

Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of *Arabidopsis thaliana*

Takashi Ueda¹, Masatoshi Yamaguchi², Hirofumi Uchimiya² and Akihiko Nakano

Molecular Membrane Biology Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198 and ²Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

¹Corresponding author
e-mail: tueda@postman.riken.go.jp

Ara6 of *Arabidopsis thaliana* is a novel member of the Rab/Ypt GTPase family with unique structural features. It resembles Rab5 GTPases best, but lacks a large part of the C-terminal hypervariable region and the cysteine motif, and instead harbors an extra stretch of amino acid residues containing myristoylation and palmitoylation sites at the N-terminus. Ara6 is tightly associated with membranes and is expressed constitutively. In contrast, the conventional Rab5 ortholog, Ara7, is highly expressed only in actively dividing cells. Examination of green fluorescent protein (GFP)-tagged proteins indicates that both Ara6 and Ara7 are distributed on a subpopulation of endosomes and suggests their roles in endosomal fusion. The endosomal localization of Ara6 requires N-terminal fatty acylation, nucleotide binding and the C-terminal amino acid sequence coordinately. Proteins similar to Ara6 are found only in higher plants and thus represent a novel class of Rab GTPases regulating endocytic function in a plant-specific manner.

Keywords: Ara6/endosome/GFP/plant-specific gene/Rab

Introduction

It is well established that the molecular mechanisms and machinery of vesicular transport are very well conserved from yeast to higher eukaryotes. However, considering that membrane trafficking is required for a variety of cellular functions, evolutionary diversion of molecular components should also be important. In higher plants, vesicular transport is required for specialized phenomena as well as common house-keeping events. For example, tip growth of pollen tubes and root hairs utilizes strictly regulated secretion mechanisms. Secretion also plays a central role in cell plate formation. Plants may well have developed plant-specific mechanisms of vesicular traffic.

The Rab/Ypt family of GTPases are known as key players of vesicular transport. They act as molecular switches regulating the fusion of vesicles with target membranes through the conformational change between GTP- and GDP-bound forms (Novick and Zerial, 1997; Martinez and Goud, 1998). The Rab/Ypt GTPases have four consensus amino acid sequences, which are necessary

for nucleotide binding and GTP hydrolysis. The C-terminal region is basically hypervariable but almost always ends in conserved motifs containing cysteine residues. These cysteine residues are the sites of isoprenylation, which is essential for membrane binding and function (Araki *et al.*, 1991; Musha *et al.*, 1992; Soldati *et al.*, 1993; Ueda *et al.*, 2000). The hypervariable region is also known to play a critical role in determining the subcellular localization of each protein (Chavrier *et al.*, 1991; Brennwald and Novick, 1993). To date many Rab GTPases have been identified from higher plants (Ma, 1994; Verma *et al.*, 1994; Uchimiya *et al.*, 1998). Ara is the name given to a group of Rab GTPases from *Arabidopsis thaliana*. ARA1 was the first RAB/YPT gene identified in plants (Matsui *et al.*, 1989). ARA2 to ARA5 were isolated subsequently by cross-hybridization using ARA1 as a probe (Anai *et al.*, 1991). Two new members, ARA6 and ARA7, are described in this study.

For correct operation of vesicle targeting and fusion, the GTPase cycles of Rab/Ypt proteins have to be strictly regulated. Many regulator molecules have been identified including GDP dissociation inhibitor (GDI), guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP) (Pfeffer *et al.*, 1995; Pan and Wessling-Resnick, 1998; Scheffzek *et al.*, 1998). We have discovered *Arabidopsis* GDI, AtGDI1 and AtGDI2, as molecules interacting with Ara4 GTPase (Ueda *et al.*, 1996b, 1998).

Here we report a novel, plant-unique type of Rab GTPase, Ara6. Ara6 shows some homology to Rab5 GTPases, which play a critical role in endosomal homotypic fusion, but has a unique structure. We isolated a conventional *Arabidopsis* ortholog of Rab5 and named it Ara7. Comparison of Ara6 and Ara7 reveals differences in distribution, expression and regulation. Characterization of Ara6 should enhance our understanding of the reason and mechanism of evolution of plants including those of a unique system for the regulation of vesicular traffic.

Results

Ara6 represents a plant-specific subtype of Rab/Ypt GTPases

A novel member of the *Arabidopsis* Rab family was isolated fortuitously from a cDNA library of *A.thaliana* during screening for other genes. The nucleotide sequence predicted a protein of 202 amino acid residues that conserves the four consensus sequences characteristic of Rab/Ypt GTPases. We thus designated this cDNA ARA6 (DDBJ/EMBL/GenBank accession No. AB007766). The Ara6 protein is judged by homology analysis to be most similar to Rab5 proteins in the family (see Supplementary data, available at *The EMBO Journal* Online). However, Ara6 has some very unique features (Figure 1A). First of

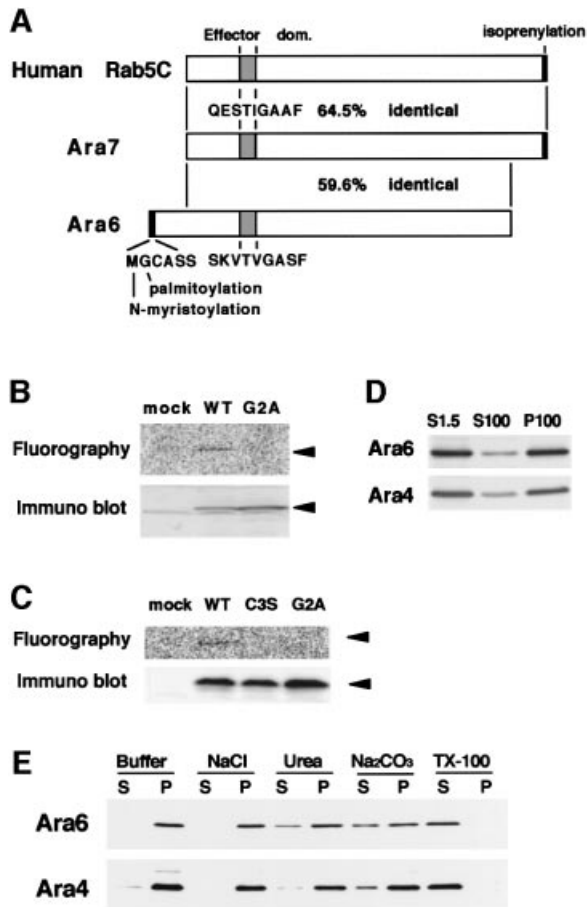


Fig. 1. Ara6 is a peripheral membrane protein with a unique structure. (A) Schematic structures of human Rab5c, Ara7 and Ara6. (B) The N-terminus of Ara6 is *N*-myristoylated *in vitro*. *In vitro* transcription and translation of wild-type *ARA6* (WT) and *ARA6*^{G2A} (G2A) were performed in the presence of ¹⁴C-labeled myristic acid. The same reaction was carried out without the template (mock). (C) The N-terminus of Ara6 is palmitoylated *in vitro*. Ara6 (WT), Ara6^{C3S} (C3S) and Ara6^{G2A} (G2A) synthesized *in vitro* were immunoprecipitated and incubated with *Arabidopsis* lysate in the presence of ¹⁴C-labeled palmitoyl-CoA. (D) Membrane binding of Ara6 and Ara4 was examined by western blotting. The total lysate prepared from *Arabidopsis* cultured cells (S1.5) was ultracentrifuged to separate the cytosolic fraction (S100) from the membrane fraction (P100), and analyzed by immunoblotting with anti-Ara6 polyclonal or anti-Ara4 monoclonal antibody. (E) The P100 fraction was incubated on ice in a buffer containing either salt, urea, alkali (Na₂CO₃) or Triton X-100. Samples were ultracentrifuged again, then the supernatant and the pellet were analyzed by immunoblotting with anti-Ara6 or anti-Ara4 antibody.

all, it lacks the C-terminal region containing part of the hypervariable region and the cysteine motif. The cysteine motif is conserved in almost all Rab/Ypt GTPases, and its isoprenylation is essential for attachment to membranes. The hypervariable region is important for their subcellular localization (Chavrier *et al.*, 1991; Brennwald and Novick, 1993). Instead of this, Ara6 has an extra stretch of amino acids at the N-terminus, which contains putative *N*-myristoylation and palmitoylation sites. The amino acid sequence of the effector domain of Ara6, which is believed to specify the function of Rab/Ypt proteins (Brennwald and Novick, 1993; Dunn *et al.*, 1993), does not match any other proteins of this family known to date. We examined whether Ara6 does indeed hydrolyze GTP

using a fusion protein of Ara6 and GST. GST–Ara6 showed GTPase activity, and the rate of GTP hydrolysis was estimated to be ~0.0065 mol GTP/min/mol (data not shown). Based on literature and database searches, homologs of Ara6 can be found in *Lotus japonicus* (Borg *et al.*, 1997), *Mesembryanthemum crystallinum* (Bolte *et al.*, 2000) and *Oryza sativa* (registered in a database), but this type of Rab/Ypt protein has never been reported in mammals or yeasts. The Rab subclass to which Ara6 belongs appears to be unique to plants.

