4e-1* plants at 25 d after germination. Bar = 3 cm.

To further characterize these *gfs* mutants, we next examined the localization of a GFP with the vacuole-targeting signal of β-conglycinin, GFP-CT24 (Nishizawa

et al., 2003). As for *gfs4-1* and *gfs5-1* (Fuji et al., 2007), the fluorescence of GFP-CT24 was observed abnormally in the extracellular matrix of seed cells in *gfs4-2*, *gfs5-2*, and *gfs6-1* (Fig. 1B). The missorting of vacuole-type GFP-CT24 out of the cell resulted in the strong GFP fluorescence in the mutant seeds (Fig. 1C). Our findings suggest that the VSR1 of *gfs4*, *gsf5*, and *gfs6* are defective in their dynamics and/or stability, causing the missorting of GFP-CT24 in seeds. In addition to the phenotypes found in seeds, the *gfs* mutants displayed growth defects during their life cycles. As shown in Figure 1D, *gfs4-1*, *gsf5-1*, and *gfs6-1* plants exhibited abnormal development and dwarfism compared with wild-type plants.

GFS4, *GFS5*, and *GFS6* Encode Putative Subunits of the AP-4 Complex

We determined the responsible genes, *GFS4*, *GFS5*, and *GFS6*, by positional cloning. They are mapped to different loci: *GFS4* is located on chromosome 5, *GFS5* is on chromosome 4, and *GFS6* is on chromosome 2. Fine-mapping and sequence analysis revealed that the mutations *gfs4-1* and *gfs4-2* were found in the At5g11490 gene, which encodes the β-subunit of the AP-4 complex (AP4B; Fig. 2A). The mutations *gfs5-1* and *gfs5-2* were identified in the At4g24550 gene, which encodes the μ-subunit of the AP-4 complex (AP4M; Fig. 2A). The *gfs6-1* mutant has a nucleotide substitution at the splicing donor site in the At2g19790 gene, which encodes the σ-subunit of the AP-4 complex (AP4S; Fig. 2A). To confirm that each of these three genes is responsible for a mutant phenotype, we obtained T-DNA-tagged lines as shown in Figure 2A: two lines for the At5g11490 gene (*gfs4-3* and *gfs4-4*), two lines for the At4g24550 gene (*gfs5-3* and *gfs5-4*), and one line for the At2g19790 gene (*gfs6-2*). These allelic mutants also exhibited growth defects (Supplemental Fig. S1). The immunoblot analysis with VSR1 antibody revealed that the VSR1 levels were increased in these T-DNA-tagged lines (Fig. 1A). Among these *gfs* mutants, *gfs6-2* exhibited a relatively weak phenotype compared with the other mutants (Fig. 1A, lane 12). A reverse transcription (RT)-PCR analysis revealed a few weak bands of *AP4S* in the *gfs6-2* mutant, which has a T-DNA insertion at the first intron of the *AP4S* gene (Fig. 2A). It is possible that some portions of the AP4S protein function in the *gfs6-2* mutant. In contrast, no full-length transcripts were detected in the T-DNA-tagged mutants *gfs4-3*, *gfs4-4*, *gfs5-3*, and *gfs5-4* (Fig. 2B). Our results suggest that putative subunits of the AP-4 complex, GFS4/AP4B, GFS5/AP4M, and GFS6/AP4S, are responsible for the VSR1-mediated vacuolar sorting.

The AP-4 complex is predicted to be composed of four subunits, AP4E, AP4B, AP4M, and AP4S. Because three subunits are thought to be critical for the accumulation of VSR1 in seeds, we investigated whether the fourth subunit, AP4E, also functions in the same manner. We obtained and characterized two T-DNA-tagged lines of the *AP4E* gene (At1g31730; Fig. 2A). RT-PCR analysis revealed that full-length *AP4E* transcripts were

