KATAMARI1/MURUS3 Is a Novel Golgi Membrane Protein That Is Required for Endomembrane Organization in Arabidopsis

Kentaro Tamura, a Tomoo Shimada, a Maki Kondo, b Mikio Nishimura, b and Ikuko Hara-Nishimura a, 1

a Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
b Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan

In plant cells, unlike animal and yeast cells, endomembrane dynamics appear to depend more on actin filaments than on microtubules. However, the molecular mechanisms of endomembrane–actin filament interactions are unknown. In this study, we isolated and characterized an Arabidopsis thaliana mutant, katamari1 (kam1), which has a defect in the organization of endomembranes and actin filaments. The kam1 plants form abnormally large aggregates that consist of endoplasmic reticulum with actin filaments in the perinuclear region within the cells and are defective in normal cell elongation. Map-based cloning revealed that the KAM1 gene is allelic to the MUR3 gene. We demonstrate that the KAM1/MUR3 protein is a type II membrane protein composed of a short cytosolic N-terminal domain and a transmembrane domain followed by a large luminal domain and is localized specifically on Golgi membranes. We further show that actin filaments interact with Golgi stacks via KAM1/MUR3 to maintain the proper organization of endomembranes. Our results provide functional evidence that KAM1/MUR3 is a novel component of the Golgi-mediated organization of actin functioning in proper endomembrane organization and cell elongation.

INTRODUCTION

The endomembrane system is composed of the endoplasmic reticulum (ER), Golgi stacks, endosomes, and vacuoles in plant cells. Membrane traffic between these endomembranes is important for plant cell division, growth, differentiation, and function during development. The ER is an essential organelle for protein assembly and lipid biosynthesis and acts as the beginning of the secretory pathway (Vitale and Denecke, 1999). The ER usually consists of a polygonal meshwork of membrane tubules and variously shaped sheet-like cisternae (Grabski et al., 1993; Boevink et al., 1996). These structures are interconnected with one another and with the continuous membrane of the outer nuclear envelope (Herman et al., 1990; Boevink et al., 1996). Unlike mammalian cells, in which Golgi stacks are practically immobile and condensed in a limited perinuclear region, plant cells contain a large number of Golgi stacks throughout the cytoplasm. Green fluorescent protein (GFP) fusions have allowed the visualization of the Golgi stacks in vivo and revealed that each stack is highly mobile, moving around the ER and actin filaments driven by myosin motors (Boevink et al., 1998; Nebenfuhr et al., 1999). Vacuoles, which are the most prominent compartment in the plant cell, also have a dynamic and complex membrane structure. Studies using a vacuolar membrane–targeted GFP found that spherical structures (bulbs) consisting of a double membrane were often observed within the lumen of vacuoles and were connected with the vacuolar membrane (Saito et al., 2002; Uemura et al., 2002). These bulbs were observed to move around within or along the outline of the membrane, mediated by the actin filaments but not by the microtubules (Uemura et al., 2002).

The structural and functional maintenance of endomembranes is also important for various aspects of plant development and signal transduction (Surpin and Raikhel, 2004). The vacuoleless (vcl1) mutant of Arabidopsis thaliana, which has no obvious vacuoles, does not survive beyond the torpedo stage of embryonic development (Rojo et al., 2001). Because Arabidopsis VCL1 is a homolog of yeast Vacular Protein-Sorting 16, which regulates the homotypic fusion of vacuoles and the docking of vesicles (Sato et al., 2000), the vcl1 mutant may have defects in the very early stages of vacuole biogenesis. Some shoot gravitropism (sgr) mutants of Arabidopsis have abnormal vacuolar and vesicular structures in several tissues, including the endodermis, in which gravity is sensed by sedimentable amyloplasts (Kato et al., 2002; Morita et al., 2002; Yano et al., 2003). Two SGR genes encode protein components of vesicle trafficking, and another SGR gene encodes a protein that regulates vacuolar membrane structure.

Two cytoskeleton systems (i.e., actin filaments and microtubules) are indispensable for the intracellular positioning and dynamic movement of organelles. In plant cells, the dynamics of endomembranes appear to depend more on actin filaments than on microtubules. Thus, it is believed that plant cells develop unique mechanisms for endomembrane organization that depend on actin filaments (Boevink et al., 1998; Brandizzi et al., 2002). To elucidate the molecular mechanisms that regulate endomembrane organization in plants, we screened Arabidopsis seedlings for mutants that have a defect in endomembrane organization.
organization. Here, we report the isolation and characterization of a mutant (termed katamari1 [kat1]) in which the endomembranes formed large aggregates. We provide evidence that KAM1 mediates the actin organization that contributes to endomembrane organization and cell elongation.

RESULTS

The kam1 Mutant, Which Has a Defect in Endomembrane Organization, Includes Abnormal Aggregates of Various Organelles in the Perinuclear Region of the Cells

To isolate Arabidopsis mutants that have an abnormal endomembrane structure within the cells, we used a transgenic Arabidopsis, GFP-2sc, which expresses vacuole-targeted GFP-2SC. Previously, we reported the light-dependent disappearance of GFP fluorescence in the vacuoles (Tamura et al., 2003). Light-grown GFP-2sc does not exhibit fluoresced vacuoles but maintains GFP fluorescence in entire endomembranes, including ER network structures and dot-like structures of the Golgi complex (Tamura et al., 2003) (Figures 1A to 1G; see Supplemental Video 1 online). In this study, all fluorescent images were taken from light-grown plants.