Membrane binding and expression of Ara6

To determine whether the N-terminus of Ara6 is in fact myristoylated, we performed *in vitro* transcription and translation of wild-type *ARA6* and *ARA6*^{G2A}, a mutant form in which the putative myristoylation site is destroyed, in the presence of ¹⁴C-labeled myristic acid. As the source of a transcription and translation system, we used wheat germ extract which contains *N*-myristoyltransferase (NMT) activity (Heuckeroth *et al.*, 1988). As shown in Figure 1B, radioactivity was incorporated into the wild-type Ara6 but not the mutant Ara6, indicating that Gly2 is indeed the site of *N*-myristoylation. The palmitoylation of Ara6 was also observed when Ara6 protein synthesized *in vitro* was incubated with the *Arabidopsis* lysate and ¹⁴C-labeled palmitoyl-CoA. Incorporation of radioactivity into the Ara6^{C3S} mutant protein was not observed, indicating that Ara6 is indeed palmitoylated at Cys3 (Figure 1C). The Ara6^{G2A} mutant was not palmitoylated either, showing that myristoylation of Ara6 is a prerequisite for palmitoylation.

Next, we examined the membrane association of Ara6. *Arabidopsis* suspension cultured cells were harvested at a log phase, homogenized and centrifuged at 1500 *g*. The supernatant (S1.5) was centrifuged further at 100 000 *g* for 1 h to separate the crude membrane fraction (P100) from the cytosol (S100), which were subjected to immunoblotting analysis. As indicated in Figure 1D, most of the Ara6 protein was detected in the membrane fraction. A small amount was also found in the cytosol, as is the case for a conventional-type Ara protein, Ara4. Ara6 was not extracted from membranes by salt but was partially extracted by urea or alkali, and almost completely by Triton X-100 (Figure 1E). This property appears to be a little different from the case of Ara4, in which extraction by urea or alkali is less efficient. Such a weaker membrane association of Ara6 may reflect its unique lipid modification.

As an authentic Rab5 homolog in *Arabidopsis*, we cloned a novel cDNA by RT-PCR and named it *ARA7* (DDBJ/EMBL/GenBank accession No. AB038491). Ara7 shares 64.5% identity at the amino acid level to human Rab5c, and is 90.5% identical to Rha1, another Rab5 homolog of *Arabidopsis* (Anuntalabhochai *et al.*, 1991). In contrast to Ara6, the effector domain sequence of Ara7 and Rha1 is identical to that of Rab5c (Figure 1A), indicating that Ara7 and Rha1 are Rab5 orthologs. In the central part, Ara7 shares 59.6% identity with Ara6.

The expression of Ara6 and Ara7 in suspension cultured cells was examined by immunoblotting analysis. As shown in Figure 2A and B, Ara6 was expressed throughout the culture phase, and the level of protein was somewhat higher in the stationary phase. In contrast, Ara7 was highly expressed only when cells were actively dividing.

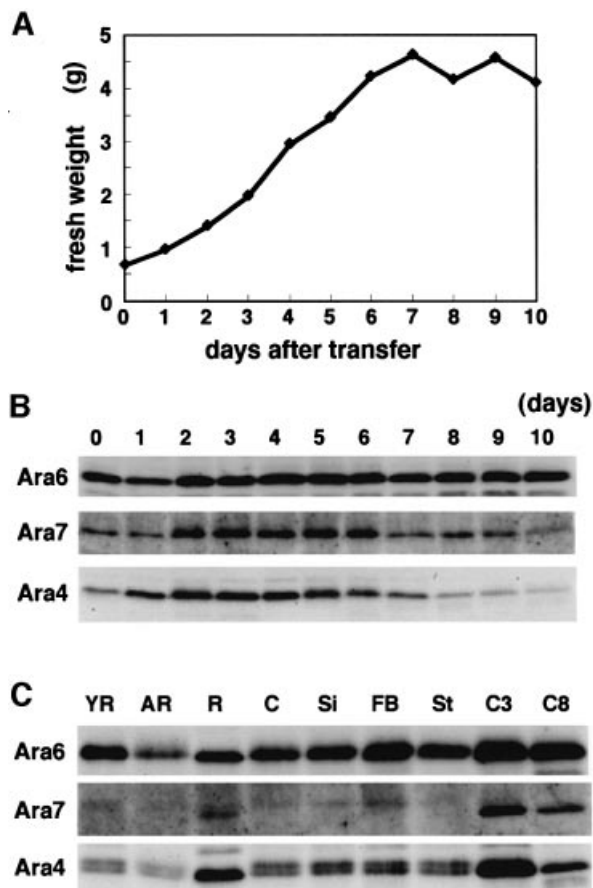


Fig. 2. Expression of Ara6, Ara7 and Ara4 in *Arabidopsis* suspension cultured cells or plants. (A) The *Arabidopsis* suspension cultured cells were collected every day after transfer to the new medium for 10 days, and fresh weights were plotted. (B) The total protein samples prepared from a series of suspension cultured cells were subjected to immunoblotting analysis with anti-Ara6, anti-Ara7 or anti-Ara4 antibody. (C) The total protein samples were prepared from young rosettes (YR), adult rosettes (AR), roots (R), cotyledons (C), siliques (Si), flower-bud clusters (FB) and stems (St). These samples and total lysates from suspension cultured cells were collected over 3 (C3) or 8 days (C8) after transfer and were subjected to immunoblotting analysis with anti-Ara6, anti-Ara7 or anti-Ara4 antibody.

A similar expression pattern was observed for Ara4, although the peak of expression was noted a little earlier than that of Ara7. Figure 2C shows the expression of these proteins in different organs of *Arabidopsis*. Ara6 was ubiquitously expressed in all organs examined, whereas Ara7 and Ara4 showed organ specificities. Ara7 and Ara4 were expressed abundantly in roots and suspension cultured cells. These results suggest that Ara6 has a more general role than Ara7 in plant cells.

Cytosolic pool of Ara6 is independent of GDI

GDI is the best known regulator molecule for the Rab/Ypt GTPases. GDI controls both the nucleotide state and the subcellular localization of Rab/Ypt proteins (Pfeffer *et al.*, 1995). The isoprenylation of Rab/Ypt proteins at their C-terminus is essential for their recognition by GDI (Araki *et al.*, 1991; Musha *et al.*, 1992; Soldati *et al.*, 1993; Ueda *et al.*, 2000). Since Ara6 lacks the C-terminal cysteine motif, its regulation should be independent of GDI. This

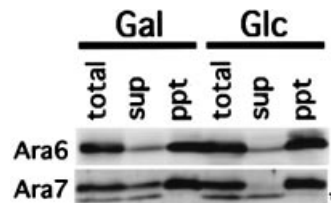


Fig. 3. Recycling of Ara6 is independent of GDI in yeast cells. *ARA6* or *ARA7* was expressed constitutively in yeast *sec19/gdi1* mutant cells harboring *AtGDI1* under control of the *GAL1* promoter. Transformant cells were cultured in galactose medium at the permissive temperature, shifted up to the restrictive temperature and then transferred to new galactose medium (Gal) or glucose medium (Glc). The total cell lysates (total) were ultracentrifuged to obtain cytosol (sup) and membrane (ppt) fractions, and samples were subjected to immunoblotting analysis with anti-Ara6 or anti-Ara7 antibody. The asterisk indicates a cross-reacting band which is not related to Ara7.

was tested by using a yeast expression system. As we described previously, *Arabidopsis* GDI, AtGDI1, can replace the function of yeast GDI, Gdi1/Sec19 (Ueda *et al.*, 1996b). Yeast temperature-sensitive *sec19/gdi1* mutant cells transformed with *AtGDI1* under the control of the galactose-inducible *GAL1* promoter depend on the function of AtGDI for growth at high temperature. These cells were transformed further with a plasmid harboring *ARA6* or *ARA7* under the control of the constitutive *TDH3* promoter. If GDI serves to recycle Ara proteins from membranes to the cytosol, depletion of GDI should lead to a decrease of Ara proteins in the soluble pool. The above transformant cells were cultured in galactose medium at 23°C for 6 h, shifted to 37°C and cultured for 4 h to inactivate yeast GDI, and then transferred to glucose medium to deplete AtGDI1. Cells were collected after 16 h culture at 37°C, lysed, separated into supernatants and pellets, and then analyzed for Ara6 or Ara7 by immunoblotting (Figure 3). When all GDI was depleted in glucose, the soluble pool of Ara7 was almost completely lost, indicating that AtGDI1 indeed serves to recycle Ara7. In contrast, almost the same amount of Ara6 was detected in the supernatant regardless of GDI depletion. Thus, GDI is not required for maintenance of the soluble pool of Ara6, suggesting that Ara6 has a unique system for the regulation of its GTPase cycle.