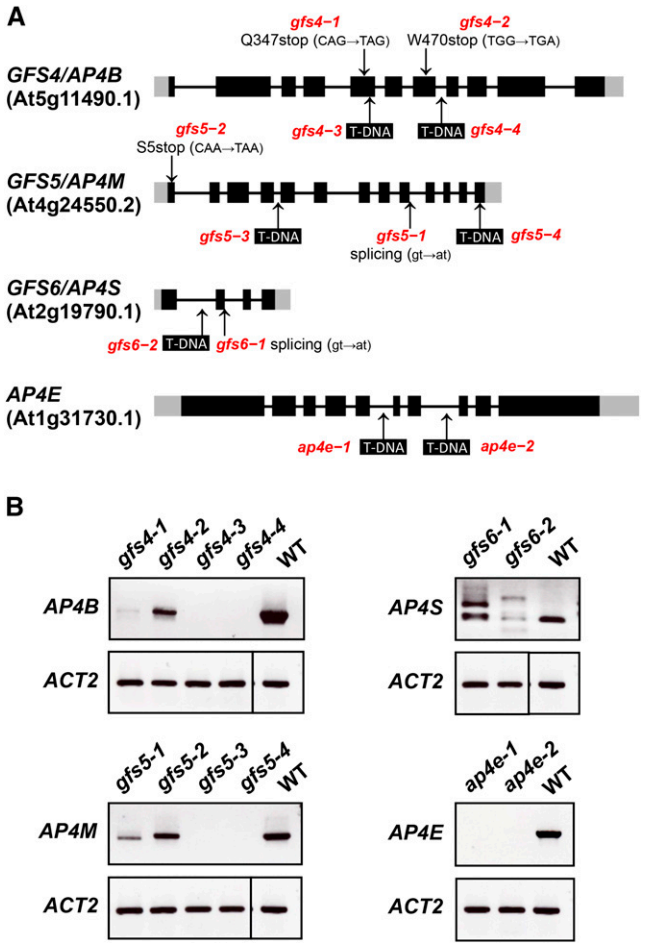

Figure 2. Characterization of Arabidopsis genes encoding four subunits of the AP-4 complex. A, Schematic representations of the *GFS4/AP4B* (At5g11490), *GFS5/AP4M* (At4g24550), *GFS6/AP4S* (At2g19790), and *AP4E* (At1g31730) gene structures. Boxes and solid lines indicate exons and introns, respectively. Black and gray boxes indicate coding and noncoding regions, respectively. The positions of point mutations and transfer DNA (T-DNA) insertions are indicated. B, RT-PCR analysis of *gfs4*, *gfs5*, *gfs6*, and *ap4e* mutants. RT-PCR showed the expression of *AP4B*, *AP4M*, *AP4S*, and *AP4E* in the respective mutants. *ACTIN2* (*ACT2*) was used as an internal control. WT, Wild type.

not accumulated in these mutants (Fig. 2B). The deficiency in the AP4E subunit caused growth defects that were similar to those caused by other AP-4 subunit mutants (Supplemental Fig. S1). As expected, the levels of VSR1 in the *ap4e* mutant seeds were increased (Fig. 1A, lanes 13 and 14). Taken together, our findings strongly suggest that the putative AP-4 complex regulates the VSR1-mediated vacuolar sorting.

The Arabidopsis AP-4 Complex Is Composed of Four Subunits, GFS4/AP4B, GFS5/AP4M, GFS6/AP4S, and AP4E

We next investigated whether GFS4/AP4B, GFS5/AP4M, GFS6/AP4S, and AP4E actually form the AP-4 complex in Arabidopsis. We generated transgenic

Table I. *Compositional analysis of the Arabidopsis AP-4 complex*

A summary of the interacting proteins with AP4M-GFP or GFP-AP4S identified by mass spectrometry analysis is shown. Arabidopsis Genome Initiative (AGI) codes were obtained from The Arabidopsis Information Resource database (http://www.arabidopsis.org). Scores were calculated by Mascot (Matrix Science). n.d., Not detected. For details, see Supplemental Table S1 to S3.

		AP4M-GFP		
Protein	AGI Code	Experiment 1	Experiment 2	GFP-AP4S
AP4B	At5g11490	1,427	842	219
AP4E	At1g31730	1,588	853	264
AP4M	At4g24550	1,363	914	30
AP4S	At2g19790	154	n.d.	107

Arabidopsis plants expressing AP4M-GFP under the control of its own promoter (*ProAP4M*:*AP4M-GFP*). This construct was able to rescue the *gfs5* phenotype (Supplemental Fig. S2), suggesting that the GFP fusion protein AP4M-GFP was functional. We also generated transgenic Arabidopsis plants expressing GFP-AP4S under the control of a cauliflower mosaic virus 35S promoter (*Pro35S*:*GFP-AP4S*). Using these transgenic lines, we performed pull-down and subsequent mass spectrometric analyses. Transgenic Arabidopsis plants expressing cytosolic GFP were used as controls. All four subunits, GFS4/AP4B, GFS5/AP4M, GFS6/AP4S, and AP4E, were repeatedly detected (Table I). No components of the other AP complexes were detected in this analysis (Supplemental Tables S1–S3), indicating that these four subunits specifically form the AP-4 complex in Arabidopsis.

