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ARL1 plays a role in the binding of the GRIP domain of a peripheral matrix protein to the Golgi apparatus in plant cells

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Abstract

ARF GTPases play a central role in regulating membrane dynamics and protein transport in eukaryotic cells. ARF-like (ARL) proteins are close relatives of the ARF regulators of vesicular transport, but their function in plant cells is poorly characterized. Here, by means of live cell imaging and site-directed mutagenesis, we have investigated the cellular function of the plant GTPase ARL1. We provide direct evidence for a role of this ARL family member in the association of a plant golgin with the plant Golgi apparatus. Our data reveal the existence of key residues within the conserved GRIP-domain of the golgin and within the GTPase ARL1 that are central to ARL1–GRIP interaction. Mutations of these residues abolish the interaction of GRIP with the GTP-bound ARL1 and induce a redistribution of GRIP into the cytosol. This indicates that the localization of GRIP to the Golgi apparatus is strongly influenced by the interaction of GRIP with Golgi-localized ARL1. Our results assign a cellular role to a member of the *Arabidopsis* ARL family in the plant secretory pathway and propose mechanisms for localization of peripheral golgins to the plant Golgi apparatus.

Introduction

The endoplasmic reticulum (ER) and the Golgi apparatus are closely associated in plant cells (Boevink *et al*., 1998; Brandizzi *et al*., 2002; daSilva *et al*., 2004). How the ER and Golgi apparatus communicate with each other and how they maintain their identity are fundamental questions in the biology of the plant secretory pathway. In general, the identity of organelles is established by their enzymatic content, which specifies their cellular function, and by features of their cytoplasmic face (Munro, 2004). Coat proteins, cytoskeletal motors, tethering-factors and SNARE proteins may recognize such features and use them to distinguish between different cell membranes (Munro, 2004; Bonifacino and Glick, 2004). With the exception of SNAREs, most of these machinery components are peripheral membrane proteins that are recruited from the cytosol (Munro, 2002). Examples of these components are golgins, a family of peripheral and integral membrane proteins. These proteins bind to effector molecules, such as activated GTP-binding proteins of Ras-protein families, and serve as Golgi vesicle tethering molecules prior to vesicle fusion with a target membrane (reviewed by Munro, 2002, 2004;

Gillingham and Munro, 2003). Most golgins share a C-terminal GRIP domain which is sufficient to target them to the *trans*-Golgi (Barr, 1999; Kjer-Nielsen *et al*., 1999; Munro and Nichols, 1999; Barr and Short, 2003). This domain is about 40 amino acids long and was named after the four proteins in which it was initially identified: golgin-97, RanBP2α, Imh1p, and p230/golgin-245 (Munro and Nichols, 1999).

In plants, knowledge of the distribution of plant golgins and their molecular interactions with effector molecules is limited. The *Arabidopsis* genome encodes homologues of mammalian and yeast peripheral golgins (Gillingham *et al*., 2002; Rose *et al*., 2004), and the Golgi localization of a green fluorescent protein fusion to the GRIP domain of a peripheral *Arabidopsis* golgin (At-GRIP) and of the full-length AtGRIP has been reported (Gilson *et al*., 2004; Latijnhouwers *et al*., 2005).

How peripheral golgins associate with the plant Golgi apparatus is only now beginning to emerge. It has been suggested that the GTPase ARL1 may be involved in recruiting GRIP to the Golgi apparatus in plant cells (Latijnhouwers *et al*., 2005). Overexpression of active and inactive mutants of this protein affected the localization of GRIP on the Golgi apparatus in live tobacco leaf epidermal cells. *In vitro* analysis also showed that active ARL1 is capable of a direct interaction with GRIP and that tyrosine residue in GRIP in position 717 may be responsible for such an interaction (Latijnhouwers *et al*., 2005). However, a complete and direct *in vivo* analysis of ARL1-mediated recruitment of GRIP to the Golgi apparatus in plant cells has yet to be provided.

In this work we aimed to contribute to the understanding of the organization of membrane traffic and identity of the plant Golgi apparatus by characterizing the targeting of peripheral membrane proteins and their effectors at the Golgi membranes. Specifically, we have tested whether ARL1 may have a function in the subcellular localization of the GRIP domain of a peripheral plant golgin in live cells as well as the requirements for this interaction. This would reveal the cellular role and the mechanism of action of ARL1 as well as GRIP domain-mediated targeting of a novel class of proteins, the golgins, in plant cells.

Materials and methods

Molecular cloning

Standard molecular techniques were used for subcloning. The fluorescent proteins used in this study were based on fusions with mGFP5 (Haseloff *et al*., 1997), ECFP or EYFP (Clonetech Inc., California, USA). The spectral properties of mGFP5 allow efficient spectral separation from YFP (Brandizzi *et al*., 2002). The coding sequence of GRIP (amino acids 578–788 of the peripheral Golgi matrix protein AtGRIP (Gilson *et al*., 2004), Genebank accession number: AF370325) was a generous gift of Dr P. Gleeson, University of Melbourne, Australia, and it was amplified with primers containing the *BamH*I and *Sac*I sites for subcloning downstream of YFP in the binary vector pVKH18-En6 (Batoko *et al*., 2000). cDNA of ARL1 (At2g24765) was obtained as an ABRC clone and fused to the N-terminus of a YFP using the *Xba*I and *Sal*I sites of the binary vector pVKH18-En6. Mutant sequences of GRIP and ARL1 were generated by site-directed mutagenesis using specific primers with encoded information for substitutions of target amino acid residues. For protein His_6- and GST-tagging, DNA sequences of wild-type and mutant proteins used as indicated in the Results section, were subcloned in recombinant *E. coli* expression vectors pET-28b(+) (Novagen) or in pGEX (Amersham), respectively. The His_6-tag was downstream of the ARL1 proteins while the GST-tag was placed upstream of the GRIP sequence. The primer sequences used for the subcloning and mutagenesis indicated above are available upon request.

Plant material and transient expression system

Four-week-old *Nicotiana tabacum* (cv Petit Havana) greenhouse plants grown at 25 °C were used for *Agrobacterium tumefaciens* (strain GV3101)-mediated transient expression (Batoko *et al*., 2000). The bacterial optical density (OD_{600}) used for plant transformation was 0.05 for GRIP, ARL1 and its mutants, and 0.2 for ERD2 constructs.

In vitro *expression*

Protein production of His_6- and GST-fusions subcloned in pET-28b(+) and pGEX vectors

was induced in *E. coli* BL21(DE3) lysogens. Positive clones were selected for low-scale protein production. A single colony was inoculated initially into 5 ml of LB containing kanamycin (100 μg/ml) for pET-28b(+) or ampicillin (100 μg/ml) for pGEX, further expanded into a 50 ml shaker culture into a 250 ml flasks. The cells were incubated with shaking at 30 °C until OD_{600}~1.0. Protein production was induced by the addition of 1 mM IPTG and further incubation of the culture for 5 h at 30 °C. Cells were then pelleted and lysed according to the manufacture's instruction: QIAGEN (The QIAexpressionist handbook; August 2002 ed.) for His_6-tagged proteins and BD Biosciences for GST-tagged proteins. In both cases *E. coli* extracts were prepared under native conditions in order to discharge insoluble proteins in the pellet and they were cleared of inclusion bodies by centrifugation (12 000g×30 min). The pellet was then eliminated and the soluble supernatants were used for further analyses. For protein-protein interaction assays, these extracts were loaded into glutathione resin columns (BD Biosciences) for binding of GST-tagged proteins. Protein binding, removal of endogenous proteins and elution of tagged proteins was performed according to the manufacturers' instructions.

Glutathione-agarose affinity chromatography of leaf extracts

One gram of leaves transformed with YFP-tagged ARL1 proteins or untransformed leaves was subjected to protein extraction in 1.25 ml of NE buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT) with protease inhibitor cocktail for plant cell extracts (Sigma) and with or without 100 μM GTPγS or GDPβS (Sigma) in liquid N_2. The resulting suspension was then centrifuged at 4 °C, 14 000 rpm for 15 min. One ml of the supernatant was added to 150 μl of glutathione-agarose beads suspension (see below) (72% in NS buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT)) previously mixed with bacterial lysates containing GST-GRIP and washed from unbound proteins. To ensure that equal amounts of GST-GRIP were mixed with leaf extracts, 400 ml of overnight *E. coli* culture expressing GST-GRIP were extracted with 25 ml of GST extraction buffer (BD Biosciences) mixed with 1 ml of agarose beads. About 150 μl of bead slurry was then aliquoted with leaf extracts expressing ARL1 proteins. The mix was kept for 3 h at 4 °C with gentle rotation. The beads were centrifuged at 4 °C, 500g for 1 min and then washed three times with NS buffer containing 10 μM of nucleotide. Bound proteins were eluted from the beads with an appropriate volume of 5× SDS-PAGE sample buffer (0.225 M Tris–HCl, pH 6.8; 50% glycerol; 5% SDS; 0.05% bromophenol blue; 0.25 M DTT (QIAGEN-QIAexpressionist kit) in a proportion sample:buffer = 1:0.4, respectively) and run on a 10% SDS-PAGE gel. Gels were developed by immunoblotting with an anti-His_6 serum (Santa-Cruz).

GTP-agarose binding assay

About 50 ml of induced *E. coli* cultures expressing $ARL1^{GTP}$, $ARL1^{GTP}$F51G and $ARL1^{GTP}$Y81G were extracted in 2.5 ml of buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$). Total bacterial extracts (0.2 mg) were then incubated at 4 °C for 45 min with 0.05 ml of GTP-agarose (Sigma). After incubation, the mixtures were centrifuged (14 000g, 1 min at 4 °C). The pelleted beads were then washed 5 times with 1 ml of buffer (HEPES 25 mM pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$). The beads were then resuspended in sample buffer prior to SDS-polyacrylamide gel electrophoresis and western blot analysis.