Ara6 functions in the endocytic pathway

To examine the subcellular localization of Ara6, we transiently expressed a fusion protein of Ara6 and green fluorescent protein (GFP) in protoplasts prepared from *Arabidopsis* suspension cultured cells. The GFP fluorescence was observed with a high-speed confocal laser scanning microscope developed in our laboratory. Still images of Ara6-GFP expressed under the control of the constitutive 35S promoter are shown in Figure 4C, E and G. Fluorescent dots moved around in the cytoplasm very rapidly. They did not look like the Golgi apparatus, because GFP-tagged Golgi proteins give larger signals in *Arabidopsis* cells that move much more slowly (Takeuchi *et al.*, 2000). Ring-like structures were also observed, often in association with a bright dot along the ring (Figure 4E and G). In addition, faint fluorescence was also recognized on the plasma membrane and the endoplasmic reticulum (ER) in some cells (Figure 4C). No fluorescence was observed in cells that were not treated with plasmids

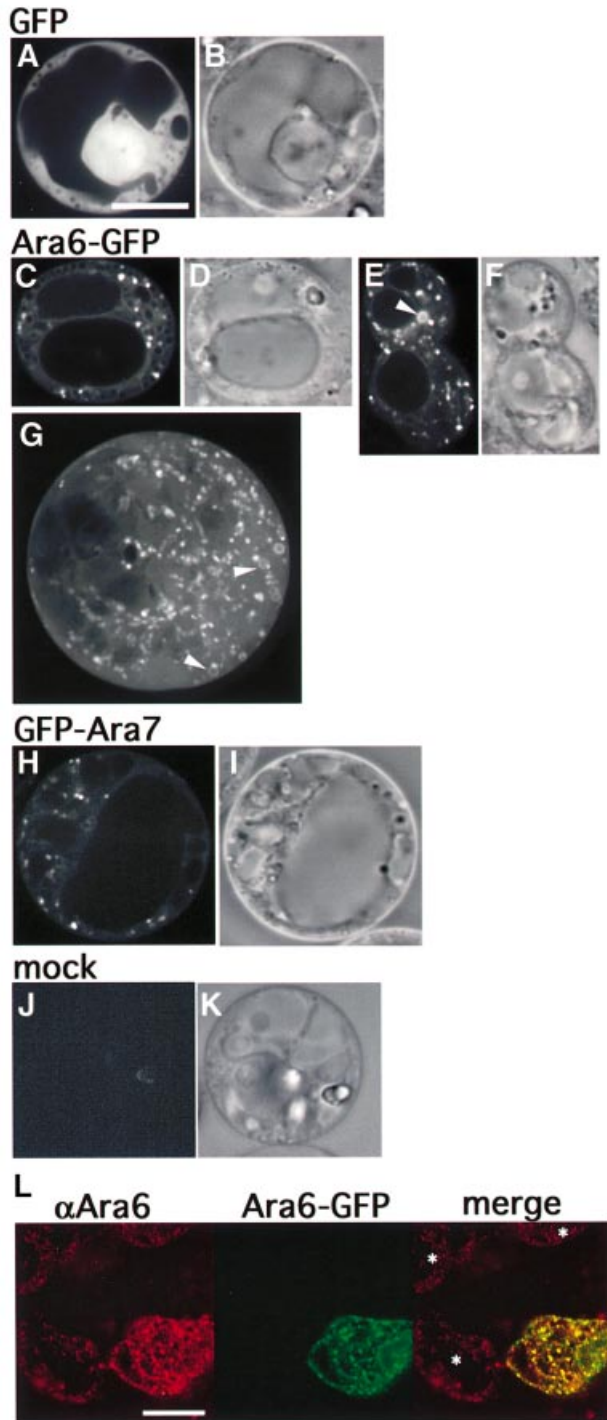


Fig. 4. Subcellular localization of Ara6-GFP and GFP-Ara7. *ARA6* and *ARA7* were tagged with GFP and expressed transiently in the protoplasts of *Arabidopsis* suspension cultured cells. (A and B) An *Arabidopsis* cell expressing GFP. (C–G) Ara6-GFP was localized on motile dots and ring-like organelles (arrowheads). (H and I) GFP-Ara7 was localized predominantly on the motile dotted organelles. (J and K) Protoplasts not treated with the plasmid showed no fluorescence. (A, C, E, H and J) Confocal images. (B, D, F, I and K) Nomarski images. (G) Projection of serial confocal images taken at 0.25 μm intervals. (B–K) Same scale as (A). Bar, 10 μm . (L) Distribution of Ara6 examined by immunofluorescence analysis. Protoplasts, some of which are expressing *ARA6*-GFP, were fixed and stained with the anti-Ara6 antibody. Asterisks indicate untransformed cells. Bar, 10 μm .

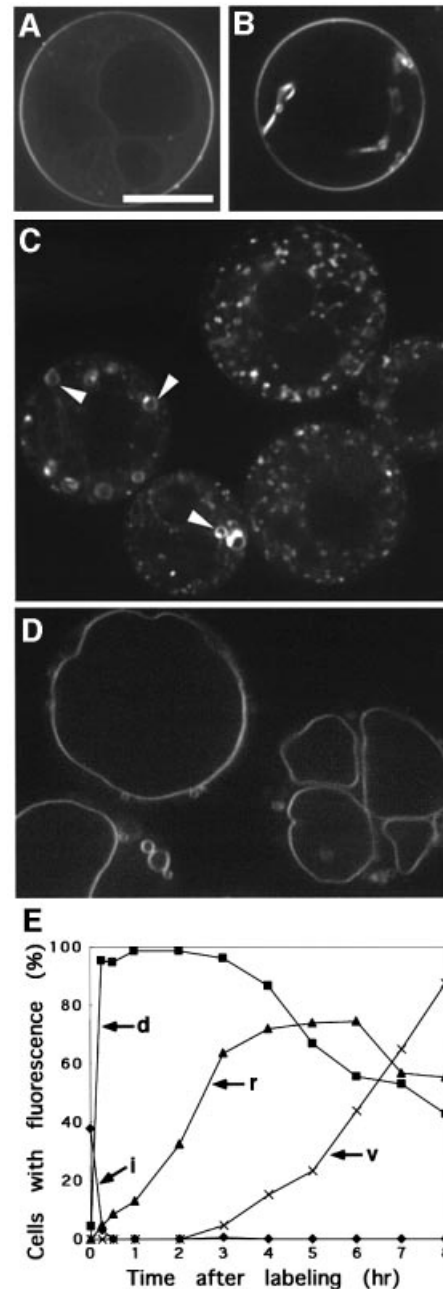


Fig. 5. Endocytic pathway of the *Arabidopsis* cell visualized with FM4-64. FM4-64 was internalized from the plasma membrane (A and B), via motile dotted organelles (C) and ring-like organelles (C, arrowheads), to the vacuole (D). Bar, 10 μm . (E) Time course of the internalization of FM4-64. Percentages of cells with stained plasma membrane invaginations (i), dotted endosomes (d), ring-like structures (r) and vacuole (v) are plotted.

(Figure 4J). To verify that the punctate localization of Ara6-GFP reflects the localization of endogenous protein, immunofluorescence analysis was carried out. To obtain the dual-color image, the cells were viewed with a confocal laser microscope fitted with a spectrophotometer for emission band wavelength selection. The anti-Ara6 antibody and Ara6-GFP stained the identical punctate organelles in transformed cells, and the staining pattern by the anti-Ara6 antibody was very similar between transformed and untransformed cells (Figure 4L). These results

led us to conclude that Ara6-GFP is localized correctly in *Arabidopsis* cells.

For comparison, we examined the localization of Ara7 tagged with GFP at the N-terminus (GFP-Ara7). If Ara7 is a true counterpart of Rab5, it is expected to reside on early endosomes. As shown in Figure 4H, GFP-Ara7 was localized predominantly on the rapidly moving punctate organelles. Ring-like structures were observed occasionally. Thus, Ara6-GFP and GFP-Ara7 yielded very similar fluorescent structures, which were most probably early endosomes.

The lipophilic styryl dye, FM4-64, is known to follow the endocytic pathway, from the plasma membrane via endosomes to the vacuole, when exogenously added to yeast cells (Vida and Emr, 1995). We tried to use this chemical to analyze endocytosis in plant cells. The protoplasts of the *Arabidopsis* suspension culture cells were incubated with 50 μ M FM4-64 for 10 min, washed and resuspended in the culture medium containing 0.4 M mannitol. Just after labeling, FM4-64 stained the plasma membrane (Figure 5A) and invaginations of the plasma membrane in some populations of cells (Figure 5B). Half an hour later, this dye was internalized and localized to small punctate structures in almost all cells (Figure 5C and E), whose size and motility were almost indistinguishable from those visualized by Ara6-GFP and GFP-Ara7. Ring-like structures emerged following the punctate organelles (Figure 5C and E), which were also seen in the case of Ara6-GFP and GFP-Ara7. At 3 h after labeling, vacuolar membranes became fluorescent and, at 8 h, >85% of cells showed vacuolar membrane staining (Figure 5E). After 16 h of incubation, FM4-64 was completely transported to the vacuole (Figure 5D). These results strongly support the notion that Ara6 and Ara7 are localized to the organelles in the early step of the endocytic pathway.

To confirm the endosomal localization of Ara6-GFP, we examined Ara6-GFP and FM4-64 simultaneously. *Arabidopsis* protoplasts transiently expressing Ara6-GFP were labeled with FM4-64, incubated for 30 min and viewed with a confocal microscope. At this time point, FM4-64 stained only the organelles in the early endocytic pathway (Figure 5E). As shown in Figure 6A-F, the small motile dots and the ring-like structures visualized by Ara6-GFP were both stained with FM4-64. This demonstrates that the organelles where Ara6-GFP resides are indeed early endosomes. Interestingly, Ara6-GFP was not localized on all early endosomes visualized by FM4-64 (Figure 6A-C). Ring-like structures, which were marked by FM4-64 but barely stained by Ara6-GFP, were also observed (Figure 6G-I). These findings suggest that *Arabidopsis* has at least two distinctive populations of early endosomes.

