AtRAB-H1\textsuperscript{b} and AtRAB-H1\textsuperscript{c} GTPases, homologues of the yeast Ypt6, target reporter proteins to the Golgi when expressed in \textit{Nicotiana tabacum} and \textit{Arabidopsis thaliana}

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Abstract

Ypt/Rab GTPases act as key regulators of intracellular traffic through the conformational differences exhibited by their GTP or GDP-bound forms. In this paper, two \textit{Arabidopsis} Ypt\textsubscript{6} homologues, AtRAB-H1\textsuperscript{b} and AtRAB-H1\textsuperscript{c} were characterized and compared. Using a live cell imaging approach, it is shown that yellow fluorescent protein-fusions (YFP) of AtRAB-H1\textsuperscript{b} and AtRAB-H1\textsuperscript{c} locate to the Golgi and to the cytosol in both \textit{Nicotiana tabacum} and \textit{Arabidopsis thaliana}. In addition, YFP-AtRAB-H1\textsuperscript{b} targets an as yet unknown compartment not labelled by YFP-AtRAB-H1\textsuperscript{c} or Golgi markers. It is also shown that the subcellular location of YFP-AtRAB-H1\textsuperscript{b} and YFP-AtRAB-H1\textsuperscript{c} is affected by the state of GTP-binding and that expression of a GTP-deficient mutant results in increased apoplastic fluorescence of a secretory form of YFP.

Key words: Confocal microscopy, GFP, Golgi apparatus, Rab GTPase.

Introduction

Since the late 1980s, small GTPases of the Rab subfamily have been known to be important regulators of intracellular membrane traffic (Olkkonen and Stenmark, 1997). They belong to the superfamily of small (21–25 kDa) Ras-related GTPases that have several structural motifs, designated PM1, PM2, PM3, G1, G2, and G3 (nomenclature according to Valencia \textit{et al.}, 1991), which are important for the binding and hydrolysis of GTP. The GTPases cycle between a GTP-bound and a GDP-bound state through the action of specific GTPase-activating (GAPs) proteins and guanine nucleotide exchange factors (GEFs) (Olkkonen and Stenmark, 1997). GTP-binding and hydrolysis is coupled to a cycle of membrane association and dissociation during the processes of vesicle formation, targeting, docking, and fusion. In addition, Rab proteins are intrinsically soluble proteins, whose activity is dependent upon the association with the cytoplasmic leaflet of cellular membranes. The membrane association is mostly mediated by post-translational C-terminal prenylation.

Fifty-seven loci that can encode Rab GTPases have been identified in the \textit{Arabidopsis} genome sequence (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002). Five putative isoforms of the Rab-H subgroup have been identified in \textit{A. thaliana}. Bednarek \textit{et al.} (1994) showed that AtRAB-H1\textsuperscript{b} (AtYpt\textsubscript{6}, AtRab\textsubscript{6}, At2g44610) was able to complement and rescue the yeast \textit{ypt6} deficient mutant. This cDNA clone was present in leaf, root, stem, and flower tissues suggesting a ubiquitous role for this protein. It was shown that a putative Rab\textsubscript{6} homologue from \textit{N. tabacum} (NtRab\textsubscript{6}) bound tightly to a membrane and could only be
solubilized with a detergent in tobacco leaf extracts (Haizel et al., 1995). Small amounts of NtRab6 were found in the soluble fraction. To date, there is no information about the functions of the Rab-H subclass in planta.

The function and location of Ypt6-related GTPase has been extensively studied in several species and members of the Rab family have been shown to decorate the Golgi and cytoplasmic vesicles in mammalian cells (Goud et al., 1990; White et al., 1999; Del Nery et al., 2006). The three known isoforms, Rab6A, Rab6A', and Rab6B have been shown to have different characteristics (Echard et al., 2000; Opdam et al., 2000; Mallard et al., 2002; Young et al., 2005; Del Nery et al., 2006). They have all been located to the Golgi, but their function and interaction with effector proteins seem to differ. Several different functions have been ascribed to both Rab6A and Rab6A'. Rab6A has been identified as a regulator of the COP-independent recycling route that carries Golgi-resident proteins and certain toxins from the Golgi to the ER (Martinez et al., 1997; Girod et al., 1999; White et al., 1999; Mallard et al., 2002). Over-expression of a GTPase-deficient Rab6A-mutant, Rab6A[Q72L], relocated Golgi-proteins from as far as the trans-Golgi network to the ER in a microtubule-dependent manner (Martinez et al., 1997; White et al., 1999; Young et al., 2005). The splice form variant of Rab6A, Rab6A', also participates in Golgi-to-ER recycling (Young et al., 2005). Both variants have been shown to act in the transport and targeting of exocytic vesicles to the cell periphery in HeLa cells (Grigoriev et al., 2007). Thus GFP-Rab6A and GFP-Rab6A' are abundantly present on exocytic vesicles that exit from the Golgi and move together with the microtubule plus-end-directed motor kinesin-1 along microtubules to the plasma membrane where they fuse to sites enriched in the Rab6-binding protein Rab6IP1.

Unlike Rab6A, Rab6A' does not bind rabkinexin6, a microtubule-binding protein important in cytokinesis (Hill et al., 2000; Fontijn et al., 2001) indicating a possible difference in their trafficking functions. In contrast to the Rab6A isoforms, which are ubiquitously expressed, Rab6B seems to have a tissue and cell-type specific role in mammalian cells (Opdam et al., 2000). Rab6B binds rabkinexin6 and locates to the Golgi and to ERGIC-53 positive vesicles in mammalian cells (Opdam et al., 2000).

The involvement of the yeast Rab6 homologue, Ypt6, in endosome-to-late Golgi trafficking has been well established (Tsukada et al., 1996; Bensen et al., 2001; Siniosoglou and Pelham, 2001). A specific role for Ypt6 in S. cerevisiae has been proposed where Ypt6 recruits the VFT complex to the late Golgi as well as on the early endosomes (Siniosoglou and Pelham, 2002), which allows for binding of endosome-derived vesicles with the late Golgi (Siniosoglou et al., 2000).

In this paper, data are presented showing differential subcellular locations and stability of the YFP-tagged plant Rab6 homologues AtRAB-H1b (At2g44610) and AtRAB-H1a (At4g39890) and mutants altered in their GTP and membrane binding capacity.

