Arabidopsis KAM2/GRV2 Is Required for Proper Endosome Formation and Functions in Vacuolar Sorting and Determination of the Embryo Growth Axis

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We isolated an Arabidopsis thaliana mutant, katamari2 (kam2), that has a defect in the organization of endomembranes. This mutant had deformed endosomes and formed abnormally large aggregates with various organelles. Map-based cloning revealed that kam2 is allelic to gravitropism defective 2 (grv2). The KAM2/GRV2 gene encodes a homolog of a DnaJ domain–containing RECEPTOR-MEDIATED ENDOCYTOSIS-8, which is considered to play a vital role in the endocytotic pathway from the plasma membrane to lysosomes in animal cells. Immunofluorescent staining showed that KAM2/GRV2 protein localizes on punctate structures, which did not merge with any markers for Golgi, trans-Golgi network, endosomes, or prevacuolar compartments. KAM2/GRV2, which does not have a predicted transmembrane domain, was peripherally associated with the membrane surface of uncharacterized compartments. KAM2/GRV2 was expressed at the early to middle stages of seed maturation. We found kam2 mis-sorted seed storage proteins by secreting them from cells, indicating that KAM2/GRV2 is involved in the transport of the proteins into protein storage vacuoles. kam2 had another defect in embryogenesis. Half of the developing kam2-1 cotyledons grew into the opposite space of the seeds before the walking stick–shaped embryo stage. Our findings suggest that KAM2/GRV2 is required for proper formation of the endosomes involving protein trafficking to the vacuoles and determination of growth axis of the embryo.

INTRODUCTION

Eukaryotic cells use many internal membrane-bound compartments, called the endomembrane system, to spatially separate metabolic reactions and to create different chemical environments. The endomembrane system in plants is composed of endoplasmic reticulum (ER), Golgi stacks, the vacuole, and intermediate organelles, such as the endosomal compartments (Hawes et al., 1999; Surpin and Raikhel, 2004). The identity of each compartment must be maintained to assure that newly synthesized proteins, polysaccharides, and lipids are correctly delivered to the proper cellular subcompartments. This is a complex task in view of the fact that organelles on the vacuolar and endocytotic pathways continually exchange membrane components. However, little is known about how membrane traffic structurally and functionally supports the endomembrane system.

The pathways of membrane traffic in plant cells can be divided into two types: vacuolar pathways and endocytotic pathways (Jurgens, 2004; Samaj et al., 2005). Most vacuolar proteins are transported to Golgi stacks from the ER and are recognized by a vacuolar sorting receptor that delivers them into the vacuoles. In the Arabidopsis thaliana vsr1 mutant, in which VACUOLAR SORTING RECEPTOR1 (VSR1/ATELP1) was disrupted, most seed vacuolar proteins are mis-sorted into extracellular space but not into the vacuoles (Shimada et al., 2003a), suggesting that the default destination for secretory soluble proteins might be the extracellular space, as in the case in animals and yeasts. A unique characteristic of plant cells is that the proteins are directly delivered from the ER to the vacuole without involvement of the Golgi stacks (Vitale and Galili, 2001; Galili, 2004), although the mechanism by which this occurs is unknown. This Golgi-independent pathway, another type of vacuolar pathway, may represent a general mechanism of plant vacuolar ontogeny (Marty, 1999; Vitale and Galili, 2001).

Endocytotic pathways, the other membrane traffic system, are involved in the internalization of molecules from the plasma membrane and extracellular environment, including uptake and the degradation of signal molecules (Baluska et al., 2002; Geldner, 2004; Samaj et al., 2005). Plant cell biologists have tended to develop their own terms, such as prevacuolar compartment (PVC), for some endosomal compartments (Geldner, 2004; Samaj et al., 2005). The PVC most likely belongs to the late endosomal system in plant cells (Jurgens, 2004; Tse et al., 2004) and is thought to function as a midway point between the endocytotic and vacuolar pathways. However, the mechanisms by which the endocytotic and vacuolar pathways communicate are unknown.

Previously, we isolated mutants whose endomembrane formed large aggregates and designated them katamari1 (kam1), after the Japanese word for aggregate. The kam1 mutant showed abnormal organization of actin filaments that resulted in aggregates of the whole endomembrane (Tamura et al., 2005). Here, we describe that identification and characterization of another Arabidopsis mutant, kam2, which has an aggregated endomembrane structure, including deformed endosomal compartments and...
defects in vacuolar transport in seed cells. We show that KAM2 protein, which is a homolog to animal RECEPTOR-MEDIATED ENDOCYTOSIS-8 (RME-8) proteins that are involved in endocytosis, is required for both the vacuolar pathway and endomembrane organization in plants.

RESULTS

The Arabidopsis kam2 Mutant Has Abnormal Cytoplasmic Aggregates with Deformed Endosomes

GFP-2sc, which is a transgenic Arabidopsis plant that expresses vacuole-targeted GFP-2SC, gave no fluorescent vacuoles when the plants were grown under light because of the light-dependent degradation of green fluorescent protein (GFP) at the acidic condition of vacuoles (Tamura et al., 2003). Light-grown GFP-2sc, however, retains GFP fluorescence in the entire endomembranes, including ER network structures and dot-like structures of Golgi stacks in various organs (Tamura et al., 2003), as shown in Figures 1A, 1C, 1E, and 1G). In this study, all fluorescent images were taken from light-grown plants.

To isolate Arabidopsis mutants that have abnormal endomembrane structures within the cells, we used M2 lines from GFP-2sc seeds that had been mutagenized with ethyl methanesulfonate previously (Tamura et al., 2005). We selected a mutant line that exhibited abnormal endomembrane structure by examining 5- to

![Figure 1](image-url) Disorganization and Aggregation of Endomembranes in Different Cells of the Isolated Arabidopsis Mutant kam2-1.