GFP-2sc seeds were mutagenized, and 669 M2 lines were obtained. Of ~12,000 M2 seedlings examined with the fluorescence microscope, we isolated a mutant whose endomembrane formed large aggregates and designated the mutant kam1 (for katamari1), after the Japanese word for aggregate. The aggregates (~10 μm) were found in most of the cells of cotyledons (Figure 1H), hypocotyls (Figure 1I), and flower stalks (Figure 1J); they were also found in some cells of siliques (Figure 1K), roots (Figure 1L), root hairs (Figure 1M), and trichomes (Figure 1N). A higher magnification of a fluorescent image showed that the aggregates were composed of many small particles (Figure 1H, inset). Small aggregates of endomembranes that moved on the ER network were also observed on the surface of leaf cells (see Supplemental Video 2 online). These observations imply that the endomembranes are fragmented and/or deformed.

To identify the components of the aggregates, we visualized various organelles in leaf epidermal cells from 15-d-old seedlings

Figure 1. Disorganization and Aggregation of Endomembranes in Different Organs of the Isolated Arabidopsis Mutant kam1.
of GFP-2sc (Figures 2A to 2E) and kam1-1 (Figures 2F to 2J) by transient expression of monomeric red fluorescent protein (mRFP)-tagged marker proteins (Campbell et al., 2002), FM4-64 staining, and 4′,6-diamidino-2-phenylindole (DAPI) staining. Most of the RFP-fluorescent ERs were completely merged with the GFP-fluorescent aggregates, causing the aggregates to become yellow fluorescent (Figure 2F). Some of the RFP-fluorescent Golgi stacks were engulfed in the aggregates (Figure 2G). Some of the RFP-fluorescent peroxisomes were detected as red fluorescent particles in the aggregates (Figure 2H). Most of the FM4-64–stained endosomes were detected as red fluorescent small particles and their assembly, which were found in the green fluorescent aggregates (Figure 2I). These results suggested that the ER is specifically aggregated, whereas the Golgi, peroxisomes, and endosomes are nonspecifically engulfed in the aggregates in kam1 cells. DAPI-stained nuclei were close to the GFP-fluorescent aggregates (Figure 2J), suggesting that the aggregates are located in the perinuclear region.

Electron microscopic analysis showed that the epidermal cells of wild-type roots were occupied by a large central vacuole surrounded by a cytosol (Figure 3A). On the contrary, epidermal cells of kam1-1 roots had a cytoplasmic aggregate abnormally at the center of the cell (Figure 3B). Higher magnification (Figure 3C) revealed that the cytoplasmic aggregate contained many fragmented vacuoles and a nucleus. These results suggest that the aggregates, which are composed of various organelles, including nuclei, are found in cells of different organs in kam1-1.

**KAM1 Is Allelic to MUR3, Encoding a Protein That Has an Exostosin-Like Domain**

To identify the KAM1 gene, we used a map-based cloning approach with codominant cleaved amplified polymorphic sequence markers (Konieczny and Ausubel, 1993) and simple sequence length polymorphism markers (Bell and Ecker, 1994) and found that KAM1 is located on chromosome 2 between markers PHYB and m421. Further mapping using 450 F2 plants that exhibited the phenotype conferred by kam1 and DNA sequencing showed a single base pair mutation from C to T in the At2g20370 gene of kam1-1, which might cause a nonsense mutation from CAA (Gln-62) to TAA (stop codon) (Figure 4A).

To confirm that the mutation in the At2g20370 gene is responsible for the phenotype conferred by kam1, we analyzed knockout mutants of the At2g20370 gene from the Salk T-DNA insertion line collections. We isolated two lines in which T-DNA was inserted into the At2g20370 coding region and named these mutants (salk_127057 and salk_141953) kam1-2 and kam1-3, respectively (Figure 4A). To visualize the endomembranes, a vacuole-targeted GFP, SP-GFP-2SC, was transiently expressed in epidermal cells of rosette leaves of each kam1 allele by particle bombardment. Figure 4B (top row) shows the aggregates of endomembranes in kam1-2 and kam1-3 cells, as seen in the kam1-1 cells. In addition, we confirmed that constitutive expression of KAM1-mRFP (see Figure 6B) rescued the phenotype conferred by kam1 (data not shown). These results indicate that KAM1 is the At2g20370 gene.

The KAM1 gene encodes a polypeptide sequence of 619 amino acids with a single putative transmembrane domain (Figure 4A).
Immunoblot analysis with the anti-KAM1LD antibody showed that the 84-kD protein was accumulated in GFP-2sc but not in kam1-1, kam1-2, or kam1-3 (Figure 4C). KAM1 had an exostosin-like domain (Solomon, 1964) (Figure 4A, green box). Exostosins act as glycosyltransferases, which catalyze the formation of heparan sulfate, an extracellular glycosaminoglycan in animal cells (Esko and Selleck, 2002). The Arabidopsis mutants mur3-1 and mur3-2, which have a missense mutation in the At2g20370 gene, lacked glycosyltransferase activity (Madson et al., 2003). To determine whether a deficiency of glycosyltransferase activity in KAM1 is responsible for the defect in endomembrane organization in kam1, we examined endomembrane organization in mur3-1 and mur3-2. The 84-kD immunopositive protein was accumulated in mur3-1 and mur3-2 as in GFP-2sc (Figure 4C). We expressed the vacuole-targeted GFP in mur3-1 and mur3-2 leaves and inspected the GFP-fluorescent endomembranes. Both mutants exhibited normal endomembrane organization in the cells, like wild-type plants (Figure 4B, bottom row). This result indicates that glycosyltransferase activity is not involved in the organization of the endomembranes.