**Materials and methods**

**Isolation of AtRAB-H1b and AtRAB-H1c**

To isolate AtRAB-H1b and AtRAB-H1c cDNAs from A. thaliana, total RNA was extracted from leaves using the RNeasy Plant Mini Kit (Qiagen) and was used in a reverse transcription PCR (RT-PCR) procedure (described in Ausubel et al., 1999) with the primers: AT3NY (AtRAB-H1b): 5'-CGCGATCCGGCTGGTAAACGTTGAGGATTTT-TTAG-3' and AF3NY (AtRAB-H1c): 5'-CGCGATCCGGCTGGTAAACGTTGAGGATTTT-TTAG-3' and AF3NY (AtRAB-H1c). Both cDNAs were cloned into the binary vector pVKH18-En6 (Batoko et al., 2000) with either mGFP5 or EYFP (Clontech, Palo Alto, CA) at the N-terminus of AtRAB-H1b and AtRAB-H1c.

Mutations in functionally important regions of AtRAB-H1b and AtRAB-H1c were created with site-directed mutagenesis using AtRAB-H1b and AtRAB-H1c as templates. AtRAB-H1b[T23N], was designed using the primers: T23N5: 5'-CAATCCGTGGCAGAATTCATTTACATTTCGATCCTG-3' and AT3NY; T23N3: 5'-GAATCGGAATTAGTGAAGAATTCGACCCAGGCATGGTCACTACGTGTTTC-3'. The AtRAB-H1b[T23N] mutant was designed with the following primers: AFT23N5: 5'-CAATCCGTGGCAGAATTCATTTACATTTCGATCCTG-3' and AT3NY; T23N3: 5'-GAATCGGAATTAGTGAAGAATTCGACCCAGGCATGGTCACTACGTGTTTC-3' and AT3NY. The AtRAB-H1c[T23N] mutant was designed with the following primers: AFQ68L5: 5'-TGCTGGATCCGCAGGTCATGCTGAGAGAATTCATTTACATTTCGATCCTG-3' and AT3NY; T23N3: 5'-GAATCGGAATTAGTGAAGAATTCGACCCAGGCATGGTCACTACGTGTTTC-3' and AT3NY. The AtRAB-H1b[Q68L] was designed using the primers: Q68L5: 5'-TTGGGAATCCGAGGTCATGCTGAGAGAATTCATTTACATTTCGATCCTG-3' and AT3NY; Q68L3: 5'-ACATCCGTGGCAGAATTCATTTACATTTCGATCCTG-3' and AT3NY. The AtRAB-H1c[Q68L] mutant was designed with the following primers: AFQ68L5: 5'-TGCTGGATCCGCAGGTCATGCTGAGAGAATTCATTTACATTTCGATCCTG-3' and AT3NY; Q68L3: 5'-ACATCCGTGGCAGAATTCATTTACATTTCGATCCTG-3' and AT3NY.
Transient and stable expression in plants

For Agrobacterium tumefaciens-mediated transformation, strain GV3101 (pMP90) was used for infiltration into Nicotiana tabacum L. cv. SR1. Before infiltration, the plants were grown in a mix of multipurpose compost (Levingtons) and vermiculite in a ratio 3:1 in a greenhouse at 20–23 °C for 6 weeks. Infiltration was performed as previously described (Batoko et al., 2000; Geelen et al., 2002; Kotzer et al., 2004) with an inoculum at OD_{600} of 0.03 if not stated otherwise. Agrobacterium-mediated transformation of YFP-AtRAB-H1b and YFP-AtRAB-H1c into the A. thaliana strain Columbia was performed as described in Clough and Bent (1998).

Confocal imaging

Agrobacterium transiently transformed N. tabacum or A. thaliana stably transformed plants were examined using an inverted Zeiss Confocal Laser Scanning Microscope (CLSM) 510 (Zeiss, Welwyn Garden City, UK). Unless otherwise stated, transiently transformed cells of the lower epidermis of tobacco leaves were analysed 48–72 h after inoculation. This corresponds to 18–42 h after the onset of expression (Zheng et al., 2005). Images were collected using ×10 (dry), ×40 and ×63 oil immersion objectives. For imaging of GFP or YFP alone, a single excitation with a standard 488 nm laser line was used. For multi-track imaging, excitation lines 458 nm for GFP and 514 nm for eYFP were used alternately with line switching in the multi-tracking mode of the microscope (Kotzer et al., 2004; Sparkes et al., 2005). Fluorescence was detected using a 458 nm/514 nm dichroic beam splitter with 515 nm dichroic filter and 475–525 nm band pass filter for GFP and 535–590 nm/514 nm dichroic filter and 475–525 nm band pass filter for YFP and 535–590 nm band pass filter for eYFP. Controls were carried out to prevent cross-talk and bleed through of fluorescence. To image Nag-EGFP and YFP-AtRAB-H1b with an inoculum at OD_{600} of 0.03 if not stated otherwise. Agrobacterium-mediated transformation of YFP-AtRAB-H1b and YFP-AtRAB-H1c into the A. thaliana strain Columbia was performed as described in Clough and Bent (1998).

Western blot analysis

Plant tissue was homogenized in 0.4 N NaOH and 4% β-mercaptoethanol. Proteins in the 10 000 g supernatant were precipitated with 10% TCA (trichloroacetic acid), washed in acetone, and solubilized for SDS-PAGE (Laemmli, 1970). Transfer to nitrocellulose membranes and antibody incubations were done according to standard protocols. The blot was probed with a polyclonal antibody raised against GFP (1:2000 dilution, Molecular Probes, Leiden, The Netherlands). The reacting polypeptides were visualized with alkaline-phosphatase-conjugated secondary antibody (Dako, Denmark) and BCIP/NBT colour development substrate (Promeja, UK) according to the manufacturer’s instructions.

E. coli expression and GTP overlay assay

For expression of AtRAB-H1b wild-type and mutants in E. coli, the AtRAB-H1b wild-type, AtRAB-H1b[T23N], AtRAB-H1b[Q68L], and AtRAB-H1b[N212I] were used as templates. The primers, ATEXPR5: 5′-CGG GATCC-CATGGCTCCGGTCTCGGCACTC-3′ and ATEXPR3: 5′-CGCGGATCC CTC GAGACAGAGACATCCTCTGATTGCTGCTGAGC-3′ and ATEXPR5: 5′-CGG GATCC-CATGGCTCCGGTCTCGGCACTC-3′ and ATEXPR3: 5′-CGCGGATCC CTC GAGACAGAGACATCCTCTGATTGCTGCTGAGC-3′ were used including the restriction sites BamHI and XhoI, respectively (underlined). The amplified products were subcloned into the expression vector pET23b(+) containing a C-terminal His-tag sequence. The constructs were transformed into E. coli strain BL21 (DE3). Two hours after induction with IPTG, proteins were extracted from the soluble and insoluble fractions. AtRAB-H1b[wt] and AtRAB-H1b[Q68L] were found both in the soluble fraction and also deposited into inclusion
bodies in an approximate 1:1 ratio (data not shown). In contrast to the expected GTP-binding of recombinant proteins (wild-type and QL mutant), the AtRAB-H1\(^{B}[T23N]\) and AtRAB-H1\(^{C}[N122I]\) were only found in the insoluble fraction (data not shown). Since the inclusion body fractions were only partially enriched in the expressed protein, the recombinant proteins were purified before further analysis. The inclusion body fractions were solubilized in 6 M guanidinium hydrochloride and loaded onto Ni-NTA agarose spin columns. Equal amounts of purified AtRAB-H1\(^{B}[wt]\), AtRAB-H1\(^{B}[T23N]\), AtRAB-H1\(^{B}[Q68L]\), and AtRAB-H1\(^{C}[N122I]\) were loaded onto a SDS-gel. BSA was added as a control for the unspecific binding of GTP. The purified recombinant proteins were blotted onto a nitrocellulose membrane before incubation with radiolabelled \([x-3^{25}P]GTP\). The GTP-binding assay was performed according to the procedure of Celis et al. (1998).

