

# The N-myristoylated Rab-GTPase m-Rab<sub>mc</sub> is involved in post-Golgi trafficking events to the lytic vacuole in plant cells

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## Summary

We report on the sub-cellular localisation and function of m-Rab<sub>mc</sub>, a N-myristoylated plant-specific Rab-GTPase previously characterised at the molecular level and also by structural analysis in *Mesembryanthemum crystallinum*. By confocal laser scanning microscopy, we identified m-Rab<sub>mc</sub> predominantly on the prevacuolar compartment of the lytic vacuole but also on the Golgi apparatus in various plant cell types. Two complementary approaches were used immunocytochemistry and cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP)-fusion proteins. Co-localisation studies of m-Rab<sub>mc</sub> with a salinity stress modulated integral calcium-ATPase suggest involvement of m-Rab<sub>mc</sub> in a plant-specific transport pathway to the prevacuolar compartment of the lytic vacuole. This

hypothesis was strengthened by the inhibition of the transport of aleurain fused to green fluorescent protein (GFP), a marker of the lytic vacuole, in the presence of the dominant negative mutant m-Rab<sub>mc</sub>(N147I) in *Arabidopsis thaliana* protoplasts. The inhibitory effect of m-Rab<sub>mc</sub>(N147I) was specific for the transport pathway to the lytic vacuole, since the transport of chitinase-YFP, a marker for the neutral vacuole, was not hindered by the mutant.

Supplemental data available online

Key words: Rab-GTPase, Myristoylation, Golgi apparatus, Lytic vacuole, Prevacuolar compartment, Plant cells

## Introduction

Plants are immobile and thus display strategies to cope with unfavourable conditions in their habitat. Environmental stress, such as high salinity of the soil, provokes loss in cell turgor, a decrease in water and nutrient uptake, and an accumulation of harmful ions in the cytosol. However, growth under these unfavourable conditions is possible, as plants have developed a palette of stress tolerance mechanisms. Physiologically, this is mainly achieved by the action of the large lytic vacuole, which is the detoxification sink for harmful ions like Na<sup>+</sup> and which maintains cell turgor. The influence of salinity stress on membrane protein composition in the tonoplast has long been reported. The levels and activities of tonoplast resident integral membrane proteins such as Na<sup>+</sup>/H<sup>+</sup>-antiporters, several subunits of the vacuolar H<sup>+</sup>-ATPase, Ca<sup>2+</sup>-ATPases and water channels have recently been shown to be affected by salinity stress (Barkla et al., 1994; Dietz and Arbing, 1996; Tsiantis et al., 1996; Geisler et al., 2000; Kirch et al., 2000; Kluge et al., 2003) and their over-expression may increase the stress tolerance of a plant (Apse et al., 1999). Two fundamental mechanisms required for the maintenance and the regulation of a functional vacuole need to be understood: (1) how is the specific transport of proteins to the vacuole achieved and (2) how is this transport pathway regulated during the plant's adaptation to environmental stress?

The transport of soluble and membrane integral proteins relies on vesicle shuttles, as described for other membrane exchanges within the endomembrane system of most eucaryotic cells (Hawes et al., 1999; Couchy et al., 2003). Different populations of vesicles might provide specificity for transport to different endomembrane destinations (Robinson et al., 1998). Monomeric G-proteins of the Rab family are implicated in budding, transport and fusion of these transport vesicles (Armstrong, 1999; Rodman and Wandinger-Ness, 2000) owing to their molecular switch properties. In mammalian cells, the activated GTP-bound Rab protein may participate in budding, coordination of cytoskeletal transport and finally orchestration of docking and fusion of the transport vesicle. At the end of the transport cycle, the Rab protein is inactivated by GTP hydrolysis that also promotes its recycling for a new round of transport (Rodman and Wandinger-Ness, 2000).

We recently identified and characterised a monomeric G-protein (Mcrab5b) from *Mesembryanthemum crystallinum* (common ice plant), a salinity-resistant plant with inducible Crassulacean acid metabolism (Bolte et al., 2000). Showing high homology to the Rab family, this protein exhibited several features so far restricted to sequences found uniquely in the plant kingdom. Thus, a new subclass of Rab proteins had to be defined (Bolte et al., 2000; Ueda et al., 2001). Sequence

comparison of the *M. crystallinum* Rab protein with other Rab5 GTPases is available in the EMBL database [*Lotus japonicus* Rab5b(Z73939) (Borg et al., 1997); *Arabidopsis thaliana* Ara6 (AB007766) (Ueda et al., 2001); *Oryza sativa* (AF304518)], and from our cloning attempts (*Mesembryanthemum crystallinum*, *Medicago truncatula*, *Nicotiana tabacum*) showed a very high conservation at the amino acid level (70–89%). In contrast to conventional Rab proteins, which carry an isoprenylation at the C terminus, this new family of Rab proteins undergoes N-myristoylation. We propose therefore to designate members of this plant-specific Rab family as m-Rab (for N-myristoylated Rab) and to add a suffix to the name indicating the species of origin. Thus, *M. crystallinum* m-Rab would be m-Rab<sub>mc</sub>. To date, no plant-specific function has been assigned for any of the plant homologues.

The transcript level and the level of membrane bound m-Rab<sub>mc</sub> is strongly upregulated after salinity stress in salinity tolerant and non-tolerant plants (Bolte et al., 2000) (S.B., unpublished results). This leads to the working hypothesis that m-Rab<sub>mc</sub> may be involved in regulating membrane traffic to adapt the vacuolar machinery to salinity stress. To test this hypothesis, we have examined the subcellular location of m-Rab<sub>mc</sub> within the endomembrane system and its function.

Attempts to localise Rab proteins in plant cells are scarce and often indirect (Ueda et al., 2001; Sohn et al., 2003). In this study, m-Rab<sub>mc</sub> was immunolocalised by confocal laser scanning microscopy (CLSM), using an m-Rab<sub>mc</sub> antiserum. Firstly, co-localisation studies with various markers of the plant endomembrane system were carried out combining immunocytochemistry in different plant cell types with transient co-expression of m-Rab<sub>mc</sub> fused to CFP and endomembrane markers in *Arabidopsis thaliana* protoplasts. Secondly, we have analysed the spatial association of m-Rab<sub>mc</sub> with a salinity stress-modulated calcium-ATPase (ACA4) (Geisler et al., 2000) to investigate their co-localisation along the secretory pathway. Furthermore, we performed functional studies of m-Rab<sub>mc</sub> based on co-expression of fluorescently labelled vacuolar reporter proteins and a dominant negative mutant m-Rab<sub>mc</sub>(N147I) in a transient *Arabidopsis thaliana* protoplast expression system (Lee et al., 2002).

m-Rab<sub>mc</sub> is predominantly localised on the prevacuolar compartment of the lytic vacuole with a partial association with the Golgi apparatus. CLSM indicates that the salinity responsive calcium-ATPase ACA4 is resident on the prevacuolar compartment and partially on the Golgi apparatus. We present evidence that m-Rab<sub>mc</sub> is specifically involved in the transport of the soluble reporter protein aleurain-GFP to the lytic vacuole in *Arabidopsis thaliana* protoplasts. We propose that m-Rab<sub>mc</sub> may be involved in the regulation of delivery of soluble proteins to the lytic vacuole.

## Materials and Methods

### Plant material

*Mesembryanthemum crystallinum* plants were grown on soil in 1 l plastic pots in the greenhouse for 8 weeks. *Nicotiana tabacum* Bright Yellow-2 (BY-2) suspension cultured cells were grown and sampled as described previously (Couchy et al., 1998). *Arabidopsis thaliana* suspension cultures were grown at 25°C for 4 days. Cells were maintained in 100 ml of liquid growth medium containing 4.6 g/l MS salts with vitamins (Murashige and Skoog, 1962) (Sigma, France), 30 g/l sucrose at 25°C with gentle agitation in the light.

### Constructs

The enhanced yellow and cyan variants of GFP were purchased from Clontech (Palo Alto, CA). The m-Rab<sub>mc</sub> coding sequence was fused in frame to CFP. The sequence was cloned into the pUC18 vector under the control of a cauliflower mosaic virus (CMV) promoter. The dominant negative mutant m-Rab<sub>mc</sub>(N147I) was generated by site directed mutagenesis. This was done by PCR using the oligonucleotide 3'-ggcttgggtggatcaaagctgatcttcaagaacgtcgg-5'. The m-Rab<sub>mc</sub>-CFP-containing plasmid served as template to synthesize single stranded DNA. The non-fluorescent dominant negative mutant m-Rab<sub>mc</sub>(N147I) was raised by PCR, introducing the restriction sites BamHI and Asp718I at the ends of m-Rab<sub>mc</sub>(N147I). The PCR product was cloned into the BamHI/Asp718I sites of the binary vector pCP60 (pBIN19 derivative) under the control of the 35S-CMV promoter. The nucleotide sequences of all constructs were confirmed by sequencing. The construction of the ST-YFP sequence, aleurain-GFP and chitinase-YFP has been described previously (Di Sansebastiano et al., 2001; Brandizzi et al., 2002a).