Protein gel and blot analysis

Where stated, proteins were quantified with a Bio-Rad Protein Assay kit using BSA as a standard. Protein extracts were diluted 1:0.4 with 5× SDS loading buffer and boiled for 5 min. Equal volumes of all extracts were loaded on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membrane by electroblotting and blocked with PBS, 0.05% Tween 20 and 5% milk powder for 2 h. The filter was then incubated in blocking buffer with either anti-GFP serum from rabbit (Molecular Probes or AbCam) at a dilution of 1:1000 overnight, or anti-His (Santa-Cruz) at a dilution of 1:200 for 4 h or anti-GST (AbCam) at a dilution of 1:500 for 4 h. The anti-GFP serum is known to recognize all the GFP variants. Further steps were performed as in Crofts *et al*. (1999).

For Coomassie Blue analysis, purified bound proteins eluted from glutathione columns were subject to boiling in sample buffer and separated on a 10% SDS-polyacrylamide gels SDS-PAGE slab gels as described above. After separation, each gel was stained with Coomassie Blue for 10 min (0.125% Coomassie Blue, 50% MeOH, 10% acetic acid) and destained (one wash with 50% MeOH + 10% acetic acid followed by several washes with 5% MeOH + 7% acetic acid).

Biochemical results presented in this work are representative of at least three independent repetitions.

Chemiluminescence signals of the blots were acquired using an Epichem3 Darkroom gel documentation system (UVP BioImaging Systems) using Labworks Image Acquisition Software without digital enhancement.

Sampling and imaging

Transformed leaves were analysed 48 h after infection of the lower epidermis. Confocal imaging was performed using an upright LSM Zeiss 510 META, and a 63× water immersion objective. For imaging co-expression of YFP and GFP/CFP constructs, excitation lines of an argon ion laser of 458 nm for GFP/CFP and 514 nm for YFP were used alternately with line switching using the multi-track facility of the microscope. A 458/514 nm dichroic beam splitter was used and a 475–525 nm bandpass filter and a 560–615 nm bandpass filter was used for GFP and YFP, respectively. In this way any cross talk and bleed through of fluorescence was eliminated. Same settings were used for imaging cells expressing one fluorescent protein fusion alone (i.e. GFP or CFP or YFP).

Confocal observations and image acquisition of plants expressing fluorescent GRIP alone/and with ARL1 constructs were performed with same imaging settings of the microscope (laser intensity, pinhole aperture, detector gains, zoom and line averaging) using the ''re-use'' function of the microscope. Images of cells with similar levels of saturation of the imaging pixels, as determined by the palette function of the microscope software, were acquired. This method allows comparison of levels of expression of fluorescent proteins among cells (daSilva *et al.*, 2004).

PaintShop Pro7 imaging suite was used for further image handling. Images reported in microscopy figures are representative of at least five independent experiments.

Molecular modeling

Molecular model building and dynamic simulations for AtGRIP, ARL1, ARL1 and their complexes were carried out using InsightII modules: Homology, Biopolymer, Discover_3, Docking and Binding Site (Accelrys Inc., San Diego, CA) on a Silicon Graphics Octane2 workstation (Haas and Plow, 1997). Secondary structural predictions were performed using Homology and Jpred. Molecular models for the amino-terminal coiled-coil region of AtGRIP were constructed by secondary structural alignment of the 1C1GA(1–284) and 1C1GB(285–568) (PDB# 1C1G) with AtGRIP(1–284) and then imposing the structure of 1C1GA onto AtGRIP. For the region between the coiled-coil region and the GRIP domain of AtGRIP, residues 276–712, no structure in the protein database align well to this region based on either sequence homology or structural similarity. Therefore, the coiled-coil structure of AtGRIP 1–275 was extended to span this region based upon secondary structural predictions. Following splicing together of these three structures, a structure for one rod of AtGRIP was generated. In the structural templates for AtGRIP 1–275 (1C1G) and AtGRIP 713–788 (1UPT), both regions are present as a dimer. Therefore, to generate a full-length dimer for AtGRIP, the dimeric interface of these two regions in their templates was preserved and some minor rearrangement in the three-dimensional structure of AtGRIP 713–788 was introduced to accommodate the preservation of the dimeric interface. The side-chain atoms of the dimeric structure were then relaxed, followed by energy minimization of the entire dimer. A molecular model for the GRIP domain of AtGRIP was constructed assigning atomic coordinates for AtGRIP(713–788) using the GRIP domain of golgin-245, 1UPTB (PDB# 1UTP) as a template. The structure of AtGRIP(713–788) was then duplicated and the A and B chains of AtGRIP(713–788) were positioned by superimposing AtGRIP(713–788) and AtGRIP(713B-788B) onto 1UPTB and 1UPTD, respectively. The complete dimeric structure for AtGRIP was generated by coupling the coiled-coil domains to the GRIP domains, followed by molecular dynamic simulations and

energy minimizations as described in Haas and Plow (1997). To support our predictions, dimerization of AtGRIP was ensured by native PAGE gel analysis on extracts of tobacco leaves expressing AtGRIP (Supplementary data 4).

To generate a molecular model for ARL1, the structures of mammalian ARLs and ARFs (PDB# 1R8Q, 1HUR and 1UPT) were screened for suitability. The structure of 1HURA provided the best fit, however, residues 10–16 of 1HUR were vibrationally active and did not fit particularly well in the rigid crystal structure. Therefore, a molecular model for *Arabidopsis* ARL1 was constructed using residues from human ARF1, A2–A19 from 1R8QA (PDB# 1R8Q) and A20–A181 from 1HURA (PDB# 1HUR) as structural templates. In the structure of human ARL1 bound to the GRIP domain of Golgin-245 (PDB# 1UPT), GTP bound to a magnesium ion was present, as GTP is a required cofactor for ARL interactions with the GRIP domain. As the structure of 1HUR contained a GDP-Mg molecule and not a GTP-Mg molecule, a GDP-Mg molecule in ARL1 was three-dimensionally aligned to its placement in 1HUR and then converted from GDP into GTP. Following molecular dynamic simulations and energy minimization, a GTP-Mg-ARL1 structure was obtained.

Two molecules of GTP-Mg-ARL1 were then docked to the GRIP domain of the AtGRIP dimer using the coordinates of 1UPTA (for ARL1) and 1UPTB (for AtGRIP) from PDB# 1UPT as an initial docking structure. Following multiple dynamic simulations and energy minimizations, 20 structures for the complex were produced. Similarly, a GTP-Mg-ARL1 structure, obtained using human ARL1 (1UPTA, PDB# 1UPT) as the structural template, was docked to the AtGRIP dimer. For structural comparison, structures of the AtGRIP dimer bound with $ARL1^{GDP}$, ARL1, $ARL1^{GDP}$ and ARL were also generated.

Results

Requirements for the subcellular localization of ARL1

The *Arabidopsis* genome encodes a sequence with 60% identity at amino acid level to the human and yeast ARL1 (Figure 1A), previously indicated as ARF3 by Lebas and Axelos (1994). To determine the subcellular distribution of ARL1 we expressed it as a yellow fluorescent protein (YFP) fusion (ARL1-YFP) in tobacco leaf epidermal cells. Laser scanning confocal microscopy revealed that ARL1-YFP was distributed at punctate structures (Figure 1B, arrowhead) and in the cytosol (Figure 1B, arrow). Dual-color experiments with a known marker of the endoplasmic reticulum (ER) and Golgi apparatus, ERD2-GFP (Boevink *et al*., 1998), showed that the ARL1-YFP-labelled punctate structures were Golgi stacks (Figure 1C–E), as also reported by Latijnhouwers *et al*. (2005). Rare punctate structures that did not co-localize with the Golgi marker were also visible (Figure 1E, arrow).

We next investigated the requirements for ARL1 binding to the Golgi apparatus. ARL1 contains a consensus myristoylation motif at its N-terminus (Figure 1A, underlined; Lowe *et al*., 1996), corresponding to data showing that the yeast homologue is myristoylated at its N-terminus (Lee *et al*., 1997). To investigate whether the association of plant ARL1 with the Golgi apparatus requires an intact myristoylation motif, we mutated the glycine in position 2 to an alanine. A YFP fusion of this mutant ($ARL1^{G2A}$-YFP) was found to localize in the cytosol (Figure 1F) with no detectable labeling of the Golgi apparatus, as shown in cells co-expressing ERD2-GFP (Figure 1G–I).

We next tested whether ARL1 requires activation to localize at the Golgi apparatus. Therefore we generated YFP fusions to ARL1 with a reduced or enhanced affinity for GTP. ARL1 restricted to the GDP-bound form ($ARL1^{GDP}$) was created by replacing a threonine residue at position 31 with an asparagine while active ARL1 restricted to the GTP-bound form ($ARL1^{GTP}$) was created by replacing a glutamine at position 71 with a leucine (Lee *et al*., 2002; Latijnhouwers *et al*., 2005). Confocal microscopy imaging of tobacco leaf epidermal cells co-expressing ARL1-YFP mutants and ERD2-GFP demonstrated that both constitutively active and inactive forms of ARL1 showed a similar subcellular distribution to the wild-type protein (Figure 1J–O).

These data indicate that ARL1 localizes at the Golgi apparatus in both the active and inactive forms and that it requires an intact glycine residue in position 2 for this association, likely for ensuring the presence of a myristoylation anchor.