**Results**

**Subcellular location of YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) in N. tabacum and A. thaliana**

To investigate the location of AtRAB-H1\(^{B}\) and AtRAB-H1\(^{C}\) in plants, green or yellow fluorescent protein (GFP/YFP) was fused to the N-terminus of AtRAB-H1\(^{B}\) (At2g44610) in plants, green or yellow fluorescent protein (GFP/YFP) to investigate the location of AtRAB-H1\(^{B}\) and AtRAB-H1\(^{C}\). This construction has proved successful with several Rab GTPases, as the fusion does not seem to obstruct the location of the protein of interest, AtRAB-H1\(^{B}\) and AtRAB-H1\(^{C}\) were generated. Fluorescence from AtRAB-H1\(^{B}\) (Fig. 1R) and the YFP-AtRAB-H1\(^{C}\) (data not shown) was more diffuse and did not co-locate with the ER (Fig. 1T).

Transgenic A. thaliana, plants expressing YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) were generated. Fluorescence from stably transformed YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) transgenics and both fusion constructs were seen to target punctate structures which were highly mobile in the main root, root hairs, and in leaves (Fig. 2A–D). In addition, cytosolic labelling was also observed, as seen in transiently transformed YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) tobacco leaves. Punctate structures labelled by YFP-AtRAB-H1\(^{B}\) extensively co-localized with the fluorescent Golgi marker Nag-GFP (Grebe et al., 2002) in root tips (Fig. 3A). To confirm that the labelled structures were Golgi, transgenic Rab-H1\(^{B}\) and Rab-H1\(^{C}\) A. thaliana were high pressure frozen, freeze-substituted, and immunogold-labelled with GFP anti-serum. Low levels of gold labelling of both constructs were observed on the Golgi. On average, three gold particles were found to be evenly distributed over mobile punctate structures with a smaller apparent diameter than the Golgi that were not labelled by ST-GFP (Fig. 1E, arrowhead). These smaller structures were not observed for YFP-AtRAB-H1\(^{C}\) (Fig. 1H); indicating a possible difference in targeting of the two AtRab-H isoforms. These smaller structures were also present when AtRAB-H1\(^{B}\) was fused to GFP (Fig. 1J–K, arrowhead) and they did not co-locate with a ST-YFP-fusion, suggesting that GFP/YFP-AtRAB-H1\(^{B}\) labels a separate compartment (Fig. 1E, K). Similar-sized structures have been observed when GONST1-GFP was transiently transformed into tobacco leaf epidermal cells (Handford et al., 2004). These were interpreted as artefacts of over-expression although here they were found at varying expression levels of GONST1-GFP. To investigate if these structures are the same as those labelled by YFP-AtRAB-H1\(^{B}\), the two constructs were co-expressed and confocal images were acquired 2 d after infiltration. YFP-AtRAB-H1\(^{B}\) co-located with the larger sized structures labelled by GONST1-GFP (Fig. 1L–N), which were earlier identified as Golgi stacks (Handford et al., 2004). However, YFP-AtRAB-H1\(^{B}\), in most instances, did not co-locate with the subpopulation of smaller structures labelled by GONST1-GFP (Fig. 1N, see arrowhead).

To investigate if the small punctate structures labelled with YFP-AtRAB-H1\(^{B}\) would co-locate with a marker of the prevacuolar compartment, YFP-AtRAB-H1\(^{B}\) was co-expressed with PS1-GFP (BP80-GFP). Little co-location was observed with this marker (Fig. 1 O–Q and insert in Q).

Residual labelling of a reticulate network was apparent when YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) were expressed in tobacco. To confirm the background labelling as cytosol and not ER, the constructs were co-expressed with the ER marker GFP-HDEL (Boevink et al., 1996) which marked as expected the polygonal network of short, interconnecting tubules and various shaped cisternae was observed (Fig. 1S). However, the fluorescence seen from the YFP-AtRAB-H1\(^{B}\) (Fig. 1R) and the YFP-AtRAB-H1\(^{C}\) (Fig. 1T) showed more diffuse and did not co-locate with the ER (Fig. 1T).

ST-GFP has previously been described in Boevink et al. (1998). PS1-GFP is a construct where GFP is fused to the transmembrane and the cytosolic sequence of pea BP80 (Paris et al., 1997). The construct is identical to BP19 described in Brandizzi et al. (2002) except that all the cytosolic amino acids are present. PS1-GFP has been shown to target the prevacuolar compartment when transiently transformed into tobacco leaf epidermal cells (Kotzer et al., 2004). FM 4-64 was added to Arabidopsis roots at 5 μM for 10–20 min prior to observation with BFA treatment at 25 μM for 30–40 min.