### Transient expression in *Arabidopsis thaliana* protoplasts

Transient expression of CFP-, GFP- and YFP-fused proteins in *Arabidopsis thaliana* suspension cultured cells was performed as follows. Cultured cells were incubated in GM solution (MS-medium containing 0.34 M mannitol, 0.34 M glucose) containing 1% (w/v) cellulase Onozuka RS and 0.2% (w/v) macerozyme R10 (Yakult, Japan) for 3 hours at 22°C under gentle agitation in the dark. Protoplasts were collected by centrifugation at 2000 g for 5 minutes, washed twice with GM solution and resuspended in MS medium containing 0.28 M sucrose. Protoplasts were centrifuged at 1000 g. Floating protoplasts were collected and diluted to a density of 4000 protoplasts/μl. 5–15 μg plasmid DNA, 150 μl of DNA uptake solution containing 25% (w/v) polyethylene glycol (PEG) 6000, 0.45 M mannitol and 0.1 M Ca(NO<sub>3</sub>)<sub>2</sub> were sequentially added to 50 μl of protoplast solution. The mixture was placed at room temperature for 30 minutes and then diluted with 1 ml of 275 mM Ca(NO<sub>3</sub>)<sub>2</sub>. Protoplasts were collected by centrifugation at 500 g for 10 minutes, re-suspended in GM solution and incubated at 22°C for 16–24 hours in the dark.

### Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed with the Pharmacia IPGphor System. 1.5 mg protein of *M. crystallinum* leaf extracts were re-suspended in a buffer containing 8 M urea, 2% (w/v) CHAPS, 40 mM Tris base, 1 mM dithiothreitol. For rehydration an equivalent of rehydration buffer containing 8 M urea, 2% (w/v) CHAPS, 0.01% (w/v) bromophenol blue and 2% (v/v) IPG buffer (Pharmacia) was added. The mixture was loaded on Immobiline-strips™ (Pharmacia) with a high range linear pH gradient embedded in a polyacrylamide matrix. Isoelectric focussing was done according to the Pharmacia protocol with 12 hours of re-hydration followed by a three-step electrophoresis (1 hour at 500 V, 1 hour at 1000 V and 5 hours, 15 minutes at 8000 V). After isoelectric focussing, Immobiline-strips™ were equilibrated in loading buffer and proteins were separated electrophoretically on a 14% SDS-polyacrylamide gel (Laemmli, 1970).

### SDS-PAGE and immunoblot analysis

Protein strips from the isoelectric focussing step were separated on 14% SDS-PAGE (Laemmli, 1970). The gels were blotted to nitrocellulose membranes (Pall, France). Immunoblots were blocked with Tris-buffered saline (TBS) (Sambrook et al., 1989) containing 5% (w/v) fat-free dry milk powder and incubated with m-Rab<sub>mc</sub> antiserum (Bolte et al., 2000). m-Rab<sub>mc</sub>-anti-rabbit polyclonal antiserum was used in a 1:1000 dilution. m-Rab<sub>mc</sub> protein levels were

detected with the Lumi-Light System (Roche, Mannheim, Germany) according to the manufacturer's protocol.

#### Immunocytochemistry and confocal laser scanning microscopy Immunofluorescence staining

Immunocytochemistry was performed as described by Satiat-Jeunemaitre and Hawes (Satiat-Jeunemaitre and Hawes, 2001) except that *Arabidopsis thaliana* protoplasts transiently expressing ST-YFP were fixed with 3.7% paraformaldehyde in GM medium.

#### Primary and secondary antibodies

m-Rab<sub>mc</sub> rabbit polyclonal antiserum (Bolte et al., 2000) was used at a dilution of 1/250. BP80 mouse-monoclonal antibody 14G7 (Paris et al., 1997) was used at a dilution of 1/100. ACA4 affinity purified rabbit-polyclonal antibody (Geisler et al., 2000) was used at a dilution of 1/100. JIM84 rat-monoclonal antibody (Horsley et al., 1993) was used undiluted. AtPep12p rabbit polyclonal antibody was used at a dilution of 1/500.

The following secondary antibodies were used according to the manufacturers' instructions: Anti-rabbit IgG coupled to fluorescein isothiocyanate (Sigma, France), anti-rabbit IgG coupled to Cyanine3 (Sigma, France), anti-rabbit F(ab')<sub>2</sub> fragments coupled to Cyanine3 (Jackson Immunochemicals, USA), anti-mouse IgG coupled to Cyanine3 (Sigma, France), anti-rat IgG coupled to Cyanine3 (Sigma, France) and anti-rat IgG coupled to Alexa568 (Molecular Probes, USA).

The double labelling with two rabbit primary antibodies was performed as described by Paris et al. (Paris et al., 1997), but in PBS buffer containing 1 mM EGTA and 1 mM MgSO<sub>4</sub> (mPBS). After incubation with the first primary antibody, and washes, cells were incubated with an excess of the first secondary antibody anti-rabbit F(ab')<sub>2</sub> fragment coupled to Cyanine3.5 at a dilution of 1/20. Cells were then washed and post-fixed for 1 hour with 3.7% paraformaldehyde in mPBS and rinsed overnight with buffer alone. Non-specific binding sites were blocked with mPBS containing 1% (w/v) BSA and the cells were then treated with the second primary antibody, followed by the second secondary anti-rabbit antibody coupled to fluorescein isothiocyanate, for 3 hours each.

#### FM4-64 staining

FM4-64 (Molecular Probes, USA) staining of living *Arabidopsis thaliana* protoplasts was performed in vivo as described previously (Ueda et al., 2001) but with 20  $\mu$ M FM4-64 instead of 50  $\mu$ M.

#### Confocal microscopy and image processing

Images were collected with a Leica TCS SP2 upright laser scanning confocal microscope (Leica Microsystems, Mannheim, Germany). Different fluorochromes were detected sequentially frame by frame with the acousto-optical tunable filter system using laser lines 454 nm (CFP), 488 nm (FITC, GFP), 514 nm (YFP) and 543 nm (Cyanine3, Alexa568). The images were coded green (FITC, GFP, CFP) and red (Cyanine3, YFP, Alexa568) giving yellow co-localisation in merged images. The oil objectives used were 40 $\times$  (NA 1.25), giving a resolution of 160 nm in the x,y-plane and 330 nm along the z-axis and 63 $\times$  (NA 1.32), giving a resolution of 150 nm in the x,y-plane and 290 nm in the z-axis (pinhole 1 Airy unit).

#### Quantification of co-localisation

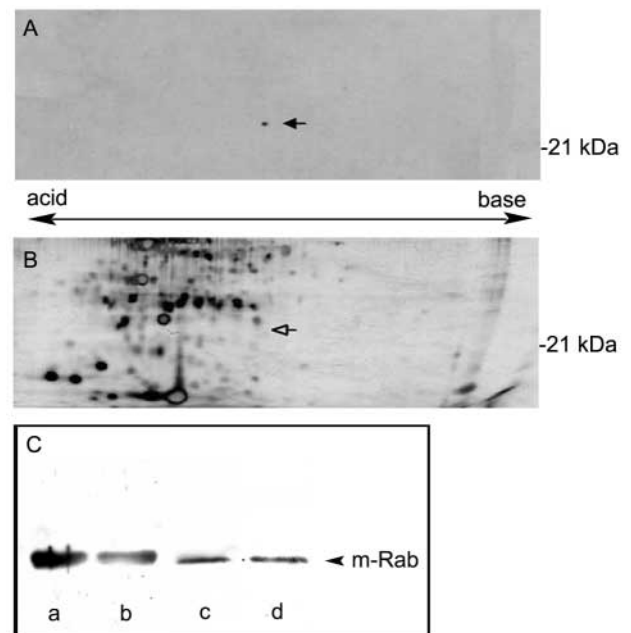
Co-localisation was quantified as described previously (Jiang and Rogers, 1998) with the following changes. Single image overlays were opened in Adobe Photoshop (Adobe Systems). After applying a median yellow filter, red and green pixels were defined with the colour

range-function. Pixel areas were then measured using the histogram function and exported to Excel (Microsoft). Values from 18-25 different cells from three different experiments were taken to calculate the co-localisation percentages and corresponding standard deviations.

## Results

### Distribution of m-Rab<sub>mc</sub> in plant cells

The antiserum against m-Rab<sub>mc</sub> was made from heterologously expressed m-Rab<sub>mc</sub> protein (Bolte et al., 2000). Since more than 50 rather homologous Rab proteins with a size around 20 kDa are known in plants (Rutherford and Moore, 2002), we had to assess the specificity of our antiserum to the N-myristoylated Rab proteins. We investigated a possible cross-reaction with conventional non-myristoylated Rab proteins by different methods. Firstly, we performed a two-dimensional western blot of *Mesembryanthemum crystallinum* protein extracts. The m-Rab<sub>mc</sub> antibody labels a single polypeptide of approximately 21 kDa with an isoelectric point (IP) of approximately 7 (Fig. 1A,B). This isoelectric point matches the calculated IP of 7.04. Secondly, we investigated the potential cross-reaction of our antibody with several closely related Rab protein homologues (plant Rab 7, plant Rab 11, animal Rab 5). Our antibody did not cross-react with any of the non-myristoylated Rab proteins (data not shown). These results indicate the specificity of our antiserum to the m-Rab protein.