![Figure 4. Analysis of kam1 Mutant Alleles.](image)

(A) Structure of the KAM1/MUR3 gene, which is an intronless gene. The site of each mutation of kam1-1, mur3-1, and mur3-2 is indicated. The site of each T-DNA insertion of kam1-2 and kam1-3 is indicated as well. The yellow box indicates the coding region for a putative transmembrane domain, and the green box indicates the coding region for an exostosin-like domain. (B) Structure of endomembranes in each kam1 allele. To visualize endomembrane structures, a chimeric gene encoding SP-GFP-2SC, a vacuole-targeted GFP, was transiently expressed after particle bombardment of rosette leaf epidermal cells of each kam1 allele. Arrowheads indicate the aggregates of endomembrane. Bars = 10 μm. (C) Immunoblot analysis of kam1 alleles. The same protein amounts of membrane fraction isolated from each kam1 allele were subjected to immunoblot analysis with anti-KAM1LD. Molecular masses are given at right.
KAM1/MUR3 Is a Type II Integral Membrane Protein That Localizes on the Golgi Stacks

A hydropathy plot of KAM1/MUR3 (Figure 5A) suggests that a transmembrane domain (bar) is located in the N-terminal region. KAM1/MUR3 was extracted from microsomes of wild-type seedlings by detergent (Triton X-100) but not by high salt (NaCl) or alkaline pH (pH 11) (Figure 5B). This profile was the same as that of an integral membrane protein, AtVSR (for *Arabidopsis thaliana* vacuolar sorting receptor) (Shimada et al., 2003). This result indicates that KAM1/MUR3 is an integral membrane protein.

To determine the membrane topology of KAM1/MUR3, we used transgenic Arabidopsis plants, which overexpress the KAM1-mRFP fusion in the *kam1-1* background. The phenotypes conferred by *kam1* were fully complemented in the transgenic plants, suggesting that KAM1-mRFP has activity for endomembrane organization, like endogenous KAM1/MUR3. Microsomes isolated from the transgenic plants were incubated with proteinase K either in the presence or the absence of detergent. Subsequently, the sensitivity of KAM1/MUR3 was evaluated by immunoblot analysis with anti-KAM1LD antibody, which was raised against the C-terminal domain of KAM1/MUR3. KAM1-mRFP became sensitive to protease digestion only when the microsomes were solubilized with detergent (Figure 5C, lane 4), suggesting that the epitope is exposed to the lumen. These results, together with the hydropathy analysis, indicate that KAM1/MUR3 has a type II topology, with a short N terminus exposed to the cytosol, a single transmembrane-spanning domain, and a C terminus inside the lumen.

In sucrose density gradient fractions of a homogenate of Arabidopsis seedlings, KAM1/MUR3 cosedimented with a Golgi marker, RGP1 (Dhugga et al., 1997), but not with a pre-vacuolar compartment/trans-Golgi network marker, AtVSR, or an ER marker, BiP (Figure 6A). This finding suggests that KAM1/MUR3 is localized on the Golgi stack. To visualize the intracellular localization of KAM1/MUR3, we constructed chimeric genes encoding modified mRFPs. KAM1-mRFP was composed of the full length of KAM1/MUR3 followed by mRFP, whereas KAM1ΔC-mRFP was composed of the C-terminal-truncated form of KAM1/MUR3 (120 amino acids) followed by mRFP (Figure 6B). Each fusion protein was transiently expressed in protoplasts prepared from Arabidopsis suspension-cultured cells. These modified mRFPs were completely merged with a GFP-fluorescent Golgi marker (GFP-AVP2) (Mitsuda et al., 2001) (Figure 6C). These results indicate that KAM1/MUR3 localizes on the Golgi stacks and that its N-terminal portion (120 amino acids) is sufficient for Golgi targeting.

KAM1/MUR3 Interacts with Actin, Whose Filaments Are Necessary for the Proper Endomembrane Organization in Cells

It is well known that the cytoskeleton is involved in the intracellular positioning and movement of organelles. To investigate how the cytoskeleton contributes to the maintenance of endomembranes, we examined the effect of cytoskeleton-disordering reagents on the endomembranes of GFP-2sc seedlings. Latrunculin B, which depolymerizes actin filaments, disordered the endomembranes in epidermal cells of cotyledons (Figures 7A and 7B). The network structure of the cortical ER was completely

Figure 5. KAM1/MUR3 Is a Type II Membrane Protein.
(A) Hydropathy plot of KAM1/MUR3 as calculated according to Kyte and Doolittle (1982). The hydrophobic region is shown as a gray bar.
(B) Microsomal fractions from wild-type seedlings were resuspended in control buffer (control; 100 mM Hepes-KOH, pH 7.5), high-salt buffer (NaCl; 1 M NaCl and 100 mM Hepes-KOH, pH 7.5), alkaline buffer (pH 11; Na2CO3, pH 11), and Triton X-100 buffer (Triton X; 1% [v/v] Triton X-100 and 100 mM Hepes-KOH, pH 7.5). These suspensions were ultracentrifuged to obtain supernatant (S) and pellet (P) fractions. Each fraction was subjected to immunoblot analysis with anti-KAM1LD and anti-AtVSR antibodies. Molecular masses are given at right.
(C) Microsomal fractions from transgenic Arabidopsis seedlings, which express the KAM1-mRFP fusion, were incubated in the presence (+) or absence (−) of Triton X-100 and proteinase K and then subjected to immunoblot analysis with anti-KAM1LD antibody. KAM1-mRFP, full-length KAM1-mRFP; KAM1-mRFP*, truncated form of KAM1-mRFP. Dots indicate degraded products or nonspecific bands. Molecular masses are given at right.
destroyed, and blobs of the endomembranes were formed on the surface of latrunculin B–treated cells (Figure 7A). Aggregates of endomembranes were found in the perinuclear region of the treated cells (Figure 7B), as in kam1 cells. On the contrary, neither of two microtubule-disordering reagents (nocodazole and colchicine) had any effect on the structure of the endomembranes (Figures 7D and 7E). We also found that the latrunculin B–induced aggregates were perfectly colocalized with the mRFP-tagged ER marker (Figure 7C). These results suggest that actin filaments, but not microtubules, are necessary for the maintenance of endomembrane structures.