**Fluorescent markers**

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**Results**

**Subcellular location of YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\)** in N. tabacum and A. thaliana

To investigate the location of AtRAB-H1\(^{B}\) and AtRAB-H1\(^{C}\) in plants, green or yellow fluorescent protein (GFP/YFP) was fused to the N-terminus of AtRAB-H1\(^{B}\) (At2g44610) and AtRAB-H1\(^{C}\) (At4g39890). This construction has proved successful with several Rab GTPases, as the fusion does not seem to obstruct the location of the protein of interest, although function can be compromised (Ueda et al., 2001; Cheung et al., 2002; Kotzer et al., 2004; Lee et al., 2004; Preuss et al., 2004; Zheng et al., 2005; Chow et al., 2008). Tobacco leaves were inoculated with serial dilutions of Agrobacterium containing either YFP-AtRAB-H1\(^{B}\) or YFP-AtRAB-H1\(^{C}\) and the abaxial epidermis was imaged 48–72 h post-inoculation. At \textit{OD}_{600} of 0.03 or less, fluorescence from YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) was observed in mobile, punctate, structures approximately 0.5 μm in apparent diameter and also as diffuse cytoplasmic fluorescence (Fig. 1A, B). Co-expression with the Golgi marker ST-GFP (Boevink et al., 1998) revealed that YFP-AtRAB-H1\(^{B}\) and YFP-AtRAB-H1\(^{C}\) (Fig. 1C–E, Fig. 1F–H) were targeted to the Golgi in this system. Interestingly, YFP-AtRAB-H1\(^{B}\) also labelled a second subpopulation of
Fig. 1. YFP-AtRAB-H1b[wt] and YFP-AtRAB-H1c[wt] locate to the Golgi and the cytosol in tobacco leaf epidermal cells. Confocal images of transiently transformed tobacco leaf epidermal cells expressing (A) YFP-AtRAB-H1b[wt] (scale bar=10 μm) and (B) YFP-AtRAB-H1c[wt] (scale bar=5 μm) at OD_{600} of 0.03, 2 d after infiltration. (C–E) High magnification confocal images of a tobacco leaf epidermal cell expressing YFP-AtRAB-H1b[wt] (magenta image) (C) and ST-GFP (green image) (D) at OD_{600} of 0.03 of both constructs, 2 d after infiltration. Merged image (E) shows that two populations of YFP-AtRAB-H1b[wt]-labelled structures are present. Punctate structures that co-locate with ST-GFP (see arrow) and a population of smaller, bright, punctate structures that are not labelled by ST-GFP (see arrowhead). Scale bar=2 μm. (F–H) Confocal images of a tobacco leaf epidermal cell expressing YFP-AtRAB-H1c[wt] (magenta image) (F) and ST-GFP (green image) (G). Merged image (H) and insert show co-location with the Golgi-marker, ST-GFP (see arrow). YFP-AtRAB-H1c[wt] also labels the cytosol. Scale bar=5 μm, 2 μm in insert. (I–K) High magnification confocal images of ST-YFP (J) and GFP-AtRAB-H1b[wt] (I). Two populations of labelled structures are also present in the GFP-version of AtRAB-H1b[wt], one that co-located with ST-YFP (arrow) and some smaller structures not labelled by the Golgi marker (arrowhead) (merged image) (K). Scale bar=5 μm. (L–N) Confocal images of a tobacco leaf epidermal cell expressing YFP-AtRAB-H1b[wt] (magenta image) (L) and GONST1-GFP (M) (green image). YFP-AtRAB-H1b[wt] co-located with the larger structures targeted by GONST1-GFP (N) (arrow). YFP-AtRAB-H1b[wt] does not co-locate with the smaller structures targeted by GONST1-GFP (N) (arrowhead). Scale bar=5 μm. (O–Q) Confocal images of a tobacco leaf epidermal cell expressing YFP-AtRAB-H1c[wt] (magenta image) (O) and PS1-GFP (P) (green image). YFP-AtRAB-H1c[wt] did not co-locate with PS1-GFP (Q) and insert in (Q). Scale bar=5 μm, 2 μm in insert. (R–T) Confocal images of a tobacco leaf epidermal cell expressing YFP-AtRAB-H1b[wt] (magenta image) (R) and GFP-HDEL (S) (green image). YFP-AtRAB-H1b[wt] does not co-locate with GFP-HDEL in the ER (T). Scale bar=5 μm.
the Golgi stacks but were not found on other organelles (Fig. 2E, F).

When Brefeldin A (BFA) was applied to Arabidopsis root tips expressing Nag-EGFP and YFP-AtRAB-H1β (Fig. 3B, C), a portion of the YFP signal apparently dissociated from the Golgi marker and accumulated in the core of the BFA body that contains endosomal and TGN markers (Geldner et al., 2003; Dettmer et al., 2006). The partial relocation of

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**Fig. 2.** YFP-AtRAB-H1β[wt] and YFP-AtRabH1c[wt] locate to punctate structures and the cytosol in A. thaliana. Confocal images of A. thaliana stably expressing YFP-AtRAB-H1β[wt] and YFP-AtRAB-H1c[wt]. Images obtained from 2-week-old seedlings. (A) Low magnification image of A. thaliana leaf cells expressing YFP-AtRAB-H1β[wt]. Scale bar=100 μm. Insert show high magnification confocal image of an A. thaliana leaf cell expressing YFP-AtRAB-H1β[wt] in punctate structures (arrow) and cytosol. Scale bar in insert=5 μm. (B) Confocal image of A. thaliana root expressing YFP-AtRAB-H1β[wt] in punctate structures (arrow). Scale bar=10 μm. Insert show high magnification image of the root hair showing YFP-AtRAB-H1β[wt] expression in punctate structures (arrow). Scale bar in insert=10 μm. (C) High magnification image of an A. thaliana leaf cell expressing YFP-AtRAB-H1c[wt] in punctate structures (arrow) and cytosol. Scale bar=5 μm. (D) High magnification image of stem cells showing YFP-AtRAB-H1c[wt] expression in punctate structures (arrow) and cytosol. Scale bar=10 μm. (E) Electron micrographs of epidermal cells from a high-pressure frozen A. thaliana leaf stably transformed with YFP-AtRAB-H1β[wt] and immunolabelled with a polyclonal anti-GFP antibody. Gold particles are distributed across the Golgi stack. (F) Electron micrographs of epidermal cells from a high-pressure frozen A. thaliana leaf stably transformed with YFP-AtRAB-H1c[wt] and immunolabelled with polyclonal anti-GFP antibody showing a similar distribution of gold. Scale bar for (E) and (F)=200 nm.
YFP-AtRAB-H1\textsuperscript{b} to the BFA bodies was confirmed by labelling the endosomes and TGN with FM4-64. As shown in Fig. 3D, prior to BFA treatment, FM4-64 labelled compartments showed limited co-localization with YFP-AtRAB-H1\textsuperscript{b}, but were often in close proximity, as reported before for Golgi markers (Geldner \textit{et al.}, 2003; Dettmer \textit{et al.}, 2006; Chow \textit{et al.}, 2008). After a 30 min treatment with 25 \(\mu\text{M}\) BFA, YFP co-localized partially with FM4-64 in the core of the BFA body (Fig. 3E, F). Significant quantities of YFP-AtRAB-H1\textsuperscript{b} were excluded from the BFA bodies or remained associated with their periphery where Golgi stacks accumulated (Fig. 3B, C). Thus, a portion of YFP-AtRAB-H1\textsuperscript{b} may exist on BFA-sensitive Golgi-associated TGN or endosomal membranes.