Transgenic Arabidopsis GFP-2sc exhibits GFP fluorescence in entire endomembranes, including ER networks and the Golgi complex (Tamura et al., 2003). Median planes of various cells of GFP-2sc (left panels) and the kam2-1 mutant (right panels) were inspected with a confocal laser scanning microscope (GFP) and a differential interference contrast (DIC) microscope. Arrows indicate aggregates of GFP-fluoresced endomembranes in kam2-1. Bars = 20 μm.

(A) and (B) Epidermal cells of 7-d-old cotyledons.
(C) and (D) Hypocotyls of 7-d-old plants.
(E) and (F) Trichomes of 14-d-old plants.
(G) and (H) Root hairs of 7-d-old plants.
The isolated kam2-1 mutant had GFP fluorescent aggregates (~10 μm) in most of the cells of cotyledons (Figure 1B) and in some cells of hypocotyls (Figure 1D), trichomes (Figure 1F), and roots hairs (Figure 1H). The same phenotype was observed in another mutant allele, kam2-6, that we isolated in this study (described below). To know the three-dimensional structure of the aggregates in kam2 mutant cells, we collected the GFP fluorescent images of a series of 0.43-μm optical sections along the z axis in kam2-1 cotyledon cells, starting from the upper surface toward the bottom of cells, and then reconstituted them as a projection movie (see Supplemental Movie 1 online; Figure 2). An aggregate attached to the cell surface by facing a part of the inner cell surface, bulged toward the inside of the cell, and connected to the other aggregate that faced the other side of the cell. These observations imply that a part of the endomembranes dilated and/or clustered in kam2-1 cells.

To identify the components of the aggregates, we visualized various organelles in 10-d-old cotyledons of GFP-2sc (Figures 3A, 3C, 3E, and 3G) and kam2-1 (Figures 3B, 3D, 3F, and 3H) by transient expression of marker proteins tagged with monomeric red fluorescent protein (mRFP) (Campbell et al., 2002) and FM4-64 staining. Most of the mRFP fluorescent ER was completely merged with ER bodies and ER-derived organelles (Matsushima et al., 2003) in the GFP-fluorescent aggregates, causing the ER bodies to become yellow fluorescent (Figure 3B). Some of the mRFP fluorescent organelles (Golgi stacks and peroxisomes) were engulfed by the aggregates (Figures 3D and 3F, respectively). Most of the FM4-64-stained endosomal compartments were detected as yellow fluorescent aggregates, which were found in the GFP fluorescent aggregates (Figure 3H).

A higher magnification of kam2-1 cotyledon cells showed that the FM4-64-stained sheet-like structures on the cell surface merged completely with GFP fluorescent aggregates but not with GFP fluorescent ER bodies (Figure 4B). In kam2 cells, endosomes swelled and aggregated, while the ER, Golgi, and peroxisomes were engulfed in the aggregates. Electron microscopy showed that epidermal cells of the GPA-2sc roots were entirely occupied by a large central vacuole surrounded by thin-layered cytosol (Figure 4C). By contrast, kam2-1 root cells appeared to have abnormal cytoplasmic aggregates that contained the nucleus, many endomembranes, and fragmented vacuoles (Figure 4D). These results suggest that the aggregates in kam2-1 are composed of various organelles, including deformed endosomes in different organs.

**KAM2 Encodes a Homolog of a DnaJ Domain–Containing RME-8**

The KAM2 gene was located in the middle of chromosome 2 between markers ER and COP1. Further mapping with 220 F2 plants that exhibited the kam2 phenotype and DNA sequencing showed a single base pair mutation from G to A in the AT2g26890 gene of kam2-1, which might cause a non-sense mutation from TGG (Trp-1767) to TGA (stop codon) (Figures 5A and 5B).

To confirm that the mutation in the AT2g26890 gene is responsible for the kam2 phenotype, we obtained three knockout mutants (salk_078570, salk_067162, and salk_132563) of the AT2g26890 gene from the Salk T-DNA insertion lines. These mutants, each of which had T-DNA inserted into an exon of the AT2g26890 gene, were named kam2-2, kam2-3, and kam2-4 (Figure 5A). To visualize the endomembranes in these mutants, a vacuole-targeted GFP, SP-GFP-2SC, was transiently expressed in epidermal cells of leaves of each kam2 allele by particle bombardment. All kam2 alleles had the aggregates of endomembranes in the cells, as in the kam2-1 cells (see Supplemental Figures 1A to 1C online). We identified one more kam2 allele having the aggregates of endomembranes by further screening of the mutagenized pool of the GFP-2sc line. We named this mutant kam2-6 (Gln-1584 to stop) (Figures 5A and 5B; see Supplemental Figure 1D online). These results indicate that the AT2g26890 gene is responsible for the kam2 phenotype, although we did not perform the complementation test with the gene.

The AT2g26890 gene was reported to be responsible for the gravitropism defective2 (grv2) mutant, which is deficient in shoot gravitropism and phototropism (Silady et al., 2004). We found that all six alleles of kam2-1 to kam2-6 exhibited a defect of shoot gravitropism, as does grv2 (data not shown). Overall results indicate that the KAM2 gene is the AT2g26890 gene.

The KAM2/GRV2 gene encodes a polypeptide sequence of 2555 amino acids with a single DnaJ domain (Figure 5B, gray box) (Miernyk, 2001). To characterize the protein, we raised specific antibody against each peptide region that is indicated by

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**Figure 2. Two Cytoplasm Aggregates That Are Connected within the kam2-1 Cell.**

We collected the GFP fluorescent images of a series of 0.43-μm optical sections along the z axis in kam2-1 cotyledon cells, starting from the surface toward the bottom of the cell, and then reconstituted them as a projection movie. The three-dimensional structure is given in Supplemental Movie 1 online. Three still images ([A] to [C]) show two connected cytoplasm aggregates in the cells. Bar = 10 μm.
KAM2I and KAM2C, respectively, in Figure 5B. An immunoblot analysis with both anti-KAM2I and anti-KAM2C antibodies showed that GFP-2sc accumulated a 280-kD KAM2/GRV2 protein (Figure 5C, open arrowheads). On the other hand, kam2-1 accumulated a slight amount of a 190-kD protein, which was recognized by anti-KAM2I antibody but not by anti-KAM2C (Figure 5C, closed arrowhead). This 190-kD protein was a C-terminal truncated form of KAM2/GRV2. This result suggests that the C-terminal region of KAM2/GRV2 is involved in protein stabilization and/or is essential for a function of KAM2/GRV2. Alternatively, introducing a premature stop codon might cause instability of KAM2/GRV2 transcripts. KAM2/GRV2 is a homolog of RME-8, a DnaJ domain–containing protein that is required for endocytosis and organization of endosomes in Caenorhabditis elegans (Zhang et al., 2001), Drosophila melanogaster (Chang et al., 2004), and human (Girard et al., 2005). These results imply that defects in membrane trafficking or endosomal organization cause the abnormal endomembrane structure in the Arabidopsis kam2 mutant.