**Fig. 1.** Characterisation of the m-Rab<sub>mc</sub> antiserum by two-dimensional gel-electrophoresis. (A) Western blot of *Mesembryanthemum crystallinum* protein extracts incubated with the m-Rab<sub>mc</sub> antiserum. The arrow indicates the single polypeptide recognized by the m-Rab<sub>mc</sub> antiserum, with a molecular mass of approximately 21 kDa and an isoelectric point near pH 7. (B) Silver-stained gel of the western blot in A. The arrow indicates the putative position of the m-Rab<sub>mc</sub>-labelled polypeptide. (C) Western blots of protein extracts of *M. crystallinum* leaf extracts (a), BY-2 cells (b), *A. thaliana* suspension cells (c) and tobacco leaves (d). m-Rab<sub>mc</sub> antiserum stains a single band of approximately 21 kDa.

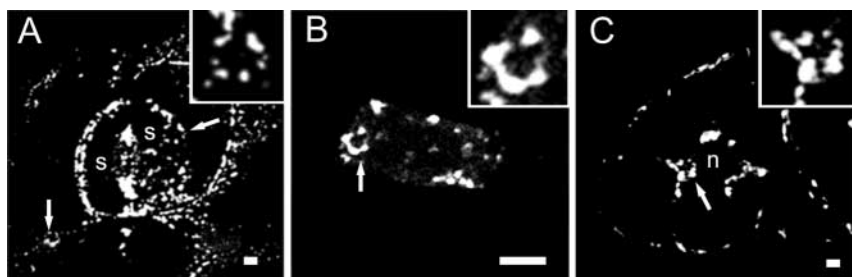
Western blot analysis of membrane protein extracts of the plants used in this study with the m-Rab<sub>mc</sub> antiserum resulted in the staining of a single band of approximately 21 kDa (Fig. 1C). This confirmed the ubiquity of the m-Rab protein throughout the plant kingdom and allowed us to investigate the localisation of m-Rab<sub>mc</sub> in plant cells by immunocytochemistry.

Localisation of m-Rab<sub>mc</sub> in situ was determined by immunocytochemistry on fixed leaf tissue and root cells of *Medicago truncatula*, *M. crystallinum* and *Zea mays*, and suspension cells of *A. thaliana*, *M. crystallinum*, *Nicotiana tabacum* and *Medicago truncatula*. CFP, GFP and YFP constructs were expressed in tobacco leaf tissue and *A. thaliana* protoplasts.

As control experiments for immunofluorescence studies, immunoreactions with the pre-immune serum and immunoreactions with omission of the m-Rab<sub>mc</sub> antibodies were tested. In these two latter cases, no staining was observed within the cell or the tissue (not shown), confirming the specificity of our m-Rab<sub>mc</sub> antiserum. Whatever the tested biological material, immunostaining of plant cells with m-Rab<sub>mc</sub> antiserum gave comparable results, with different intensities according to the cell type, as described below.

In the leaf epidermis of *M. crystallinum* (Fig. 2A), stomatal guard cells, subsidiary cells and epidermal cells were all labelled. Small punctuate structures with an apparent diameter of less than 0.5  $\mu$ m (sub-micron structures) were observed in the three cell types. Some of the labelling was arranged in a ring-like fashion in the cytoplasm, both in stomatal guard cells and in the cells of the surrounding tissue.

This typical m-Rab<sub>mc</sub> labelling pattern was also observed in root cells of *M. crystallinum* (Fig. 2B) and in BY-2 suspension culture cells (Fig. 2C). However, confocal analysis of different root cell types showed that the amount and distribution of labelling was heterogeneous. The number of m-Rab<sub>mc</sub>-labelled structures varied from 0–30 per cell in different root cell types. Meristematic cells without developed vacuoles were not labelled by m-Rab<sub>mc</sub> antibody (T. Coba de la Peña and S.B., unpublished data). Similar results were also obtained for root cells of other plant species like *Medicago truncatula* and *Zea mays*. These results may be correlated with the lower expression of the m-Rab<sub>mc</sub>-transcript and protein in roots (data not shown).



**Fig. 2.** Confocal microscopy of immunofluorescence staining with m-Rab<sub>mc</sub> antiserum in various plant tissues. m-Rab<sub>mc</sub> antiserum reveals a punctuate pattern throughout the cytoplasm, some of the structures being typically organised in a ring-like pattern (arrows, also see insets). (A) m-Rab<sub>mc</sub> labelling of upper leaf epidermal cells of *M. crystallinum*. Single optical section of the upper epidermal layer with stomatal guard cells (s) surrounded by pavement cells. (B) *M. crystallinum* root cell projected from 15 single optical sections of 0.5  $\mu$ m. (C) Image stack of 20 optical sections of 0.5  $\mu$ m of a BY-2 cell. n, nucleus. Scale bars: 5  $\mu$ m.

The punctuate labelling pattern was evocative of Golgi apparatus (GA) or prevacuolar compartment (PVC) staining. Therefore, a potential co-localisation of m-Rab<sub>mc</sub>-labelled organelles with Golgi markers and PVC markers was investigated.

#### m-Rab<sub>mc</sub>-labelled structures versus the prevacuolar marker BP80

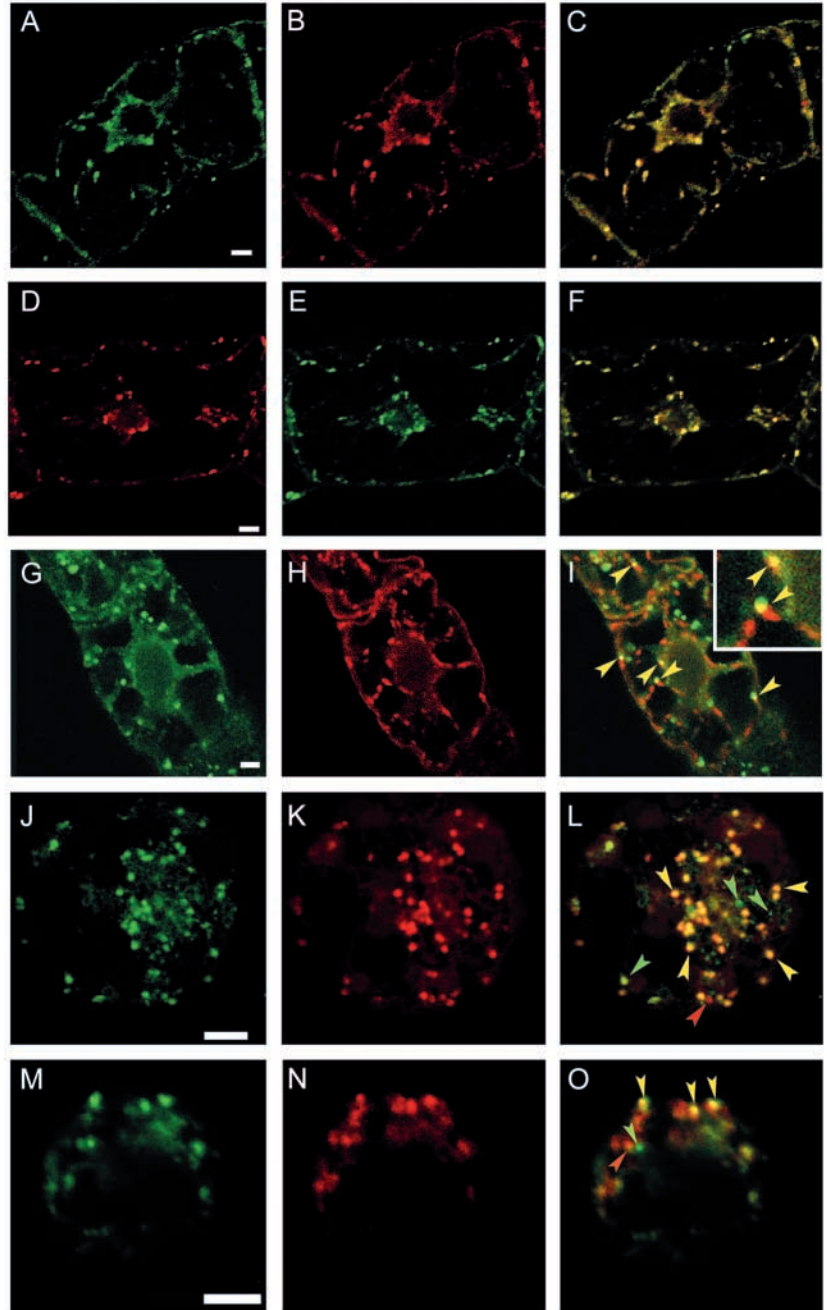
To study the potential association of m-Rab<sub>mc</sub> with the PVC, we used the BP80 monoclonal antibody. BP80 has been described as a marker for the PVC of the lytic vacuole (Paris et al., 1997; Li et al., 2002).