AP4M-GFP and GFP-AP4S Localize at the TGN

To investigate the subcellular localization of the AP-4 complex, the GFP fluorescence of AP4M-GFP and GFP-AP4S was observed in transgenic Arabidopsis root tip cells. Confocal microscopy showed that both GFP-tagged proteins were observed on punctate structures together with a cytosolic distribution (Fig. 3; Supplemental Fig. S3). These puncta were colocalized with FM4-64, an endocytosis tracer, indicating that AP-4 localizes to endomembrane compartments (Fig. 3A). To confirm the localization of AP-4, a series of colocalization experiments were performed using well-established fluorescent markers, including a TGN marker (monomeric red fluorescent protein [mRFP]-SYP43 [SYNTAXIN OF PLANTS43; Uemura et al., 2012]), trans-Golgi markers (ST-mRFP [Boevink et al., 1998] and KAM1ΔC-mRFP [Tamura et al., 2005]), and prevacuolar compartment (PVC) markers (mCherry-ARA7 [Geldner et al., 2009] and SORTING NEXIN1 [SNX1]-mRFP [Jaillais et al., 2006]; Fig. 3B). Among these organelle markers, the TGN marker mRFP-SYP43 was preferentially colocalized with the AP4M-GFP puncta (Pearson's coefficient [Rp] = 0.44; Supplemental Fig. S4). The trans-Golgi marker ST-mRFP was in close proximity to the AP4M-GFP puncta and showed a lower degree of colocalization with AP4M-GFP (*Rp* = 0.33; Supplemental Fig. S4). By contrast, the PVC marker mCherry-ARA7 was essentially independent from the AP4M-GFP puncta (*Rp* = 0.01; Supplemental Fig. S4). A similar localization pattern was observed with the GFP-AP4S puncta (Supplemental Fig. S3). Our results indicate that the AP-4 complex principally localizes to the TGN.

Recently, the μ-subunit of the AP-1 complex, AP1M2, was shown to localize to the TGN (Park et al., 2013; Teh et al., 2013). To investigate whether AP4M and AP1M2 are colocalized, the transgenic lines expressing