KAM2/GRV2 Is Peripherally Associated with the Membrane Surface of Uncharacterized Endosomal Compartments

To determine whether KAM2/GRV2 is associated with the membrane fraction, we prepared the microsomal and soluble fractions from Arabidopsis seedlings by ultracentrifugation and subjected them to immunoblot analysis. Most of KAM2/GRV2 protein was detected in the microsomal fraction (Figure 6A, P100), and a small portion was detected in the soluble fraction (Figure 6A, S100). This result suggests that most of KAM2/GRV2 protein localizes on the membrane and a small amount in cytosol. To determine how KAM2/GRV2 was associated on the membrane, we extracted KAM2/GRV2 from the microsomes under various conditions, as shown in Figure 6B. A portion of KAM2/GRV2

Figure 3. Aggregates in kam2-1 Include Various Organelles.

Organelles were visualized in epidermal cells from 10-d-old cotyledons of GFP-2sc (left panels) and kam2-1 (right panels) by transient expression of mRFP-tagged organelle markers and by FM4-64 staining. The cells were inspected with a confocal laser scanning microscope. Shown are GFP channel (green), RFP or FM4-63 channel (red), and merged images (merge). Arrows indicate aggregates including the organelles. Bars = 10 μm. (A) and (B) GFP and ER-targeted mRFP in a single cell. (C) and (D) GFP and Golgi-targeted mRFP in a single cell. (E) and (F) GFP and peroxisome-targeted mRFP in a single cell. (G) and (H) GFP and FM4-64 red fluorescence of endosomes and plasma membrane.
was extracted from the microsomal fraction of wild-type seedlings by alkaline pH (pH 11) or nonionic detergent (Triton X) but not by high salt (NaCl). All of the KAM2/GRV2 was extracted with an ionic detergent (SDS). This profile was the different from that of an integral membrane protein, *Arabidopsis VACUOLAR SORTING RECEPTOR* (VSR) (Shimada et al., 2003a) (Figures 6A and 6B). This is consistent with a hydropathy plot analysis predicting that KAM2/GRV2 has no transmembrane domain (data not shown). These results suggest that KAM2/GRV2 is a peripheral membrane protein rather than an integral membrane protein.

Microsomes from seedlings were further fractionated on a sucrose density gradient. Figure 7A shows that the sedimentation pattern of KAM2/GRV2 was similar but not identical to those of VSR (a PVC/trans-Golgi network [TGN] marker) (Shimada et al., 2003a) and VAM3 (a PVC/vacuolar membrane marker) (Sato et al., 1997) and was completely different from that of PROTEIN DISULFIDE ISOMERASE (PDI; an ER marker). To determine the intracellular localization of KAM2/GRV2, we immunostained protoplasts of *Arabidopsis* cultured cells that expressed a fluorescent-tagged marker for each organelle. Anti-KAM2I antibody stained many punctate structures within the cells (Figures 7B to 7H, left panels), while preimmune serum for KAM2I did not stain any structures (data not shown). These punctate structures merged with none of KAM1ΔC-mRFP (Figure 7B, Golgi complex), Ara6-GFP (Figure 7C, endosome), GFP-Ara7 (Figure 7D, endosome), GFP-PEP12 (Figure 7E, PVC), GFP-VAM3 (Figure 7F, PVC and vacuolar membrane), and GFP-SYP41 (Figure 7G, TGN). Brefeldin A (BFA) treatment, which inhibits guanine-nucleotide exchange factor of ADP-ribosylation G protein activity, induced the formation of the BFA compartment that includes the Golgi complex (Figure 7H, KAM1ΔC-mRFP) and endosomes (Geldner et al., 2001). The KAM2/GRV2 punctate structures did not change their structures by BFA treatment (Figure 7H, KAM2). These results suggest that KAM2/GRV2 localizes on the unknown punctate compartments.

**kam2 Mis-Sorts Storage Proteins by Secreting Them from Cells**

It has been shown that RME-8, a homolog of KAM2/GRV2, plays a vital role in endocytosis in animal cells (Zhang et al., 2001; Chang et al., 2004; Girard et al., 2005). This implied that KAM2/GRV2 is involved in intracellular trafficking in *Arabidopsis*. For investigating intracellular trafficking, maturing seeds are an ideal material because they actively synthesize a large amount of storage proteins on ER as a precursor form and then deliver them to the protein storage vacuoles (PSVs), in which the precursors are converted into the mature form (Shimada et al., 2003a). Vacular sorting deficiency should cause the accumulation of storage protein precursors in dry seeds.

Wild-type seeds accumulated the mature forms of major storage proteins: 12S globulin (α- and β-subunits) and 2S albumin (the large and small subunits) (Figure 8, CBB, lane 1). On the other hand, seeds of kam2 alleles accumulated 17-, 49-, 51-, and 54-kD proteins in addition to the mature storage proteins (Figure 8, CBB, lanes 3 to 6). An immunoblot analysis showed that these additional proteins in kam2 seeds were the precursors of storage proteins (Figure 8, anti-12S and anti-2S, lanes 3 to 6). *kam2* seeds abnormally accumulated the precursors, as did seeds of **atvsr1-2** that lacks a vacuolar sorting receptor for storage proteins (Shimada et al., 2003a). This result suggests that kam2 has a defect in the vacuolar sorting of storage proteins in maturing seeds.