\textbf{Location of AtRAB-H1\textsuperscript{b} and AtRAB-H1\textsuperscript{c} chimeras}

Rab GTPases cycle between an active, membrane-bound, GTP-bound form and an inactive cytosolic GDP-bound form. To investigate the nucleotide dependency of YFP-AtRAB-H1\textsuperscript{b} and YFP-AtRAB-H1\textsuperscript{c} labelling pattern, mutants were generated that were predicted to be deficient in GTP-binding, GTP hydrolysis, or nucleotide-binding. Exchanging the serine (S) or threonine (T) of the end of the PM1 region decreases the affinity for both GTP and GDP, but the affinity for GDP has been shown to be higher than for GTP (Jones \textit{et al.}, 1993; Stenmark \textit{et al.}, 1994; Macara and Brondyk, 1995; Olkkonen and Stenmark, 1997). To determine the effect of GDP-binding on the location of the two Rab-H isoforms, T23N mutants of YFP-AtRAB-H1\textsuperscript{b} and YFP-AtRAB-H1\textsuperscript{c} were transiently expressed in tobacco epidermal cells. Two to three days after expression of YFP-AtRAB-H1\textsuperscript{b}\textsuperscript{T23N}, mobile structures of approximately 0.5 \(\mu\text{m}\) were apparent, together with diffuse labelling of the cytosol (Fig. 4A). The YFP-AtRAB-H1\textsuperscript{b}\textsuperscript{T23N} labelled punctate structures co-located with the Golgi marker ST-GFP (Fig. 4B–D). However, introduction of the T23N mutation appeared to eliminate targeting to the smaller structures labelled by YFP-AtRAB-H1\textsuperscript{b}\textsuperscript{wt} (Fig. 4B–D).

Interestingly, when YFP-AtRAB-H1\textsuperscript{c}\textsuperscript{T23N}, was transiently transformed into tobacco leaf epidermal cells at the same expression level as the wild type, only the cytosolic
network was labelled (Fig. 4E). No other structures in the cell were observed to be targeted by this construct, irrespective of the expression level or the time (2–5 d after infiltration) of confocal analysis. When YFP-AtRAB-H1b[T23N] was co-expressed with GFP-HDEL, little or no accumulation on the ER could be detected (Fig. 4F–H). Thus YFP-AtRAB-H1b[T23N] is apparently cytosolic in contrast to the Golgi-labelling observed for YFP-AtRAB-H1b[T23N] (Fig. 4B–E).

GTPase-deficient mutants of several small GTPases have been generated by altering glutamate [Q] of the PM3 region to leucine [L] (Ueda et al., 2001; Kotzer et al., 2004; Lee et al., 2004). To investigate the effect of this mutation on the subcellular location of YFP-AtRAB-H1b, YFP-AtRAB-H1c, YFP-AtRAB-H1b[Q68L] and YFP-AtRAB-H1c[Q68L] were expressed in tobacco leaf epidermal cells. At 48 h post-inoculation, both mutants targeted Golgi and the cytosol (Fig. 4I–P). As with YFP-AtRAB-H1b[wt], YFP-AtRAB-H1c[Q68L] labelled a second structure (see arrowhead) not labelled by ST-GFP. Scale bar=5 μm. (M) Confocal micrograph of a tobacco leaf epidermis cell expressing YFP-AtRAB-H1c[Q68L] transiently transformed into tobacco leaf epidermal cells at OD600 of 0.03, 2 d after infiltration. The construct only labelled the cytosol. Scale bar=10 μm. (S) Confocal micrograph of a tobacco leaf epidermis cell expressing YFP-AtRAB-H1b[Q206,208S]. The construct is only in the cytosol. Scale bar=10 μm.
glutamate (Q) to leucine (L) at position 68 did not markedly change the distribution of YFP-AtRAB-H1). Similarly, YFP-AtRAB-H1\(^{\text{Q68L}}\) located like its wild-type counterpart, to the Golgi but not to the second population of smaller structures labelled by YFP-AtRAB-H1\(^{\text{b}}\) (Fig. 4N–P).

In mammalian cells, over-expression of Rab6A\(^{\text{Q72L}}\) and Rab6A\(^{\text{Q72L}}\) causes Golgi-located proteins to relocate to the ER in a microtubule-dependent manner (Martinez et al., 1997; Young et al., 2005). To determine if the same would occur in plants, Agrobacterium strains harbouring the YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{Q68L}}\) and YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{Q68L}}\) constructs were infiltrated at increased titres (up to OD\(_{600}\) 0.1) and co-expressed with the Golgi-markers ST-GFP, GONST1-GFP, and ERD2-GFP. No effect was observed on the Golgi-location of any of these marker proteins for either RabH construct (data not shown).

Mutation of asparagine to isoleucine in the G2 motif is predicted to reduce the affinity of the protein for both GDP and GTP (Olkkonen and Stenmark, 1997). Two days post-inoculation of tobacco leaves with YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{N122I}}\) and YFP-AtRAB-H1\(^{\text{c}}\)\(^{\text{N123I}}\), each protein exhibited purely cytosolic labelling (Fig. 4Q, R) irrespective of the relative expression level or the time of analysis. However, compared to the wild type and other mutants, only a few cells were observed to express YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{N122I}}\) and YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{N123I}}\), indicating that these mutants might be unstable when transiently expressed.

YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{wt}}\) is dependent on post-translational addition of geranylgeranyl groups for location to the Golgi

Most Rab proteins are tightly associated with membranes through the post-translational addition of two geranylgeranyl (20-carbon polyisoprenoid) groups to two conserved cysteines in the extreme C-terminus. (Olkkonen and Stenmark, 1997). These two cysteines (C206 and C208) in YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{wt}}\) were replaced with serine (S), creating YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{C206,208S}}\). When transiently expressed in tobacco leaf epidermal cells YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{C206,208S}}\) was found to be completely cytosolic 2 d after infiltration (Fig. 4S). This result is consistent with the lack of insertion into a membrane owing to the absence of isoprenyl groups.

**Stability of YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{wt}}\) is dependent on the state of GTP and GDP-binding**

To investigate the stability of YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{wt}}\) and the mutants, protein extracts were isolated after 2 d and 4 d from Agrobacterium-transformed YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{wt}}\), YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{T23N}}\), YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{Q68L}}\), YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{N122I}}\), and YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{C206,208S}}\) tobacco plants. The extracts were probed with anti-GFP antibodies to determine the stability of the YFP-fusions. In all cases, except for YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{N122I}}\), a single band of full-length protein including the YFP-fusion was detected indicating that each fusion-construct was stable and that cytosolic labelling did not reflect free YFP (Fig. 5A, B). By contrast, little or no cross-reacting protein was detected in extracts of YFP-AtRAB-H1\(^{\text{b}}\)\(^{\text{N122I}}\) expressing cells, consistent with the general lack of fluorescence (Fig. 5A, lane 8,9) Similar results were observed for YFP-AtRAB-H1\(^{\text{c}}\) wild type and mutants (data not shown).