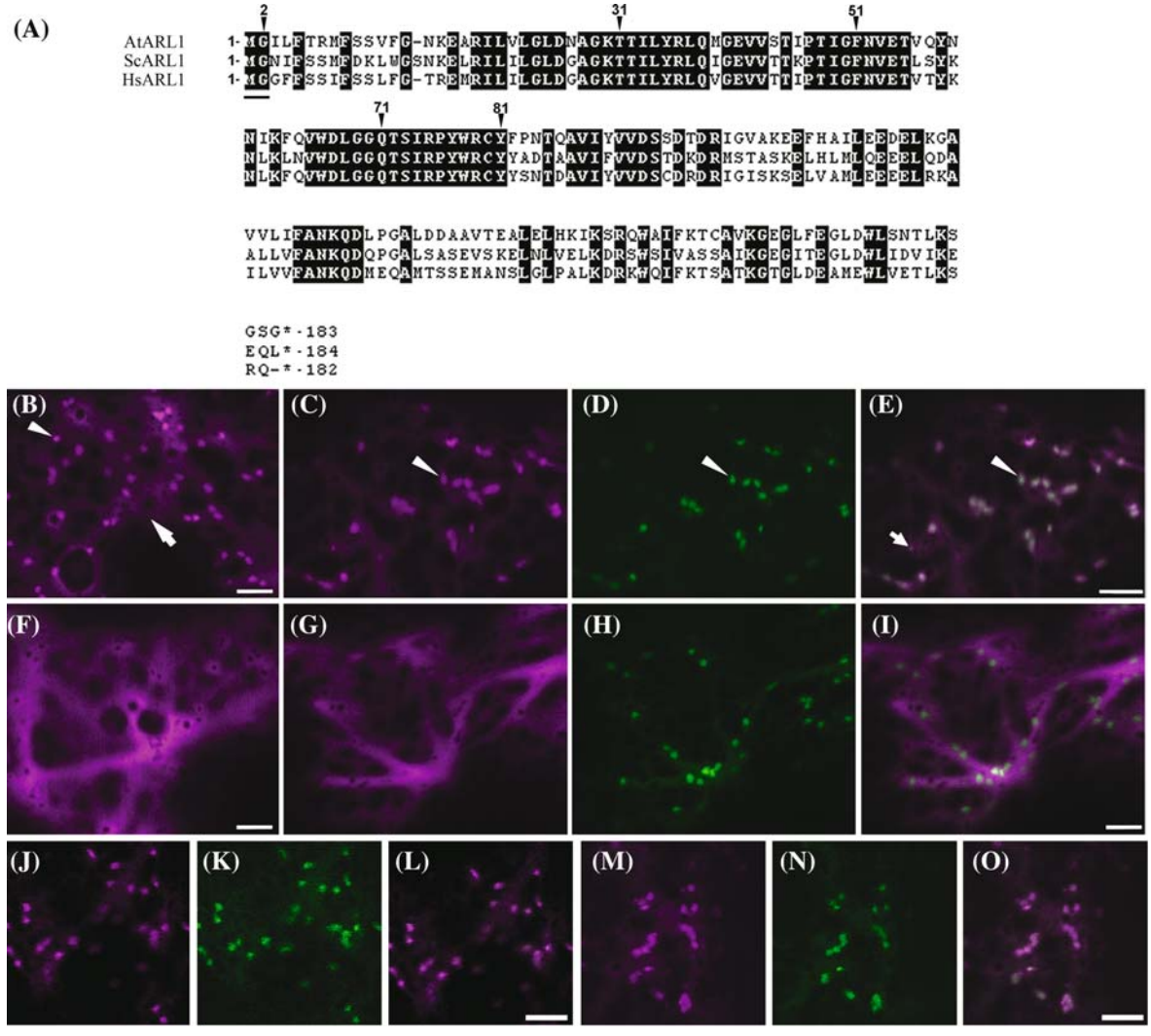

Figure 1. ARL1 is a homologue of yeast and human ARL1 and associates with the plant Golgi apparatus. (A) Amino acid sequence alignment (ClustalW) of *Arabidopsis* ARL1 (AtARL1) with *Saccharomyces cerevisiae* (Sc; P38116) and human (Hs; P40616) ARL1. ARL1 shares 60% identity and 20% similarity with ScARL1, and 64% identity and 18% similarity with HsARL1. Conserved amino acids of interest for this work are annotated and marked by arrowheads. Identical amino acids are black shadowed. The myristoylation motif encompassing glycine residue in position 2 is underlined. (B) Confocal image of a tobacco leaf epidermal cell expressing ARL1-YFP alone showing that YFP fluorescence distributed to punctate structures (arrowhead) and in the cytosol (arrow). (C)–(E) Confocal images of a tobacco leaf epidermal cell co-expressing ARL1-YFP (C) and ERD2-GFP (D) show that ARL1-YFP-labelled punctate structures are Golgi bodies (arrowhead). (E) Merged image of (C) and (D). Note the presence of a small ARL1-YFP punctate structure that does not co-localize at a Golgi stack (arrow). (F)–(I) Confocal images of cells expressing $ARL1^{G2A}$-YFP either alone (F) or with ERD2-GFP (G) show that the mutant is distributed in the cytosol. (I) Merged image of (G) and (H). (J)–(O) Confocal images of cells co-expressing ERD2-GFP (K–N) with either $ARL1^{GDP}$-YFP (J) or $ARL1^{GTP}$-YFP (M) (L) and (O). Merged images of (J)–(K) and (M)–(N), respectively. Scale bars = 5 μm.

Association of GRIP with the Golgi apparatus

It has been shown by means of fluorescence microscopy that the last 211 amino acids of AtGRIP, which include a short coiled-coil portion of this golgin and the GRIP domain, are capable of mediating targeting to the Golgi apparatus in mammalian cells (Gilson *et al*., 2004). This portion of AtGRIP therefore seemed the ideal candidate to investigate whether ARL1 could have a role in the binding of a GRIP domain to the Golgi apparatus in plant cells. Furthermore, by eliminating most of

the coiled-coil region of AtGRIP we would confirm that the recruitment of the protein to the Golgi apparatus is due to the domains of this portion of AtGRIP. To verify that this region of AtGRIP, residues 578–788 (Figure 2A), was associated with the Golgi apparatus of plants, we expressed a YFP fusion protein (YFP-GRIP) in tobacco leaf epidermal cells. For simplicity, the region of AtGRIP used in this work is indicated as 'GRIP' (Figure 2A). Laser scanning confocal imaging of YFP-GRIP showed that this protein was distributed at punctate structures and in the cytosol (Figure 2B). The latter were identified as Golgi stacks by co-expression analysis with the Golgi marker, ERD2-GFP (Figure 2C–E). Thus, similarly to ARL1, GRIP localizes at the Golgi apparatus in plant cells.

Direct association of GRIP with ARL1

To determine if ARL1 could be involved in the binding of GRIP to the Golgi apparatus and to characterize fully the requirements for the interaction, we developed a glutathione-agarose affinity chromatography assay based on the interaction of a recombinant GST-GRIP with wild-type and mutant ARL1-YFP proteins expressed in tobacco leaves (Figure 3A,B). Expression of ARL1 proteins was first tested by western blot on a fraction of total extracts of leaves expressing the

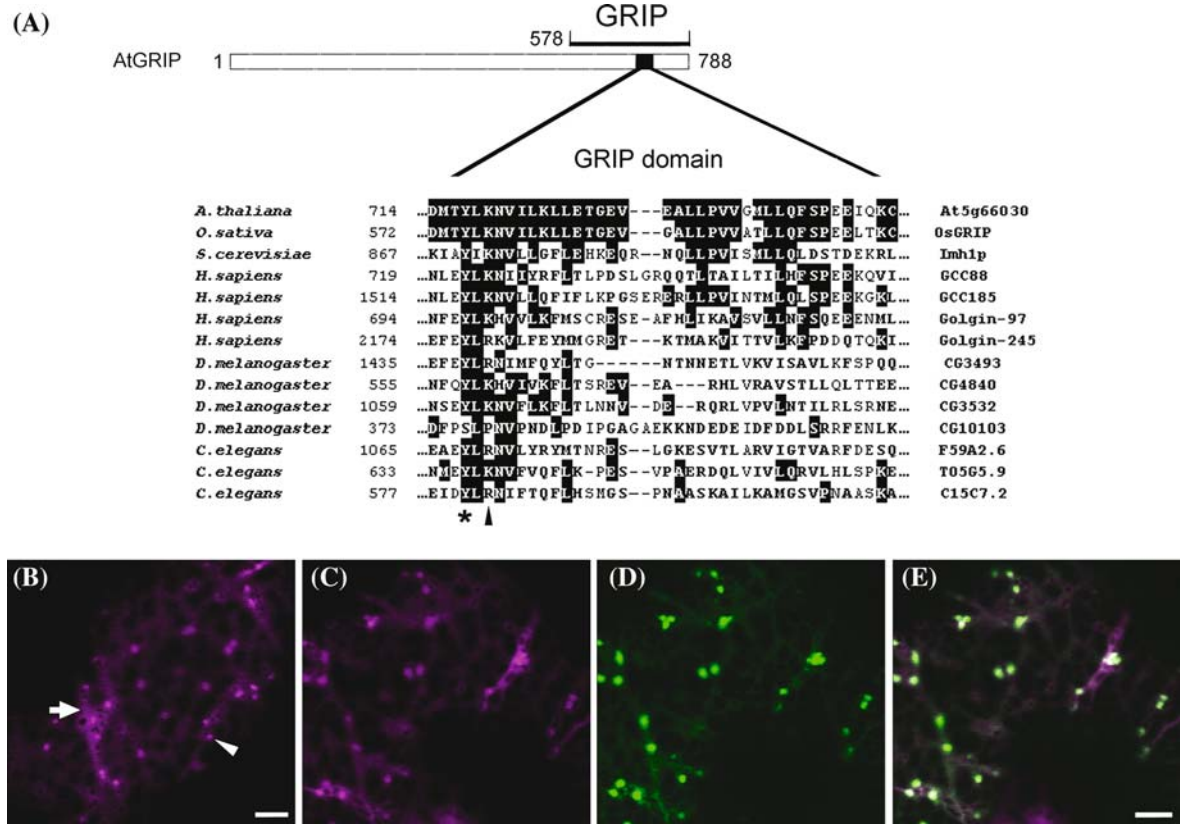

Figure 2. GRIP localizes at the plant Golgi apparatus in tobacco leaf epidermal cells. (A) Diagram of the plant golgin AtGRIP (Gilson *et al*., 2004) and sequence alignment of GRIP domains of golgins from *Arabidopsis* (At5g66030, alias AtGRIP; Gilson *et al*., 2004), rice, yeast, human, fly and worm (see also Barr (1999), Munro and Nichols (1999), Kjer-Nielsen *et al*. (1999), McConville *et al*. (2002), Gilson *et al*. (2004); Latijnhouwers *et al*. (2005)). Identical amino acids are black shadowed. The relative position of the GRIP domain is shown by a black rectangle in the diagram of AtGRIP. A line above the AtGRIP diagram is positioned over the AtGRIP amino acid residues (578–788) used in this study, here collectively named as "GRIP". Conserved tyrosine (717) and lysine (719) residues used in this study are marked by an asterisk and an arrowhead, respectively. (B) A confocal image of a cell expressing YFP-GRIP alone shows that the fusion is distributed at punctate structures (arrowhead) and in the cytosol (arrow). (C)–(E) Confocal images of a cell co-expressing YFP-GRIP (C) and ERD2-GFP (D) show that the punctate structures of YFP-GRIP correspond to Golgi stacks. (E) Merged image of C and D. Scale bars = 5 μm.

fluorescent fusions (Figure 3A, lanes 1–4). Then, ARL1-fusion proteins from leaf extracts were loaded onto glutathione-agarose beads. All ARL1 proteins were found to interact with recombinant GST-GRIP (Figure 3A, lanes 7–9) but not with GST alone (Figure 3A, lane 5).