**Figure 4. Abnormal Aggregation of Cytoplasm in kam2-1.**

(A) GFP-fluoresced endomembranes and FM4-64 red-fluoresced endosomes in epidermal cells of GFP-2sc cotyledons. (B) GFP-fluoresced aggregate in epidermal cells of kam2-1 cotyledons. FM4-64 stains a sheet-like structure composed of deformed endosomes. Bright-green structures are ER bodies. (C) Electron micrograph of a root epidermal cell of 7-d-old plants of GFP-2sc. (D) Electron micrograph of a root epidermal cell of 7-d-old plants of kam2-1. cw, cell wall; n, nucleus; v, vacuole. Bars = 10 μm.
The morphologies of autofluorescent PSVs showed that kam2-1 PSVs were deformed (Figure 9B), suggesting that KAM2/GRV2 is essential for the proper formation of PSVs. Electron microscopy revealed that the extracellular space of kam2-1 (Figure 9D, arrows) was abnormally enlarged and filled with electron-dense material compared with GFP-2sc (Figure 9C). Immunogold analysis of kam2-1 seeds indicated that this electron-dense material has high concentrations of the storage proteins 12S globulin (Figure 9F, arrow) and 2S albumin (Figure 9H, arrow). The storage proteins were distributed in the PSVs and the electron-dense extracellular space in the kam2-1 seeds. These observations indicate that the kam2-1 mutant mis-sorts the storage proteins by secreting them from cells, resulting in a swelled and electron-dense extracellular space in the seeds (Figures 9D, 9F, and 9H). These results indicate that KAM2/GRV2 is involved in the trafficking of both 12S globulin and 2S albumin to PSVs in the cells of maturing seeds.

KAM2/GRV2 Is Involved in Determination of the Growth Axis of the Embryo during Embryogenesis

Figure 10 shows the developmental change in the level of KAM2/GRV2 during embryogenesis and seed maturation. Arabidopsis siliques were harvested at sequential stages from young flowers (lane 1) to mature yellow siliques (lane 12) and then were subjected to immunoblots. The amount of KAM2/GRV2 reached a maximum before the accumulation of VSR, 12S globulin, and αTIP (KAM2, lanes 5 to 7). VAM3, which is required for vacuolar trafficking from the PVC, accumulated in association with the accumulation of KAM2/GRV2. The stages of developing embryos for lanes 5 to 7 were estimated to be between the torpedo-shaped and the full-sized embryo stages, considering the accumulation profile of δVPE, which is known to reach a maximum at the heart-shaped embryo stage (Nakaune et al., 2005). This result suggests that KAM2/GRV2 functions in intracellular trafficking at torpedo-shaped to full-sized embryo stages.

Figure 5. KAM2 Encodes a Homolog of Animal RME-8.

(A) Schematic representation of the KAM2/GRV2 gene (AT2g26890), which consists of 22 exons, and the position of each point mutation of kam2-1 and kam2-6 and of each T-DNA insertion of kam2-2, kam2-3, and kam2-4. Closed boxes represent exons, and solid lines represent introns. (B) KAM2/GRV2 is composed of 2555 amino acids. Gray box indicates a DnaJ domain. Mutations of kam2-1 and kam2-6 on the KAM2/GRV2 polypeptide are indicated. Anti-KAM2I and anti-KAM2C antibodies were generated against the polypeptides derived from the KAM2I and KAM2C regions, respectively. (C) Immunoblot analysis of the same protein amounts of microsomes from GFP-2sc and kam2-1 with either anti-KAM2I (left) or anti-KAM2C (right). GFP-2sc accumulated 280-kD KAM2/GRV2, while kam2-1 accumulated a 190-kD truncated polypeptide that was recognized by anti-KAM2I but not by anti-KAM2C due to the lack of the C-terminal domain of KAM2/GRV2. The molecular masses are given at the right in kilodaltons.

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Figure 6. Association of KAM2/GRV2 with Membranes.

(A) The extract (total) from Arabidopsis seedlings was ultracentrifuged at 100,000 g for 1 h to obtain supernatant (S100) and pellet (P100, microsomal fraction). Each fraction was subjected to immunoblot analysis with anti-KAM2I and specific antibodies for organelle markers: anti-VSR1 (a PVC/TGN) and anti-ALEU (a marker for vacuoles). (B) The microsomal fraction from seedlings was suspended in control buffer (100 mM Hepes-KOH, pH 7.5), high-salt buffer (1 M NaCl and 100 mM Hepes-KOH, pH 7.5), alkaline solution (100 mM Na2CO3, pH 11), Triton X buffer (1% [v/v] Triton X-100 and 100 mM Hepes-KOH, pH 7.5), and SDS buffer (1% [w/v] SDS and 100 mM Hepes-KOH, pH 7.5). These suspensions were ultracentrifuged to obtain supernatant (sup) and pellet (ppt). Each fraction was subjected to immunoblot analysis with anti-KAM2I and anti-VSR1.
We found that kam2 showed an embryo-defective phenotype (Figure 11). The kam2 embryo (Figures 11E and 11F) grew normally from the heart-shaped to the torpedo-shaped embryo stages, as did wild-type embryo (Figures 11A and 11B). Afterward, however, approximately half of the kam2-1 embryos started to bend retrorsely before the walking stick–shaped embryo stage (Figure 11G), and the developing cotyledons grew into the opposite space within the seeds (Figure 11H). The other half of the kam2-1 embryo grew normally (Figures 11I and 11J), as did the wild-type embryo (Figures 11C and 11D). Consequently, almost half of kam2 seeds acquired an aberrant shape (Figure 11L, asterisks). The defect of growth axis of embryo was observed in other kam2 alleles (kam2-2, kam2-3, kam2-4, and kam2-6) (see Supplemental Figure 2 online). The deformed seeds normally germinated and grew as did wild-type seeds (data not shown). This result suggests that KAM2/GRV2 is required for endomembrane organization that supports determination of the growth axis of the embryo between the late torpedo-shaped embryo stage and the walking stick–shaped embryo stage.