Next we investigated whether the mutations of Ara6 and Ara7 that alter their characteristics as GTPases affect their intracellular localization. We first constructed GFP fusions of Ara6^{Q93L} and Ara7^{Q69L}, which correspond to Ras^{Q61L} (Der *et al.*, 1986) and are expected to be stabilized in the GTP-bound state. When these fusion proteins were expressed in *Arabidopsis* cells, predominant fluorescence was observed on aggregates of ring-like structures, the vacuole membrane and small dots (Figure 7). Fluorescence on the plasma membrane was also observed in some populations of cells expressing GFP-Ara6^{Q93L} (Figure 7E).

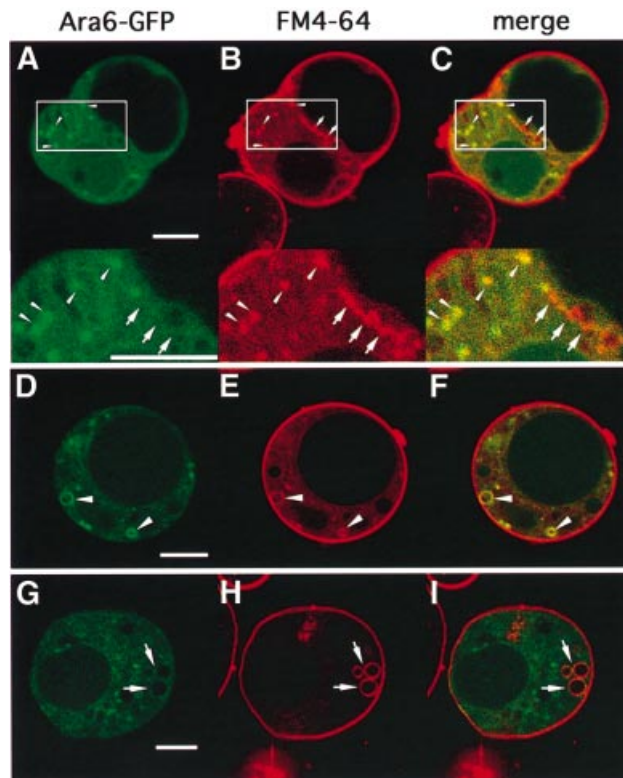


Fig. 6. Ara6-GFP resides on early endosomes. Protoplasts of *Arabidopsis* cultured cells expressing Ara6-GFP were labeled with FM4-64. (A-C) The motile dots with Ara6-GFP (arrowheads) were also stained by FM4-64. Note that some dots labeled by FM4-64 were not marked by Ara6-GFP (arrows). The lower panels are high magnification images of the boxed regions. (D-F) Ring-like organelles where Ara6-GFP resides (arrowheads) were also labeled with FM4-64. (G-I) Some structures (arrows) were marked by FM4-64 but barely by Ara6-GFP. Bar, 10 μ m.

In contrast, Ara7^{Q69L}-GFP never stained the plasma membrane. We again performed a double-labeling experiment, and confirmed that aggregating vesicles were positive for both markers before the large central vacuole became stained with FM4-64 (Figure 7M). These results suggest that the constitutive active mutants of Ara6 and Ara7 accelerate the fusion of early endosomes to form aggregates of larger membrane structures. These aggregating membranes may well represent late endosomes or the pre-vacuolar compartment, which are promoting fusions further and forming even larger vacuoles. Alternatively, this fluorescence may reflect faulty recycling of the mutant proteins. In the case of mammalian cells, the dominant-active mutant of Rab5 (Rab5^{Q79L}), which is known as a key regulator of homotypic fusion of early endosomes, also promotes the formation of larger endosomes when overexpressed (Stenmark *et al.*, 1994).

Taken together, the above results led us to propose that the novel Rab/Ypt protein, Ara6, functions as a regulator of membrane fusion in the endocytic pathway of *Arabidopsis* cells. Ara7 should also be a regulator of endocytic membrane fusion. Currently we are examining whether Ara6 and Ara7 function on the same endosome. The endosomal localization of Ara7 seems more stringent than that of Ara6. We will discuss this point further below. The dominant effects of the GTP-bound mutants also

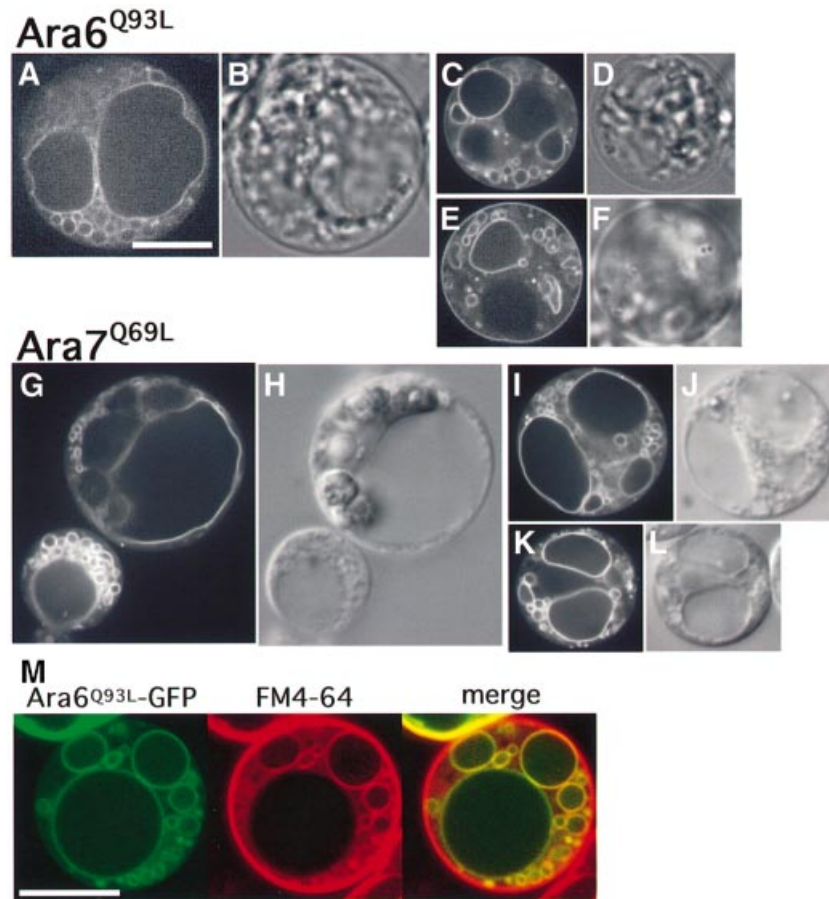


Fig. 7. GTP-bound mutant of Ara6 (Ara6^{Q93L}) or Ara7 (Ara7^{Q69L}) tagged by GFP resides on aggregates of ring-like structures, dots and the vacuole membrane. (A–F) Protoplasts of *Arabidopsis* suspension cultured cells expressing Ara6^{Q93L}-GFP. (G–L) Protoplasts of *Arabidopsis* suspension cultured cells expressing Ara7^{Q69L}-GFP. (A, C, E, G, I and K) Confocal images. (B, D, F, H, J and L) Nomarski images. (B–L) Same scale as (A). Bar, 10 μm. (M) The protoplast expressing Ara6^{Q93L} was labeled with FM4-64 and viewed after 30 min. Bar, 10 μm.

suggest that GFP fusions of Ara6 and Ara7 retain their functions, as is the case for other Rab-GFP fusion proteins (Roberts *et al.*, 1999; White *et al.*, 1999; Bucci *et al.*, 2000; Sönnichsen *et al.*, 2000).

***N*-terminal modification is essential for correct localization of Ara6**

We also examined the role of N-terminal fatty acylation in the subcellular localization of Ara6. We constructed GFP-tagged mutant Ara6 which contained an amino acid substitution in Gly2 and/or Cys3, and expressed them transiently in *Arabidopsis* cells. Ara6^{C3S}-GFP, which has Gly2 where *N*-myristoylation occurs but cannot be a substrate for palmitoylation, was localized mainly on the ER, but not on the endosomes (Figure 8A and C). This result indicates that *N*-myristoylation can direct Ara6 to the membrane, but palmitoylation is essential for the correct localization to the endosomes. Both Ara6^{G2A}-GFP and Ara6^{G2A,C3S}-GFP were localized in the cytosol and nucleus (Figure 8E–J), which is the same pattern as found for free GFP (Figure 4A), indicating that *N*-myristoylation is essential for membrane binding. Thus, Ara6 is attached to the membrane via N-terminal fatty acylation, and both *N*-myristoylation and palmitoylation are essential for localization of Ara6 on the endosomes. Then, is the N-terminal region sufficient to locate Ara6 on the

endosomes? To answer this question, we analyzed whether the N-terminal 34 amino acids of Ara6 containing fatty acylation sites could direct the GFP fusion protein to the endosomes. The N-terminal 34 amino acids of Ara6 were added to the N-terminus of GFP (N34-GFP), which was then expressed in the suspension cultured cells. As shown in Figure 9A–D, it was localized mainly on the plasma membrane and occasionally on punctate structures. These results indicate that the N-terminal region of Ara6 is not sufficient for the predominant endosomal localization.