To ensure that the interaction of GST-GRIP with GTP and GDP-restricted mutants was not due to these proteins behaving as partially constitutively activated and inactivated mutants, extracts from tobacco leaves expressing YFP fusions of $ARL1^{GDP}$ and $ARL1^{GTP}$ were preloaded with non-hydrolysable analogues of GDP or GTP and then incubated with GST-GRIP on glutathione-agarose beads (Figure 3B, lanes 6 and 7). We found that both proteins were retained by GST-GRIP. To ensure that these results were independent from the mutations that could have malformed ARL1, we charged wild-type ARL1 with non-hydrolysable nucleotides and tested its interaction with GST-GRIP (Fig-

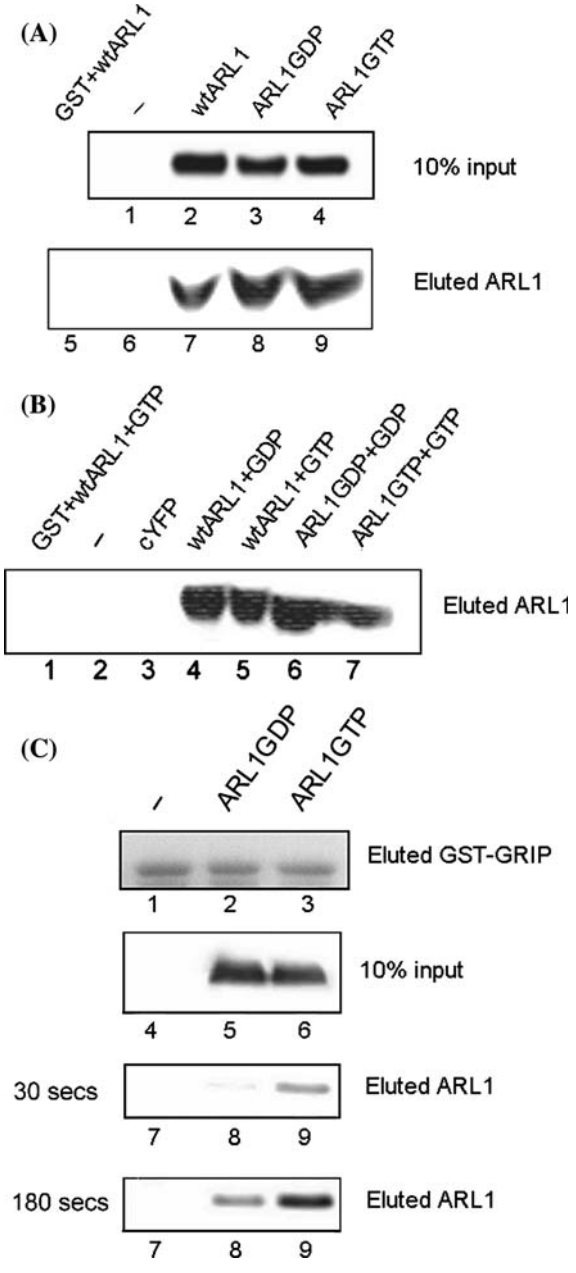

Figure 3. ARL1 Interacts with GRIP. (A) *Lanes 1–4*: Western blot analysis with anti-GFP serum of extracts of leaves expressing the YFP fusions of wtARL1 (*lane 2*), ARL1GDP (*lane 3*), and ARL1GTP (*lane 4*). *Lane 1*, negative control: untransformed leaf extracts. *Lanes 5–9*: Extracts of tobacco leaves expressing YFP-tagged wild-type ARL1 and ARL1 mutants were incubated with glutathione agarose beads preloaded with equal volumes of extracts of *E. coli* expressing GST-GRIP. For this, identical total protein concentrations of GST-GRIP extracts from the same *E. coli* culture were used and the beads washed to remove nonspecifically bound proteins. Proteins retained by the GST-GRIP agarose beads were eluted and then boiled in SDS sample buffer for immunoblot analysis with anti-GFP serum. Wild-type ARL1 (*lane 7*), $ARL1^{GDP}$ (*lane 8*) and $ARL1^{GTP}$ (*lane 9*) mutants were retained by GST-GRIP. Negative controls: GST beads alone did not retain wtARL1-YFP (*lane* 5); extracts of untransformed tobacco leaves incubated with GST-GRIP bound to glutathione agarose beads were used to test the specificity of the GFP antibody (*lane 6*). B YFP-tagged wild-type ARL1 (*lanes 4, 5*), $ARL1^{GDP}$ (*lane 6*) and $ARL1^{GTP}$ (*lane 7*) mutants from tobacco leaf extracts charged with either GDP or GTP were tested for their interaction with GST-bound GRIP using anti-GFP serum. Negative controls: GST-resin did not retain wtARL1 loaded with GTP (*lane 1*); extracts of untransformed tobacco leaves incubated with GST-GRIP bound to glutathione agarose beads were used to test the specificity of the GFP antibody (*lane 2*); extracts from leaves expressing cytosolic YFP (cYFP; daSilva *et al*., 2004) were used to test if the fluorescent protein fusion was responsible for an interaction with GST-GRIP. YFP did not interact with GST-GRIP (*lane 3*). (C) Interaction of recombinant ARL1 with GST-GRIP *in vitro*. *Lanes 1–3*: Coomassie Blue analysis on the glutathione column eluates used for testing the GST-GRIP-ARL1 interaction shows similar quantities of GST-GRIP. *Lanes 4–6*: To test whether comparable amounts of $ARL1^{GDP}$ (*lane 5*) and $ARL1^{GTP}$ (*lane 6*) mutants were used for the experiment, a western blot with a His_6-antibody on a 10% fraction (90 μg total proteins/lane) of *E. coli* extracts that were loaded onto the GST columns was performed. *Lane 4*: Negative control: Extracts from *E. coli* expressing His_6-tag alone. *Lanes 7–9*: Western blot with anti-His_6 serum on column eluates shows a weaker signal of the His_6-tagged ARL1GDP (*lane 8*) in comparison to the GTP mutant (*lane 9*). *Lane 7*: Negative control: Extracts from *E. coli* expressing His_6-tag alone.

ure 3B, lanes 4 and 5). We verified that wild-type ARL1 interacted with GST-GRIP in the presence of both nucleotides.

These results confirm that ARL1 from leaf extracts binds to GRIP; however, our data do not allow us to establish whether the binding of ARL1 to GRIP is direct. Therefore, to determine if the interaction between ARL1 and GRIP required the presence of other cytosolic or Golgi-associated proteins, we produced ARL1 and GRIP in *E. coli* and tested if ARL1 and purified GRIP could interact with each other *in vitro* (Figure 3C). To do so, we aimed to use extracts of *E. coli* at identical total protein concentration expressing His_6-fusions of $ARL1^{GDP}$ or $ARL1^{GTP}$ and to load them onto glutathione columns, which had been preloaded with equal volumes of extracts of *E. coli* at identical total protein concentration expressing GST-GRIP and pre-washed from unbound proteins. Proteins would then be eluted and subject to immunoblot analysis with anti-His_6 or Coomassie Blue staining. The result would allow to test the nature of the interaction of ARL1 proteins with GRIP and to establish the association of ARL1 proteins with GRIP quantitatively. To perform this experiment, we first ensured similar loading of GST-GRIP onto columns by aliquoting equal volumes of extracts of the same culture of *E. coli* expressing GST-GRIP in each glutathione column, by Coomassie Blue analysis on the column eluates (Figure 3C, lanes 1–3), and by western blot with anti-GST serum (not shown). To ascertain that similar quantities of $ARL1^{GDP}$ and $ARL1^{GTP}$ were loaded on GST-GRIP columns, the same concentration of total proteins in extracts of *E. coli* expressing the His_6-tagged ARL1 proteins was used, and a western blot on a fraction of these extracts was also performed (Figure 3C, lanes 5–6). This showed comparable amounts of the ARL1 proteins in *E. coli* extracts, ruling out the possibility that the result could be affected by differential *E. coli* expression of the ARL1 proteins. A similar ability of the two ARL1 mutants to bind to a Ni-column was also ascertained (data not shown). Then, these extracts of *E. coli* expressing His_6-tagged ARL1GDP (Figure 3C, lane 8) and GTP mutants (Figure 3C, lane 9) were loaded onto glutathione columns preloaded with GST-GRIP. Non-specifically bound proteins were washed away and all bound proteins were eluted. Eluates of these columns were subject to immunoblot analysis with anti-His_6 serum. The chemiluminescence signal was acquired on the same membrane with a short exposure (30 s) and long exposure (180 s) to allow comparison of the signals of the two ARL1 protein bands without oversaturation of pixels (30 s) and clear detection of $ARL1^{GDP}$ signal (180 s). This experiment demonstrate not only that the association of ARL1 with GRIP occurs preferentially with active ARL1 but also that the interaction of active ARL1 with GRIP is not dependent on other cytosolic proteins and must be due to a direct association of the two molecules.