**DISCUSSION**

**KAM2/GRV2 Is Involved in the Step of Protein Trafficking between the Golgi Complex and the Post-Golgi Compartment**

KAM2/GRV2 is required for protein sorting to PSVs in maturing seed cells (Figures 8 and 9). What step of the protein sorting is regulated by KAM2/GRV2? A characteristic kam2 phenotype that the storage proteins are secreted from the cells (Figure 9) is similar to that of atvsr1, which lacks a vacuolar sorting receptor of storage proteins (Shimada et al., 2003a). It has been shown that subjected to immunoblot analysis with anti-KAM2I and specific antibodies for organelle markers: anti-VSR1 (a PVC/TGN), anti-VAM3 (a marker of prevacuolar compartments and the vacuolar membrane), and anti-PDI (an ER marker). Sucrose concentration (% [w/w]) of each fraction measured is given at the top.

(B) to (H) Protoplasts that had transiently expressed GFP- or mRFP-tagged organelle markers were subjected to immunofluorescent staining with anti-KAM2I. Cells were inspected with a confocal laser scanning microscope. Bars = 10 μm.

(B) Protoplasts expressing KAM1ΔC-mRFP (a Golgi marker) were stained with anti-KAM2I (green).

(C) Protoplasts expressing Ara6-GFP (an endosome marker) were stained with anti-KAM2I (red).

(D) Protoplasts expressing GFP-Ara7 (an endosome marker) were stained with anti-KAM2I (red).

(E) Protoplasts expressing GFP-PEP12 (a marker of prevacuolar compartments) were stained with anti-KAM2I (red).

(F) Protoplasts expressing GFP-VAM3 (a marker of PVC and the vacuolar membrane) were stained with anti-KAM2I (red).

(G) Protoplasts expressing GFP-SYP41 (a TGN marker) were stained with anti-KAM2I (red).

(H) Protoplasts expressing KAM1ΔC-mRFP (a Golgi marker) were treated with 100 μg/mL of BFA (+BFA) for 2 h followed by immunofluorescent staining with anti-KAM2I (green).
VSR1 functions between the Golgi complex and the PVC (Shimada et al., 2003a). These results suggest that KAM2/GRV2 is involved in protein traffic between the Golgi complex and the post-Golgi compartment. This is consistent with the results of yeast vps mutants that mis-sort vacuolar proteins into extracellular space (Marcusson et al., 1994). These mutants are known to have a defect in Golgi-to-post-Golgi traffic.

How can KAM2/GRV2 be involved in ER-to-Golgi traffic? Yeast sec20 and tip1 mutants that have a defect in ER-to-Golgi traffic accumulate clusters of small transport vesicles (Sweet and Pelham, 1992, 1993). However, no clusters of small transport

Figure 8. kam2 Mutants Abnormally Accumulate the Precursors of Storage Proteins in Dry Seeds.

Dry seeds (two grains) of the wild type (Col; lane 1), GFP-2sc (lane 2), kam2-1 (lane 3), kam2-2 (lane 4), kam2-3 (lane 5), kam2-4 (lane 6), and atvsr1-2 (lane 7) were subjected to SDS-PAGE followed by either Coomassie blue staining (CBB; top panel) or immunoblot analysis with anti-12S globulin (anti-12S; middle panel) and anti-2S albumin (anti-2S; bottom panel). p12S, 12S globulin precursors; α and β, 12S globulin subunits; p2S, 2S albumin precursor; L and S, 2S albumin subunits. The molecular masses are given at the left in kilodaltons.

Figure 9. kam2-1 Mis-Sorts Storage Proteins by Secreting Them from Cells, Showing That KAM2/GRV2 Is Involved in Vacuolar Sorting of Storage Proteins to PSVs.

Dry seeds of GFP-2sc (left panels) and kam2-1 (right panels) were inspected with a confocal laser scanning and an electron microscope. (A) and (B) Autofluorescence of PSVs in dry seeds. kam2-1 seeds have distorted cells. Bars = 5 μm. (C) and (D) Electron micrographs of seed cells. The extracellular space of the kam2-1 seeds was abnormally enlarged and was filled with electron-dense materials (arrows). Bars = 5 μm. (E) and (F) Immunogold analysis of dry seeds with anti-12S globulin. 12S globulin was mis-sorted into the electron-dense extracellular space of the kam2-1 seeds (arrow). cw, cell wall. Bars = 2 μm. (G) and (H) Immunogold analysis of dry seeds with anti-2S albumin. 2S albumin was mis-sorted into the electron-dense extracellular space of the kam2-1 seeds (arrow). Bars = 2 μm.
vesicles were found in \textit{kam2} seeds (Figure 9). We therefore propose that KAM2/GRV2 regulates protein traffic of the Golgi complex to post-Golgi compartments but not protein traffic of the ER to Golgi complex.

Similar phenotypes in vacuolar sorting between \textit{kam2} and \textit{atvsr1} raise the possibility that KAM2/GRV2 interacts with VSR1 for vacuolar targeting of cargo proteins. However, the direct interaction between KAM2/GRV2 and VSR1 is unlikely due to the following four reasons. First, the intracellular localization of VSR1 in a PVC/TGN is different from that of KAM2/GRV2, which is localized in the uncharacterized compartments (Figure 7). Second, the intracellular localization of VSR1 did not change in the \textit{kam2} mutant (data not shown). Third, the morphologies of autofluorescent PSVs of \textit{kam2} seeds differed from those of \textit{atvsr1} seeds: the \textit{kam2} PSVs were deformed and swollen compared with wild-type PSVs (Figure 9B), while the \textit{atvsr1} PSVs are small (Shimada et al., 2003a). Fourth, the ratio of the accumulation level of 2S albumin precursors to 12S globulin precursors in \textit{atvsr1} is higher than that in \textit{kam2} (Figure 8).