Treatment with the actin-depolymerizing reagent caused GFP-2sc cells to develop a kam1-like phenotype. This implies that actin is disorganized in the phenotype conferred by kam1. Staining with phalloidin revealed that some of actin filaments coaggregated with the endomembranes in kam1-1 cotyledon cells (Figure 8A, right panels, arrows), whereas no aggregates of actin were found in GFP-2sc cells (Figure 8A, left panels). To confirm the organization of actin filaments in living cells, we transiently expressed the ABD2-GFP construct, which encodes an actin binding domain of fimbrin protein followed by GFP (Wang et al., 2004), in the wild type (Figure 8B, left panels) and kam1-2, which has no GFP (Figure 8B, right panels). The actin filaments were aggregated and the cortical network partially disappeared in kam1-2. However, a few intact actin filaments were still found in kam1 cells (Figures 8A and 8B, right panels),

Figure 6. KAM1/MUR3 Localizes on the Golgi Stacks.
(A) Microsomal fractions from Arabidopsis seedlings were further fractionated on a sucrose density gradient (10 to 50%, w/w). The fractions obtained were numbered from top to bottom of the gradient. Each fraction was subjected to immunoblot analysis with anti-KAM1LD and specific antibodies for various organelle markers: anti-RGP1 (a Golgi marker), anti-AtVSR (a trans-Golgi network and prevacuolar compartment marker), and anti-BiP (an ER marker). The sucrose concentration of each fraction measured is given at top.
(B) Two mRFP-tagged KAM1/MUR3 fusion proteins. mRFP was fused to full-length KAM1/MUR3 (619 amino acids) and the C-terminal truncated form of KAM1/MUR3 (120 amino acids). The fusion proteins were designated KAM1-mRFP and KAM1ΔC-mRFP, respectively. Yellow boxes indicate a putative transmembrane domain of KAM1/MUR3.
(C) A Golgi-localized GFP fusion (GFP-AVP2) was coexpressed with KAM1-mRFP (top row) and KAM1ΔC-mRFP (bottom row) transiently in protoplasts of Arabidopsis cultured cells. The cells were inspected with a confocal laser scanning microscope and a differential interference contrast (DIC) microscope. Bars = 5 μm.
suggestions that the mutant phenotype is not completely equivalent to the result of actin depolymerization.

These results raise the possibility that KAM1/MUR3 interacts with actin and regulates the proper distribution of actin filaments in wild-type plants. To investigate this possibility, we immuno-precipitated detergent-solubilized extracts from Arabidopsis cultured cells with both anti-KAM1LD and anti-KAM1CT antibodies. The immunoprecipitates were subjected to immunoblot analysis with anti-KAM1LD or anti-actin antibodies. The results clearly show that KAM1/MUR3 coimmunoprecipitated with actin (Figure 8C). On the contrary, actin did not precipitate in the control experiments with each preimmune serum (Figure 8C) or anti-BiP and anti-AtVSR1 antibodies (see Supplemental Figure 1 online). This result suggests that KAM1/MUR3 interacts with actin within the cells.

**kam1** Has a Defect in Cell Elongation

*kam1-1* showed a dwarf phenotype at different developmental stages (Figure 9). The *kam1-1* seedlings were significantly smaller than the GFP-2sc seedlings (Figure 9A). The *kam1-1* plants had wrinkled leaves and short petioles (Figure 9B) and exhibited dwarf stature (Figure 9C). When grown in the dark, the length of the *kam1-1* hypocotyl was only one-third that of the GFP-2sc hypocotyl (Figure 9D). These phenotypes were also observed in *kam1-2* and *kam1-3* plants but not in *mur3-1* or *mur3-2* plants (data not shown). The *kam1-1* cells in hypocotyls were significantly smaller and fatter than GFP-2sc cells and exhibited abnormal shape (Figures 9E and 9F). On the contrary, there were no differences in the number of epidermal cells of hypocotyls between GFP-2sc and *kam1* (data not shown). Therefore, the dwarf stature of *kam1* is largely or exclusively attributable to a failure of individual cells to elongate rather than to a defect in cell division.

**DISCUSSION**

Interaction of KAM1/MUR3 and Actin Is Responsible for Proper Endomembrane Organization

The isolated *kam1* mutant showed abnormal actin distribution (Figures 8A and 8B). Several groups have reported that normal organization of the actin cytoskeleton is essential for cell morphogenesis and elongation in a variety of plant cell types and organs (Staiger, 2000; Baluska et al., 2001a; Dong et al., 2001; Chen et al., 2002; Ringli et al., 2002; Li et al., 2003; Mathur et al., 2003; Nishimura et al., 2003). Actin-interfering drugs, which perturb actin filaments, reduce the cell elongation of various organs, including hypocotyls and roots, and cause a dwarf stature (Baluska et al., 2001b). These phenotypes are similar to the phenotype conferred by *kam1*, which is characterized by a defect in the elongation of hypocotyl cells (Figures 9E and 9F) and dwarf stature (Figures 9A, 9C, and 9D). This fact suggests that the phenotype conferred by *kam1* is closely associated with the altered actin organization.