The C-terminal domain of Ara6 plays a crucial role in its localization

As hitherto demonstrated, the N-terminal sequence of Ara6 is important but not sufficient for localization on early endosomes. In the mutational analysis of Ara6, we found that the nucleotide-free mutant of Ara6, Ara6^{N147I}, corresponding to the N116I mutant of Ras^H, did not localize to early endosomes. Interestingly, Ara6^{N147I}-GFP resided predominantly on the plasma membrane like N34-GFP, suggesting that nucleotide binding is also important for the endosomal localization of Ara6 (Figure 9E–H). To determine the minimal regions of Ara6 that direct endosomal localization, we constructed a chimeric protein consisting of the N-terminal 34 amino acids of Ara6, the central part of Ara4 (from Lys14 to

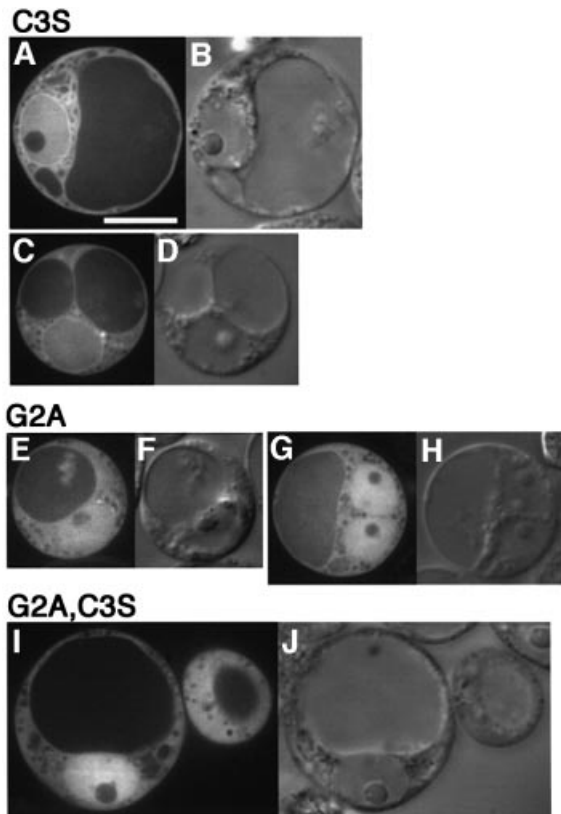


Fig. 8. N-terminal fatty acylation is essential for the correct localization of Ara6. (A–D) Protoplasts of *Arabidopsis* cells expressing Ara6^{C3S}-GFP, which retain Gly2 to be N-myristoylated but which cannot be the substrate for palmitoylation. (E–H) Protoplasts of *Arabidopsis* cells expressing Ara6^{G2A}-GFP, which cannot be myristoylated. (I and J) Protoplasts of *Arabidopsis* cells expressing Ara6^{G2A,C3S}-GFP, a double mutant of fatty acylation sites. (A, C, E, G and I) Confocal images. (B, D, F, H and J) Nomarski image. Bar, 10 μm.

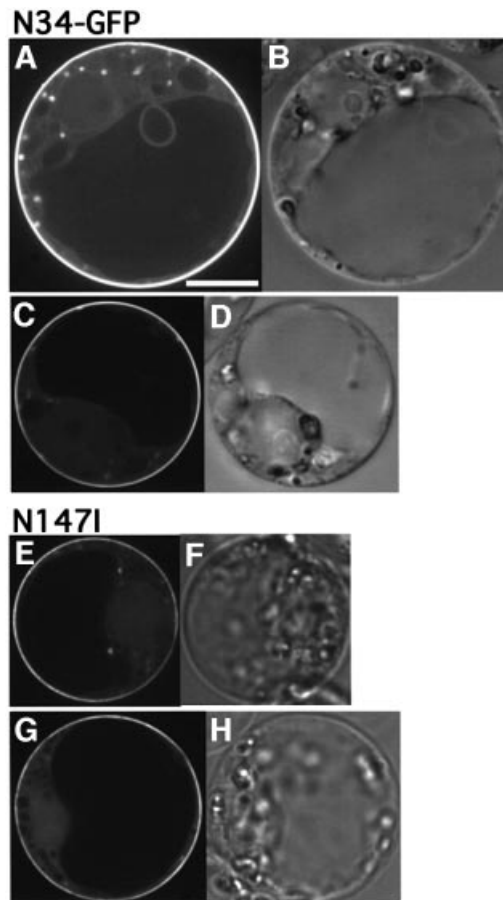


Fig. 9. (A–D) The N-terminal region of Ara6 is not sufficient for endosomal localization. A fusion of the N-terminal 34 amino acids of Ara6 with GFP (N34-GFP) was expressed transiently in *Arabidopsis* suspension cultured cells. (E–H) Nucleotide binding is required for the endosomal localization of Ara6. The nucleotide-free mutant of Ara6 tagged by GFP, Ara6^{N147I}-GFP, was expressed transiently in *Arabidopsis* suspension cultured cells. (A, C, E and G) Confocal images. (B, D, F and H) Nomarski images. Bar, 10 μm.

Ser184) and GFP (N34-A4-GFP; Figure 10A). This chimera was not localized to the endosomes but on unknown types of small structures (Figure 11E and G). GFP-Ara4 yielded patterns of ring-like structures and bright dots (Figure 11A and C). These dots most probably represent the Golgi apparatus as reported previously (Ueda *et al.*, 1996a), and the rings may be identical to those visualized by Ara6^{WT}-GFP or FM4-64. The structures on which N34-A4-GFP was localized also often looked like rings, but they were smaller in size than the ring-like FM4-64-stained organelles. The identities of these organelles have yet to be determined.

As a possible region of Ara6 that contains additional targeting information, we looked at its C-terminal part. The C-terminal hypervariable region is known to be the determinant of localization for conventional Rab/Ypt proteins (Chavrier *et al.*, 1991; Brennwald and Novick, 1993; Dunn *et al.*, 1993). To examine the role of this part, we replaced the C-terminal part of Ara4 in the N34-A4-GFP chimera by the corresponding region of Ara6 containing either eight or 16 amino acid residues (Figure 10B). We expressed these chimeric proteins in *Arabidopsis* cells, and found that the C-terminal eight amino acids of Ara6 can localize the chimeric protein

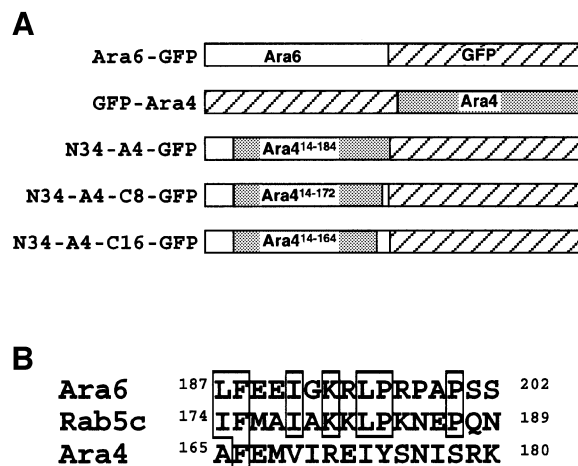


Fig. 10. (A) Schematic structures of chimeric proteins constructed to determine the minimal region required for endosome localization of Ara6. (B) Sequence comparison among the C-terminal 16 amino acids of Ara6, the corresponding region of human Rab5c and that of Ara4. Conserved amino acids are boxed.