**KAM2/GRV2 Regulates the Formation of Endosomal Compartments to Promote Membrane Traffic**

KAM2/GRV2 is a homolog of RME-8 in \textit{Drosophila} (Chang et al., 2004), \textit{Caenorhabditis} (Zhang et al., 2001), and human (Girard et al., 2005). Animal \textit{rme-8} mutants were found to be defective in endocytosis, indicating that RME-8 plays a vital role for endocytosis. We examined the rate of endocytosis with FM4-64 dyes, which are widely used fluorescent markers for the endocytotic pathway. However, we were unable to see a significant difference in the rate of endocytosis between wild-type and \textit{kam2} cells (see Supplemental Figure 3 online). This suggests that KAM2/GRV2 is not essential for efficient endocytosis in plants.

It has been shown that animal \textit{rme-8} mutants have abnormally shaped endosomes (Chang et al., 2004). Similarly, the \textit{kam2} mutant also had the deformed endosomes. \textit{kam2} endosomes form a sheet-like structure on the inner surface of cells (Figures 2 and 4B; see Supplemental Movie 1 online), suggesting that \textit{kam2} mutants have a defect in proper budding of endosomes from other endosomes and/or plasma membranes. The deformed endosomes involved other endomembranes and organelles, resulting in abnormally large aggregates that faced the inner surface of the cells (Figure 3). These results suggest that KAM2/GRV2 functions in formation of endosomal compartments.

Disruption of the formation of endosomal compartments by \textit{kam2} mutations might cause a depletion of the cytosolic or membrane proteins needed for vesicular trafficking, apparently resulting in the blocking of vacuolar trafficking. How does KAM2/GRV2 function in protein trafficking? KAM2/GRV2 has a single DnaJ domain (Figure 5B). The DnaJ domain is known to interact with heat shock protein 70 (Hsp70), which is a molecular chaperone essential for proper folding of substrate proteins. In vitro analyses reported that the DnaJ domains of RME-8 of \textit{Drosophila} (Chang et al., 2004) and human (Girard et al., 2005) interacted with Hsp70 in ADP- and ATP-dependent manners, respectively. DnaJ-containing proteins promote the interaction of substrate proteins with Hsp70 so that they can be properly folded (Miernyk, 2001). These results implied that KAM2/GRV2 is involved in the folding of proteins needed for membrane trafficking.

The \textit{Drosophila} \textit{rme-8} mutant was isolated as an enhancer mutant for the dominant negative form of dynamin that blocked endocytosis (Chang et al., 2004). Dynamin is a large molecular weight GTP binding protein on the cytosolic side of the membrane. Dynamin has been shown to constrict or tabulate membranes and to facilitate the release of vesicles from the membranes of donor organelles (Praefcke and McMahon, 2004). Considering the fact that an \textit{Arabidopsis} dynamin-like protein (ADL6) is involved in the trafficking from TGN to the vacuoles (Jin et al., 2001), it is possible that KAM2/GRV2 controls the function of a dynamin and/or a dynamin-like protein through inducing a conformational change to promote vesicle budding.
Further studies are required to assess the relationships of KAM2/GRV2 with dynamins, dynamin-like proteins, or other trafficking proteins.

KAM2/GRV2 Might Be Involved in Auxin Distribution for Determination of the Growth Axis of the Embryo and in Vacuolar Membrane Dynamics for Gravitropism

We found approximately half of the growing kam2 cotyledons bent in the opposite direction before the walking stick–shaped embryo stage (Figure 11). During embryogenesis, a growing cotyledon properly bends just before the walking stick–shaped embryo stage so that the full embryo can fill the seed space. However, little is known about the mechanism underlying the determination of the growth axis of embryo to bend growing cotyledons in the proper direction during embryogenesis. Our finding suggests that formation of endosomal compartments and vesicular traffic are involved in the proper bending of the cotyledons.

Bending of growing cotyledons is caused by asymmetric growth of the embryo, which might be mediated by asymmetric distribution of auxin. Auxin is transported through the polar localization of efflux carriers (PINs) on the plasma membrane. Asymmetric auxin translocation, which occurs during gravitropism, is correlated with decreased PIN internalization from the plasma membrane (Paciorek et al., 2005). Recycling PINs between the plasma membrane and Golgi complex are conducted by vesicular traffic to produce asymmetric distribution of auxin (Geldner, 2004). These observations suggest that KAM2/GRV2 deficiency leads to disordered vesicular traffic of auxin carriers, possibly resulting in disruption of asymmetric distribution of auxin in the kam2 embryo cells. Nonasymmetric distribution of auxin might make the cotyledons grow into any direction in the kam2 seeds. Consequently, half of the cotyledons might bend into the opposite direction, and the other half might bend normally.

Gravitropism is known to be dependent not only on the auxin distribution but also on amyloplast sedimentation through the transvacuolar strand (Morita and Tasaka, 2004). Amyloplasts in the endodermal cells of the shoot of Arabidopsis gravitropic mutants (sgr2, sgr3, and zig/sgr4) do not sediment according to the gravity vector (Morita et al., 2002; Yano et al., 2003). These

Figure 11. Approximately Half the Number of kam2-1 Embryos Develop in the Opposite Direction before the Walking Stick–Shaped Embryo Stage, Resulting in Abnormal Shapes of Dry Seeds.