Many reports have also shown that the actin filaments contribute to the structural maintenance of endomembranes in plant cells. Depolymerization of actin filaments but not microtubules led to the inhibition of Golgi movement and the clustering of Golgi stacks on small islands of lamellar ER in tobacco leaves (Boevink et al., 1998; Brandizzi et al., 2002) and to the disassembly of the structures of ER tubules in Arabidopsis hypocotyls (Zheng et al., 2004). In the Arabidopsis *rh3* mutant, actin organization is altered but cortical microtubule organization is not (Hu et al., 2003). The *rh3* mutant is characterized by disorganization of the ER and Golgi stacks (Zheng et al., 2004), an abnormal distribution of vesicles in the subapical region, and an altered vacuolar organization in the root hairs (Galway et al., 1997). The Arabidopsis *crooked* mutant, in which the structure of actin filaments is altered, has an abnormal accumulation of some Golgi stacks at certain regions in trichomes (Mathur et al., 2003).

Although many studies have examined actin organization, no factor has yet been identified that involves the interaction between the endomembranes and actin filaments. *kam1*, which exhibits the same subcellular phenotypes described above, forms endomembrane aggregates within the cells (Figures 1 to 3). The endomembrane aggregates might be caused by the formation of aggregates of actin filaments in *kam1*. We suggest that KAM1/MUR3 plays an essential role in proper actin organization, which is known to be important for endomembrane organization and cell elongation. Although the structures of endomembranes were largely destroyed in *kam1* cells, vacuolar proteins, which include storage proteins in seeds and vacuolar proteases in vegetative tissues, were accurately transported to vacuoles and processed into their mature forms (data not shown). This finding suggests that KAM1/MUR3 is
not involved in the trafficking of proteins from the ER to the vacuoles.

KAM1/MUR3 Is a Key Component in the Golgi-Mediated Organization of Actin in Plant Cells

Two features of plant Golgi stacks that are different from those of animal and yeast are their mobility along the actin filaments and their wide distribution throughout the cytoplasm (Boevink et al., 1998; Saint-Jore et al., 2002). Therefore, it has been proposed that plant cells have novel mechanisms for the secretory pathway (Nebenfuhr and Staehelin, 2001; Neumann et al., 2003). For example, in tobacco cells, Golgi stacks move to specific sites on the surface of the ER to pick up secretory proteins (daSilva et al., 2004). This function has not been observed in yeast and animal cells.

Our findings provide another reason why plant Golgi stacks are mobile along actin filaments and distributed throughout the cytoplasm. It is possible that the plant Golgi stacks serve not only as complex carbohydrate factories and as sorting stations for the processed molecules but also as an organizer of actin filaments that run throughout cells. The finding that KAM1/MUR3 is localized on the Golgi membrane (Figures 5 and 6) implies that KAM1/MUR3 may play a role in the organization of actin filaments in plant cells.

Figure 8. KAM1/MUR3 Interacts with Actin.

(A) Actin filaments in cotyledon cells of GFP-2sc (left panels) and kam1-1 (right panels) were stained with Alexa Fluor 546 phalloidin. They were inspected with a confocal laser scanning microscope. Actin filaments coaggregated with GFP-fluoresced endomembranes in kam1-1. Arrows indicate aggregates of endomembranes and actin filaments. Bars = 5 μm.

(B) Actin filaments in stem cells of the wild type (left panels) and kam1-2 (right panels) were visualized by transient expression of the ABD2-GFP construct, which encodes an actin binding domain of fimbrin protein followed by GFP. They were inspected with a confocal laser scanning microscope. The top panels show images of a single confocal section. The bottom panels show the three-dimensional structures of actin filaments that were reconstituted with sequential confocal images taken along the optical z axis throughout the cells. Arrows indicate aggregates of actin filaments. Bars = 10 μm.

(C) Detergent-solubilized extracts from Arabidopsis cultured cells were immunoprecipitated with anti-KAM1LD or anti-KAM1CT antibody (IP). The immunoprecipitates were subjected to immunoblot analysis with either anti-KAM1LD or anti-actin antibody. KAM1/MUR3 was coimmunoprecipitated with actin protein (IB). As negative controls, preimmune sera for each anti-KAM1 antibody were also used for the immunoprecipitation. Molecular masses are given at right.
that plant cells have a Golgi-mediated mechanism for actin organization. Although there is no evidence that actin is linked to the function of the Golgi stacks in plant cells, in mammal cells, actin binding proteins and actin regulatory proteins have been found on the Golgi membrane (Godi et al., 1998; Fucini et al., 2002; Stamnes, 2002). The overall results suggest that KAM1/MUR3 is a key component for the Golgi-mediated organization of actin in plant cells.