$ARL1^{GTP}$ interacts with GRIP via specific amino acid residues

To analyze the relevance of the ARL1–GRIP interaction for the localization of GRIP to the Golgi apparatus, we mutated conserved residues of GRIP that have been shown to mediate ARL1–GRIP interaction in mammals, yeast and protozoan (Kjer-Nielsen *et al*., 1999; McConville *et al*., 2002; Lu and Hong, 2003; Wu *et al*., 2004). Two different mutants of the GRIP domain were constructed by replacement of the highly conserved tyrosine at position 717 and lysine at position 719 (Figure 2A) with alanine ($GRIP^{Y717A}$ and $GRIP^{K719A}$, respectively) and expressed as GST-fusions in *E. coli* (GST-$GRIP^{Y717A}$ and GST-$GRIP^{K719A}$). GST-$GRIP^{Y717A}$ and GST-$GRIP^{K719A}$ were then immobilized on glutathione columns and were tested for their capacity to interact with His_6-$ARL1^{GTP}$ (Figure 4), which shows direct binding and a higher affinity to GST-GRIP in comparison to His_6-$ARL1^{GDP}$ (Figure 3C).

Individual mutations of GRIP residues Y717A and K719A reduced the interaction of GRIP with $ARL1^{GTP}$ in comparison to wild-type GRIP, with the tyrosine mutant showing a stronger effect than lysine. These results establish the relevance of two conserved residues in the GRIP domain in mediating direct interactions of GRIP for the GTP-restricted form of ARL1.

Golgi-association of GRIP depends on Y717 and K719

To characterize whether the subcellular distribution of GRIP mutants that have an impaired ability to interact with ARL1 would be affected by the Y717 and K719 mutations, we expressed them as YFP fusions (YFP-$GRIP^{Y717A}$ and YFP-

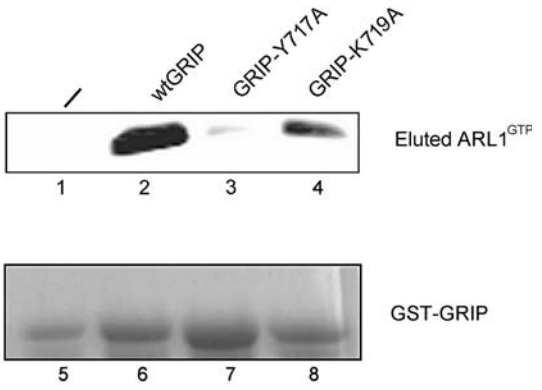

Figure 4. Mutations of conserved residues of GRIP reduce the ability of GRIP binding to active ARL1. We tested the ability of recombinant GST-GRIP^{Y717A} and GST-GRIP^{K719A} to interact with ARL1 using glutathione columns in which the two mutants were bound. To do so, equal volumes of extracts of *E. coli* at identical total protein concentration expressing wild-type GST-GRIP (*lane 2*), GST-GRIP^{Y717A} (*lane 3*) or GST-GRIP^{K719A} (*lane 4*) were loaded onto glutathione columns and unbound proteins were removed. Then equal volumes of extracts of *E. coli* at identical total protein concentration expressing ARL1^{GTP}-His$_6$ were passed through each column. Unbound proteins were washed away and bound proteins were then eluted and subject to immunoblot analysis with anti-His$_6$ or Coomassie Blue staining. *Lane 1* – Negative control: GST-wtGRIP with His$_6$-tag encoded in empty pET28(+). *Lanes 5–8*: Loading of the glutathione columns with GST-GRIP proteins was done with extracts of *E. coli* expressing the GST-GRIP proteins at identical total protein concentrations. Coomassie Blue analysis (*lanes 5–8*) and western blot with anti-GST serum (not shown) on total purified eluates of columns containing GST-GRIP proteins bound to ARL1^{GTP}-His$_6$ allowed monitoring of the amounts of GST-GRIP proteins bound to glutathione columns.

GRIP^{K719A}) in leaf epidermal cells with ERD2-GFP as a Golgi marker (Figure 5). Similarly to the wild-type YFP-GRIP (Figure 5A–C), the fluorescent signals of YFP-GRIP^{Y717A} (Figure 5D-F; see also Latijnhouwers *et al*., 2005) and that of YFP-GRIP^{K719A} (Figure 5G–I) were distributed in the cytosol but the association of the mutants with the Golgi apparatus was less detectable when compared with the wild-type YFP-GRIP (Figure 5A–C). This effect was more pronounced for YFP-GRIP^{Y717A} than YFP-GRIP^{K719A}, consistent with the lower level of interaction of the GRIP^{Y717A} with ARL1^{GTP} in comparison to GRIP^{K719A} *in vitro* (see Figure 4).

Our results reveal that the integrity of certain conserved residues of GRIP is a requirement for the direct interaction of this domain with an active form of ARL1 on the Golgi apparatus. A correlation exists between the interaction of these GRIP mutants with ARL1^{GTP} and their ability to bind to the Golgi apparatus. Mutations of the tyrosine 717 and, to a lesser extent, of the lysine 719 residues abrogate the ability of GRIP not only to interact with ARL1^{GTP} but also to bind to the Golgi apparatus.

ARL1 Phenylalanine 51 and Tyrosine 81 are at the ARL1–GRIP Interface

The results of the previous section indirectly suggest that ARL1 is involved in the localization of GRIP on the Golgi apparatus in plants. To confirm the role of ARL1 in the recruitment of GRIP to the Golgi apparatus, we wanted to obtain direct evidence and create single point mutations in ARL1 that would disrupt ARL1–GRIP binding. An altered distribution of GRIP in the presence of such mutants in live cells would provide direct evidence for a role of ARL1 in the binding of GRIP to the Golgi apparatus *in vivo*.

Panic *et al*. (2003a) have predicted a role for a subset of amino acid residues mapped in molecular models at the GRIP–ARL1 interface for the interaction of the mammalian ARL1 homologue with the GRIP domain. These residues, in particular, the analogues of the *Arabidopsis* ARL1 F51 and Y81, are highly conserved across species (Figure 1A). To gain further insights into ARL1–GRIP interaction, we have generated a molecular model of the plant ARL1-GRIP complex using crystal structures of homologous proteins as a template (Figure 6).

To produce a molecular model for the *Arabidopsis* ARL1–GRIP complex, models for each individual protein were first generated and then the two structures were docked. The final minimized structure for the two molecules of GTP-bound ARL1 plus dimeric-AtGRIP is shown in Figure 6A. AtGRIP Y717 and K719 form part of the predicted interface between ARL1 and GRIP, in agreement with our functional data presented in Figures 4 and 5. Furthermore, AtGRIP Y717 makes many more contacts with ARL1 than does AtGRIP K719 (Figure 6B), which correlates with the diminished capacity of their two corresponding mutants (Y717A and K719A) to interact with ARL1. This correlation indicates the relevance of our structural model and supports its use in predicting residues in ARL1 that may be important for the ARL1–GRIP interaction. We there-

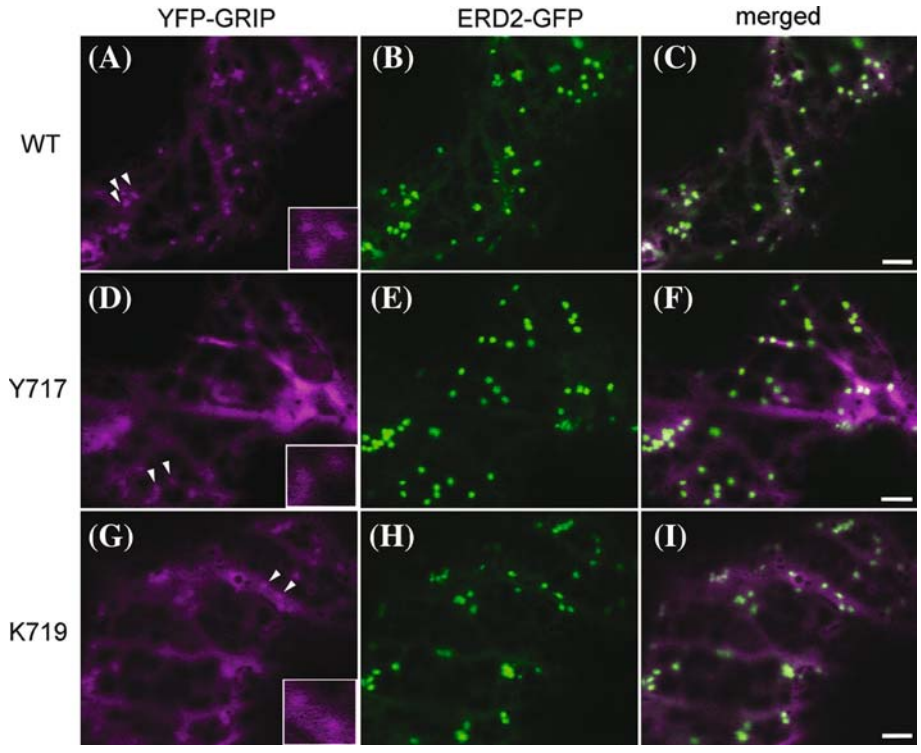

Figure 5. GRIP^{Y717A} and GRIP^{K719A} mutants show reduced association with the Golgi apparatus in live cells in comparison to wild-type GRIP. (A) Confocal images of epidermal cells co-expressing ERD2-GFP (B, E, and H) with YFP fusions of wild-type YFP-GRIP (A), GRIP^{Y717A} (D) or GRIP^{K719A} (G). Note that the punctate accumulation of YFP fluorescence at the Golgi bodies of GRIP^{Y717A} mutant and to a lesser extent of GRIP^{K719A} mutant is reduced in comparison to that of the wild-type GRIP (see insets for detailed view of Golgi stacks pointed by arrowheads). (C), (F) and (I) are merged images of A and B, D and E, G and H, respectively. Scale bars = 5 μm.

fore examined the model to identify which residues appear to be involved in the interaction. Similarly to Y717, these residues are also aromatic – ARL1 F51, W66, W78 and Y81 (Figure 6C). As the side chain of tryptophan residues occupies more space than the side chains of phenylalanine or tyrosine residues, ARL1 F51 and GRIP Y81 were chosen as sites for site-directed mutagenesis in order to minimize the chance of a mutation-induced conformational change in ARL1. Furthermore, as ARL1 F51 is located within a loop region it was mutated to a glycine residue, a residue that is abundant in loop and turn structures. To be consistent with this substitution, ARL1 Y81 was also mutated to glycine.