Observations were made of 20 different BY-2 cells in three separate experiments. Fig. 3A shows a single image of the characteristic labelling pattern of m-Rab<sub>mc</sub> antiserum in a BY-2 cell. We observed a labelling of sub-micron structures very similar to the staining pattern with the BP80 antiserum in this BY-2 cell (Fig. 3B). The m-Rab<sub>mc</sub> and BP80 labelling resulted in a punctuate pattern, distributed all over the cytoplasm and sometimes arranged in circular patterns similar in size and distribution to that described by Paris et al. (Paris et al., 1997) for pea root tip cells, and identified as PVC staining. The overlay of m-Rab<sub>mc</sub> and BP80 (Fig. 3C) resulted in an almost complete co-localisation of the m-Rab<sub>mc</sub> and BP80 signal. In order to quantify the degree of co-localisation, calculations of the red, green and yellow pixel areas in single sections were performed as described in the experimental procedures. 87 $\pm$ 4% ( $n=18$ ) of m-Rab<sub>mc</sub> and BP80 stained objects were co-localised, 7 $\pm$ 0.6% red pixels and 6 $\pm$ 0.4% green pixels were observed. These results indicate that m-Rab<sub>mc</sub> is co-localising almost completely with BP80 on the PVC. Similar co-localisation results (89 $\pm$ 4%,  $n=25$ ) were obtained using the m-Rab<sub>mc</sub> antiserum (Fig. 3D) and the AtPep12p antiserum (Fig. 3E), recognising a SNARE homologue located exclusively on PVC [AtSyp21p (Sanderfoot et al., 2000)].

#### m-Rab<sub>mc</sub>-labelled structures versus Golgi markers

The double immunolabelling of BY-2 cells with m-Rab<sub>mc</sub> antiserum and the GA marker JIM84 (Horsley et al., 1993) is shown in Fig. 3G–I. A BY-2 cell labelled with m-Rab<sub>mc</sub> shows the characteristic m-Rab<sub>mc</sub> staining (Fig. 3G; see also Fig. S1A, <http://jcs.biologists.org/supplemental/>). JIM84 immunolabelling of the same cell resulted in the characteristic Golgi staining pattern with lots of sub-micron sized labelled bodies dispersed within the cytoplasm (Fig. 3H; see also Fig. S1B, <http://jcs.biologists.org/supplemental/>) as previously described by Couchy et al., (Couchy et al., 1998). After merging the images (Fig. 3I; see also Fig. S1C, <http://jcs.biologists.org/supplemental/>) some objects seemed to be co-located. It appeared that 21 $\pm$ 8% ( $n=23$ ) of the m-Rab<sub>mc</sub>-stained objects were associated with Golgi stacks. This lower rate of Golgi association of m-Rab<sub>mc</sub> in contrast to its PVC association poses a question: is the partial overlapping of JIM84 and m-Rab<sub>mc</sub> staining due to

**Fig. 3.** Co-localisation of m-Rab<sub>mc</sub> with markers for the prevacuolar compartment and Golgi apparatus in BY-2 cells and *A. thaliana* protoplasts. Antibody staining was done in BY-2 cells using FITC-labelled or Cy3-labelled secondary antibodies and in *A. thaliana* protoplasts expressing sialyl transferase-YFP (ST-YFP) using an Alexa568-labelled secondary antibody. Co-expression of m-Rab<sub>mc</sub>-CFP and ST-YFP was performed in *A. thaliana* protoplasts. Images were colour-coded using Adobe Photoshop. Confocal images represent single images of BY-2 cells or *Arabidopsis* protoplasts as stated. Scale bars: 5 µm. (A-C) m-Rab<sub>mc</sub>-Cy3/BP80-FITC dual labelling of a BY-2 cell. (A) Single image of a cell stained with m-Rab<sub>mc</sub> antiserum. (B) Prevacuolar staining by BP80 of the same cell. (C) Merged image of the dual labelling reveals an almost complete co-localisation of the two proteins (yellow). (D-F) m-Rab<sub>mc</sub>-Cy3/Pep12-FITC dual labelling of a BY-2 cell. (D) Single image of a cell stained with m-Rab<sub>mc</sub> antiserum. (E) The same cell stained with Pep12. (F) Merged image shows a high level of co-localisation of the two proteins (yellow). (G-I) Single image of a BY2 cell showing m-Rab<sub>mc</sub>-FITC/JIM84-Cy3 dual labelling. (G) m-Rab<sub>mc</sub>-FITC; (H) Golgi-marker JIM84-Cy3; (I) merged image of the dual labelling. Note co-localisation (yellow arrowheads, inset). (J-L) Single image of an *A. thaliana* protoplast co-transfected with m-Rab<sub>mc</sub>-CFP and the trans-Golgi marker ST-YFP. (J) m-Rab<sub>mc</sub> CFP staining; (K) ST-YFP staining; (L) the merged image shows the single labelling of m-Rab<sub>mc</sub>-stained prevacuoles (green arrowheads), ST-YFP-stained GA (red arrowheads) and co-localisation of the two proteins (yellow arrowheads). (M-O) Single image of a fixed protoplast expressing ST-YFP and labelled with m-Rab<sub>mc</sub> antiserum. (M) m-Rab<sub>mc</sub> staining; (N) ST-YFP staining; (O) the merged image shows the single labelling of m-Rab<sub>mc</sub>-stained prevacuoles (green arrowheads) and ST-YFP-labelled GA (red arrowhead) and co-localisation of both proteins on some Golgi structures (yellow arrowheads).



inadequate resolution in the  $x,y$ -plane or  $z$ -axis? To answer this question, the fluorescence intensity profiles of overlapping regions were analysed in successive single sections from an image stack as described in supplementary data (Fig. S1, <http://jcs.biologists.org/supplemental/>). This analysis confirmed a real overlap of the JIM84- and the m-Rab<sub>mc</sub> signals on some GA.

At this stage, it cannot be excluded that the m-Rab<sub>mc</sub> antiserum recognises other N-myristoylated isoforms or m-Rab<sub>mc</sub> homologues like Ara6 (Ueda et al., 2001). To better discriminate m-Rab<sub>mc</sub> from these other N-myristoylated proteins, we transiently co-expressed m-Rab<sub>mc</sub> fused to CFP with various endomembrane markers. Furthermore, the transient expression should test the reality of the association of m-Rab<sub>mc</sub> with the GA. Accordingly, we co-transfected *A. thaliana* protoplasts with m-Rab<sub>mc</sub>-CFP and sialyl transferase-YFP [ST-YFP (Brandizzi et al., 2002a)], a marker staining predominantly the trans-half of the GA (Boevink et al., 1998). Fig. 3J-L shows single images of a protoplast co-transfected with the two constructs. m-Rab<sub>mc</sub>-CFP labelled structures were

distributed all over the cell (Fig. 3J) in a manner similar to that seen by immunofluorescence. These structures were highly mobile in the living protoplast. The staining of the GA by ST-YFP (Fig. 3K) was as described for ST-GFP (Boevink et al., 1998), i.e. punctuate motile structures distributed throughout the cytoplasm. After merging the two images (Fig. 3L), we observed sub-micron structures labelled by m-Rab<sub>mc</sub>-CFP alone, however, m-Rab<sub>mc</sub> was also resident on many GA labelled by ST-YFP. We rarely observe single ST-YFP-labelled structures, suggesting in this case a strong association of m-Rab<sub>mc</sub> with the GA. This observation differed from the BY-2 immunofluorescence data, where a co-localisation of m-Rab<sub>mc</sub> with only  $21 \pm 8\%$  of the Golgi bodies was observed. It still could not be excluded that this increase in the association of

m-Rab<sub>mc</sub> with the Golgi bodies may be linked to the mis-targeting of m-Rab<sub>mc</sub>-CFP, or ST-YFP, or both as a result of over-expression. To test if this increased GA/m-Rab<sub>mc</sub> association was due to m-Rab<sub>mc</sub> over-expression, we labelled endogenous m-Rab<sub>mc</sub> by immunocytochemistry on protoplasts expressing only ST-YFP (Fig. 3M-O). Still we found endogenous m-Rab<sub>mc</sub> (Fig. 3M) associated with 84±5% ( $n=21$ ) of the Golgi stacks (Fig. 3N,O) in *A. thaliana* protoplasts.