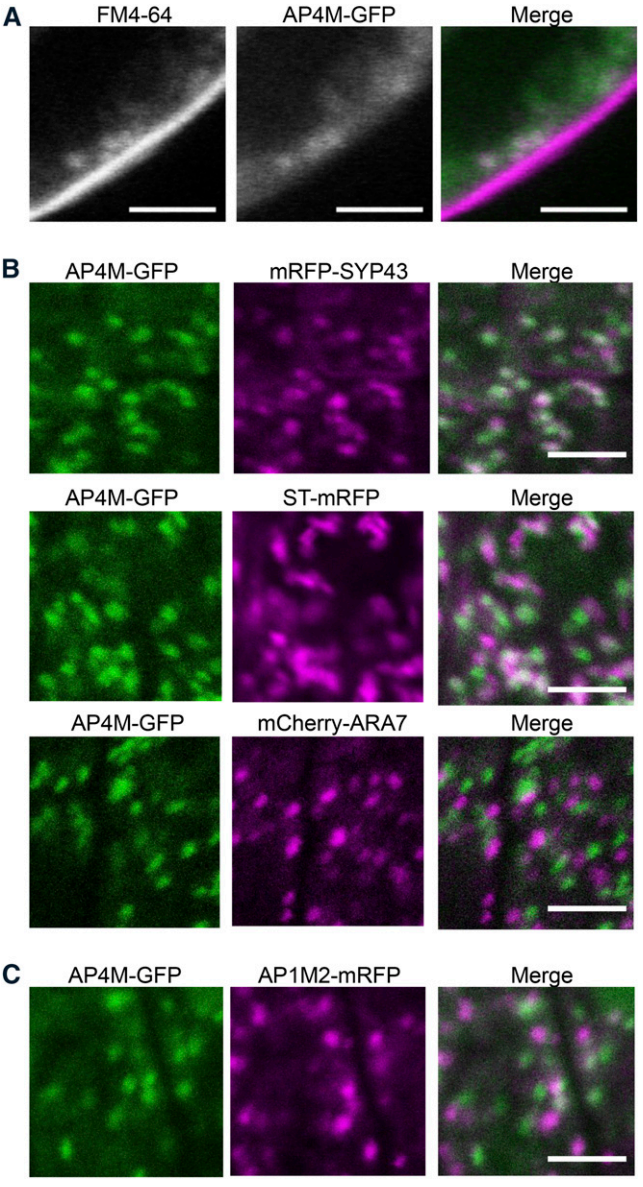

Figure 3. The AP subunit AP4M localizes to the TGN in Arabidopsis roots. A, Rapid colocalization of AP4M-GFP with the endocytic tracer FM4-64 after incubation for 6 min. B, AP4M-GFP colocalizes preferentially with the TGN marker mRFP-SYP43 and to a lower degree with the trans-Golgi marker ST-mRFP but is independent from the prevacuolar marker mCherry-ARA7. C, AP4M-GFP shows only limited colocalization with AP1M2-mRFP. Bars = 5 μm.

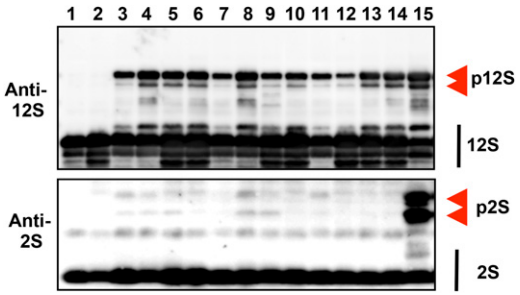

Figure 4. Abnormal accumulation of seed storage proteins in Arabidopsis AP-4 mutant seeds. Immunoblots of dry seeds with anti-12S globulin antibody (top) and anti-2S albumin antibody (bottom) are shown. Seeds of the wild type (lane 1) and the parental line GFP-CT24 (lane 2) accumulated the mature forms of the storage proteins 12S globulin (12S) and 2S albumin (2S), whereas *vsr1-2* (lane 15) seeds accumulated precursors (p12S and p2S) of the storage proteins. The AP-4 mutants, *gfs4-1* to *gfs4-4* (lanes 3–6), *gfs5-1* to *gfs5-4* (lanes 7–10), *gfs6-1* and *gfs6-2* (lanes 11 and 12), and *ap4e-1* and *ap4e-2* (lanes 13 and14), abnormally accumulated the precursors of 12S globulin in their seeds.

AP4M-GFP and *AP1M2-mRFP* were crossed. Interestingly, only limited colocalization was observed between AP4M-GFP and AP1M2-mRFP (*Rp* = 0.18; Fig. 3C; Supplemental Fig. S4), suggesting that the two AP complexes, AP-1 and AP-4, localize at different subdomains of the TGN. Distinct distribution patterns of AP-1 and AP-4 have been observed in animals. Although the distribution of AP-4 closely matched that of AP-1 in the juxtanuclear area (Hirst et al., 1999), a detailed inspection revealed that the staining patterns for AP-4 and AP-1 were not completely overlapping in HeLa cells (Dell'Angelica et al., 1999).

Missorting of Vacuolar Storage Proteins in AP-4 Mutant Seeds

The missorting of the vacuole-type GFP-CT24 in *gfs4*, *gfs5*, and *gfs6* (Fig. 1B) suggests that endogenous storage proteins are missorted and accumulated as precursors in these mutant seeds. As shown previously, *vsr1-2* seeds abnormally accumulated the unprocessed precursor forms of two major storage proteins, 12S globulin and 2S albumin, whereas wild-type and GFP-CT24 seeds contained only the mature storage proteins (Fig. 4, lanes 1, 2, and 15). The AP-4 mutants abnormally accumulated 12S globulin precursors in the seeds (Fig. 4, lanes 3–14, anti-12S). To our surprise, the 2S albumin precursors were hardly detectable in the AP-4 mutants (Fig. 4, lanes 3–14, anti-2S). These results suggest that these two types of storage proteins have distinct transport pathways or manners. The vacuolar sorting of 12S globulin is partly AP-4 dependent, whereas that of 2S albumin is almost AP-4 independent. A similar phenotype was observed previously in Arabidopsis mutants defective in SNXs, which form a subcomplex of the retromer complex (Pourcher et al., 2010).