ARL1 Phenylalanine 51 and Tyrosine 81 are important for the interaction with GRIP

Our model suggests that GRIP interacts with ARL1 residues that include F51 and Y81, similarly to models proposed for human GRIP domain and ARL1 proteins (Panic *et al*., 2003a; Wu *et al*., 2004). However, the relevance of the side-chains of the F51 and Y81 residues in the ARL1–GRIP interaction has yet to be tested experimentally. Therefore, we generated mutants of the active ARL1 that binds to GRIP directly (ARL1^{GTP}F51G and ARL1^{GTP}Y81G) to provide support to our hypothesis. These mutants were then expressed as recombinant His_6-fusions and tested for the efficiency of their interaction with GST-GRIP on a glutathione column (Figure 7). We found that the interaction of GST-GRIP with these ARL1^{GTP} mutants was reduced (Figure 7, lanes 10–12), confirming the relevance of these conserved amino acid residues to the interaction with GRIP. In particular, ARL1^{GTP}Y81G had a more reduced ability to bind to GST-GRIP in comparison with ARL1^{GTP}F51G.

To rule out the possibility that the two new mutations could indeed induce a malformation of

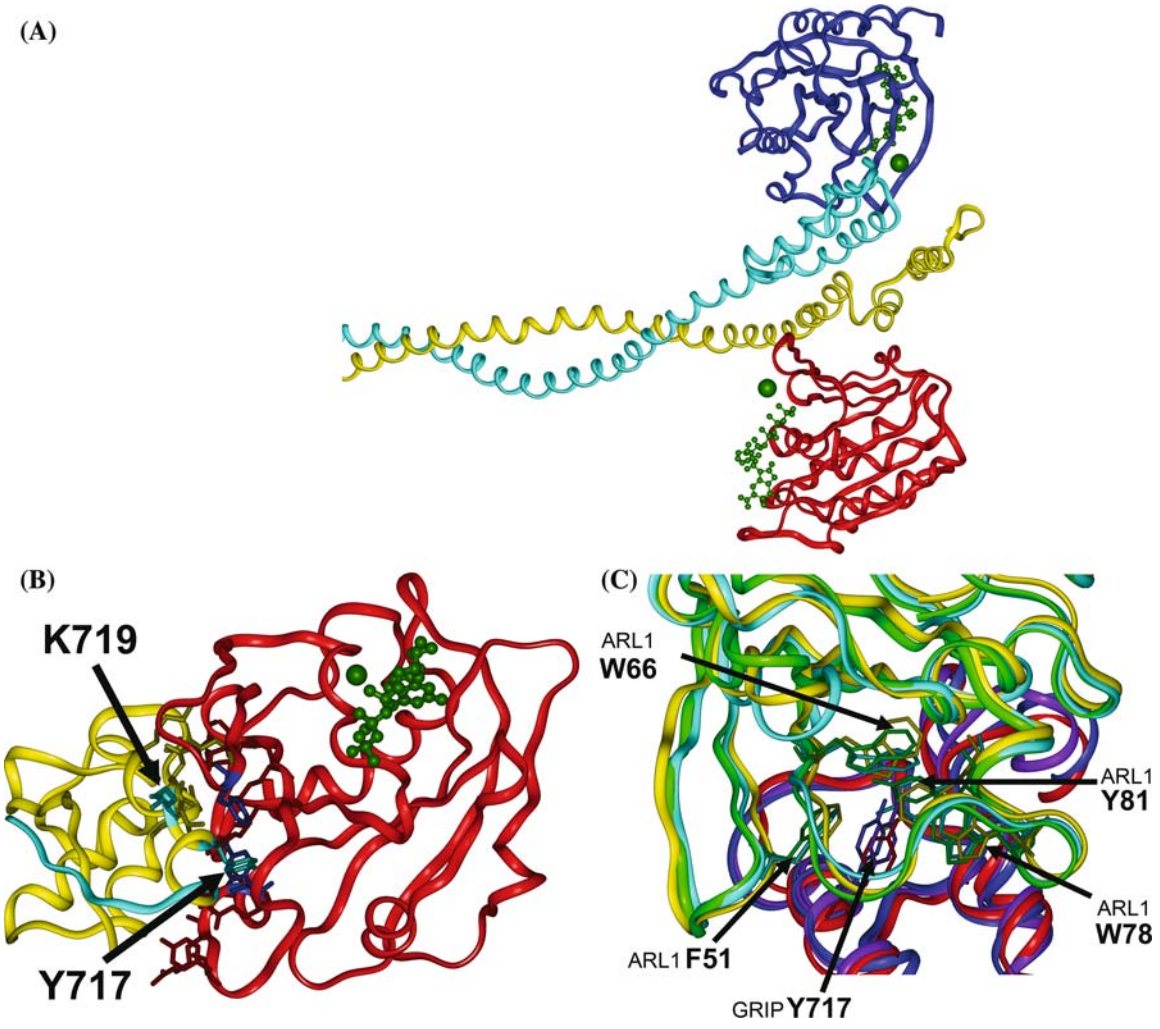

Figure 6. Structure of GTP-bound AtARL1 in Complex with the AtGRIP Dimer. (A) Structure of the two GRIP domains in the AtGRIP dimer interacting with two GTP-bound ARL1 proteins. Molecular modeling and docking was carried out using InsightII modules (Accelrys Inc.). The backbone atoms of AtGRIP and ARL1 are displayed as ribbons with the A and B chains of the AtGRIP dimer colored light blue and yellow and the two ARL1 proteins colored red and blue. The two GTP-Mg molecules, each bound to one ARL1 protein, are colored green and displayed in the ball-and-stick style. The complete coiled-coil region of the AtGRIP dimer is not displayed. (B) Detailed view of side-chain interactions involved in ARL1 binding to the GRIP domain of AtGRIP. The heavy atoms of AtGRIP 713–788 and ARL1 protein are colored yellow and red, respectively. The GTP-Mg molecule bound to ARL1 is colored green and displayed in the ball-and-stick style. For clarity, only one ARL1-AtGRIP interface is displayed. Residues coordinating the binding of ARL1 to AtGRIP are highlighted using thicker sticks. Residues where point mutations were made, AtGRIP 717 and 719 and ARL1 51 and 81, are highlighted in blue and light blue, respectively. The GTP-Mg molecule bound to ARL1 does not interact with AtGRIP. (C) Detailed view of the interactions of AtGRIP Y717 with ARL1. Three superimposed structures of the complex are displayed to show the variability in protein structure. The heavy atoms of AtGRIP 704–788 and ARL1 are displayed in stick fashion. For clarity, only one ARL1–AtGRIP interface is displayed. AtGRIP Y717, and residues in ARL1 interacting with AtGRIP Y717 are highlighted using thicker sticks. These residues are ARL1 51, 66, 78 and 81.

$ARL1^{GTP}$, we tested the ability of $ARL1^{GTP}$F51G and $ARL1^{GTP}$Y81G to associate with GTP (Figure 7, lanes 13–20). Therefore, recombinant His-tagged $ARL1^{GTP}$, $ARL1^{GTP}$F51G and $ARL1^{GTP}$Y81G from total extracts of *E. coli* at identical total protein concentration were incubated on GTP-agarose beads and unbound proteins were removed upon extensive washing. Bound proteins were separated by SDS-PAGE followed by western blotting. This revealed that

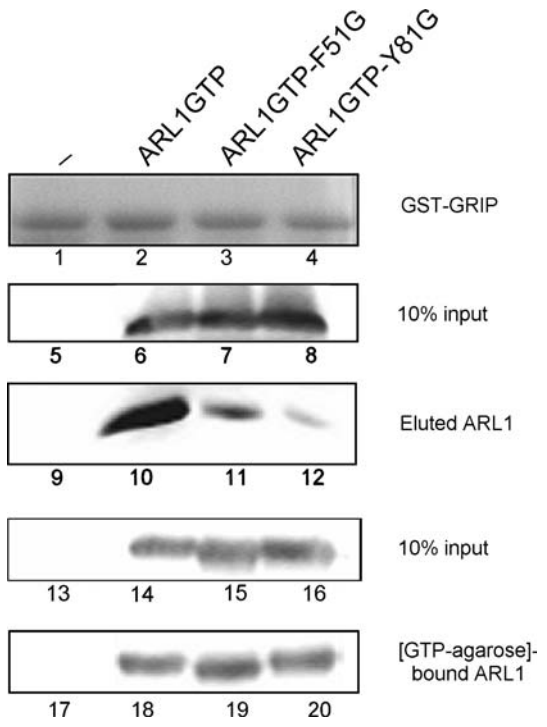

Figure 7. ARL1^{GTP} proteins with mutated F51 and Y81 show a reduced interaction with GRIP. To test the interaction of ARL1 proteins bearing mutations of the amino acid residues in position 51 and 81 with GRIP, extracts of *E. coli* at identical total protein concentration expressing His$_6$-fusions of ARL1^{GTP}, ARL1^{GTP}F51G and ARL1^{GTP}Y81G were loaded onto glutathione columns, which had been preloaded with equal volumes of extracts of *E. coli* at identical total protein concentration expressing GST-GRIP and pre-washed from unbound proteins. All proteins were then eluted and subject to immunoblot analysis with anti-His$_6$ or Coomassie Blue staining. *Lanes 1–4*: Similar loading of GST-GRIP onto columns was ascertained by aliquoting equal volumes of extracts of the same culture of *E. coli* expressing GST-GRIP in each glutathione column, by Coomassie Blue analysis and by Western blot with anti-GST serum (not shown). *Lanes 5–8*: A fraction of *E. coli* extracts expressing ARL1 proteins (130 μg total proteins/lane) was subject to western blot analysis to verify the presence of similar quantities of ARL1 proteins in the extracts. *Lanes 9–12*: Western blot with anti-His$_6$ serum on shows that ARL1^{GTP}F51G and ARL1^{GTP}Y81G were present in less quantity in the GST-GRIP column eluates in comparison to ARL1^{GTP}. This indicates that ARL1^{GTP}F51G and ARL1^{GTP}Y81G have a reduced ability to interact with GRIP in comparison to ARL1^{GTP}. The ability of ARL1^{GTP}F51G and ARL1^{GTP}Y81G to associate with GTP was tested on GTP-agarose beads. *Lanes 13–16*: Western blot with anti-His$_6$ serum on a fraction of extracts of *E. coli* cultures expressing His$_6$-tagged ARL1^{GTP}, ARL1^{GTP}F51G or ARL1^{GTP}Y81G that were used for the GTP-agarose binding experiment. *Lanes 17–20*: Western blot with anti-His$_6$ serum of the proteins bound to the GTP-agarose shows that ARL1^{GTP}F51G and ARL1^{GTP}Y81G retain their ability to associate with GTP. Negative controls: Western blots with anti-His$_6$ serum of extracts of *E. coli* transformed with empty vector [pET28(+)] expressing His$_6$-tag alone (*lanes 5, 9, 13,17*).