Furthermore, immunocytochemistry in *A. thaliana* protoplasts with m-Rab<sub>mc</sub> antiserum and JIM84 antibody also showed a significant level of m-Rab<sub>mc</sub>/Golgi association (Fig. S2). In another set of experiments of co-expression of m-Rab<sub>mc</sub>-CFP and ST-YFP in tobacco leaf epidermal cells, the association of m-Rab<sub>mc</sub> with the GA was weaker than in *A. thaliana* protoplasts (data not shown). This suggests that the degree of Golgi association of m-Rab<sub>mc</sub> varies according to the biological material used. Clearly, m-Rab<sub>mc</sub> has a certain potential to associate with Golgi structures.

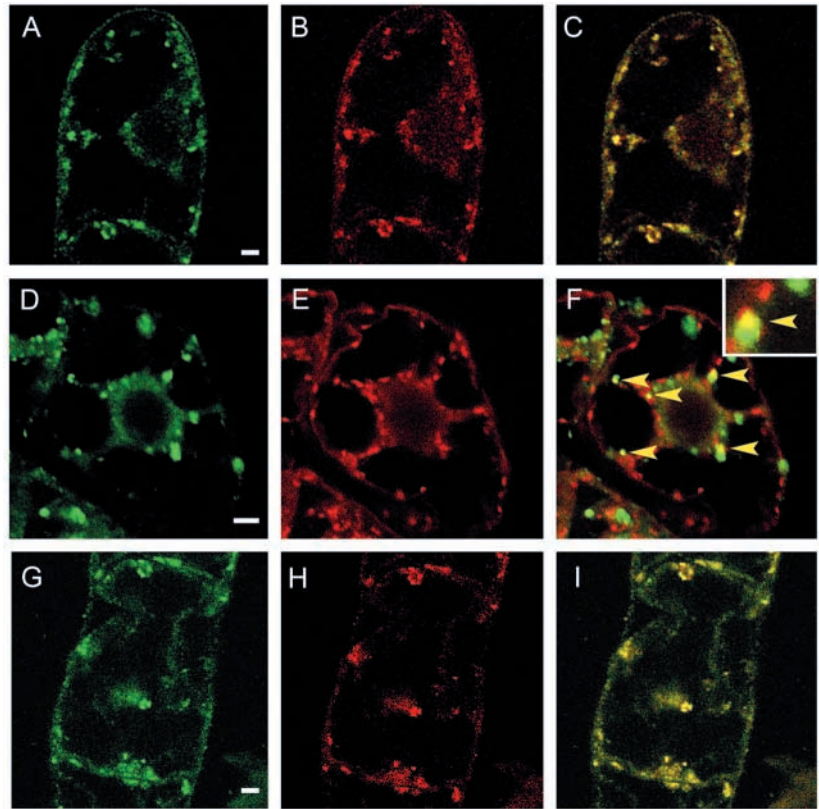
#### m-Rab<sub>mc</sub> and the salinity stress modulated calcium-ATPase ACA4 co-localise along the vacuolar pathway

Previous results raised the hypothesis that m-Rab<sub>mc</sub> might be involved in stress-modulated alterations of membrane transport to the vacuole (Bolte et al., 2000). To further test this hypothesis, we studied the location of a salinity responsive Ca<sup>2+</sup>-ATPase, ACA4 (Geisler et al., 2000), and investigated its potential association with m-Rab<sub>mc</sub> along the vacuolar pathway.

In a single image of a BY-2 cells, the ACA4 antiserum stained sub-micron structures (Fig. 4A). Geisler et al. (Geisler et al., 2000) described similar structures expressing ACA4-GFP in protoplasts of *A. thaliana*. This labelling pattern was very similar to the staining of BP80 epitopes on PVC (Fig. 4B, and compare with Fig. 3B). The prevacuolar location of ACA4 was confirmed in overlay images (Fig. 4C) where the two antisera were associated with the same sub-micron structures. The percentage of co-localisation was determined in single images by pixel area measurements as before, and confirms a predominant location on the PVC. 82±4% ( $n=18$ ) co-locating pixels, 7±0.5% red pixels and 10±0.8% green pixels were observed.

Co-labelling of a BY-2 cell with ACA4 (Fig. 4D) and the GA marker JIM84 (Fig. 4E) suggested that the ACA4 protein was associated with some Golgi stacks (Fig. 4F, compare with Fig. 3I). The amount of co-localisation was 11±2%. This co-localisation was again confirmed by analyses of profile measurements in single images as described before (data not shown).

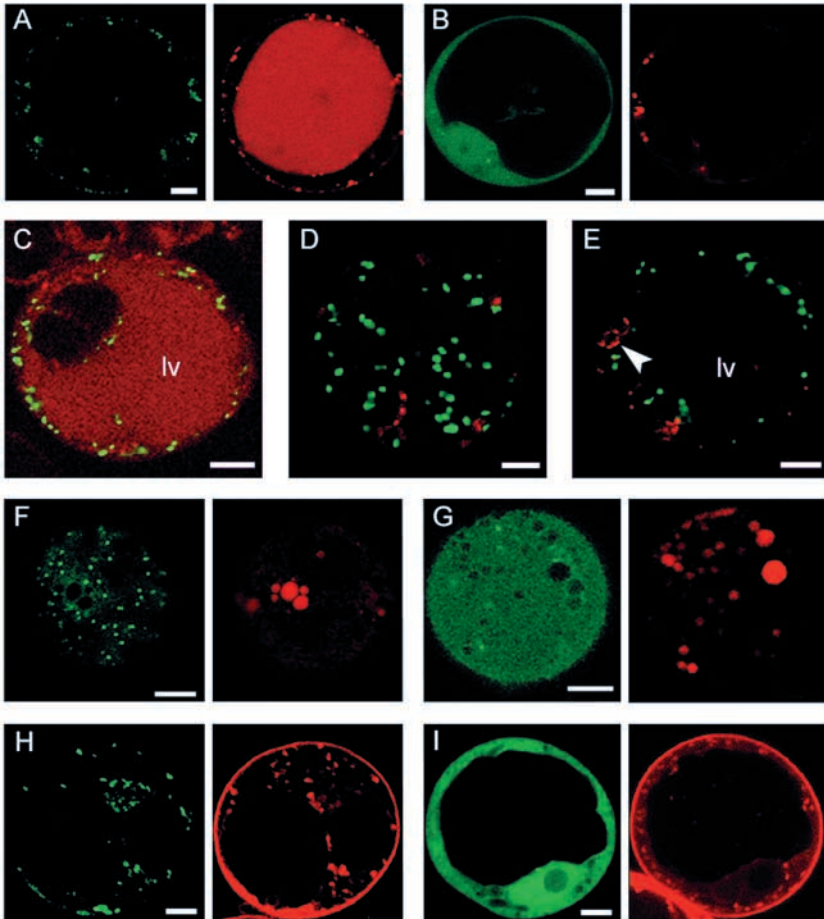
ACA4 staining (82±4% PVC, 11±2% GA) was similar to m-Rab<sub>mc</sub> staining (87±4% PVC, 21±8% GA). We thus expected a high degree of co-localisation of ACA4 with m-Rab<sub>mc</sub>. For dual labelling of cells with ACA4 and m-Rab<sub>mc</sub> (Fig. 4G-I)



**Fig. 4.** Co-localisation of the Ca<sup>2+</sup>-ATPase ACA4 and m-Rab<sub>mc</sub> with the PVC and the GA. Secondary antibodies used were as described in Fig. 3. Images were colour-coded with Adobe Photoshop. Single confocal images of isolated BY-2 cells. Scale bars: 5 μm. (A-C) Single image of a cell co-labelled with ACA4-Cy3/BP80-FITC. (A) ACA4 staining; (B) BP80 staining; (C) merged image reveals an almost complete co-localisation of ACA4 and BP80 (yellow). (D-F) Single image of a cell co-labelled with ACA4-FITC/JIM84-Cy3. (D) ACA4 labelling; (E) labelling of the GA recognised by JIM84; (F) merged image of the dual labelling. Note some co-localisation (yellow arrowheads, inset). (G-I) Image stack of a single cell labelled with ACA4-FITC/m-Rab<sub>mc</sub>-Cy3. The secondary Cy3-coupled antibody was a F(ab')<sub>2</sub> fragment. (G) ACA4-labelling; (H) m-Rab<sub>mc</sub> labelling; (I) merged images. Note the almost complete co-localisation of ACA4 and m-Rab<sub>mc</sub> (yellow).

careful control experiments had to be set up because both antisera were made in rabbit. To eliminate unspecific binding of secondary antibodies, we used a protocol described by Paris et al. (Paris et al., 1997). Cells were incubated with m-Rab<sub>mc</sub> antiserum, followed by incubation with an excess of anti-rabbit F(ab')<sub>2</sub>-Cyanine3. After fixation of the complexes with 3.7% paraformaldehyde, cells were treated with IgG anti-rabbit-FITC. No FITC staining could be detected in this control experiment, indicating that all binding sites for IgG on the m-Rab<sub>mc</sub> complexes were saturated by F(ab')<sub>2</sub> fragments (data not shown). The same negative control results were obtained using ACA4 as first antibody.