To reveal the localization of 12S globulin precursors that abnormally accumulated in AP-4 mutants, we performed immunoelectron microscopy of the dry seeds. In wild-type and GFP-CT24 seed cells, the storage proteins 12S globulin and 2S albumin were localized in electron-dense PSVs within the cells, and the extracellular matrices (ECMs) were electron lucent (Fig. 5; Supplemental Fig. S5, wild type [WT] and GFP-CT24). By contrast, the ECMs of the AP-4 mutants (*gfs4-1*, *gfs5-1*, *gfs6-1*, and *ap4e-1*) were filled with electron-dense materials (Fig. 5, red arrowheads). Immunogold analyses of the AP-4 mutants revealed the presence of 12S globulin in the ECMs (Fig. 5) but not 2S albumin (Supplemental Fig. S5). Our observations indicate that the AP-4 complex is required for the vacuolar sorting of 12S globulin precursors.

AP4M Interacts with the Tyr-Based Motif of VSR1

To reveal the role of the AP-4 complex in sorting the storage proteins, we investigated the interaction between AP4M and the Tyr-based motif of VSR1. Among the four subunits of the AP complex, μ-adaptin has been shown to recognize the Tyr-based motif of cargo proteins (Ohno et al., 1995). One putative Tyr-based motif (YMPL in VSR1) is conserved at the cytosolic tail among VSR

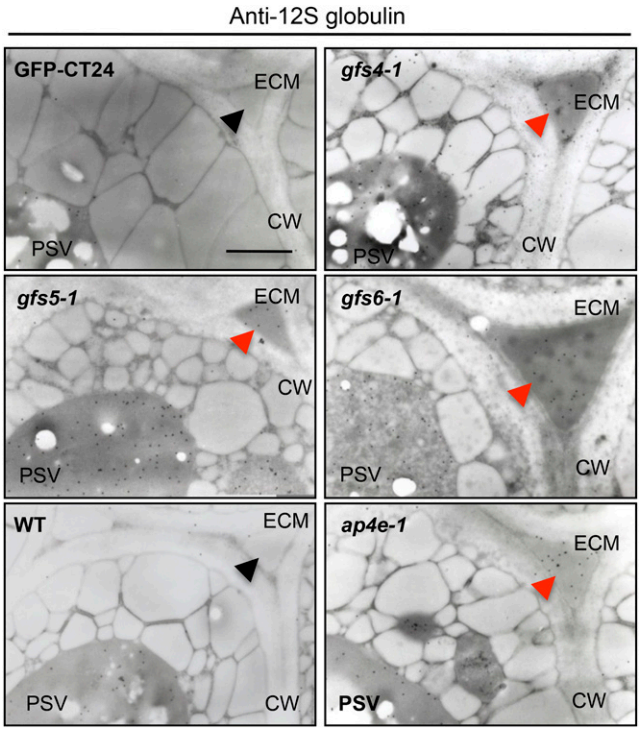

Figure 5. Missorting of vacuolar storage proteins in Arabidopsis AP-4 mutant seeds. Immunoelectron micrographs of the seeds of the wild type (WT), GFP-CT24, *gfs4-1*, *gfs5-1*, *gfs6-1*, and *ap4e-1* with anti-12S globulin antibodies are shown. Red arrowheads show abnormal secretion of 12S globulin in the ECMs of these mutant seeds. CW, Cell wall. Bar = 5 μm.

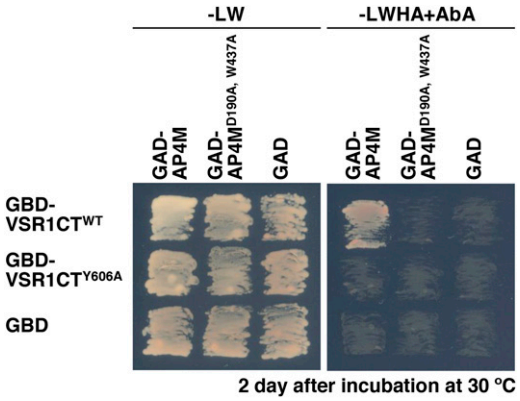

Figure 6. Arabidopsis AP4M interacts with the Tyr-based motif of VSR1. Yeast two-hybrid analysis shows GBD-fused VSR1CT^{WT} and the mutant VSR1CT^{Y606A} with GAD-fused AP4M and the mutant AP4M^{D190A,W437A}. Transformants were plated on medium lacking Leu, Trp, His, and adenine supplemented with AureobasidinA (-LWHA+AbA) to test for their growth or on medium lacking Leu and Trp (-LW) for 2 d at 30°C. pGADT7 and pGBKT7 vectors that had no inserts were used as negative controls (shown as GAD and GBD).