(A) to (D) DIC images of developing embryos of the wild type (Col) from heart-shaped embryo stage to full-sized embryo stage. Bars = 100 μm. (E) to (J) DIC images of developing embryos of kam2-1 from the heart-shaped embryo stage to the full-sized embryo stage. All of the kam2-1 embryos normally grew until torpedo-shaped embryo stages ([E] and [F]). Afterward, approximately half the number of embryos grew retrorsely ([G]) and the developing cotyledons grew into the opposite space within the seeds ([H]). The rest of the kam2-1 embryos grew normally ([I] and [J]). Bars = 100 μm. (K) Dry seeds of the wild type. (L) Dry seeds of kam2-1. Approximately half the number of the kam2-1 seeds exhibit abnormal shapes (asterisks).
mutations are thought to primarily affect the vacuolar membrane, causing alterations in vacuolar morphology and dynamics. The grv2 mutant, which is allelic to kam2, has a defect in shoot gravitropism and phototropism (Silady et al., 2004), which may be caused by disorder of the endomembrane structure, especially in vacuolar membranes. To see the development of vacuolar membranes, we expressed GFP-VAM3 transiently in the petiole cells and epidermal cells of 10-d-old cotyledons of kam2 and the wild type. kam2 had no vacuolar strands or bulb-like structures, showing a simpler structure of vacuolar membrane than the wild type (see Supplemental Figures 4C and 4D online). kam2 had small-fluoresced aggregates composed of the vacuolar membrane and/or PVC (see Supplemental Figure 4B online). Our results suggest that KAM2/GRV2 is involved in trafficking not only the vacuolar contents but also vacuolar membrane constituents, which are needed for vacuolar dynamics in gravitropism and phototropism of plants.

Endomembrane Organization

The kam2 aggregates differ from the aggregates of kam1, which was isolated previously (Tamura et al., 2005). The kam2 aggregates contained normal ER bodies (Figure 4), while the kam1 aggregates contained completely deformed ER bodies. kam2 had sheet-like structures of endosomes on the cell surface that was responsible for vesicular trafficking (Figures 3 and 4), which have not been seen in kam1. kam2 had normal organization of actin filaments (see Supplemental Figure 5 online), while kam1 exhibited the disorganization of actin filaments. These differences between the kam1 and kam2 mutants suggest that the maintenance of endomembrane structure is supported by multiple mechanisms. Further isolation of the kam mutants should permit us to clarify the molecular mechanism underlying the maintenance of endomembrane organization in plant cells.

METHODS

Plant Materials and Growth Conditions

We used wild-type plants of Arabidopsis thaliana (ecotype Columbia [Col] and Landsberg erecta) and a transgenic plant of Arabidopsis (ecotype Col) that expresses SP-GFP-2sc, which is composed of a signal peptide and GFP followed by vacuolar targeting signal derived from pumpkin (Cucurbita sp) 2S albumin (Tamura et al., 2003). We designated this transgenic plant GFP-2sc. Growth conditions of the plants were the same as described previously (Tamura et al., 2005). Protoplasts were prepared from Arabidopsis cultured cells that had been subcultured and were incubated in medium containing Murashige and Skoog salts, B5 vitamins, 1% (w/v) sucrose, and 0.4 M mannitol, as described previously (Tamura et al., 2005).

kam2 Mutants

We used M2 lines from GFP-2sc seeds that had been mutagenized previously (Tamura et al., 2005). We examined 5- to 7-d-old seedlings with a fluorescence microscope and selected a mutant line that exhibited abnormal endomembrane structure, and we named the mutants kam, after the Japanese word for aggregate. We isolated two kam2 mutants (kam2-1 and kam2-6). We obtained T-DNA insertion mutants from the ABRC at Ohio State University: salk_078570 (kam2-2), salk_067162 (kam2-3), and salk_132563 (kam2-4).

Immunofluorescent Staining of Fluorescent Marker–Expressed Protoplasts

We transiently transformed protoplasts from Arabidopsis cultured cells with each of the chimeric genes for expression of GFP or mRFP-tagged organelle markers (KAM1-C-mRFP, Ara6-GFP, Ara7-GFP, PEP12-GFP, VAT1-GFP, and SYP41) using polyethylene glycol as described previously (Shimada et al., 2002). The protoplasts were fixed with 3.7% (w/v) paraformaldehyde and 5 mM EGTA in 50 mM phosphate buffer, pH 7.5, for 1 h. The fixed cells were treated with 0.2% (v/v) Triton X-100 for 20 min and then with 2% BSA and 1% sheep serum (Chemicon) in Tris-buffered saline plus 0.05% Tween 20 for 1 h. The cells were incubated for 1 h with anti-KAM2I antibody or preimmune serum (diluted 1:100). After washing with Tris-buffered saline plus 0.05% Tween 20, the cells were incubated for 30 min with goat anti-rabbit IgG (H+L) (1:300; Alexa Fluor 546; Molecular Probes). All antibodies were diluted with Solution B of an immunostaining kit (Can Get Signal Immunostain; Toyobo). The cells were washed and then inspected with a confocal laser scanning microscope and a DIC microscope. Alternatively, we treated protoplasts expressing KAM1-C-mRFP with 100 μg/mL of BFA for 2 h and then stained with anti-KAM2I antibody.

Transient Expression of mRFP-tagged Organelle Markers and FM4-64 Staining

Rosette leaves of 1- to 2-week-old GFP-2sc and kam2-1 plants were transformed with each chimeric gene for transient expression of mRFP-tagged organelle markers (ER-targeted mRFP, Golgi-targeted mRFP, and peroxisome-targeted mRFP) by particle bombardment, as described previously (Tamura et al., 2005). For staining of the plasma membrane and endosomes, 15-d-old seedlings were incubated in Murashige and Skoog medium containing 10 μM FM4-64 for 15 min. Cells were inspected with a confocal laser scanning microscope and a DIC microscope.

Confocal Laser Scanning Microscopy

The fluorescent images were inspected with a confocal laser scanning microscope (model LSM510 META; Carl Zeiss) using the 488-nm line of a 40-mW Ar/Kr laser or the 544-nm line of a 1-mW He/Ne laser with either a ×100 oil immersion objective (1.4 numerical aperture) or a ×40 dry objective (0.75 numerical aperture). Image analysis was performed using LSM image examiner software (Carl Zeiss). The data were exported as 8-bit TIFF files and processed using Adobe Photoshop 5.5 (Adobe Systems).