A single image of a BY-2 cell shows that co-localisation of sub-micron structures labelled by ACA4 antiserum (Fig. 4G) and m-Rab<sub>mc</sub> antisera (Fig. 4H) is almost total (Fig. 4I). These results clearly show that ACA4 and m-Rab<sub>mc</sub> proteins share the same location within the cell. They are predominantly located on the PVC but also associated with some Golgi stacks.



**Fig. 5.** Effects of the m-Rab<sub>mc</sub>(N147I) mutant. *A. thaliana* protoplasts were transformed with either wild-type m-Rab<sub>mc</sub>-CFP (A,F,H), the dominant negative mutant m-Rab<sub>mc</sub>(N147I)-CFP (B,G,I), untagged wild-type m-Rab (C) or m-Rab<sub>mc</sub>(N147I) (D,E). Fluorescent markers for the different cellular destinations were pseudocoloured in red (aleurain-GFP, chitinase-YFP, FM4-64) with the exception of ST-YFP (C-E, green). Expression of the wild-type and mutant m-Rab<sub>mc</sub> protein was assessed by monitoring the CFP fluorescence (A,B and F-I). Confocal analysis was performed 16 hours after transformation. Scale bars: 5 µm. The white arrowhead indicates a ring-like structure. Lv, lytic vacuole. (A,B) Wild-type m-Rab<sub>mc</sub>-CFP punctate pattern (A, green) and aleurain-GFP vacuolar signal (A, red) in *A. thaliana* protoplasts. The expression pattern of the dominant negative mutant m-Rab<sub>mc</sub>(N147I)-CFP is cytosolic (B, green) and causes a punctate aleurain-GFP pattern (B, red). (C-E) ST-YFP-labelled Golgi stacks (green) and aleurain-GFP labelling (red) in protoplasts expressing wild-type m-Rab<sub>mc</sub> (C) or mutant-expressing protoplasts (D,E). (D) Top view of a cell and (E) an image taken from the middle of the cell. Note the lack of aleurain-GFP staining in the lytic vacuole in the mutant-expressing protoplasts (D,E). (F,G) Wild-type punctate m-Rab<sub>mc</sub>-CFP staining (F, green) or cytosolic mutant m-Rab<sub>mc</sub>(N147I)-CFP pattern (G, green) and chitinase-YFP vacuolar pattern (F,G, red). The mutant has no effect on the transport of chitinase-YFP. (H,I) Internalisation of FM4-64 (H,I, red) in sub-micron structures in wild-type (H, green) or mutant-expressing protoplasts (I, green). The staining pattern is not affected by the expression of the mutant m-Rab<sub>mc</sub>(N147I).

#### Effect of the dominant negative mutant m-Rab<sub>mc</sub>(N147I) on the trafficking of aleurain-GFP in *A. thaliana* protoplasts

The location of m-Rab<sub>mc</sub> on a prevacuolar compartment of the lytic vacuole and on the Golgi apparatus suggests that m-Rab<sub>mc</sub> might be involved in the trafficking of proteins between these two compartments. To investigate this hypothesis, we generated a dominant inhibitory m-Rab<sub>mc</sub>(N147I) mutant containing a single amino acid substitution, asparagine to isoleucine, in the conserved GTP binding motif GNKxD. Another plant Rab-GTPase mutant, AtRab1b(N121I), has been shown to have a dominant negative effect when expressed in plant cells by inhibiting the activity of the wild-type GTPase (Batoko et al., 2000). We selected aleurain-GFP that is targeted to an acidic lytic vacuole (Di Sansebastiano et al., 2001) as a soluble marker protein to investigate the effect of m-Rab<sub>mc</sub>(N147I) on intracellular trafficking events.

Before examining the vacuolar trafficking of aleurain-GFP in the presence of the mutant protein m-Rab<sub>mc</sub>(N147I), we checked protoplasts transformed with wild-type m-Rab<sub>mc</sub> and aleurain-GFP. The staining pattern of the wild-type m-Rab<sub>mc</sub>-CFP was punctate (Fig. 5A, left panel) and the protoplasts showed a vacuolar distribution pattern of aleurain-GFP similar to that described in the literature with most of the GFP staining present in the central vacuole (Di Sansebastiano et al., 2001; Sohn et al., 2003) and some more patchy staining in the cytosol (Di Sansebastiano et al., 2001), probably representing pro-

vacuoles (Fig. 5A, right panel). Protoplasts expressing wild-type m-Rab<sub>mc</sub>-CFP were fixed and labelled with the BP80 antibody to investigate the effect of m-Rab<sub>mc</sub>-CFP over-expression on the prevacuolar compartment. It seems that these protoplasts have a normal prevacuolar pattern (Fig. S3, <http://jcs.biologists.org/supplemental/>). Next we examined the aleurain-GFP pattern in the presence of the dominant negative mutant m-Rab<sub>mc</sub>(N147I) fused to CFP. The mutant m-Rab<sub>mc</sub>(N147I)-CFP shows a cytosolic labelling pattern (Fig. 5B, left panel). Protoplasts that expressed m-Rab<sub>mc</sub>(N147I)-CFP lacked completely the vacuolar staining pattern of aleurain-GFP with a few punctate stains in the cytosol (Fig. 5B, right panel). This finding indicates that m-Rab<sub>mc</sub>(N147I) might inhibit the transport of aleurain-GFP to the central vacuole.

#### Effect of m-Rab<sub>mc</sub>(N147I) on the trafficking of the Golgi marker ST-YFP and the neutral vacuole marker chitinase-YFP in *A. thaliana* protoplasts

In order to dissect in more detail the blockage site, we co-expressed the mutant protein m-Rab<sub>mc</sub>(N147I) with the Golgi marker ST-YFP in *A. thaliana* protoplasts. First, we monitored the ST-YFP staining pattern in protoplasts expressing the wild-type m-Rab<sub>mc</sub>, (Fig. 5C) and in protoplasts expressing either the CFP-tagged m-Rab<sub>mc</sub>(N147I) (not shown) or the untagged mutant protein (Fig. 5D,E). Neither the wild-type m-Rab<sub>mc</sub>,

nor mutant m-Rab<sub>mc</sub>(N147I) affected the distribution and motility of the Golgi-marker ST-YFP. We observed a normal Golgi pattern with highly motile Golgi stacks. These findings indicate mutant m-Rab<sub>mc</sub>(N147I) does not affect the transport of proteins from the ER to the Golgi apparatus.

We then investigated the identity of aleurain-GFP-labelled structures by co-expression of the mutant m-Rab<sub>mc</sub>(N147I) with aleurain-GFP and ST-YFP. We first checked the co-expression of aleurain-GFP and ST-YFP to determine whether the expression of ST-YFP may influence the aleurain-GFP targeting to the vacuole. We observed a normal vacuolar pattern for aleurain-GFP as described before (Fig. 5C, red). After co-expressing the unlabelled mutant m-Rab<sub>mc</sub>(N147I) with aleurain-GFP (Fig. 5D,E, red) and ST-YFP (Fig. 5D,E, green), the vacuolar staining pattern of aleurain-GFP disappeared completely. We observed a punctuate aleurain-GFP staining sometimes arranged around ring-like structures that were identified as small vacuoles by differential interference contrast microscopy (not shown). We did not observe a co-localisation of aleurain-GFP and ST-YFP after expressing the mutant protein. These findings indicate that the inhibition of aleurain-GFP transport does not happen in the GA but in an unidentified post-Golgi compartment.

To investigate if m-Rab<sub>mc</sub>(N147I) specifically inhibits the transport of proteins targeted to the lytic vacuole, we examined the targeting of chitinase-YFP in the presence of m-Rab<sub>mc</sub>(N147I). Tobacco chitinaseA is targeted to a vacuolar compartment with a neutral pH (Di Sansebastiano et al., 2001). In protoplasts co-transformed with wild-type m-Rab (Fig. 5F, left panel), and chitinase-YFP, a typical vacuolar staining pattern of several small vacuoles was observed with the chitinase-YFP (Fig. 5F, right panel). This pattern was similar to that recently described as peripheral vacuoles in tobacco mesophyll protoplasts (Di Sansebastiano et al., 2001). The differential interference contrast images revealed larger vacuoles not stained by chitinase-YFP, which are probably lytic vacuoles (data not shown). Co-expression of the mutant m-Rab<sub>mc</sub>(N147I)-CFP (Fig. 5G, left panel) did not affect the chitinase vacuolar staining pattern (Fig. 5G, right panel) in any *A. thaliana* protoplasts. This observation confirms that m-Rab<sub>mc</sub>(N147I) blocks neither the transport of proteins from the ER to the GA, nor the transport of proteins to neutral vacuoles. It indicates that m-Rab<sub>mc</sub>(N147I) acts specifically in the transport of cargo to the lytic vacuole.