AtRAB-H1\(^{\text{b}}\)\(^{\text{T23N}}\) and AtRAB-H1\(^{\text{b}}\)\(^{\text{N122I}}\) are unable to bind GTP

Bednarek et al. (1994) showed that both the wild-type and AtRAB-H1\(^{\text{b}}\)\(^{\text{C206,208S}}\) were able to bind GTP in a GTP overlay assay. Interestingly, it is shown here that the
putatively GDP-bound mutant, YFP-AtRAB-H1b[T23N], is able to bind to the Golgi while the YFP-AtRAB-H1b[Q68L] mutant was completely cytosolic. These results could indicate that the two Rab-H isoforms bind to the Golgi in a different manner, where AtRAB-H1b is able to bind the Golgi also in a GDP-bound state. Alternatively, AtRAB-H1b[T23N] may retain normal GTP-binding activity. To establish whether AtRAB-H1b[T23N] is indeed compromised in its ability to bind GTP, AtRAB-H1b[wt], AtRAB-H1b[Q68L], and AtRAB-H1b[N122I] were expressed in E. coli and analysed in a GTP overlay assay.

AtRAB-H1b[wt] and AtRAB-H1b[Q68L] were found to bind detectable amounts of [γ-32P]GTP (Fig. 6, lanes 1, 3). No binding of [γ-32P]GTP was observed for AtRAB-H1b[T23N] or AtRAB-H1b[N122I] (Fig. 6, lanes 2, 4) demonstrating that the specific mutations introduced into the proteins had indeed reduced their GTP-binding activities in this assay.

**Function of AtRAB-H1b** and **AtRAB-H1c**?

As an initial attempt to identify the function of AtRAB-H1b and AtRAB-H1c in plants, the two constructs were expressed in tobacco leaf epidermal cells in both a fluorescent-tagged form and untagged in case the tag inhibited or altered the function of the proteins. The constructs in either their wild-type or mutant forms were expressed along with a number of fluorescent markers locating to different organelles in the cell; ST-GFP (Golgi), GONST1-GFP (Golgi), ERD2-GFP (Golgi/ER), GFP-HDEL (ER), PS1-GFP (prevacuolar compartment), BobTIP-GFP (tonoplast), aleurain-GFP (lytic vacuole), and sporamin-GFP (lytic vacuole). Both AtRAB-H1b and AtRAB-H1c showed no observable effect in over-expression experiments on the distribution of markers targeted to the ER, the Golgi, the prevacuolar compartment, the vacuole, and to the plasma membrane (data not shown).

The effect of AtRAB-H1b and AtRAB-H1c on biosynthetic membrane traffic was investigated using a transport assay based on the transport of a secreted YFP, N-secYFP/pc (Geelen et al., 2002) from the ER to the apoplast where it accumulates poorly and exhibits weak fluorescence. This assay is similar to a transport assay using secreted GFP, secGFP, which has previously been used to characterize mutants of AtRAB-D2a, AtRAB-F2b, AtRAB-E1d, and SAR1 (Batoko et al., 2000; daSilva et al., 2004; Kotzer et al., 2004, 2005).

When an untagged form of AtRAB-H1b[N122I] was co-expressed with a plasmid that expresses both a secreted YFP marker (N-secYFP/pc; Geelen et al., 2002) and the ER-localized GFP marker GFP-HDEL from the same T-DNA (pVKH18-En6-GFP-HDEL/N-secYFP/pc (Fig. 7A) a significant increase in fluorescence of N-secYFP/pc was observed (Fig. 7B). Clear apoplastic fluorescence was observed at higher magnification (Fig. 7C). When the marker construct was expressed alone, most cells accumulated little or no detectable N-secYFP/pc as expected (Geelen et al., 2002), even though GFP-HDEL was readily detected (Fig. 7D, E). By contrast, co-expression of N-secYFP/pc with AtRAB-D2a[N122I] resulted in an increase in intracellular N-secYFP/pc fluorescence owing to the accumulation of N-secYFP/pc fluorescence in the ER (Fig. 7F, G). A similar result was obtained with the secreted GFP (data not shown). No increase in N-secYFP/pc fluorescence was observed when AtRAB-H1b[wt], AtRAB-H1b[T23N], AtRAB-H1b[Q68L], AtRAB-H1b[N122I], AtRAB-H1b[N123I] were co-expressed with the pVKH18-En6-GFP-HDEL/N-secYFP/pc plasmid (data not shown).

**Discussion**

Rab GTPases play an important role in membrane trafficking. However, in plants, we are only beginning to unravel the function of a few of the Rab proteins (Woollard and Moore, 2008). A Rab6 homologue from *A. thaliana* and *N. tabacum* was previously identified (Bednarek et al., 1994; Haizel et al., 1995). Bednarek and co-workers demonstrated that the plant Rab6 was functionally able to complement the yeast *yp60* mutant, suggesting a potentially similar role for the plant Rab6 in retrograde protein transport from endosomes to Golgi (Bednarek et al., 1994). Since then, four other members of this subclass have been identified and classified in *A. thaliana* as RAB-H1α to RAB-H1ε (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002).
Fig. 7. AtRAB-H1[N122I] causes a secreted fluorescent marker, N-secYFPΔc, to accumulate in the apoplast. (A) A schematic representation of a plasmid for expression of a secreted fluorescent marker (N-secYFPΔc, Geelen et al., 2002) and a fluorescent endoplasmic reticulum marker (GFP-HDEL) from the same T-DNA. GFP-HDEL is expressed from a promoter with six copies of the cauliflower mosaic virus (CaMV) 35S enhancer (35S-En6) while N-secYFPΔc is expressed from a simple 35S promoter. Both fusions carry a translational enhancer from Tobacco Mosaic virus (TMV Ω) in their 5' UTR and a polyadenylation signal from CaMV (polyA). (B) Low magnification confocal image of tobacco leaf epidermal cells expressing pVKH18-En6-GFP-HDEL/N-secYFPΔc and untagged AtRAB-H1[N122I]. Scale bar=100 μm (C) High magnification confocal image of tobacco leaf epidermal cells expressing pVKH18-En6-GFP-HDEL/N-secYFPΔc and untagged AtRAB-H1[N122I]. N-secYFPΔc fluorescence is clearly in the apoplast. Scale bar=20 μm (D) Low magnification confocal image of tobacco leaf epidermal cells expressing pVKH18-En6-GFP-HDEL/N-secYFPΔc alone. Scale bar=100 μm (E) High magnification confocal image of tobacco leaf epidermal cells expressing pVKH18-En6-GFP-HDEL/N-secYFPΔc alone. Only GFP-HDEL fluorescence can be seen in the ER with no apoplastic fluorescence of secreted YFP. Scale bar=20 μm (F) Low magnification confocal image of tobacco leaf epidermal cells expressing pVKH18-En6-GFP-HDEL/N-secYFPΔc and untagged AtRAB-D2[N1]. Scale bar=100 μm (G) High magnification confocal image of tobacco leaf epidermal cells expressing pVKH18-En6-GFP-HDEL/N-secYFPΔc and untagged AtRAB-D2[N1]. N-secYFPΔc fluorescence is restricted to the ER. Scale bar=20 μm.
Using Agrobacterium-mediated transient expression into tobacco leaf epidermal cells and stable expression in A. thaliana, it is shown that GFP/YFP-AtRAB-H1\textsuperscript{b}[wt] (AtYpt6; AtRab6; At2g44610) and YFP-AtRAB-H1\textsuperscript{c}[wt] locate to the Golgi. A background level of fluorescence in the cytosol of YFP-AtRAB-H1\textsuperscript{b}[wt] and YFP-AtRAB-H1\textsuperscript{c}[wt] was expected, due to the partially cytosolic nature of Rab GTPases. The Golgi location of the Rab6A protein in mammalian cells has been shown by immunogold labelling and by light microscopy (Goud et al., 1990; Antony et al., 1992). The addition of a fluorescent tag to the N-terminus of the protein did not obstruct the targeting of Rab6A to the Golgi in HeLa cells (White et al., 1999). Thus, it is reasonable to propose that the same would be true in plant cells.