KAM1/MUR3 Is a Dual-Function Protein Responsible for Actin Organization and the Synthesis of Cell Wall Materials

Although KAM1/MUR3 interacts with actin (Figure 8C), how it interacts is unclear. We showed that the N-terminal domain of KAM1/MUR3 was exposed in the cytosol (Figure 5); therefore, it is possible that KAM1/MUR3 interacts with actin via the cytosolic N-terminal domain. However, the N-terminal domain has no known motifs for actin binding. An in vitro binding assay showed that the domain did not interact directly with actin (data not shown). These observations suggest that the interaction between actin and the N-terminal domain of KAM1/MUR3 requires other unidentified cytosolic factors. Profilin, which is an actin-polymerizing protein that prevents monomeric actin from forming abnormal assemblies (Baluska et al., 2001b), is a candidate for a cytosolic factor that interacts with KAM1/MUR3 on the Golgi membrane. It was reported that plant Golgi stacks move along actin filaments on the ER network using myosin motors (Boevink et al., 1998; Nebenfuhr et al., 1999). We observed that Golgi stacks were mobile along the ER network not only in wild-type leaf cells (see Supplemental Video 1 online) but also in kam1-1 cells (see Supplemental Video 2 online). This finding suggests that KAM1/MUR3 is not involved in the Golgi stack movement driven by myosin motors and that the interaction between KAM1/MUR3 and actin filaments is independent of myosin.

On the other hand, the lumenal C-terminal domain of KAM1/MUR3 has an exostosin-like domain, which contributes to the activity of xyloglucan galactosyltransferase, an enzyme involved in the biosynthesis of the cell wall. Both the mur3-1 and mur3-2 mutants, each of which has a mutation in the exostosin-like domain (Madson et al., 2003), have a defect in xyloglucan galactosyltransferase activity but show normal endomembrane organization (Figure 4B). Therefore, the endomembrane organization does not require xyloglucan galactosyltransferase activity. We suggest that KAM1/MUR3 is a dual-function protein that is responsible for actin organization and the synthesis of cell wall materials. KAM1/MUR3 could interact in some way between the cytoskeleton organization and the biogenesis of the cell wall, because proper actin organization is required for the secretion of polysaccharides and enzymes from the Golgi stacks to the cell wall (Blancaflor, 2002; Hu et al., 2003).

Transgenic Arabidopsis GFP-2sc Plants Are an Ideal Tool for Studying Endomembrane Organization

Several groups have used GFP-based screens to isolate Arabidopsis mutants that have abnormally shaped organelles, including mitochondria (Logan et al., 2003), peroxisomes (Mano et al., 2004), vacuolar membranes (Avila et al., 2003), and ER bodies (Matsushima et al., 2003, 2004). Thus, GFP screening is a useful method for studying organelle morphology and biogenesis. However, a genetic approach to investigate the entire endomembrane system has been prevented by the lack of a simple phenotypic trait that can be used as a visual marker for the entire endomembrane system. Previously, we demonstrated that vacuole-targeted GFP (SP-GFP-2SC) can act as a visual marker for monitoring the dynamics of the entire endomembrane system in Arabidopsis cells (Tamura et al., 2003). By screening mutagenized GFP-2sc plants, we have identified several mutants showing abnormal endomembrane organization, which is different from the phenotype conferred by kam1. A phenotypic analysis of these mutants and identification of the mutated gene responsible for the abnormal phenotype should help to identify the mechanisms that control endomembrane organization, which, in turn, controls fundamental aspects of plant development.

Figure 9. kam1 Plants Exhibit a Dwarf Phenotype.
Morphological comparison at various developmental stages between GFP-2sc and kam1-1 plants.
(A) Seven-day-old seedlings. Bar = 1 cm.
(B) Four-week-old plants. Bar = 1.5 cm.
(C) Six-week-old plants. Bar = 3 cm.
(D) Five-day-old seedlings grown in continuous dark. Bar = 1 cm.
(E) and (F) Hypocotyl cells of 5-d-old seedlings of GFP-2sc (E) and kam1-1 (F) were examined with a scanning electron microscope. Bars = 100 μm.
METHODS

Plant Materials and Growth Conditions

We used wild-type plants of Arabidopsis thaliana (ecotypes Columbia and Landsberg erecta) and a transgenic plant of Arabidopsis (ecotype Columbia) that expresses SP-GFP-2SC, which is composed of a signal peptide (SP) from pumpkin (Cucurbita pepo) 2S albumin followed by GFP and the vacuole-targeting signal of the C-terminal 18-amino acid sequence from pumpkin 2S albumin (Tamura et al., 2003). We designated this transgenic plant GFP-2sc. We also used the Arabidopsis mutants mur3-1 and mur3-2 (Madson et al., 2003). Information about T-DNA insertion mutants, salk_127057 (kam1-2) and salk_141953 (kam1-3), was obtained from the Salk Institute Genomic Analysis Laboratory website (http://signal.salk.edu). All seeds were provided by the ABRC at Ohio State University. Surface-sterilized seeds were sown onto 0.5% Gellan Gum (Wako, Osaka, Japan) with MS medium (Wako) supplemented with B5 vitamins and 1% sucrose and were grown at 22°C under continuous light. Protoplasts were prepared from Arabidopsis cultured cells, which had been subcultured, and were incubated in medium containing MS salts, B5 vitamins, 1% (w/v) sucrose, and 0.4 M mannitol as described previously (Tamura et al., 2003).

Plasmid Construction

The mRFP1 gene was kindly provided by R.Y. Tsien of Howard Hughes Medical Institute (Campbell et al., 2002). We amplified mRFP with a specific primer harboring a XhoI site and a specific primer harboring a SacI site by PCR to produce pmRFP. The amplified DNA was inserted into the XhoI-Sacl site of pBI221 (Clontech, Palo Alto, CA) to generate mRFP/pBI221. Based on pmRFP/pBI221, we constructed a chimeric gene encoding each of two modified mRFPs as follows.