#### Effect of the m-Rab<sub>mc</sub>(N147I) on the internalisation of the fluorescent dye FM4-64

In addition, we investigated the effect of the dominant negative mutant m-Rab<sub>mc</sub>(N147I) on the endocytic pathway. FM4-64 has been reported as an internalisation marker in plant cells (Ueda et al., 2001; Emans et al., 2002). We transformed *A. thaliana* protoplasts either with wild-type m-Rab<sub>mc</sub>-CFP or mutant m-Rab<sub>mc</sub>(N147I)-CFP and, 16-24 hours later, monitored the internalisation of FM4-64 after a 30-minute incubation. In wild-type m-Rab<sub>mc</sub>-expressing protoplasts, we observed a punctuate cytosolic FM4-64 staining and staining of the plasma membrane similar to the patterns described by Ueda et al. (Ueda et al., 2001) (Fig. 5H, right panel). The FM4-64 internalisation was then monitored in the presence of the mutant protein m-Rab<sub>mc</sub>(N147I) (Fig. 5I). FM4-64 was again

seen in a patchy cytosolic pattern in addition to the plasma membrane staining similar to that observed in wild-type-expressing cells (Fig. 5I, right panel). This finding indicates that m-Rab<sub>mc</sub>(N147I) does not inhibit the internalisation of the fluorescent dye FM4-64. Interestingly, when FM4-64 internalisation studies were performed either on BY-2 cells or in *A. thaliana* protoplasts expressing ST-GFP, or m-Rab<sub>mc</sub>-CFP, co-location of the dye with both GA and m-Rab<sub>mc</sub>-stained structures were observed (Bolte et al., 2004).

#### Discussion

m-Rab<sub>mc</sub> is a putative plant-specific Rab-GTPase with high homology to conventional Rab proteins but with some unique features that distinguish it strongly from this protein family. The most striking difference is the lack of the C-terminal isoprenylation motif characteristic of conventional Rab proteins. This isoprenylation motif that allows membrane attachment of the protein is replaced by a N-terminal myristoylation motif (Borg et al., 1997; Bolte et al., 2000) and a N-terminal palmitoylation motif (Ueda et al., 2001). These findings have recently been confirmed by Ueda and co-workers, who have shown that in a closely related m-Rab homologue from *A. thaliana* (Ara6), N-myristoylation and palmitoylation occurs and that it is responsible for membrane attachment of the protein (Ueda et al., 2001). The fact that N-myristoylated Rab proteins do not exist in animals and yeast leads to the fundamental question of their plant-specific function. A plant-specific transport pathway may be related to the specific compartmentation of plant cells in the occurrence of at least two functionally distinct vacuoles (Paris et al., 1996; Jauh et al., 1999). Indeed, sorting and targeting mechanisms for soluble and membranous vacuolar proteins in plant cells are distinct from those of yeast and mammals (Vitale and Chrispeels, 1992; Jiang and Rogers, 1998; Brandizzi et al., 2002b). Intracellular location of m-Rab<sub>mc</sub> and its potential association with endomembrane markers, together with the effects of the mutated form on reporter proteins lend support to the discussion of a plant-specific function of this protein.

#### m-Rab<sub>mc</sub> is predominantly located on the PVC of the lytic vacuole

m-Rab<sub>mc</sub> co-localised with 87±4% of BP80-marked structures, and 89±4% of AtPep12p, two markers for the PVC (Paris et al., 1997; Conceição et al., 1997; Sanderfoot et al., 2000; Li et al., 2002). This indicates a preferential location of m-Rab<sub>mc</sub> on the PVC.

The staining pattern described for m-Rab<sub>mc</sub> and other markers related to vacuole dynamics have been given different names, such as 'prevacuolar compartments' (Paris et al., 1997) or 'hot spots on small vacuoles' (Geisler et al., 2000). Ueda and co-workers also described a similar staining pattern for the *A. thaliana* m-Rab<sub>mc</sub> homologue, Ara6. A GFP-labelled Ara6 protein stained highly motile structures, comparable to m-Rab<sub>mc</sub>-CFP staining. Because they were stained by FM4-64, they were named 'early/late endosomes' (Ueda et al., 2001). These terms may cover the same membranous compartment involved in the sorting of proteins towards their final destination, the lytic vacuole. As discussed below, we suggest



that m-Rab<sub>mc</sub> may be implicated in the dynamics of these prevacuolar compartments.

### Is m-Rab<sub>mc</sub> also associated with the Golgi apparatus?

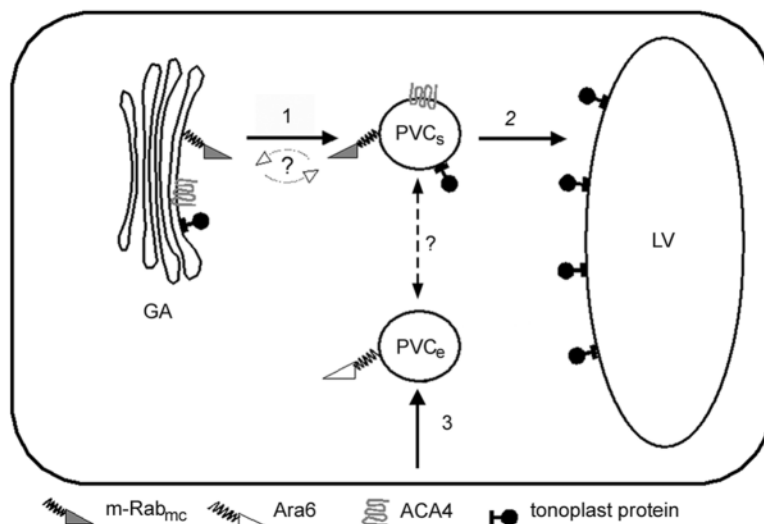
Immunocytochemistry has shown that the association of m-Rab proteins recognised by the m-Rab<sub>mc</sub> antiserum with the GA appears to differ between cell types. The association varies from 20% in BY-2 cells to 85% in *A. thaliana* protoplasts. This variation may be due to physiological differences or to the presence of different isoforms or homologues of m-Rab proteins in various plant cells. However, when using transient expression in *A. thaliana* protoplasts, similar GA associations of m-Rab<sub>mc</sub>-CFP were observed. This 85% GA association was true for protoplasts co-expressing m-Rab<sub>mc</sub>-CFP and ST-YFP as well as for endogenously detected m-Rab<sub>mc</sub> in ST-YFP-expressing protoplasts. Indeed, our biochemical assays indicate that the expression of m-Rab<sub>mc</sub> varies in different plant tissues (data not shown).

The partial Golgi association of m-Rab<sub>mc</sub> is in accordance with data from the literature showing that a fraction of PVC markers may be found with the GA. Studies using confocal laser scanning microscopy have shown BP80 location on ~10% of the Golgi stacks (Li et al., 2002): it was proposed that this mirrored a very transient association of BP80 on the GA (Li et al., 2002). Electron microscopy studies also revealed that BP80 was located on the most *trans*-face of the GA and on the PVC (Paris et al., 1997). It was further shown that BP80 was associated with a fraction enriched in clathrin-coated vesicles (Hinz et al., 1999). These data strongly suggested that BP80 was involved in the regulation of vacuolar proteins on the way to the lytic vacuole, shuttling between the most *trans*-face of the Golgi and the prevacuolar compartment (Kirsch et al., 1994; Humair et al., 2001). We hypothesize that m-Rab<sub>mc</sub> may also shuttle between the most *trans*-face of the GA and the PVC similar to BP80 (Fig. 6). Then in accordance with the model of vesicle traffic coordination by Rab proteins (reviewed by Rodman and Wandinger-Ness, 2000), m-Rab<sub>mc</sub> might regulate sequentially the budding, transport and docking of vesicles between these two compartments.

### m-Rab<sub>mc</sub> blocks specifically the transport of cargo to the lytic vacuole

m-Rab<sub>mc</sub> shows highest homology to members of the Rab5 family. Several members of this family, such as the Rab5 homologues Rha1 and Ara7, have been described so far in plants (Anuntalabhochai et al., 1991; Ueda et al., 2001; Sohn et al., 2003).