**Location of YFP-AtRAB-H1\textsuperscript{b} and YFP-AtRAB-H1\textsuperscript{c} to the Golgi is dependent on the state of GTP-binding**

The GDP-bound forms of Rab GTPases are mainly cytosolic and associated with the GDP dissociation inhibitor (Olkkonen and Stenmark, 1997). By disrupting the specific threonine involved in GTP-binding in the conserved PM1 region it was shown that AtRAB-H1\textsuperscript{b} and AtRAB-H1\textsuperscript{c} would preferentially bind GDP and, thereby, be located to the cytosol rather than to the Golgi. The disruption of GTP-binding of the AtRAB-H1\textsuperscript{b}[T23N] mutant was demonstrated by expression of the mutant in E. coli, followed by a GTP overlay assay where the specific mutation rendered the protein unable to bind GTP.

Interestingly, in contrast to what may be expected, a substantial fraction of YFP-AtRAB-H1\textsuperscript{b}[T23N] was still targeted to the Golgi, while YFP-AtRAB-H1\textsuperscript{c}[T23N] was completely cytosolic when transiently transformed into tobacco leaf epidermal cells. This indicates that YFP-AtRAB-H1\textsuperscript{b}[T23N] may be recruited to the Golgi in its GDP-bound form. Luan et al. (1999) have shown that in S. cerevisiae it is possible for Rab GDI to be membrane-bound independently of Rabs. Similarly, in N. tabacum, YFP-AtRAB-H1\textsuperscript{b}[T23N] may bind a membrane-associated Rab GDI or alternatively YFP-AtRAB-H1\textsuperscript{b}[T23N] may interact with other Golgi-located proteins such as Golgi-localized members of the AtPRA1 family (Alvim-Kamei et al., 2008). A change in subcellular location was also observed for the PVC-located AtRAB-F2\textsuperscript{b}, and TGN/endosome-localized AtRAB-A2\textsuperscript{a} which were found to relocate to the Golgi in their GDP-bound form (Kotzer et al., 2004; Lee et al., 2004; Chow et al., 2008). By contrast, another Golgi-located RAB, AtRAB-E1\textsuperscript{d} has been demonstrated to be fully cytosolic when the equivalent mutation was introduced (Zheng et al., 2005). These differences may simply reflect the abundance of the binding partner of the GDP-form on the membrane.

Many studies of Rab proteins in higher eukaryotes have demonstrated that a specific point mutation within the region of the GTP-binding site results in a dominant active mutant protein, which has dramatically reduced GTPase activity but retains the affinity for GTP. Such mutants were designed for AtRAB-H1\textsuperscript{b} and AtRAB-H1\textsuperscript{c}. The continuing ability of AtRAB-H1\textsuperscript{b}[Q68L] to bind GTP was demonstrated by a GTP overlay assay where AtRAB-H1\textsuperscript{b}[Q68L] bound GTP to a similar extent as AtRAB-H1\textsuperscript{b}[wt]. When AtRAB-H1\textsuperscript{b}[Q68L] and AtRAB-H1\textsuperscript{c}[Q68L] were transiently transformed in tobacco leaf epidermal cells, the targeting of the mutant constructs was not found to be significantly different to that of the wild-type proteins. The mutant constructs were both observed to target the Golgi and to be present in the cytosol. In addition, YFP-AtRAB-H1\textsuperscript{b}[Q68L] labelled smaller structures as observed for the tagged wild-type protein YFP-AtRAB-H1\textsuperscript{b}[wt]. The consequence of reduced GTP hydrolysis in vivo is that the protein will remain active and membrane-associated longer than the wild type and be likely to recycle to the cytoplasm less efficiently than the wild type. Therefore it was rather surprising that the intracellular distribution observed for YFP-AtRAB-H1\textsuperscript{b}[Q68L] and YFP-AtRAB-H1\textsuperscript{c}[Q68L] was so similar to that of their wild-type proteins when observed by confocal microscopy. In other plant Rab GTPases, the GTP-bound mutant has been observed to have a different steady-state localization to that of the wild type (Ueda et al., 2001; Kotzer et al., 2004; Lee et al., 2004; Chow et al., 2008).

In mammalian HeLa cells, over-expression of the Rab6A wild-type and the GTP-bound mutants, Rab6A[Q72L] and Rab6A[Q72L] induced redistribution of the trans-Golgi protein β-1,4-galactosyltransferase (Gal-T) and α-mannosidase II into the ER and allowed the addition of sialylated O-glycans on an ER-retained protein, the major histocompatibility complex class II-associated invariant chain (Martinez et al., 1997). This redistribution was shown to be abolished in the presence of microtubule-disrupting drugs, which indicates that the effects of Rab6A[Q72L] require the integrity of microtubules (Martinez et al., 1997). Martinez and co-workers concluded that these results suggested that increased levels of the GTP-bound Rab6A affect the dynamics and homeostasis of the medial late Golgi trans-Golgi network compartment. As a further consequence, resident proteins of these compartments such as galactosyl transferase might be relocated to earlier Golgi compartments and from there be included in active retrograde traffic between the Golgi and ER (Martinez et al., 1997). However, in this study, no evidence of Golgi-marker relocation was observed when AtRAB-H1\textsuperscript{b}[Q68L] and AtRAB-H1\textsuperscript{c}[Q68L] were over-expressed and co-infiltrated with the Golgi-markers ST-GFP, ERD2-GFP, or GONST1-GFP.

