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AGD5 is a GTPase-activating protein at the *trans*-Golgi network

Giovanni Stefano[1,*,†,‡], Luciana Renna[1,†,‡], Marika Rossi[1], Elisa Azzarello[1], Susanna Pollastri[1], Federica Brandizzi[2], Frantisek Baluska[3] and Stefano Mancuso[1]

[1]*Department of Plant, Soil and Environmental Science, viale delle Idee, University of Florence, Sesto Fiorentino, FI 50019, Italy,*
[2]*Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA, and*
[3]*Institut für Zelluläre und Molekuläre Botanik, Universität Bonn, Kirschallee 1, D-53115 Bonn, Germany*

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*For correspondence (fax +1 517 353 9168; e-mail gstefano@msu.edu).
†These authors contributed equally to this work.
‡Present address: Department of Energy Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA.

SUMMARY

ARF-GTPases are important proteins that control membrane trafficking events. Their activity is largely influenced by the interplay between guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which facilitate the activation or inactivation of ARF-GTPases, respectively. There are 15 predicted proteins that contain an ARF-GAP domain within the *Arabidopsis thaliana* genome, and these are classified as ARF-GAP domain (AGD) proteins. The function and subcellular distribution of AGDs, including the ability to activate ARF-GTPases *in vivo*, that remain largely uncharacterized to date. Here we show that AGD5 is localised to the *trans*-Golgi network (TGN), where it co-localises with ARF1, a crucial GTPase that is involved in membrane trafficking and which was previously shown to be distributed on Golgi and post-Golgi structures of unknown nature. Taking advantage of the *in vivo* AGD5–ARF1 interaction at the TGN, we show that mutation of an arginine residue that is critical for ARF-GAP activity of AGD5 leads to longer residence of ARF1 on the membranes, as expected if GTP hydrolysis on ARF1 was impaired due to a defective GAP. Our results establish the nature of the post-Golgi compartments in which ARF1 localises, as well as identifying the role of AGD5 *in vivo* as a TGN-localised GAP. Furthermore, *in vitro* experiments established the promiscuous interaction between AGD5 and the plasma membrane-localised ADP ribosylation factor B (ARFB), confirming that ARF-GAP specificity for ARF-GTPases within the cell environment may be spatially regulated.

Keywords: ARF-GAP, GTPase, ARF1, *trans*-Golgi network, Golgi apparatus, protein transport.

INTRODUCTION

The regulation of membrane traffic is essential for the functioning of all eukaryotic cells, and small GTPase proteins largely control this process. GAP proteins influence the activity of small GTPases by facilitating GTP hydrolysis events (Donaldson and Jackson, 2000). Based on their substrate proteins, GAPs can be divided into several groups, including ADP ribosylation factor (ARF) GAPs, Rab GAPs and Rho GAPs. GAPs play a particularly crucial role for ARFs because ARF proteins do not have detectable intrinsic GTPase activity and bind tightly to GTP (Randazzo and Kahn, 1994); thus GAPs are needed for conversion of ARFs from the GTP-bound form (active) to the GDP-bound form (inactive). Furthermore, because ARFs bind most of their effectors in the GTP-bound form, ARF-GAPs also play a critical role in terminating ARF–effector protein interactions (Spang *et al.*, 2010).

In higher eukaryotes, ARF-GAP families consist of a large number of diverse proteins (Donaldson and Jackson, 2000; Jensen *et al.*, 2000) that may be grouped into subfamilies depending on domain structure and phylogenetic analyses. Generally, GAPs share a common N-terminal GAP homology domain with a Cys4 zinc finger motif (Cukierman *et al.*, 1995) that promotes the GTPase activity of ARFs. The structure of GAPs mostly varies at the C-terminus, which may contain conserved pleckstrin homology (PH) or Ca^{2+} binding domains that may serve to specify the target membranes (Donaldson and Jackson, 2000; Jensen *et al.*, 2000).

In yeast and humans, ARF-GAPs have been described in detail and their function has been demonstrated for membrane trafficking (Poon *et al.*, 1999; Szafer *et al.*, 2000; Lanoix *et al.*, 2001; Tanabe *et al.*, 2005; Yahara *et al.*, 2006; Spang *et al.*, 2010); however, the role of ARF-GAP proteins in plant cells is not very well defined. In the *Arabidopsis thaliana* genome, 15 proteins have been identified and classified as ARF-GAP domain (AGD) proteins (Vernoud *et al.*, 2003). Based on phylogenetic analyses and domain organisation, ARF-GAP proteins are grouped into four distinct classes. Three groups are related and similar to the yeast ARF-GAPs Age2p, Gcs1p and Glo3p that act at the *trans*-Golgi network (TGN), while the members of the fourth group show similarities to human ARF-GAP1, which functions at the Golgi (Donaldson, 2000; Jensen *et al.*, 2000; Jurgens and Geldner, 2002).