Map-Based Cloning of KAM2

Map-based cloning of the KAM2 gene was performed essentially as described previously (Tamura et al., 2005). We used codominant cleaved amplified polymorphic sequence markers (Konieczny and Ausubel, 1993) and simple sequence length polymorphism markers (Bell and Ecker, 1994). Twenty recombinants of the F2 progeny were screened for the kam2 phenotype for rough mapping. The position of the KAM2 gene was located in the middle of chromosome 2. For fine-scale mapping, DNA was isolated from 220 plants of the F2 progeny. Nucleotide sequences were determined from both strands using an ABI Prism Big Dye Terminator Cycle Sequence Reaction kit (Applied Biosystems) and a DNA sequencing (model 3100-Avant Genetic Analyzer; Applied Biosystems).

Subcellular and Suborganelar Fractionations

Ten-day-old Arabidopsis seedlings (~3 g fresh weight) were minced on ice in 10 mL of buffer A (100 mM Heps-KOH, pH 7.5, 0.3 M sucrose,
5 mM EGTA, 5 mM MgCl₂, and proteinase inhibitors [Complete; Roche]). The homogenate was filtered through cheesecloth and centrifuged at 2000g for 20 min at 4°C to remove cellular debris. The supernatant was ultracentrifuged at 100,000 g for 1 h at 4°C to obtain a microsomal pellet.

The microsomal pellets were resuspended in 200 μL of each solution of buffer A, high salt buffer (1% [v/v] Triton X-100 plus buffer A), alkaline buffer (0.1 M Na₂CO₃, pH 11, 0.3 M sucrose, 5 mM EGTA, and 5 mM MgCl₂), Triton X-100 buffer (1% [v/v] Triton X-100 plus buffer A), and SDS buffer (1% [v/v] SDS plus buffer A). These suspensions were incubated for 10 min and then ultracentrifuged at 100,000 g for 1 h at 4°C to obtain supernatant and pellet fractions.

Subcellular fractionation of the seedlings was performed with a linear sucrose density gradient (10 to 50% [w/w]) essentially as described previously (Tamura et al., 2005). Centrifugation was performed in an SW28.1 rotor (Beckman) at 27,000 rpm for 13 h at 4°C, and 0.75-mL fractions were collected with a piston gradient fractionator (TOWA LABO).

Antibody Production
We bacterially synthesized two polypeptides that were derived from KAM2: KAM2I (Leu-700 to Gly-1030) and KAM2C (Thr-2282 to Pro-2554). These polypeptides were conjugated with BSA and then injected into a rabbit subcutaneously with complete Freund’s adjuvant. After 3 weeks, two booster injections with incomplete adjuvant were given at 7-d intervals. Two weeks after the booster injections, blood was drawn and the antiserum was prepared. We purified the antiserum and preimmune serum from dry seeds (two grains) of wild-type (Col-0), SW28.1 rotor (Beckman) at 27,000 rpm for 13 h at 4°C, and 0.75-mL fractions were collected with a piston gradient fractionator (TOWA LABO).

SDS-PAGE and Immunoblot Analysis
Protein extracts from dry seeds (two grains) of wild-type (Col-0), GFP-2sc, atvsr1, and kam2 mutants were subjected to SDS-PAGE followed by either Coomassie Brilliant Blue staining or immunoblot analysis. The wild-type siliques were harvested at various stages of seed development, and one-tenth of total proteins from each siliques was subjected to immunoblot analysis. Subcellular fractions from seedlings were subjected to immunoblot analysis. Immunoreactive signals were detected with the ECL detection system (Amersham).

Electron Microscopy
Dry seeds and roots from 7-d-old seedlings of GFP-2sc and kam2-1 were fixed for 2 h with 10% (v/v) dimethyl sulfoxide, 4% (w/v) paraformaldehyde, and 1% (v/v) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. Procedures for electron microscopy were essentially the same as those described previously (Shimada et al., 2003a). The ultrathin sections were examined with a transmission electron microscope (model JEM-1011; JEOL) at 100 kV. Immunochemistry was performed as described previously (Shimada et al., 2003a), except for the dilutions of antibodies anti-12S globulin (1:2000), anti-2S albumin (1:1000), and AuroProbe EM Anti-Rabbit IgG (H-I-L) (1:30, 15 nm gold; Amersham).

Inspection of the Embryo
Maturing seeds from GFP-2sc and kam2-1 were cleared by incubation in Hoyé’s solution overnight (7.5 g gun arabic, 100 g chloral hydrate, and 5 mL glycerol in 30 mL water) and then inspected with a fluorescence microscope (Axioplan 2; Carl Zeiss) equipped with a CCD camera (model DXM1200; Nikon) as described previously (Nakaune et al., 2005). To observe PSVs, dry seeds from GFP-2sc and kam2-1 were pressed in glycerol between the slide glass and cover slip to push out cotyledons. The cotyledons were inspected with a confocal laser scanning microscope (model LSM510 META; Carl Zeiss) using the 488-nm line as described previously (Shimada et al., 2003a).

Accession Numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT2g26890 (KAM2/GRV2) and AT3g52850 (VSR1).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Movie 1. Three-Dimensional Structure of Two Cytoplasm Aggregates That Are Connected within the kam2-1 Cell.

Supplemental Figure 1. Disorganization and Aggregation of Endomembranes in Different kam2 Alleles.

Supplemental Figure 2. Abnormal Shapes of Dry Seeds of Different kam2 Alleles.

Supplemental Figure 3. Similar Uptake of FM4-64 into Protoplasts of the Wild Type (Col) and kam2-2.

Supplemental Figure 4. Abnormal Structures of the Vacular Membrane in kam2 Plants.

Supplemental Figure 5. kam2 Has Normal Organization of Actin Filaments.

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Correction

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An incorrect image was published for Figure 9E. A corrected figure showing immunolocalization of 12S globulin in GFP-2sc seeds (panel 9E) appears below.

![Corrected Figure](data:image/png;base64,iVBORw0KGgoAAAANSUhEUgAAAA4AAABCAQAAABXvqCgAAAAElVEX...)

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Arabidopsis KAM2/GRV2 Is Required for Proper Endosome Formation and Functions in Vacuolar Sorting and Determination of the Embryo Growth Axis
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