ARL1^{GTP}F51G and ARL1^{GTP}Y81G retained the ability to associate with GTP. This experiment strongly suggests that mutation of the residues F51 and Y81 residues are unlikely to malform ARL1^{GTP}. Instead, the results indicate that the reduced interaction of these mutants with GRIP *in vitro* is most likely due to alteration of the ARL1-GRIP interface induced by the mutations of the F51 and Y81 residues of ARL1.

Mutations of key residues of ARL1 for the interaction with GRIP abrogate the binding of GRIP to the Golgi apparatus

Having demonstrated the reduced ability of ARL1^{GTP} F51G and Y81G to bind to GRIP *in vitro*, we next wanted to examine the *in vivo* effects of these mutants on the targeting of GRIP to the Golgi apparatus. Fluorescence recovery after photobleaching (FRAP) assays showed that both ARL1 and GRIP cycle on and off the Golgi apparatus at a very high rate, consistent with the notion that they cycle from a cytosolic pool (daSilva *et al*., 2004; Supplementary data 1 and 2). Therefore, we postulated that mutant ARL1 molecules that are unable to recruit GRIP would still be capable of binding to the Golgi apparatus. They would thus rapidly displace endogenous wild-type ARL1 from the Golgi apparatus and possibly prevent GRIP association.

We first confirmed that ARL1 F51 and Y81 mutants would associate with the Golgi apparatus in live cells. For this purpose, the wild type ARL1 (Figure 8A–C) and the two mutants ARL1^{GTP}F51G-CFP and ARL1^{GTP}Y81G-CFP (Figure 8D–F and G–I) were co-expressed with the Golgi marker ERD2-YFP. Confocal microscopy analysis revealed that the two mutants were distributed to the Golgi apparatus in the same way as the GTP-bound ARL1 with intact phenylalanine and tyrosine. This confirmed our hypothesis that ARL1-Golgi association was independent of the ability to interact with GRIP.

When YFP-GRIP was co-expressed with wild-type ARL1^{GTP}-CFP, YFP-GRIP was strongly associated with the Golgi apparatus (Figure 9A–C).

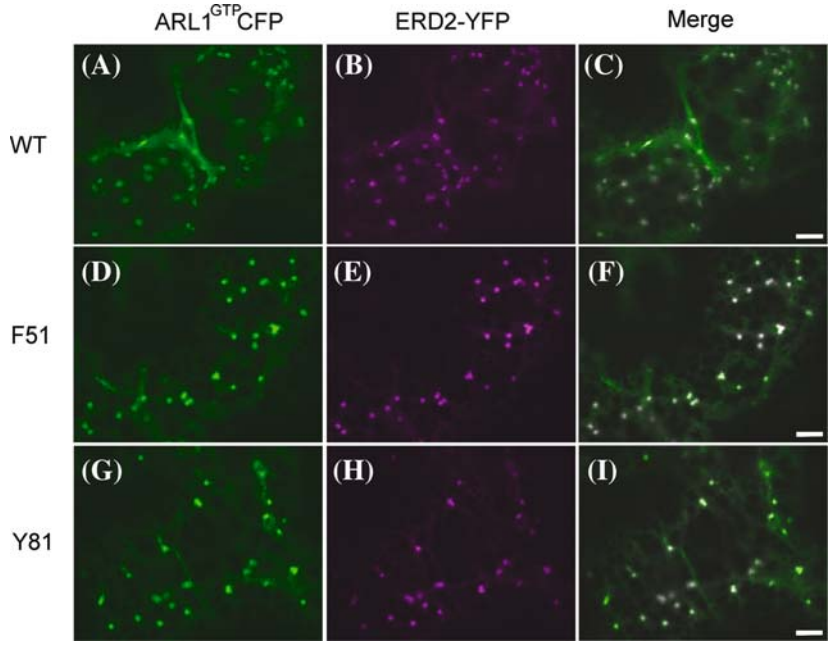

Figure 8. Mutations on F51 and Y81 of GTP-bound ARL1 do not affect the localization of the GTPase at the Golgi apparatus. Confocal images of cells coexpressing ERD2-YFP (B, E, H) with CFP-tagged $ARL1^{GTP}$ (A, WT), $ARL1^{GTP}$F51 (D) or ARF^{GTP}-Y81 (G). Note that the association of $ARL1^{GTP}$F51 and ARF^{GTP}-Y81 to the Golgi apparatus is similar to that of $ARL1^{GTP}$. (C), (F), and (I): Merged images of (A)–(B), (D)–(E) and (G)–(H), respectively. Scale bars = 5 μm.

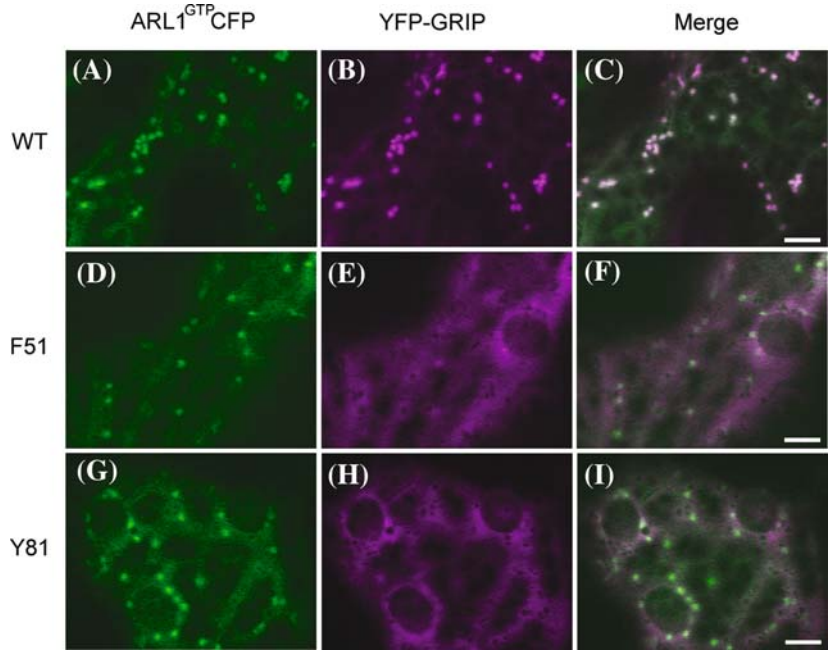

Figure 9. Mutations on key residues of arl1 affect the localization of GRIP at the Golgi apparatus. Confocal images of tobacco leaf epidermal cells coexpressing wild-type YFP-GRIP (B, E, H) with either $ARL1^{GTP}$ (A, WT), $ARL1^{GTP}$F51G-CFP (D) or $ARL1^{GTP}$Y81G-CFP (G). (C), (F) and (I): Merged images of (A)–(B), (D)–(E) and (G)–(H), respectively. Note that in the presence of the $ARL1^{GTP}$-CFP with intact F51 and Y81 residues, YFP-GRIP resides mostly on the Golgi apparatus and that the cytosolic distribution of the marker is reduced in comparison to images (C) and (F). Scale bars = 5 μm.

Such an association was similar to that in the presence of wild-type ARL1-CFP (Supplementary data 3). In contrast, co-expression with the two mutants led to a re-distribution of the GRIP fusion to the cytosol (Figure 9D–F and G–I). Taken together, these results are consistent with our prediction that intact ARL1 F51 and Y81 residues are involved in the ARL1-mediated binding of YFP-GRIP and directly support the hypothesis that ARL1 plays a role in the recruitment of GRIP to the Golgi apparatus in live plant cells.

Discussion

In this work, we have used a live-cell imaging approach combined with molecular modeling, biochemical assays and mutagenesis to analyze the cellular function and molecular mechanism of action of a member of the plant ARL family, ARL1. Our data provide direct evidence for a role of ARL1 in the binding of a conserved region of a plant golgin to the plant Golgi apparatus.

Intracellular Localization of ARL1

Our results confirm that ARL1 associates with the Golgi apparatus in plant cells (Latijnhouwers *et al*., 2005). We also show that this association depends on the integrity of a myristoylation motif at the N-terminus of ARL1. Post-translational modification with myristoyl or prenyl groups is essential for membrane association of many small GTPases in the Ras-superfamily (Johnson *et al*., 1994). The myristoylation of ARL1 is also necessary for Golgi association in mammalian cells (Lu *et al*., 2001). Conversely, not all ARL proteins are myristoylated (Burd *et al*., 2004); for example, an amino-terminal acetylation rather than myristoylation of ARL3 is needed for Golgi targeting in yeast (Setty *et al*., 2004; Behnia *et al*., 2004). This indicates that the mechanism of membrane targeting among ARLs is not entirely conserved.