To date, the role of most AGDs is largely unexplored. Recent studies have shown that four AGDs (NEV/AGD5, VAN3/SCARFACE, OsAGAP and AGD1) play important roles in membrane trafficking, with important implications in hormone signalling, polarised cell growth or organ separation (Koizumi *et al.*, 2005; Sieburth *et al.*, 2006; Zhuang *et al.*, 2006; Yoo *et al.*, 2008; Liljegren *et al.*, 2009).

In addition to the biological role of most AGDs, fundamental questions remain regarding the role of AGDs in relation to plant ARFs. These relate to the ARF/AGD ratio and the possibility of promiscuous interactions between ARF and AGD proteins. Plant ARFs are targeted to various subcellular compartments, including the Golgi apparatus, post-Golgi organelles and the plasma membrane (Stefano *et al.*, 2006; Matheson *et al.*, 2007, 2008). It is therefore plausible that the various AGDs may be distributed on distinct membranes to specifically activate different ARFs. However, the ratio of detectable ARF/ARF-like proteins to AGDs in the Arabidopsis genome is 18:15 (Vernoud *et al.*, 2003; Min *et al.*, 2007); therefore, plant AGDs with different GTPases must be promiscuous to satisfy GTPase activation in cells. This concept is further complicated by the fact that a single GTPase may have dual localization, and that distinct GAPs may be localised on two compartments. This is exemplified by ARF1, one of the best studied plant GTPases. ARF1 has been shown to be involved in several traffic routes, including regulation of protein traffic at the Golgi apparatus/endoplasmic reticulum (ER) interface (Lee *et al.*, 2002; Takeuchi *et al.*, 2002; Stefano *et al.*, 2006; Matheson *et al.*, 2007) and vacuolar protein traffic (Pimpl *et al.*, 2003). These findings suggest that ARF1 may play a role in traffic routing into post-Golgi organelles. In support of this hypothesis, ARF1 has not only been localised to the Golgi complex, but also to organelles of undefined nature that detach from the Golgi (Stefano *et al.*, 2006) and can be stained using the endocytic marker FM4-64 (Xu and Scheres, 2005; Matheson *et al.*, 2007). Furthermore, although ARF1 has been shown to recruit coatomer on Golgi membranes in live plant cells, it also recruits a coiled-coil protein, GDAP1, on the Golgi and post-Golgi organelles. These data suggest that, in order to perform its various roles (Matheson *et al.*, 2007), ARF1 may interact with protein machineries (effectors and GTP hydrolysis regulators) on distinct organelles. Thus, an intriguing possibility is that ARF1 may accomplish its multiple roles under spatial regulation by specific AGD proteins located in different compartments. In this way, AGDs may regulate specific ARF1 activities within the cell. In support of this, AGD7 has been shown to be associated with the ARF1-GAP at the Golgi (Min *et al.*, 2007), but the identity of the ARF-GAPs that are involved in the activity of ARF1 on post-Golgi compartments remains unknown. Nonetheless, an interaction of ARF1 with various plant ARF-GAPs has been shown *in vitro* [i.e. VAN3 (AGD3), RPA (AGD10) and AGD7; Koizumi *et al.*, 2005; Song *et al.*, 2006; Min *et al.*, 2007]. These findings, coupled with the evidence that there is a higher number of plant ARFs compared to AGDs, suggest the possibility of a promiscuous interaction between ARF1 and various AGDs when the GTPase is outside its natural subcellular context. It has been shown that recombinant plant AGDs can act on human and yeast ARF substrates (Koizumi *et al.*, 2005; Liljegren *et al.*, 2009), further supporting the possibility that the specificity of an AGD/ARF interaction may depend on additional factors present on target membranes.

Despite the prevailing view that ARF-GAPs act as inactivators of ARF-GTPases, it has been shown that at least two mammalian GAPs do not have detectable GAP activity, and that the GAP activity of other ARF-GAPs is dispensable for some cellular functions. This raises the intriguing question as to whether all plant AGDs behave as *bona fide* GAP proteins on their target membranes *in vivo*.