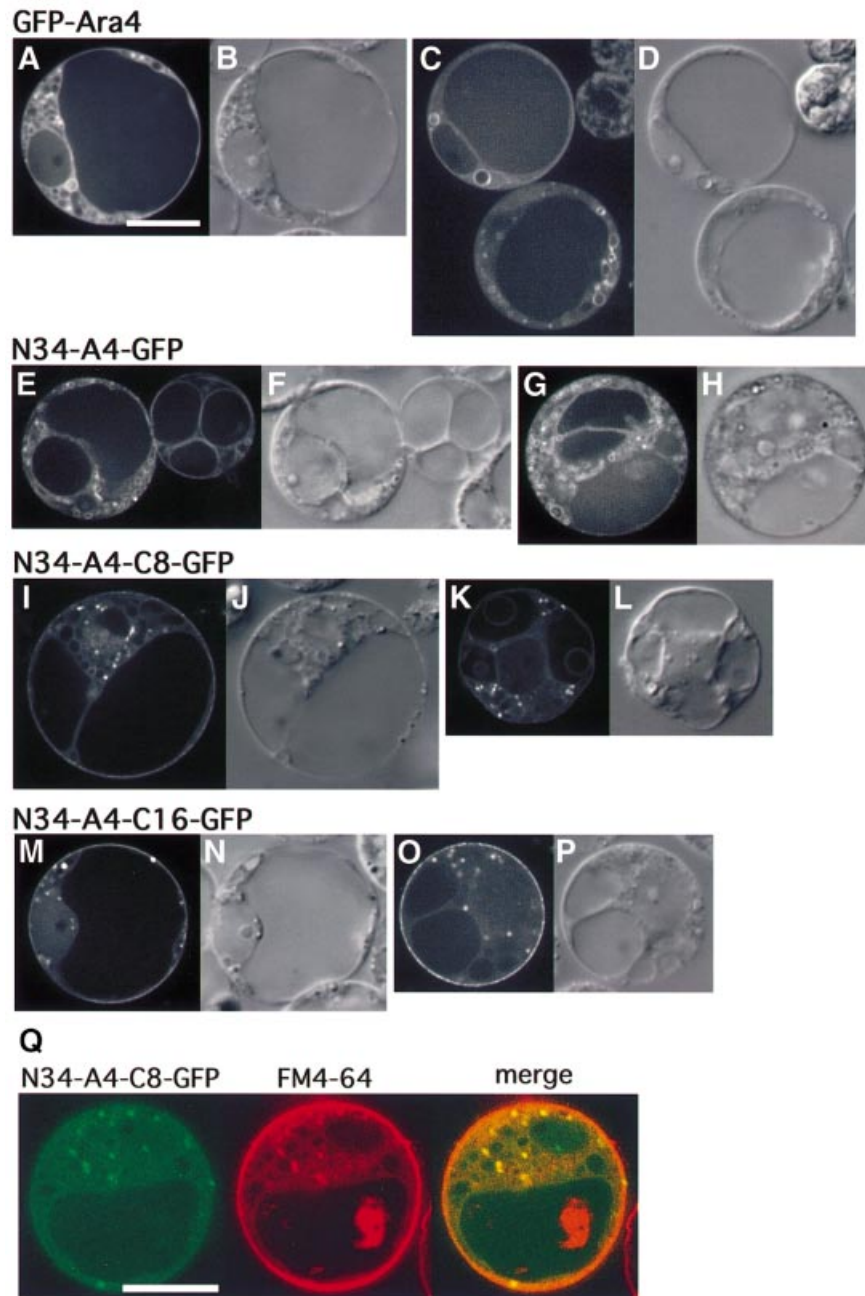


Fig. 11. Subcellular localization of chimeric proteins expressed transiently in the protoplasts of *Arabidopsis* suspension cultured cells. (A–D) *Arabidopsis* cells expressing GFP–Ara4. (E–H) *Arabidopsis* cells expressing N34–A4–GFP. (I–L) *Arabidopsis* cells expressing N34–A4–C8–GFP. (M–P) *Arabidopsis* cells expressing N34–A4–C16–GFP. (A, C, E, G, I, K, M and O) Confocal images. (B, D, F, H, J, L, N and P) Nomarski images. (B–P) Same scale as (A). Bar, 10 μ m. (Q) The protoplast expressing N34–A4–C8–GFP was labeled with FM4–64. Bar, 10 μ m.

(N34–A4–C8) to punctate organelles of high motility (Figure 11I and K). These motile organelles were labeled by FM4–64 after 30 min (Figure 11Q), demonstrating that N34–A4–C8 is localized on early endosomes. The chimera with the C-terminal 16 amino acids of Ara6 (N34–A4–C16) was also localized on the motile endosomes, although some populations occasionally marked the plasma membrane (Figure 11M and O). Thus, the C-terminal eight amino acids of Ara6 could locate the chimeric protein to the early endosomes. In this region, Ara6 shows some

similarity to Rab5; three out of eight residues are conserved (Figure 10B). This conserved region may contain common information for early endosome localization.

Taking the above results together, Ara6 employs a unique mechanism for localization on early endosomes. N-terminal fatty acylations, nucleotide binding and the C-terminal region all appear to be required to determine the subcellular localization of Ara6 in a coordinated fashion.

Discussion

Membrane binding and subcellular localization of Ara6

The Rab/Ypt family of GTPases, key regulators of membrane fusion, have attracted the interest of many researchers, and much information continues to accumulate on their molecular functions. This family is extremely well conserved among divergent eukaryotes, including vertebrates, invertebrates, fungi and plants, indicating their essential roles in all eukaryotic kingdoms. The structures of Rab/Ypt GTPases are also highly conserved, which was thought to indicate that the rigidity of the structure was essential for their function. For example, C-terminal cysteine residues, which are conserved in virtually all previously known members of this family, and their isoprenylation were shown to be essential for membrane binding, interaction with GDI and, thus, for their functions (Araki *et al.*, 1991; Musha *et al.*, 1992; Soldati *et al.*, 1993; Ueda *et al.*, 2000). The hypervariable region, which is located just before the cysteine motif, was also known to have an important role in their subcellular localization (Chavrier *et al.*, 1991; Brennwald and Novick, 1993). However, Ara6 has neither a cysteine residue at the C-terminus nor a complete hypervariable region; nevertheless, it is localized to the endosomes and probably regulates their fusion. This indicates that C-terminal isoprenylation as a membrane anchor is dispensable at least for this type of plant-specific Rab protein. In the case of Ara6, N-terminal myristoylation and palmitoylation appear to be employed as a substitute membrane anchoring system. Both of these modifications are essential for the correct localization of Ara6. This is different from the case of Arf GTPases, another class of small GTPases regulating vesicular traffic. In the case of Arfs, the myristoylation of the N-terminus is essential for their function, but the Cys3 residue to be palmitoylated is lacking. The non-myristoylated G2A mutant of human Arf6 is functionless, and C-terminal isoprenylation cannot remedy its defect (D'Sousa-Schorey and Stahl, 1995). Thus only N-myristoylation should be sufficient for the correct localization and functions of Arf GTPases. On the other hand, the substitution of isoprenylation by N-myristoylation also abolished the function of Rab5 (Li *et al.*, 1995). The reason for such a difference in the dependence on fatty acylation remains unknown.

GFP-tagged Ara6 and Ara7 are localized predominantly on early endosomes. GFP-Ara6 (both wild-type and mutant versions) resides on the plasma membrane and also on the ER, whereas Ara7 never shows such distribution. The broad range of localization sites exhibited by a series of Ara6-GFP variants would represent different stages of the authentic localization of Ara6. In subcellular localization experiments using sucrose density gradient fractionation, Ara6 was detected in multiple peaks, whereas Ara7 showed a single peak (T.Ueda and A.Nakano, unpublished results). This apparent multi-localization of Ara6 may reflect its targeting pathway. As demonstrated in Figure 9, the fusion protein comprising the N-terminal 34 amino acids of Ara6 and GFP is targeted predominantly to the plasma membrane. In mammalian cells, a myristoylated plus palmitoylated GFP variant is reported to be localized mainly to the plasma membrane

(McCabe and Berthiaume, 1999). These results suggest that the plasma membrane is the default destination of such fatty acylated proteins in both plant and animal cells. Ara6^{C3S}-GFP is localized mainly on the ER, suggesting that a myristoylated but not palmitoylated intermediate of Ara6 is formed on the ER. Little is known about the localization of palmitoyl transferase in plants, but it is possible that Ara6 may be targeted from the ER to the plasma membrane to be palmitoylated. Apparently, further information is necessary for Ara6 to be localized to the endosomes. Correct nucleotide binding is critical because the nucleotide-free mutant, Ara6^{N147I}-GFP, is left behind in the plasma membrane. Whereas the GTP-bound mutant Ara6^{Q93L} appears to cause aberrant fusion of endosomes, the GDP-bound mutant Ara6^{S47N} again is located predominantly on the plasma membrane (T.Ueda and A.Nakano, unpublished), suggesting the cycling of Ara6 between the plasma membrane and the endosome during the GTPase cycle.

We have also demonstrated that the C-terminal eight amino acids of Ara6 are very important for its localization. This region shows some similarity between Ara6 and conventional Rab5 proteins. For Rab5, Chavrier *et al.* (1991) demonstrated that the C-terminal 35 amino acids containing highly variable C-terminal sequence followed by the cysteine motif specify its localization. Not all amino acid residues in this region are required for the determination of its localization, because eight or 13 C-terminal residues are exchangeable with the corresponding residues of Rab7 without affecting the localization on early endosomes. These pieces of evidence imply that Ara6 and Rab5 may share, at least in part, a common mechanism of localization on early endosomes.

The regulation system of Ara6 GTPase is also different from that of other Rab/Ypt proteins. The cytosolic pool of Ara6 is unaffected by depletion of GDI. GDI was reported to interact with all Rab/Ypt GTPases examined previously and to detach them from membranes and keep them soluble until the next GTPase cycle starts. Since GDI requires the C-terminal prenylation of Rab for interaction, it is reasonable that Ara6, which lacks this motif, should employ a different mechanism. Identification of regulatory components that act specifically on Ara6 would provide an exciting clue to understanding the role of such plant-specific Rab GTPases. A preliminary result of a gel filtration experiment indicated that Ara6 forms a large complex in the cytosol (T.Ueda and A.Nakano, unpublished). Characterization of this complex is currently underway. Other screening studies for Ara6-interacting molecules should also reveal how different mechanisms have evolved to regulate this type of Rab GTPases.

Do Ara6 and Ara7 function in the same way?

Both Ara6 and Ara7 appear to regulate fusion of early endosomes. However, several lines of evidence suggest that these molecules are involved in distinctive phenomena. First, temporal and spatial patterns of expression of these molecules are different. Ara6 is expressed constitutively all through the growth phases of suspension cultured cells, and ubiquitously in all organs examined. In contrast, Ara7 is expressed only when cells are actively dividing, and in an organ-specific manner in plant bodies. These expression patterns may imply that Ara6 plays a more

general role than conventional Rab5 in vesicular traffic, which is required even in resting cells. The functional difference between Ara6 and Ara7 is also suggested by their amino acid sequences in the effector domain. The effector domain specifies the function of Rab/Ypt proteins (Brennwald and Novick, 1993; Dunn *et al.*, 1993), and is regarded as a marker to classify the members of Rab/Ypt family into subfamilies. The effector domain of Ara7 is identical to that of human Rab5c. In contrast, the effector domain of Ara6 does not coincide with that of any subtype of Rab/Ypt GTPases identified previously, suggesting that Ara6 belongs to a bona fide novel subtype. The three plant homologs of Ara6 have exactly the same effector sequence. Based on this information, we speculate that the Ara6 subfamily is a novel class of Rab/Ypt GTPases which evolved in higher plants in parallel with the Rab5 group to regulate endosome fusion in a distinctive way.