The cDNAs of KAM1/MUR3 and KAM1ΔC were amplified by PCR from a cDNA library prepared from Arabidopsis roots using the primer pairs 5'-CGCTAGAATGTGTTCCAAGGTTCGTATGA-3' and 5'-GGCGCCGGTGGAGTGGCGGCCCTC-3' for KAM1/MUR3 and 5'-CGCTAGAATGTGTTCCAAGGTTCGTATGA-3' and 5'-CGTCGAGCAATGGTGCGGTAGGCGTA-3' for KAM1ΔC. The amplified fragments were inserted into the XbaI-XhoI site of mRFP/pBI221. These chimeric genes encode the full length of KAM1/MUR3 followed by mRFP1 (KAM1-mRFP) and the N-terminal 119-amino acid sequence of KAM1/MUR3 followed by mRFP1 (KAM1ΔC-mRFP).

The chimeric gene encoding SP-mRFP-HDEL was produced by three rounds of PCR amplification as follows. First, the DNA fragment for a SP was amplified using SP-GFP (Mitsushashi et al., 2000) as a template and a set of oligonucleotide primers, 5'-CTCGAGATGGCCAGACTCACAAGCATCATT-3' and 5'-CATGCGGGCGGTGCGGTTACGGA-3'. Second, the SP-mRFP-HDEL DNA fragment was amplified using pmRFP as a template and a set of oligonucleotide primers, 5'-ATGCGCAGCATGCCGCAATTGGACGCGTGCACTT-3' and 5'-AGATCTCAGCAGCCGCAATTGGACGCGTGCACTT-3'. Third, the SP-mRFP-HDEL DNA fragment was amplified using both PCR fragments as templates and a set of oligonucleotide primers, 5'-ATGCGCAGCATGCCGCAATTGGACGCGTGCACTT-3' and 5'-AGATCTCAGCAGCCGCAATTGGACGCGTGCACTT-3'. The amplified fragment SP-mRFP-HDEL was inserted into the XhoI-BglII site of SP-GFP-2SC/pBI221 (Tamura et al., 2003). The chimeric gene encodes a SP and mRFP followed by a 12-amino acid sequence including an ER-retention signal, HDEL.

The DNA fragment for mRFP-peroxisome-targeting signal 1 (PTS1) was produced by PCR amplification using pmRFP as a template and the primer pair 5'-GCTCGAGATGGCCAGACTCACAAGCATCATT-3' and 5'-AGATCTCAGCAGCCGCAATTGGACGCGTGCACTT-3'. This amplified fragment was inserted into the XhoI-Sacl site of pBI221. This chimeric gene encodes mRFP followed by a 12-amino acid sequence including PTS1 (Mano et al., 2002).

Transient and Stable Expression in Arabidopsis

Protoplasts from Arabidopsis cultured cells were transformed with each of the chimeric genes using polyethylene glycol as described previously (Shimada et al., 2002). Arabidopsis plants (1 to 3 weeks old) were transformed with each chimeric gene by particle bombardment as described previously (Matsushima et al., 2004). To generate transgenic Arabidopsis plants, we transformed kam1-1 with a chimeric gene encoding KAM1-mRFP by the in planta method (Bechtold and Pelletier, 1998).

Isolation of the kam1 Mutant

GFP-2sc seeds were mutagenized by soaking them for 16 h in 0.2 or 0.25% (v/v) methanesulfonic acid ethyl ester (Sigma-Aldrich, Tokyo, Japan) and then washed for 11 h in running water to obtain M1 seeds. The M1 seeds were grown after self-fertilization, and the M2 seeds were collected from individual M1 plants to generate the M2 lines (669 lines). Thirty seed grains from each M2 line were grown to obtain 5- to 7-d-old seedlings. We examined each seedling with a fluorescence microscope and selected a mutant line that exhibited abnormal endomembrane structure. We named the mutant katamar1-1 (kam1-1).

Map-Based Cloning of the KAM1 Gene

The kam1-1 homozygous mutant (ecotype Columbia) was crossed with Landsberg erecta wild-type plants to generate a mapping population. In the F2 generation, the kam1-1 mutants were selected and DNA was isolated from the leaf tissues. The polymorphism between Columbia and Landsberg erecta was analyzed using a combination of cleaved amplified polymorphic sequence and simple sequence length polymorphism markers (Konieczny and Ausubel, 1993; Bell and Ecker, 1994) with data obtained from The Arabidopsis Information Resource (http://www.arabidopsis.org). Approximately 20 recombinant plants of the F2 progeny were screened for the kam1 phenotype for rough mapping. The position of the KAM1 gene was located in the middle of chromosome 2. For fine-scale mapping, DNA was isolated from 450 plants of the F2 progeny. Nucleotide sequences were determined from both strands using the ABI Prism Big Dye Terminator cycle sequence reaction kit (Applied Biosystems, Foster City, CA) and a DNA sequencer (Prism 3100; Applied Biosystems).

Specific Antibodies and Immunoblot Analysis

Two polypeptides derived from KAM1/MUR3, Ser-57 to Gln-619 (KAM1LD: lumenal domain) and Arg-542 to Gln-619 (KAM1CT: C-terminal domain), were bacterially produced and injected into an each 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 antibodies were prepared.

Immunoblot analysis was performed essentially as described previously (Shimada et al., 2002), except that the dilutions of the antibodies were as follows: anti-actin (1:1,000; ICN, Aurora, OH), anti-AELP against Arabidopsis VSR1 (1:1,000) (Shimada et al., 2003), anti-BIP (1:4,000) (Hatano et al., 1997), anti-KAM1LD (1:500), anti-KAM1CT (1:500), and anti-RGP1 (Dhugga et al., 1997).