In contrast to the AtRAB-H1\textsuperscript{b} wild-type, the GTP-deficient dominant negative mutant of AtRAB-H1\textsuperscript{b}, AtRAB-H1\textsuperscript{b}[N122I], was not able to rescue the S. cerevisiae ypt6 null mutant (Bednarek et al., 1994). The change of asparagine to isoleucine had obviously resulted in an alteration of the biochemical properties of the AtRAB-H1\textsuperscript{b} protein. In this current study, the E. coli expressed AtRAB-H1\textsuperscript{b}[N122I] protein was found to be unable to bind GTP in a GTP overlay assay. This instability of the Rab to bind GTP also seemed to make the fluorescent tagged protein, YFP-AtRAB-H1\textsuperscript{b}[N122I], unstable. A similar instability of the
dominant negative mutant has been reported for YFP-AtRAB-D2[N121I] (Rab1 homologue) and YFP-AtRAB-B1[N191I] (Rab2 homologue) (U Neumann, I Moore, unpublished results). This rapid degradation of the dominant negative N121I mutant is unlikely to be due to poor prenylation, as the form lacking the two prenylated cysteines was stable. Alternatively, the mutation could reduce the stability of the tertiary structure of the mutant protein as small GTPases can be unstable in the transient nucleotide-free state that is promoted by mutations of the N121I type (Olkkonen and Stenmark, 1997).

The AtRAB-H1b mutant, which lacked the C-terminal CXC residues, AtRAB-H1b[C206,208S], did not have altered nucleotide properties as demonstrated by Bednarek et al. (1994) where the AtRAB-H1b[C206,208S] mutant bound GTP to a similar extent as AtRAB-H1b[wt] in a GTP overlay blot assay. The cytosolic localization of this protein is consistent with the expectation that membrane binding of AtRAB-H1b[wt] is dependent on post-translational isoprenylation at the cysteine residues near the C-terminus.

What is the function of RAB-H1b and RAB-H1c in plants?

No observable effect could be seen in co-expression experiments with AtRAB-H1b and AtRAB-H1c with a range of different markers locating to the ER, Golgi, prevacuolar compartment, vacuole, or the plasma membrane. It could be that AtRAB-H1b and AtRAB-H1c have a more subtle effect on the intracellular markers tested than is detected by confocal microscopy. Young et al. (2005) showed that removal of either Rab6A or Rab6A' in mammalian cells through RNAi interference showed no marked difference on the Golgi distribution of several marker proteins, while reduced levels of Rab6A and Rab6A' together resulted in a clear effect on the Golgi distribution of GalNAC-T2, GM130, and TGN46. Similar knockout experiments have, to our knowledge, not been performed for the Rab-H isoforms in plants.

Recently, interactions between AtRAB-H1b and two plant trans-Golgi matrix proteins, a GRIP domain protein and a TMF homologue, have been reported by GST-pull downs, yeast-2-hybrid assays (Latijnhouwers et al., 2007; Osterrieder et al., 2009) and by fluorescence resonance energy transfer using fluorescence life-time imaging (Osterrieder et al., 2009). This is similar to what has been reported for mammalian cells (Fridmann-Sirkis et al., 2004, Burguete et al., 2008) and suggests a role for AtRAB-H1b in recruiting matrix proteins to the trans-Golgi cisternae.

In this study, N-secYFPΔc was used to analyse the effect of AtRAB-H1b and AtRAB-H1c in intracellular protein trafficking in a similar manner as described in Geelen et al. (2002), Kotzer et al. (2004), and Zheng et al. (2005). When dominant negative mutants of AtRAB-D2b, AtRAB-D2b[N121I], were co-expressed with secGFP, a clear increase in intracellular fluorescence were observed in the ER (Batoko et al., 2000). An increase in intracellular secGFP fluorescence intensity was also detected when AtRAB-E1b[N122I] was co-expressed with secGFP, however, this intensity was around half of what was observed in the presence of AtRAB-D2b[N122I] and accumulation of secGFP fluorescence was observed in the Golgi and in the PVC owing to inhibition of secretory traffic after the Golgi (Zheng et al., 2005). AtRAB-H1b[N122I] caused an increase in accumulated N-secYFPΔc fluorescence but, in contrast to other Rab GTPase mutants, this was observed exclusively in the apoplast. Neither AtRAB-H1b[wt] nor any form of the AtRAB-H1c isoform caused a similar effect on the secretion of N-secYFPΔc.

Why does N-secYFPΔc accumulate in the apoplast in the presence of the dominant negative mutant of AtRAB-H1b?

It has been shown that, in tobacco leaf epidermis, a significant fraction of secGFP is trafficked to the vacuole via the PVC (Zheng et al., 2005). One possibility therefore is that AtRAB-H1b[N122I] inhibits sorting into the vacuolar pathway resulting in increased secretion to the apoplast. Such a role would perhaps be similar to that of its yeast parologue Ypt6. However, AtRAB-H1b[N122I] failed to alter the targeting of GFP-based soluble vacuolar markers and no significant increase in apoplastic secGFP was observed in the same system when a dominant-negative RAB-F2b mutant was used to inhibit vacuolar sorting (Kotzer et al., 2004).

Alternatively, AtRAB-H1b[N122I] may alter the condition of the apoplast to favour fluorescence from secreted GFP and YFP. In Arabidopsis, the pool of secreted secGFP is not readily detected owing to the low pH and high rates of turnover in that environment (Zheng et al., 2004). The same is likely to be true in tobacco leaf epidermis and, consistent with this, it was observed that infiltration of the leaf with mildly alkaline buffers or incubation in the dark markedly increased the apoplastic fluorescence from secreted GFP and YFP proteins (our unpublished data). Therefore an alternative explanation for the increase of apoplastic fluorescence in the presence of AtRAB-H1b[N122I] is that this mutant, directly or indirectly, disrupts the flux of protons or proteases to the apoplast creating a more alkaline and less hydrolytic environment in which secGFP or N-secYFPΔc can accumulate in the fluorescent form. Indeed, YFP has a significantly higher pK than GFP, so its fluorescence in the apoplast may respond more markedly to an increase in pH.

The partial accumulation of the YFP-tagged AtRAB-H1b in the BFA-bodies of Arabidopsis root tips is consistent with its location at trans-Golgi compartments that, in plant cells, can also act as early endosomes (Dettmer et al., 2006; Chow et al., 2008). If AtRAB-H1b[N122I] acts at such a compartment it may affect secretory, endocytic, and vacuolar trafficking pathways that collectively alter the prevailing conditions in the apoplast.

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