The question remains as to whether Ara6 and Ara7 are on the same endosomes or on different populations. As demonstrated in the double-staining experiment, *Arabidopsis* cells have at least two distinct populations of early endosomes. One comprises the endosomes in which Ara6 resides, and the other the endosomes to which Ara6 is not targeted. It is an attractive idea that Ara6 and Ara7 may regulate the fusion of different endosomes, and we are planning more elaborate and careful experiments to address this hypothesis. The use of GFP variants with different colors and their simultaneous observation will give us much more conclusive information.

That higher plants have evolved a unique system of endocytic vesicular trafficking stimulates our imagination as to why it is required and how it is utilized. Attempts to answer these questions will be made *in planta* utilizing forward and reverse genetics.

Materials and methods

Antibody preparation

The full-length open reading frame (ORF) of *ARA6* was amplified by PCR to generate *Bam*HI sites at both ends and subcloned into pGEX-2T (Pharmacia Biotech, Piscataway, NJ). This recombinant plasmid, which directs synthesis of the Ara6 protein fused to the C-terminus of GST, was introduced into *Escherichia coli*. GST-Ara6 was induced by isopropyl- β -D-thiogalactopyranoside (IPTG), purified from the lysate by affinity chromatography on a glutathione-Sepharose 4B column (Pharmacia Biotech) and used to raise a polyclonal antibody in rabbits. The cross-reactivity of this antibody was tested for lysates from yeast cells expressing *ARA6* and *ARA7*. Immunoblotting analysis detected Ara6 but not Ara7. For the anti-Ara7 antibody, a synthetic Leu185-Val194 peptide, in which Ara7 shows no homology to any other known *Arabidopsis* Rab proteins, was used to immunize rabbits. The antibody obtained was purified by protein G affinity column chromatography (Pharmacia Biotech) and was confirmed to have no cross-reactivity with Ara6.

In vitro modification and fractionation of Ara6

In vitro transcription and translation was carried out with the TNT Coupled Wheat Germ Extract System (Promega, Madison, WI) according to the manufacturer's instructions. For myristoylation assay, linearized *ARA6* and *ARA6*^{G2A} cloned in the pBSIISK⁺ vector (Stratagene, La Jolla, CA) were used as templates, and [¹⁴C]-labeled myristic acid was added to the reaction. After incubation, samples were separated by SDS-PAGE, dried and exposed to the imaging plate for 2 weeks. For palmitoylation assay, linearized *ARA6*, *ARA6*^{C3S} and *ARA6*^{G2A} in pBSIISK⁺ were used as templates. After the transcription-translation reaction was completed, the Ara6 protein was immunoprecipitated and incubated with the *Arabidopsis* lysate and 40 μ M [¹⁴C]palmitoyl CoA (53 mCi/mmol) for 45 min. The immunocomplex was washed, separated by SDS-PAGE, dried and

exposed to the imaging plate for 2 weeks. Autoradiograms were visualized with an imaging plate scanner BAS1000 (Fuji Film Co., Tokyo, Japan). As for fractionation, *Arabidopsis* suspension cultured cells (from Dr M.Umeda) were ground in GR buffer [50 mM HEPES-KOH pH 8.0, 330 mM sorbitol, 2 mM EDTA, 100 μ M phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml leupeptin and 10 μ g/ml pepstatin A], filtered through four layers of cheesecloth and centrifuged at 1500 *g* to remove cell debris. Supernatant (S1.5) was centrifuged at 100 000 *g* for 1 h to separate the crude membrane fraction (P100) from the cytosol (S100). For solubilization of Ara6, the P100 fraction was resuspended in GR buffer plus 1 M NaCl, 2 M urea, 0.1 M Na₂CO₃ or 1% Triton X-100 and allowed to stand on ice for 1 h. Samples were then centrifuged at 100 000 *g* for 1 h to separate soluble from insoluble fractions, and subjected to immunoblotting analysis with an anti-Ara6 polyclonal or an anti-Ara4 monoclonal antibody (Anai *et al.*, 1995).

Expression pattern of Ara6 protein

Total cell lysates (S1.5) were prepared as described above from the *Arabidopsis* suspension cultures collected daily after medium change. Samples were also prepared from young rosettes, adult rosettes, roots, cotyledons, siliques, flower-bud clusters and stems. Proteins (30 μ g for Ara6 and Ara4, and 50 μ g for Ara7) were separated by SDS-PAGE and analyzed by immunoblotting. The immune complexes were visualized with the ECL+Plus system (Amersham Pharmacia Biotech, Amersham, UK).

Expression of ARA6 and ARA7 in yeast and the effect of GDI depletion

Media for yeast cultures were described previously (Ueda *et al.*, 2000). For the expression of *ARA6* and *ARA7* in yeast, their ORFs were first placed under control of the constitutive *TDH3* promoter in pTU1 (Ueda *et al.*, 2000). The fragment containing the *TDH3* promoter, *ARA6* or *ARA7*, and the *CMK1* terminator was cut out by *Bam*HI and *Sal*I, and subcloned into pRS315, which is a single-copy plasmid containing the *LEU2* marker. Cells of ANS19-4A (*MATa sec19-1 ura3 leu2 his*) (Nakano and Muramatsu, 1989) were transformed with this plasmid, and further transformed with a plasmid containing *ARGDII* under control of the *GAL1* promoter and the *URA3* marker (Ueda *et al.*, 1998). Transformants were cultured in minimal galactose medium (MVGS minus uracil and leucine) at 23°C for 6 h, shifted to 37°C for 4 h and then transferred to minimal glucose medium (MVD minus uracil and leucine) or again to minimal galactose medium. Cells were collected after 16 h culture at 37°C, resuspended in Tris-buffered saline (TBS) containing 1% protease inhibitor cocktail (Sigma, St Louis, MO) and vortexed vigorously with glass beads. After centrifugation at 800 *g* for 5 min to eliminate debris, samples were adjusted to 5 mg protein/ml and centrifuged at 100 000 *g* for 30 min to obtain supernatant and pellet fractions. Each sample was analyzed by SDS-PAGE and immunoblotting.

Transient transformation of Arabidopsis suspension culture and immunofluorescent labeling

Wild-type and mutant versions of *ARA6* and *ARA7*, which were constructed by PCR-directed mutagenesis, were fused to the ORF of GFP (from Dr Y.Niwa) in the directions 5'-*ARA6*-GFP-3' and 5'-GFP-*ARA7*-3'. The fusion genes were subcloned into pHTS13 (from Dr H.Tsukaya), which is a derivative of pBSIISK⁺ containing the cauliflower mosaic virus (CaMV) 35S promoter and the Nos terminator. Transient expression of GFP-fused Ara proteins in *Arabidopsis* suspension cultured cells was performed by the method described previously (Forreiter *et al.*, 1997), with minor modifications. Cultured cells were incubated in enzyme solution [0.4 M mannitol, 5 mM EGTA, 1% (w/v) cellulase Y-C and 0.05% (w/v) pectolyase Y-23] for 1 h at 30°C under gentle agitation and then passed through a nylon mesh (125 μ m pore). Protoplasts were collected by centrifugation at 500 *g* for 10 min, washed twice with solution A (0.4 M mannitol, 70 mM CaCl₂ and 5 mM MES pH 5.7) and resuspended in MaMg solution (0.4 M mannitol, 15 mM MgCl₂ and 5 mM MES pH 5.7). After the addition of plasmid and carrier DNA to 100 μ l of protoplast solution, 0.4 ml of DNA uptake solution containing 40% (w/v) polyethylene glycol (PEG) 6000, 0.4 M mannitol and 0.1 M Ca(NO₃)₂ was added. The mixture was placed on ice for 20 min and then diluted with 10 ml of 0.4 M mannitol, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose and 1.5 mM MES pH 5.7. Protoplasts were collected by centrifugation at 500 *g* for 10 min, resuspended in Murashige and Skoog (MS) cell culture medium (Murashige and Skoog, 1962) supplemented with 0.4 M mannitol, and incubated under gentle agitation at 23°C for 16 h in the

dark. Transformed cells were observed with a confocal laser microscope system (Olympus BX60 fluorescence microscope equipped with a confocal scanner Model CSU10 of Yokogawa Electric, Tokyo). For immunofluorescent labeling, *Arabidopsis* protoplasts transiently expressing Ara6-GFP were fixed with 3.7% formaldehyde and permeabilized by 1% NP-40. The cells were incubated in phosphate-buffered saline containing 0.1 M glycine and 1% bovine serum albumin and subsequently with anti-Ara6 antibody. Immunocomplexes were detected by Alexa 568 anti-rabbit IgG (Molecular Probes, Eugene, OR) and observed with a Leica (Heerbrugg, Switzerland) DMIRB/E microscope equipped with a TCS4D confocal scanner, TCS NT software and an Ar/Kr laser.

FM4-64 staining

Protoplasts of *Arabidopsis* suspension cultured cells were incubated with 50 μ M FM4-64 (Molecular Probes) in MS cell culture medium plus 0.4 M mannitol for 10 min on ice, washed with MS medium plus 0.4 M mannitol, and resuspended in the same medium. Cells were incubated for appropriate periods and observed by confocal laser microscopy.