To address these questions, and thus contribute to knowledge of the plant AGDs *in vivo*, we investigated the role of AGD5, a member of the class 2 GAP sub-family, which contains five ARF-GAP proteins (AGD5–10) with an ARF-GAP domain at the N-terminus. Understanding the mechanisms by which ARFs are controlled *in vivo* may clarify the role of these proteins during regulation of cargo protein sorting and vesicle coat protein recruitment. Here we demonstrate that AGD5 functions as a GAP at the TGN, thus ascribing a functional role *in vivo* to one of the 15 putative members of the Arabidopsis ARF-GAP family and confirming that the specificity of the AGD/ARF interaction is controlled by the *in vivo* environment.

RESULTS

Identification of AGD5 and its domain structure

To identify a putative ARF-GAP protein localised in the post-Golgi structures, a bioinformatics search of the Arabidopsis genomic database was performed. AGD5

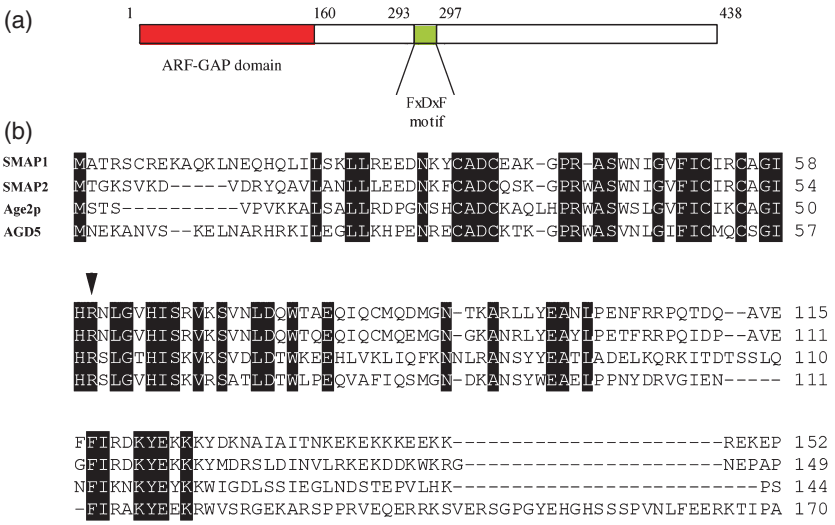

Figure 1. AGD5 and its domain structure.
(a) Functional domain and motif of the AGD5 protein. These regions include a GAP activity domain (amino acids 1–160) and a FxDxF motif that is important for binding of accessory endocytic proteins to the α-subunit of adaptor protein complex AP-2 (amino acids 293–297).
(b) Multiple sequence alignment of ARF-GAPs by ClustalW: Human SMAP1 (Q8IYB5), SMAP2 (Q8WU79), *Saccharomyces cerevisiae* Age2p (YIL044C) and *Arabidopsis thaliana* AGD5 (At5g54310). The conserved arginine amino acid is indicated by an arrowhead.

(At5g54310) appeared to be the closest homologue to yeast Age2p (51% identity, 65% homology), which resides on the TGN (Jurgens and Geldner, 2002; Natsume *et al.*, 2006) (see also Figure S1). Protein alignment of AGD5 with human SMAP1/2 and yeast Age2p showed that residues within the ARF-GAP domain are conserved (Figure 1b and Figure S2). Furthermore, primary protein sequence analysis indicated the presence of an FxDxF motif in positions 293–297 (Figure 1a). In animal cells, this motif is responsible for the binding of accessory endocytic proteins to the appendage of the α-subunit of adaptor protein complex AP-2 (Mishra *et al.*, 2004; Ritter *et al.*, 2007; Olesen *et al.*, 2008). Proteins of the endocytic machinery have been found on endosomes in plants, where the exocytic and endocytic pathways may converge (Holstein, 2002). These findings support our hypothesis that AGD5 could be an ARF-GAP involved in post-Golgi trafficking.

AGD5 is distributed on a sub-population of TGN structures, where it co-localises with ARF1

To establish whether AGD5 is indeed localised to post-Golgi compartments, we expressed this protein as a yellow fluorescent protein (YFP) fusion (YFP–AGD5) in tobacco leaf epidermal cells. Confocal microscopy analyses of leaf epidermal cells showed that YFP–AGD5 is localised at punctate structures (Figure 2a, arrowhead) and in the cytosol (Figure 2a, arrow). Despite the punctate distribution of YFP–AGD5, co-localization analyses with a known ER/Golgi marker, ERD2–CFP (Brandizzi *et al.*, 2002b), revealed that the AGD5 fusion was not localised to the Golgi (Figure 2b–d), although it was in close association with this organelle at times (Figure 2e).