ARL1 was found to associate with the Golgi apparatus in both the inactive and active forms (see also Latijnhouwers *et al*., 2005). This suggests that ARLs may have different requirements for membrane associations compared with that of ARFs. A widely accepted model of the association of the largely studied GTPase ARF1 with Golgi membranes indicates that it undergoes cycles of binding and release on Golgi membranes (Vasudevan *et al*., 1998). Inactive ARF1 has a cytosolic distribution (Vasudevan *et al*., 1998; Xu and Scheres, 2005; Stefano *et al*., In press). GEF-mediated activation of the GTPase occurs on Golgi membranes and GAP-mediated inactivation precedes release of inactive ARF1 to the cytosol (Vasudevan *et al*., 1998; Teal *et al*., 1994). The different subcellular distributions of inactive forms of ARF1 and ARL1 probably reflect characteristics of the GTPase activity of the two proteins and/or GAP activities for the two GTPases at the Golgi apparatus.

We found that inactive ARL1 ($ARL1^{GDP}$) does not affect the distribution of the Golgi marker ERD2 at the expression levels used in this study. Our observations support those of Lee *et al*. (2002) and of Latijnhouwers *et al*. (2005), who reported that the GDP-restricted mutant of ARL1 did not alter the distribution of Golgi markers in plants. In mammalian cells, inactive ARL1 is known to disrupt the Golgi localization of certain Golgi enzymes but not of Golgi matrix proteins such as p115 and GM130 (Lu *et al*., 2001). Our observations may be explained by proposing that in plant cells the ARL1-mediated pathway may be redundant, or that different factors are required for the ARL1–GEF interaction in plants in comparison to mammalian cells. Ongoing studies in our laboratory on the role of GRIP proteins in plants will provide data for further interpretation of this result.

Our data reveal that ARL1 also localizes at rare non-Golgi organelles. The biological relevance of this association is unclear (see also Latijnhouwers *et al*., 2005, for a discussion). In particular, Latijnhouwers *et al*. (2005) found that these additional structures were numerous in cells overexpressing a fluorescent protein fusion of an active ARL1 mutant. It is possible that the targeting of ARL1 to the non-Golgi structures may be due to additional functions besides an interaction with the GRIP domain on the Golgi apparatus, and that it was not as noticeable in our studies due to the much lower bacterial OD_{600} used for GRIP expression (0.05 in this work and 0.2 in Latijnhouwers *et al*., 2005).

ARL1 is involved in the binding of the GRIP domain of a plant Golgin to the Golgi apparatus

Our data prove that ARL1 has a role in the binding of a conserved GRIP domain to the Golgi

apparatus, based on several lines of evidence gathered *in vitro* and *in vivo*. First, ARL1 from leaf extracts was able to bind to GRIP. Binding of recombinant ARL1 to GRIP *in vitro* showed that this interaction is direct. Second, ARL1 interacts with GRIP via specific residues of the GRIP domain. Third, we have proved directly that disruption of the interaction of GRIP with ARL1 by reducing the affinity of ARL1 for GRIP via mutation of specific ARL1 amino acid residues determines a release of GRIP into the cytosol from the Golgi apparatus.

Our data do not exclude that other proteins beside ARL1 may function in localization of GRIP proteins to the Golgi apparatus. Although several members of the plant ARL subfamily have been identified (Gebbie *et al*., 2005), their cellular roles remain completely obscure. With the localization and function of ARL1 now established, it is reasonable to propose that at least ARL1 among the ARLs is involved in membrane traffic and in the binding of a GRIP domain of golgins to the Golgi apparatus.

Molecular dissection of the ARL1–GRIP interaction

To provide direct evidence for a role of ARL1 in the secretory pathway of plants we aimed to identify amino acid residues that would be central for the ARL1–GRIP interaction. We postulated that a disruption of the interaction between putative key amino acid residues of ARL1 and GRIP would interfere with the molecular interface between the two proteins and, as a consequence, with the binding of GRIP to the Golgi apparatus. Our studies have identified ARL1 F51 and Y81 and GRIP Y717 and K719 as being central in the predicted plant ARL1–GRIP interface. These residues were identified at the ARL1–GRIP interface by molecular modeling, and their specificity in disrupting the ARL1–GRIP binding was ensured *in vitro*, followed by an *in vivo* analysis of the effects of the mutants on the targeting of GRIP to the Golgi apparatus.

The ARL1 mutants are particularly relevant to our aims as, unlike GTP binding or GTPase mutants of ARL1 that may give rise to pleiotropic effects, ARL1 F51 and Y81 provide direct evidence for the role of ARL1 in the GRIP targeting to the Golgi apparatus. Based on our molecular models and that of the mammalian ARL1 (Panic *et al*., 2003a), the abrogation of an interaction of ARL1 with GRIP is most likely due to disruption of these amino acid residues at the ARL1–GRIP interface. Although a role for ARL1 F51 and Y81 at the ARL1-GRIP interface was predicted for the mammalian homologue (Panic *et al*., 2003a), no experimental data were presented for the relevance of these conserved residues (Panic *et al*., 2003a). We have now provided experimental evidence that mutations of ARL1 F51 and Y81 residues at the ARL1–GRIP interface abolish the interaction of GRIP with the GTPase; ARL1 F51 and, to a greater extent, Y81 are required for the ARL1–GRIP interaction and consequently for the localization of GRIP to the Golgi apparatus. This was tested with the active form of ARL1 as it has a strong direct interaction with GRIP *in vitro*. In live cells the presence of the F51 and Y81 mutants determined a redistribution of most of YFP-GRIP to the cytosol, although the ability of ARL1 mutants to bind to the Golgi apparatus was not affected. In addition, these mutations did not alter the ability of ARL1 to associate with GTP in comparison to the GTP-blocked ARL1, as demonstrated by a GTP-agarose binding experiment. Our experiments demonstrate directly in live cells that ARL1 is involved in the binding of GRIP to the Golgi apparatus, and suggests that ARL1 mutants most likely compete for the binding of GRIP at the Golgi with wild-type ARL1.

Mutation of GRIP K719 and, to a greater extent, of Y717 reduced the binding of GRIP to $ARL1^{GTP}$ *in vitro* and affected the localization of GRIP at the Golgi apparatus in comparison to wild-type GRIP in live cells. GRIP Y717, is almost invariant across species (this work; Barr; 1999; Kjer-Nielsen *et al*., 1999; Munro and Nichols, 1999; McConville *et al*. 2002; Gilson *et al*., 2004; Latijnhouwers *et al*., 2005). Similarly, the equivalent residue in mammalian, yeast and protozoan GRIP domains has proved important for GRIP to bind to an active ARL1 (Kjer-Nielsen *et al*., 1999; McConville *et al*., 2002; Lu and Hong, 2003; Wu *et al*., 2004). The weaker association of GRIP Y717 and K719 mutants with the Golgi apparatus in comparison with wild-type GRIP may be explained by a possible synergistic effect of Y717

and K719, required for association with $ARL1^{GTP}$, whereby a double mutant would show no binding with the GTPase. Future analyses based on testing the interaction of $ARL1^{GTP}$ with a double Y717/K719 mutant will provide further details on the interaction of GRIP with ARL1.

Recruitment of Golgins at the Golgi Apparatus is based on features that are partially conserved across kingdoms

We found that in a cell-free system GTP is required for optimal GRIP–ARL1 interaction, as without GTP binding the interaction between the two recombinant proteins is reduced, in agreement with studies with yeast and mammalian homologues of ARL1 and GRIP proteins (Panic *et al*., 2003a, b; Setty *et al*., 2003; Lu and Hong, 2003) and *in vitro* with plant homologues (Latijnhouwers *et al*., 2005). In contrast to reports in mammalian cells on the interaction of the GRIP domain with ARL1 (Lu and Hong, 2003), the assay based on extracts of plant cells expressing ARL1 and recombinant GST-GRIP showed an interaction of GST-GRIP with $ARL1^{GDP}$, besides that with $ARL1^{GTP}$. The glutathione binding assay based on recombinant proteins proves that the activation of ARL1 is sufficient for the interaction of the GTPase with GRIP and that interaction of $ARL1^{GTP}$ with GRIP is direct. Therefore, the interaction of $ARL1^{GDP}$ from leaf extracts with recombinant GRIP may be a consequence of the association of $ARL1^{GDP}$ with other plant-specific cytosolic or Golgi-associated factors, such as ARL1-GRIP adaptors, that would indirectly contribute to the interaction of the mutant GTPase with GRIP prior to its activation.

Our results were obtained using low levels of expression of $ARL1^{GDP}$. Previous studies have shown that overexpression of $ARL1^{GDP}$ causes partial displacement of GRIP from the Golgi apparatus (Latijnhouwers *et al*., 2005). Our data suggest that $ARL1^{GDP}$ may indirectly interact with GRIP on the Golgi apparatus in conditions of low expression of the protein. However, overexpression of $ARL1^{GDP}$ may affect the availability or functionality of factors that mediate a fruitful ARL1–GRIP interaction on the Golgi. As a consequence a loss of GRIP binding to the Golgi apparatus may take place in condition of overexpression of the mutant (Latijnhouwers *et al*., 2005).

To characterize the role of additional factors for the binding of GRIP to the Golgi apparatus further, it will be important to investigate plant homologues of the cytosolic protein ARL3 and of the membrane associated protein SYS1 that have been shown to have an indirect role in the ARL1-GRIP interaction in mammals and yeast (Behnia at al., 2004; Setty *et al*., 2004).

Concluding remarks

Our results open investigations of the role of ARL-family GTPases in protein transport along the plant secretory pathway and in the maintenance of the identity of the Golgi apparatus via interactions with proteins such as golgins. Based on differences between systems across kingdoms (Ward and Brandizzi, 2004; Nakano, 2004), it cannot be excluded that the plant early secretory pathway may also have unique mechanisms that govern its peculiar architecture and the dynamics of protein transport through small GTPases such as ARLs despite the conservation of the molecular interaction of ARL1 with a GRIP domain. ARLs share a high degree of homology with ARF-GTPases, but the two subfamilies are known to have different functions and bind different effectors in mammals and yeast (Memon, 2004; Munro, 2005). Characterization of members of the Ras-superfamily and determination of their subcellular localization, along with investigation of the Golgi-targeting mechanisms of plant golgins, represent an important step in understanding the mechanisms that regulate protein transport along the plant secretory pathway.

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