family proteins (see Fig. 7A below; De Marcos Lousa et al., 2012). In a yeast two-hybrid assay, the interaction between AP4M and the cytosolic tail of VSR1 (VSR1CT^{WT}) was observed (Fig. 6). Recently, a similar interaction between AP4M and the cytosolic tail of VSR4 (At2g14720) was reported (Gershlick et al., 2014), although its specificity has not been evaluated. We confirmed the specificity of the interaction by introducing point mutations to both the Tyr-based motif of VSR1 and its putative binding sites on AP4M. No interaction was observed between AP4M and VSR1CT^{Y606A}, in which the conserved Tyr-606 was substituted with an Ala (Fig. 6). In addition, a mutant form of AP4M (AP4M^{D190A,W437A}) failed to interact with VSR1CT^{WT} (Fig. 6). These two residues of AP4M, Asp-190 and Trp-437, correspond to the conserved binding sites for the Tyr-based motif (Owen and Evans, 1998), and an equivalent double mutant of mammal μ2 has been shown to abolish the interaction with the Tyr-based motif of its cargo (Nesterov et al., 1999). Our results suggest that AP4M specifically interacts with VSR1 by recognizing its Tyr-based motif.

Next, we investigated the significance of VSR1's Tyr-based motif in sorting the storage proteins in planta. We expressed the wild type and mutant forms of VSR1 under the control of the native *VSR1* promoter in *vsr1-1* seeds. As shown previously (Shimada et al., 2003a), the expression of wild-type VSR1 in *vsr1-1* suppressed the accumulation of 12S globulin precursors, indicating the complementation of the *vsr1* sorting defect (Fig. 7B, bottom). The wild-type VSR1 produced two bands, VSR1-L and VSR1-S, on the blot as reported previously (Fig. 7B, top). By contrast, VSR1 mutant forms, ΔTMD+CT, ΔCT, and Δ21, each of which has a premature stop at the indicated position (Fig. 7A), did not complement the *vsr1* phenotype. However, the mutant VSR1Δ11, which possesses the Tyr-base motif YMPL (Fig. 7A), complemented the *vsr1* phenotype (Fig. 7B, bottom). Like the wild-type VSR1, VSR1Δ11 also produced two bands. To confirm the significance of the Tyr-based motif, we expressed the full-length VSR1 with a Tyr-606-to-Ala substitution (Y606A in Fig. 7A). VSR1 with Y606A no longer complemented the *vsr1-1* sorting defect (Fig. 7B, bottom). These results indicate that the Tyr-based motif of VSR1 plays an important role in the sorting of storage proteins.

CONCLUSION

Here, we propose that the Arabidopsis AP-4 complex regulates the vacuolar sorting of 12S globulins at the TGN by interacting with VSR1. The abnormal accumulation of VSR1 in AP-4 mutant seeds suggests that AP-4 is required for the appropriate VSR1 localization, which is necessary for the efficient sorting to vacuoles. A similar abnormal accumulation of VSR1 was reported previously in retromer mutants (Yamazaki et al., 2008). The lack of regulation by AP-4 or the retromer may change the localization of VSR1 in the TGN/PVC to the plasma membrane, where VSR1 is stably accumulated.

To our knowledge, this is the first report showing that AP-4 plays a prominent role in sorting vacuolar proteins in plants. The adaptor complex-dependent vacuolar/lysosomal protein sorting is different between plants and other organisms. In animals, the AP-4 complex is expressed at much lower levels than the