Very recently, using a transient *A. thaliana* protoplast assay, Sohn et al. (Sohn et al., 2003) have shown an involvement of Rha1 and Ara7 in the transport of sporamin-GFP and AALP-GFP, two markers for lytic vacuoles. Our study, based on the same *A. thaliana* protoplast assay, indicates a similar function of m-Rab<sub>mc</sub>. Since m-Rab<sub>mc</sub> co-located with markers for the vacuolar pathway such as BP80 or Pep12, and the dominant



**Fig. 6.** Location and putative function of m-Rab<sub>mc</sub> in plant cells. The compartment marked by BP80, m-Rab<sub>mc</sub> and the calcium ATPase ACA4 is named the secretory prevacuolar compartment (PVC<sub>s</sub>). The location of m-Rab<sub>mc</sub> on the PVC, its partial association with the GA and its function in aleurain transport to the lytic vacuole suggest an involvement in GA/PVC trafficking. m-Rab<sub>mc</sub> might regulate the transport of vacuolar proteins from the GA to the PVC (1) that in turn would fuse with the lytic vacuole (LV) to deliver cargo and presumably tonoplast proteins (2). Dotted arrows with open arrowheads indicate a putative recycling event of m-Rab<sub>mc</sub> between the GA and the prevacuolar compartment. Ara6, the *A. thaliana* homologue of m-Rab<sub>mc</sub>, has been assigned to FM4-64-labelled sub-micron structures called endosomes (Ueda et al., 2001) (3). These structures are named endocytic prevacuolar compartments (PVC<sub>e</sub>) in this model. The broken double-headed arrow respects the possibility that the PVC<sub>s</sub> and PVC<sub>e</sub> may be the same compartment.

negative mutant blocked the transport of aleurain-GFP to the lytic vacuole, we favour the idea that m-Rab<sub>mc</sub> is indeed specifically associated with the regulation of transport of vacuolar proteins coming from the *trans*-side of the GA en route to the lytic vacuole. Two features still remain to be elucidated: the nature of the aleurain-GFP accumulating compartments and the low level of aleurain-GFP after expression of the mutant m-Rab<sub>mc</sub>(N147I). It is possible that the compartments where the transport of aleurain-GFP is blocked might represent a form of post-Golgi/prevacuolar compartment. Attempts to check this by immunocytochemistry failed so far because of the instability of the mutant-expressing protoplast during fixation. Co-expression of m-Rab<sub>mc</sub>(N147I)-CFP with BP80-YFP resulted in a normal prevacuolar pattern (S.B. et al., unpublished results), indicating that the PVC formation seems not to be affected by the mutant. However, in this co-expression experiment, we cannot exclude that BP80-YFP might be mis-targeted by the mutant. Another possibility is that the level of aleurain-GFP in protoplasts expressing the dominant negative m-Rab<sub>mc</sub> mutant is very small. However, m-Rab<sub>mc</sub> (N147I) does not cause a build up of aleurain-GFP in the ER. Such a build up was described by Batoko et al. (Batoko et al., 2000) for sec-GFP in tobacco epidermal cells expressing a dominant negative mutant of Rab1, a protein regulating ER to Golgi transport. m-Rab<sub>mc</sub>(N147I) might instead cause a secretion of aleurain-GFP into the medium. This effect was described for a dominant negative mutant form of Rha1 (Sohn

et al., 2003) in *Arabidopsis* protoplast. It is very likely that the m-Rab<sub>mc</sub> dominant negative mutant has a similar effect, as it seems to act in the same transport pathway.

#### The vacuolar pathway versus the endocytic pathway

The N-myristoylated Rab protein Ara6, showing 84% homology to m-Rab<sub>mc</sub> has been described in *A. thaliana*, but it is not known whether the location and function of m-Rab<sub>mc</sub> is different from the location and function of Ara6.

Regarding the location, the question is still open. The putative endosomal localisation of Ara6 has been mainly assessed by co-labelling with structures that were labelled with FM4-64 after 30 minutes of internalisation. However, FM4-64 fails to be a specific marker for endosomes, as it stains ST-YFP-labelled Golgi stacks after 30 minutes of internalisation in *A. thaliana* protoplasts and also in BY-2 cells (Bolte et al., 2004). Since FM4-64 co-localisation is not sufficient to assess the nature of an endomembrane compartment, it may well be that the PVC stained by BP80/AtPep12p/m-Rab<sub>mc</sub> is the same compartment as that stained by Ara6. The exact relationship between endosomes and PVC is still a subject of debate (Jürgens and Geldner, 2002). The prevacuolar compartment might function as a junction between the endocytic pathway and the vacuolar route of the secretory pathway (Fig. 6). This is the case for the multivesicular bodies in developing pea cotyledons, which accumulate tracers for endocytosis and vacuolar proteins (Robinson and Hinz, 1999). Similarly, the cisternae of the GA may also accumulate tracers for endocytosis (Tanchak et al., 1984), besides their activity of exporting cargo towards the plasma membrane or the vacuole. Interestingly, several plant homologues to mammalian endocytosis regulators have been localised on the pathway between the GA and PVC in plants (Conceição et al., 1997; Sanderfoot et al., 1998; Kim et al., 2001; Jin et al., 2002). Furthermore it is reported that some Rab proteins may regulate two vectorial pathways (Press et al., 1998; Gerrard et al., 2000a; Gerrard et al., 2000b). In yeast for instance, the Rab homologue Vps21 and the syntaxin homologue Pep12p regulate vacuolar delivery of transport vesicles deriving from the *trans*-Golgi network and endocytosis (Gerrard et al., 2000a; Gerrard et al., 2000b). Endocytosed nanogold particles are found in a Pep12-positive prevacuolar compartment in yeast cells (Prescianotto-Baschong and Riezman, 2002). The mammalian Rab7 is supposed to regulate the flux of proteins into and out of the late endosome (Press et al., 1998).

Regarding the function of m-Rab proteins, much has still to be determined. First reports (Ueda et al., 2001; Sohn et al., 2003) (this work) suggest that m-Rab<sub>mc</sub> and Ara6 may assume distinct functions. Our functional data based on aleurain transport indicate that m-Rab<sub>mc</sub> is involved in the regulation of an anterograde movement from the GA to the lytic vacuole. In contrast, functional studies with a different dominant negative mutant of Ara6, (S47N), based on sporamin transport, showed that Ara6 might not be involved in this secretory pathway to the lytic vacuole. However, aleurain and sporamin are targeted to the vacuole with different kinetics (Sohn et al., 2003) and the affinity of the two proteins to vacuolar sorting receptors like AtELP and BP80 was shown to differ strongly (Kirsch et al., 1994, Ahmed et al., 2000). Whether these differences in the block by either Ara6 or m-Rab<sub>mc</sub> reflects these differences

in kinetics and receptor affinities remains to be explored. These observations indicate differences in the transport of these proteins to the lytic vacuole and might explain the differences in the block by either Ara6 or m-Rab<sub>mc</sub>. Since the potential association of Ara6 with the PVC and the GA has not yet been investigated, it cannot be excluded that Ara6 and m-Rab<sub>mc</sub> may label the same compartments. Despite their high homology, they may also have discrete functions (Ueda et al., 2001) as it is the case for some yeast Rab5 homologues (Singer-Krueger et al., 1994). Furthermore, EST databases indicate that m-Rab<sub>mc</sub> has another isoform in *Mesembryanthemum crystallinum*. It may well be that this second m-Rab<sub>mc</sub> isoform exhibits Ara6 functions.

#### The possible plant specific physiological function of m-Rab<sub>mc</sub>

Two lines of evidence suggest that PVC compartments in plant cells may have a regulatory role in stress-induced adaptation of the vacuolar system, namely in salinity stress. Firstly, it was recently shown that phosphatidylinositol 3-phosphate plays a role in vesicle trafficking from the GA to the lytic vacuole via the PVC in plant cells (Kim et al., 2001). Phosphatidylinositol 3-phosphate derivatives are elements of the osmosensory signal pathway in plants (Kearns et al., 1998; Monks et al., 2001). Secondly, the prevacuolar compartment is supposed to play a large role in salinity tolerance in yeast (Apse et al., 1999; Gaxiola et al., 1999). Over-expression of the Ca<sup>2+</sup>-ATPase ACA4 in yeast provided salinity tolerance (Geisler et al., 2000). In this paper we show for the first time that ACA4 is located preferentially on the PVC of the lytic vacuole. This is reminiscent of the *A. thaliana* homologue of a yeast Na<sup>+</sup>/H<sup>+</sup>-antiporter, which is localised on the yeast PVC (Gaxiola et al., 1999) and was also demonstrated to provide salinity tolerance to *A. thaliana* after over-expression, showing that the PVC might be an important regulator of salinity tolerance in plants as in yeast.

ACA4 and m-Rab<sub>mc</sub> co-localise almost perfectly. Furthermore, previous data show that salinity stress substantially increased the level of m-Rab<sub>mc</sub> and ACA4 in different plant species and also increased the number of m-Rab<sub>mc</sub>-labelled prevacuoles [(Bolte et al., 2000) S.B., unpublished results] (Geisler et al., 2000)]. We are currently investigating whether m-Rab<sub>mc</sub> is also regulating the transport of membrane proteins implicated in salinity stress adaptation at the tonoplast. We have evidence that the dominant negative mutant of m-Rab<sub>mc</sub> blocks the transport of several of these tonoplast-located proteins (S.B. and S. Thomine, unpublished results). These data suggest that m-Rab<sub>mc</sub> might be responsible for modulating transport events at the PVC level to allow the lytic vacuole and namely the tonoplast to adapt to altered growth conditions. m-Rab<sub>mc</sub>(N147I) and its homologues will thus provide valuable tools to investigate the targeting and transport of vacuolar proteins.

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