To determine the identity of these non-Golgi structures, YFP–AGD5 was co-expressed with post-Golgi markers.

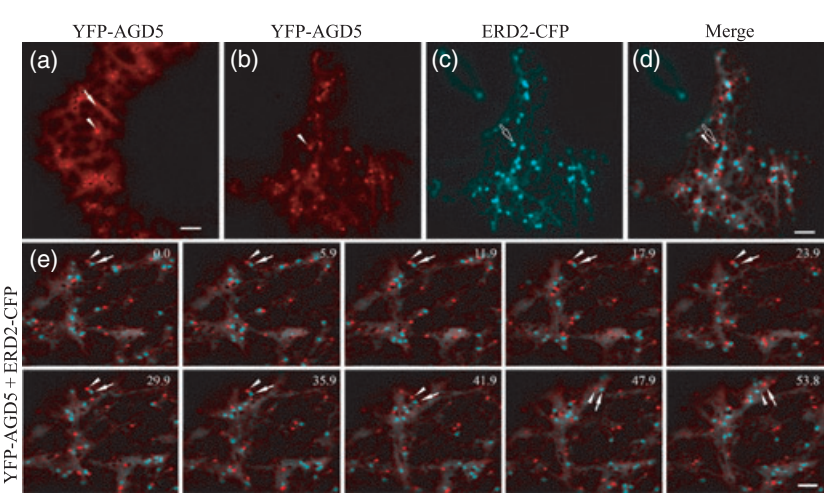

Figure 2. Subcellular distribution of AGD5 in *Nicotiana tabacum* plant cells.
(a–d) Confocal images of tobacco leaf epidermal cells 2 days after *A. tumefaciens* infiltration. Images are of YFP–AGD5 alone (a) or with the Golgi marker and ERD2–CFP (b–d). YFP-AGD5 alone (a) shows fluorescence distributed as punctate structures (white arrow), and in the cytosol (white arrowhead). YFP-AGD5 (b) fluorescence is closely associated with the Golgi apparatus (c, ERD2-CFP) (black arrow). (d) Merged image of (b) and (c).
(e) Images from a time-lapse sequence of tobacco leaf epidermal cells expressing YFP–AGD5 and ERD2–CFP.
Scale bars = 5 μm.