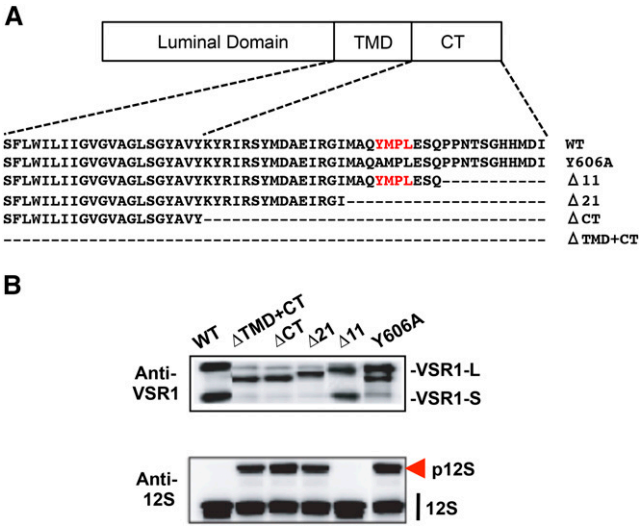

Figure 7. Significance of the Tyr-based motif of VSR1 in vacuolar protein sorting. A, Schematic representation of VSR1 and amino acid sequences of the transmembrane domain (TMD) and the cytosolic tail (CT) of VSR1 in the wild type (WT) and the transformants Y606A, Δ11, Δ21, ΔCT, and ΔTMD+CT. Truncated regions are indicated by dashed lines. A putative Tyr-based motif (YMPL) is shown in red. B, Immunoblots of dry seeds with anti-VSR1 antibody (top) and anti-12S globulin antibody (bottom). Dry seeds of the wild type and the transformant VSR1Δ11 accumulated only mature 12S globulin (12S). Dry seeds of the transformants ΔTMD+CT, ΔCT, Δ21, and Y606A accumulated the 12S globulin precursors (p12S) in addition to the mature form.

AP-1 complex (Hirst et al., 2013) and could serve as a backup for lysosomal protein sorting that is mediated by AP-1 (Hirst et al., 2012). Yeasts have no AP-4 complex and use the AP-1 complex for vacuolar protein sorting. An interesting aspect of our finding is that AP-4 has a role in 12S globulin sorting but not in 2S albumin sorting. In Arabidopsis, the expression levels of the AP-4 subunits are comparable to those of the AP-1 subunits (Park et al., 2013). Additionally, the AP-1 subunit AP1M2 is reported to be required for vacuolar protein sorting (Park et al., 2013) and to interact with the cytosolic domain of VSR4 (Gershlick et al., 2014). It is possible that AP-1 preferentially functions in the 2S albumin sorting. Plants might use two AP complexes (AP-1 and AP-4) that localize to distinct subdomains of the TGN to differentially regulate the sorting of vacuolar proteins.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used as the wild-type plant. The transgenic Arabidopsis plant expressing GFP-CT24, a GFP fusion with the C-terminal 24 amino acids of the soybean (*Glycine max*) β-conglycinin α′-subunit, was reported previously (Nishizawa et al., 2003). *gfs4*, *gfs5*, and *gfs6* mutants were obtained from ethylmethane sulfonate-mutagenized GFP-CT24 seeds as described previously (Fuji et al., 2007). The following T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center: SALK_104326 (*gfs5-3*), SALK_044748 (*gfs5-4*) SAIL_796A10 (*gfs4-3*), SAIL_781H01 (*gfs4-4*), SAIL_525A07 (*gfs6-2*), SAIL_866C01 (*ap4e-1*), and SAIL_60E03 (*ap4e-2*). *vsr1-1* and *vsr1-2* mutants were described previously (Shimada et al., 2003a). The transgenic Arabidopsis plant expressing cytosolic GFP (Mano et al., 1999) was used as a control for immunoprecipitation. Surface-sterilized seeds were sown onto 0.5% (w/v) Gellan Gum (Wako) with Murashige and Skoog medium supplemented with 1% (w/v) Suc and were grown at 22°C under continuous light. Plants were transferred to vermiculite for subsequent growth at 22°C with a 16-h-light/8-h-dark cycle.

Plasmid Construction and Transformation

To construct *ProAP4M:AP4M-GFP*, a genomic *AP4M* fragment was amplified and subcloned into pENTR/D-TOPO using the Gateway TOPO cloning kit (Invitrogen). The fragment was transferred into a binary vector, pGWB4 (Nakagawa et al., 2007a), by the LR reaction of the Gateway system (Invitrogen). To construct *Pro35S:GFP-AP4S*, an *AP4S* fragment was amplified, subcloned into pENTR/D-TOPO, and transferred into a binary vector, pGWB406 (Nakagawa et al., 2007b). To construct the mutant forms of *VSR1*, site-directed mutagenesis was performed using pG_pAtVSR1-cDNA as the template (Shimada et al., 2003a). The constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the transformed bacteria were then infiltrated into Arabidopsis plants using the floral dip method (Clough and Bent, 1998). The sequences of the primers used are shown in Supplemental Table S4.

RT-PCR Analysis

Total RNA was isolated from Arabidopsis seedlings using the RNeasy Plant Mini Kit (Qiagen). Total RNA was used for the synthesis of complementary DNA (cDNA) with Ready-To-Go RT-PCR beads (GE Healthcare) and an oligo(dT)$_{12-18}$ primer. PCR was performed using the cDNA and ExTaq polymerase (Takara). *ACTIN2* was used as the internal control. Gene-specific primers are shown in Supplemental Table S4.