Suborganellar and Subcellular Fractionation

Ten-day-old Arabidopsis seedlings (~3 g fresh weight) were minced on ice in 10 mL of buffer A (100 mM Hepes-KOH, pH 7.5, 0.3 M sucrose, 5 mM...
EGTA, 5 mM MgCl₂, and Complete protease inhibitors (Roche, Mannheim, Germany). The homogenate was filtered through cheesecloth and centrifuged at 2000 g 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.

To perform subcellular fractionation, the microsomal pellets were resuspended in 200 µL of each solution of buffer A, high salt-buffer (1 M NaCl, 100 mM Hepes-KOH, pH 7.5, 0.3 M sucrose, 5 mM EGTA, and 5 mM EDTA), alkaline buffer (0.1 M Na₂CO₃, pH 11, 0.3 M sucrose, 5 mM EGTA, and 5 mM EDTA), and Triton X-100 buffer (1% [v/v] Triton X-100, 100 mM Hepes-KOH, pH 7.5, 0.3 M sucrose, 5 mM EGTA, and 5 mM EDTA). After incubation for 10 min, these suspensions were ultracentrifuged at 100,000 g for 1 h at 4 °C to obtain supernatant and pellet fractions. Each fraction was subjected to immunoblot analysis.

To determine membrane topology of KAM1/MUR3, the microsomal pellets were resuspended in buffer A in the presence or absence of 1% (v/v) Triton X-100 and then were incubated with 10 ng/µL proteinase K (Sigma-Aldrich) for 15 min on ice. The reactions were terminated by the addition of 5 mM phenylmethylsulfonyl fluoride and then subjected to immunoblot analysis.

To perform subcellular fractionation, the microsomal pellets were resuspended in 0.7 mL of buffer A and layered directly on top of a 16-mL linear sucrose density gradient (10 to 50%, w/w). Centrifugation was performed in an SW28.1 rotor (Beckman, Palo Alto, CA) at 27,000 rpm for 20 min at 4 °C to remove cellular debris. The extracts were incubated with each antibody for 16 h at 4 °C and then incubated with Protein G–Sepharose FF (Amersham Pharmacia Biotech, Tokyo, Japan). After extensive washing, the Sepharose beads were resuspended in 0.7 mL of buffer A and layered directly on top of a 16-mL linear sucrose density gradient (10 to 50%, w/w). Centrifugation was performed in an SW28.1 rotor (Beckman, Palo Alto, CA) at 27,000 rpm for 20 min at 4 °C to remove cellular debris. The supernatant and pellet fractions were each resuspended in 200 µL of each of the above solutions and then centrifuged at 10,000 g for 15 min on ice. The supernatant and pellet fractions were subjected to immunoblot analysis.

Immunoprecipitation

The cells were homogenized and solubilized in buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% [v/v] 3-[3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, and Complete protease inhibitors) and then centrifuged at 10,000 g for 20 min at 4 °C to remove cellular debris. The extracts were incubated with each antibody for 16 h at 4 °C and then incubated with Protein G–Sepharose FF (Amersham Pharmacia Biotech, Tokyo, Japan). After extensive washing, the Sepharose beads were resuspended in SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% [v/v] SDS, 20% [v/v] glycerol, and 5% [v/v] 2-mercaptoethanol). The resulting immunoprecipitates were subjected to immunoblot analysis.

DAPI Staining

Fifteen-day-old seedlings were stained with 1 µg/mL DAPI (Wako) in 3.7% (v/v) formaldehyde for 30 min.

Phalloidin Staining

The stock solution of fluorescent staining reagent used was 200 units/mL Alexa Fluor 546 phalloidin (Molecular Probes, Eugene, OR) in methanol. Seven-day-old seedlings were fixed in a fixation buffer containing 3.7% (v/v) formaldehyde, 10% (v/v) DMSO, and 0.1% (v/v) Nonidet P-40 for 1 h and then incubated with 20 units/mL Alexa Fluor 546 phalloidin for 1 h in MS medium.

Electron Microscopy

Roots from GFP-2sc and kam1-1 seedlings were fixed with 4% (w/v) paraformaldehyde and 1% (v/v) glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. The tissues were then cut into slices with a razor blade and fixed for another 2 h. Procedures for electron microscopic analysis was essentially the same as those described previously (Hara-Nishimura et al., 1993). After staining, sections were examined with a transmission electron microscope (model 120WX; JEOL, Tokyo, Japan). Hypocotyls from GFP-2sc and kam1-1 seedlings were examined with a scanning electron microscope (model VE-7800; Keyence, Osaka, Japan).

Confocal Laser Scanning Microscopy

The fluorescent images were inspected with a confocal laser scanning microscope (LSM510 META; Carl Zeiss, Jena, Germany) 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 × 1.4 numerical aperture oil-immersion objective or a 40 × 0.75 numerical aperture dry objective. Image analysis was performed using LSM image examiner software (Carl Zeiss). The data were exported as eight-bit TIFF files and processed using Adobe Photoshop 5.5 (Adobe Systems, Tokyo, Japan).

Treatments with Latrunculin B, Nocodazol, and Colchicine

Stock solutions of reagents used were 5 mM latrunculin B in DMSO, 10 mM nocodazol in DMSO, and 100 mM colchicine in DMSO. Seven-day-old GFP-2sc seedlings were incubated in MS medium containing 15 µM latrunculin B, 10 µM nocodazol, 1 mM colchicine, and 1% (v/v) DMSO for 12 h.

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