Double staining with GFP and FM4-64

ARA6-GFP or its mutant versions was introduced into the protoplasts of *Arabidopsis* suspension cultured cells as described above. After 16 h incubation in MS cell culture medium containing 0.4 M mannitol, protoplasts were collected by centrifugation, resuspended in MS cell culture medium containing 0.4 M mannitol and 50 μ M FM4-64, and placed on ice for 10 min. After labeling, cells were washed twice, resuspended in the same medium minus FM4-64 and incubated for 30 min at 23°C. Samples were viewed by confocal laser microscopy.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

Acknowledgements

We are grateful to Masaaki Umeda (University of Tokyo) for the *Arabidopsis* suspension culture cell line, to Yasuo Niwa (University of Shizuoka) and Hirokazu Tsukaya (National Institute for Basic Biology, Okazaki) for GFP and expression plasmids, respectively, and to Chieko Saito of our laboratory for technical help in microscopy. This work was supported by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan, by a Special Coordination Fund for Promotion of Science and Technology from the Science and Technology Agency of Japan, by a grant from the Bioarchitect Project of RIKEN, and by a President's Special Research Grant from RIKEN.

References

- Anai,T., Hasegawa,K., Watanabe,Y., Uchimiya,H., Ishizaki,R. and Matsui,M. (1991) Isolation and analysis of cDNAs encoding small GTP-binding proteins of *Arabidopsis thaliana*. *Gene*, **108**, 259–264.
- Anai,T., Aspuria,E.T., Fujii,N., Ueda,T., Matsui,M., Hasegawa,K. and Uchimiya,H. (1995) Immunological analysis of a small GTP-binding protein in higher plant cells. *J. Plant Physiol.*, **147**, 48–52.
- Anuntalabhochai,S., Terryn,N., Van Montagu,M. and Inze,D. (1991) Molecular characterization of an *Arabidopsis thaliana* cDNA encoding a small GTP-binding protein, Rha1. *Plant J.*, **1**, 167–174.
- Araki,S., Kaibuchi,K., Sasaki,T., Hata,Y. and Takai,Y. (1991) Role of the C-terminal region of smg p25A in its interaction with membranes and the GDP/GTP exchange protein. *Mol. Cell. Biol.*, **11**, 1438–1447.
- Bolte,S., Schiene,K. and Dietz,K.J. (2000) Characterization of a small GTP-binding protein of the rab5 family in *Mesembryanthemum crystallinum* with increased level of expression during early salt stress. *Plant Mol. Biol.*, **42**, 923–936.
- Borg,S., Brandstrup,B., Jensen,T.J. and Poulsen,C. (1997) Identification of new protein species among 33 different small GTP-binding proteins encoded by cDNAs from *Lotus japonicus* and expression of corresponding mRNAs in developing root nodules. *Plant J.*, **11**, 237–250.
- Brennwald,P. and Novick,P. (1993) Interactions of three domains distinguishing the Ras-related GTP-binding proteins Ypt1 and Sec4. *Nature*, **362**, 560–563.
- Bucci,C., Thomsen,P., Nicoziani,P., McCarthy,J. and van Deurs,B. (2000) Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell*, **11**, 467–480.
- Chavrier,P., Grovel,J.P., Stelzer,E., Simons,K., Gruenberg,J. and Zerial,M. (1991) Hypervariable C-terminal domain of rab proteins acts as a targeting signal. *Nature*, **353**, 769–772.
- Der,C.J., Finkel,T. and Cooper,G.M. (1986) Biological and biochemical properties of human *rash* genes mutated at codon 61. *Cell*, **44**, 167–176.
- Dunn,B., Stearns,T. and Botstein,D. (1993) Specificity domains distinguish the Ras-related GTPases Ypt1 and Sec4. *Nature*, **362**, 563–565.
- D'Sousa-Schorey,C. and Stahl,P.D. (1995) Myristoylation is required for the intracellular localization and endocytic function of ARF6. *Exp. Cell Res.*, **221**, 153–159.
- Forreiter,C., Kirschner,X. and Nover,L. (1997) Stable transformation of an *Arabidopsis* cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity *in vivo*. *Plant Cell*, **9**, 2171–2181.
- Heuckeroth,R.O., Towler,D.A., Adams,S.P., Glaser,L. and Gordon,J.I. (1988) 11-(Ethylthio)undecanoic acid. A myristic acid analogue of altered hydrophobicity which is functional for peptide N-myristoylation with wheat germ and yeast acyltransferase. *J. Biol. Chem.*, **263**, 2127–2133.
- Li,G., Barbieri,M.A. and Stahl,P.D. (1995) Myristoylation cannot functionally replace the isoprenylation of Rab5. *Arch. Biochem. Biophys.*, **316**, 529–534.
- Ma,H. (1994) GTP-binding proteins in plants: new members of an old family. *Plant Mol. Biol.*, **26**, 1611–1636.
- McCabe,J.B. and Berthiaume,L.G. (1999) Functional roles for fatty acylated amino-terminal domains in subcellular localization. *Mol. Biol. Cell*, **10**, 3771–3786.
- Martinez,O. and Goud,B. (1998) Rab proteins. *Biochim. Biophys. Acta*, **1404**, 101–112.
- Matsui,M., Sakamoto,S., Kunieda,T., Nomura,N. and Ishizaki,R. (1989) Cloning of *ara*, a putative *Arabidopsis thaliana* gene homologous to the *ras*-related gene family. *Gene*, **76**, 313–319.
- Murashige,T. and Skoog,F. (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.*, **15**, 473–497.
- Musha,T., Kawata,M. and Takai,Y. (1992) The geranylgeranyl moiety but not the methyl moiety of the smg25A/rab3A protein is essential for the interactions with membrane and its inhibitory GDP/GTP exchange protein. *J. Biol. Chem.*, **267**, 9821–9825.
- Nakano,A. and Muramatsu,M. (1989) A novel GTP-binding protein, Sar1p, is involved in transport from the endoplasmic reticulum to the Golgi apparatus. *J. Cell Biol.*, **109**, 2677–2691.
- Novick,P. and Zerial,M. (1997) The diversity of Rab proteins in vesicle transport. *Curr. Opin. Cell Biol.*, **9**, 496–504.
- Pan,J.Y. and Wessling-Resnick,M. (1998) GEF-mediated GDP/GTP exchange by monomeric GTPases: a regulatory role for Mg²⁺? *Bioessays*, **20**, 516–521.
- Pfeffer,S.R., Dirac-Svejstrup,B. and Soldati,T. (1995) Rab GDP dissociation inhibitor: putting Rab GTPases in the right place. *J. Biol. Chem.*, **270**, 17057–17059.
- Roberts,R.L., Barbieri,M.A., Pryse,K.M., Chua,M., Morisaki,J.H. and Stahl,P.D. (1999) Endosome fusion in living cells overexpressing GFP-rab5. *J. Cell Sci.*, **112**, 3667–3675.
- Scheffzek,K., Ahmadian,M.R. and Wittinghofer,A. (1998) GTPase-activating proteins: helping hands to complement an active site. *Trends Biochem. Sci.*, **23**, 257–262.
- Soldati,T., Riederer,M.A. and Pfeffer,S.R. (1993) Rab GDI: a solubilizing and recycling factor for rab9 protein. *Mol. Biol. Cell*, **4**, 425–434.
- Sönnichsen,B., De Renzis,S., Nielsen,E., Rietdorf,J. and Zerial,M. (2000) Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5 and Rab11. *J. Cell Biol.*, **149**, 901–913.
- Stenmark,H., Parton,R.G., Steele-Mortimer,O., Lütcke,A., Gruenberg,J. and Zerial,M. (1994) Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. *EMBO J.*, **13**, 1287–1296.
- Takeuchi,M., Ueda,T., Sato,K., Abe,H., Nagata,T. and Nakano,A. (2000) A dominant mutant of Sar1 GTPase inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus in tobacco and *Arabidopsis* cultured cells. *Plant J.*, **23**, 517–525.
- Uchimiya,H., Anai,T., Aspuria,E.T., Matsui,M., Nakano,A. and Ueda,T. (1998) The biological roles of small GTPases and interacting proteins in plants. *J. Plant Res.*, **111**, 257–260.

- Ueda,T., Anai,T., Tsukaya,H., Hirata,A. and Uchimiya,H. (1996a) Characterization and subcellular localization of a small GTP-binding protein (Ara4) from *Arabidopsis*: conditional expression under control of the promoter of the gene for heat-shock protein HSP81-1. *Mol. Gen. Genet.*, **250**, 533–539.
- Ueda,T., Matsuda,N., Anai,T., Tsukaya,H., Uchimiya,H. and Nakano,A. (1996b) An *Arabidopsis* gene isolated by a novel method for detecting genetic interaction in yeast encodes the GDP dissociation inhibitor of Ara4 GTPase. *Plant Cell*, **8**, 2079–2091.
- Ueda,T., Yoshizumi,T., Anai,T., Matsui,M., Uchimiya,H. and Nakano,A. (1998) *AtGDI2*, a novel *Arabidopsis* gene encoding a Rab GDP dissociation inhibitor. *Gene*, **206**, 137–143.
- Ueda,T., Matsuda,N., Uchimiya,H. and Nakano,A. (2000) Modes of interaction between the *Arabidopsis* Rab protein, Ara4 and its putative regulator molecules revealed by a yeast expression system. *Plant J.*, **21**, 341–349.
- Verma,D.P.S., Cheon,C.I. and Hong,Z. (1994) Small GTP-binding proteins and membrane biogenesis in plants. *Plant Physiol.*, **106**, 1–6.
- Vida,T.A. and Emr,S.D. (1995) A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J. Cell Biol.*, **128**, 779–792.
- White,J. *et al.* (1999) Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell Biol.*, **147**, 743–759.

*Received February 8, 2001; revised June 11, 2001;
accepted July 9, 2001*