Immunoprecipitation and Mass Spectrometric Analysis

Approximately 1 g of tissue from 5-d-old seedlings expressing AP4M-GFP, GFP-AP4S, or GFP-BE was homogenized. Immunoaffinity complexes were isolated using the μMACS Epitope Tag Protein Isolation Kit (Miltenyi Biotec). The complexes were separated by SDS-PAGE, subjected to in-gel digestion, and examined by mass spectrometry as described previously (Tamura et al., 2010).

Antibodies and Immunoblot Analysis

Immunoblot analysis of Arabidopsis seeds was performed essentially as described previously (Shimada et al., 2003b). The dilutions of the antibodies were as follows: anti-2S albumin (1:10,000), anti-12S globulin α-subunit (1:10,000), and anti-VSR1 (1:5,000; Yamada et al., 2005).

Confocal Laser Scanning Microscopy and Electron Microscopy

Confocal images of the root tip cells of 7-d-old seedlings were obtained using laser scanning microscopes (Zeiss LSM 780 and Zeiss LSM 510 META; Carl Zeiss) equipped with a 488-nm, 40-mW argon/krypton laser or a 544-nm, 1-mW helium/neon laser and a 63× 1.2 numerical aperture (N.A.) water-immersion objective, 40× 0.95 N.A. dry objective, or 20× 0.8 N.A. dry objective. Confocal images were analyzed with ZEN2009 software (Carl Zeiss) to obtain scatter-plots and *Rp* values. All images were processed using either LSM image examiner software (Carl Zeiss) or ImageJ 1.47k software (National Institutes of Health). The GFP fluorescence of dry seeds was observed as described previously (Fuji et al., 2007). The immunoelectron microscopic analysis of dry seeds was performed as described previously (Li et al., 2013; Shimada et al., 2014).

Yeast Two-Hybrid Assay

The coding region of AP4M cDNA was cloned into the entry vector pENTR/D-TOPO (Invitrogen). The entry vector of the mutant type AP4M (AP4M^{D190A,W437A}) was generated from the entry vector of wild-type AP4M by site-directed mutagenesis. The resulting entry vectors were transferred into the pDEST-GADT7 vector (Rossignol et al., 2007) by the LR reaction of the Gateway system (Invitrogen), generating the prey vectors. To generate bait vectors, the VSR1 C-terminal sequences of the wild type (VSR1CT) and the Tyr motif (VSR1CT^{Y606A}) mutant were cloned into the pGBKT7 vector linearized with *Eco*RI and *Bam*HI using the in-fusion cloning method (Clontech). The primers used in this construct are shown in Supplemental Table S4.

The prey and bait vectors were cotransformed into yeast (*Saccharomyces cerevisiae*) strain Y2HGold (Clontech), and then, the transformants were grown on solid synthetic dextrose medium lacking Leu and Trp. To examine interactions between the prey and bait proteins, the transformants were grown on solid synthetic dextrose medium lacking Leu, Trp, His, and adenine with AureobasidinA for 2 d at 30°C. pGADT7 and pGBKT7 vectors were used as negative controls (empty).

Sequence data from this article can be found in the Arabidopsis Genome Initiative under accession numbers ACT2 (At3g18780), AP4E (At1g31730), ARA7 (AT4G19640), GFS4/AP4B (At5g11490), GFS5/AP4M (At4g24550), GFS6/AP4S (At2g19790), KAM1 (At2g20370), SNX1 (AT5G06140), SYP43 (At3g05710), and VSR1 (AT3G52850).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Growth defects of *gfs4*, *gfs5*, *gfs6*, and *ap4e* plants.

Supplemental Figure S2. Complementation test of *gfs5-4* mutants.

Supplemental Figure S3. AP4S localizes to the TGN in Arabidopsis roots.

Supplemental Figure S4. AP4M localizes to the TGN in Arabidopsis roots.

Supplemental Figure S5. Localization of 2S albumin in Arabidopsis AP4 mutants.

Supplemental Table S1. The first trial of mass spectrometry of AP4M-GFP immunoprecipitates.

Supplemental Table S2. The second trial of mass spectrometry of AP4M-GFP immunoprecipitates.

Supplemental Table S3. Mass spectrometry of GFP-AP4S immunoprecipitates.

Supplemental Table S4. Primers used in this study.

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