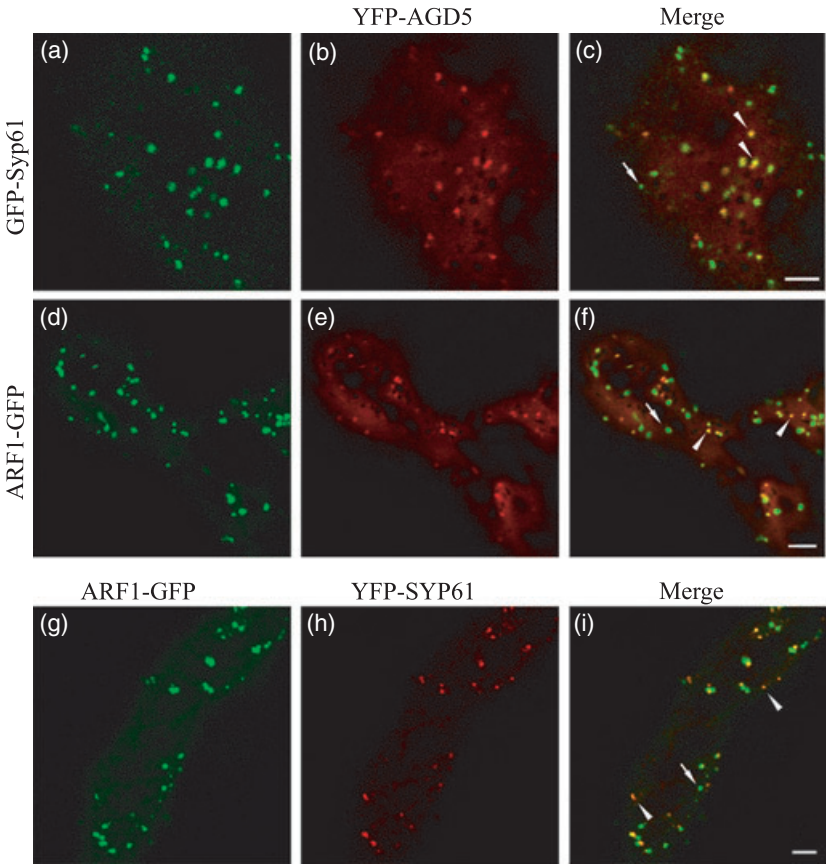

Figure 3. Subcellular distribution of AGD5, SYP61 and ARF1 in *Nicotiana tabacum* plant cells.
(a–c) Confocal images of tobacco leaf epidermal cells 2 days after *A. tumefaciens* infiltration. Images of YFP–AGD5 (b) with the TGN marker GFP–SYP61 (a), showing fluorescence distributed as punctate structures that co-localise with the TGN (white arrowhead), and additional dots corresponding to SYP61 (white arrow) (c). (c) Merged image of (a) and (b).
(d–f) Confocal images of tobacco leaf epidermal cells 2 days after *A. tumefaciens* infiltration. YFP–AGD5 fluorescence (e) partially overlaps with ARF1–GFP (d) (white arrowhead), but additional dots corresponding to ARF1 are also visible (white arrow) (f). (f) Merged image of (d) and (e).
(g–i) Confocal images of tobacco leaf epidermal cells 2 days after *A. tumefaciens* infiltration. ARF1–GFP fluorescence (g) partially overlaps with YFP–SYP61 (h) (white arrowhead), but additional dots corresponding to ARF1 are also visible (white arrow) (i). (i) Merged image of (g) and (h).
Scale bars = 5 μm.

YFP–AGD5 was found to co-localise with the known TGN marker GFP–SYP61 (Figure 3a–c) (Sanderfoot *et al.*, 2001). The localization was confirmed in Arabidopsis YFP–AGD5 transgenic lines in which fluorescence of AGD5 was detected as punctate structures along the lower epidermal cells (Figure S3a–c). Transient transformation of an Arabidopsis YFP–AGD5 transgenic line with GFP–SYP61 confirmed co-localization with the TGN marker (Figure S3d–f). However, the co-localization was only partial, as not all the TGN structures labelled with GFP–SYP61 were positive for YFP–AGD5. These findings indicate that YFP–AGD5 is localised on a sub-population of TGN structures in plant cells, confirming recent findings (Liljegren *et al.*, 2009). These results also give support to earlier data suggesting that the TGN is not uniform, but instead is functionally differentiated in plant cells (Koizumi *et al.*, 2005).

We next wished to establish whether AGD5 could function as a GAP protein *in vivo*. To do so, we wished to identify a GTPase that could function as a substrate for AGD5. The localization pattern of AGD5 partially mirrors that of ARF1, previous studies having shown that ARF1 is localised to a sub-population of post-Golgi organelles labelled by FM4-64 (Matheson *et al.*, 2007). Therefore, we sought to establish the localization of AGD5 in relation to ARF1. Co-localization analyses with ARF1–GFP (Stefano *et al.*, 2006) and YFP–AGD5 showed that localization of ARF1–GFP partially overlapped with that of YFP–AGD5 (Figure 3d–f) as well as that of a TGN marker, YFP–SYP61 (Figure 3g–i). We expected this co-localization pattern as AGD5 is present at the TGN but ARF1 is localised at the Golgi, in addition to the small post-Golgi compartments (Xu and Scheres, 2005; Matheson *et al.*, 2007). These data not only define the ARF1 post-Golgi compartments of unknown nature described previously (Xu and Scheres, 2005; Matheson *et al.*, 2007) as TGN structures, but also suggest that if AGD5 is a GAP for ARF1, then interaction between the two proteins must occur specifically on the TGN.

AGD5 exhibits promiscuous interaction with ARF-GTPases localised to various compartments *in vitro*

Given the co-localization of AGD5 and ARF1, we investigated whether ARF1 is a possible substrate for AGD5 *in vivo*. First we determined whether ARF1 could interact with AGD5 *in vitro* using a glutathione–agarose affinity chromatography assay based on interaction of recombinant GST–AGD5 with a fluorescent fusion of the mutant ARF1 in the GTP-bound form expressed in tobacco leaves. We used a GTP-bound form of ARF1 as GAP proteins assist GTPase molecules in stimulating GTP hydrolysis. Therefore in the GTP locked form, GTPase should interact tightly with GAPs. First, we

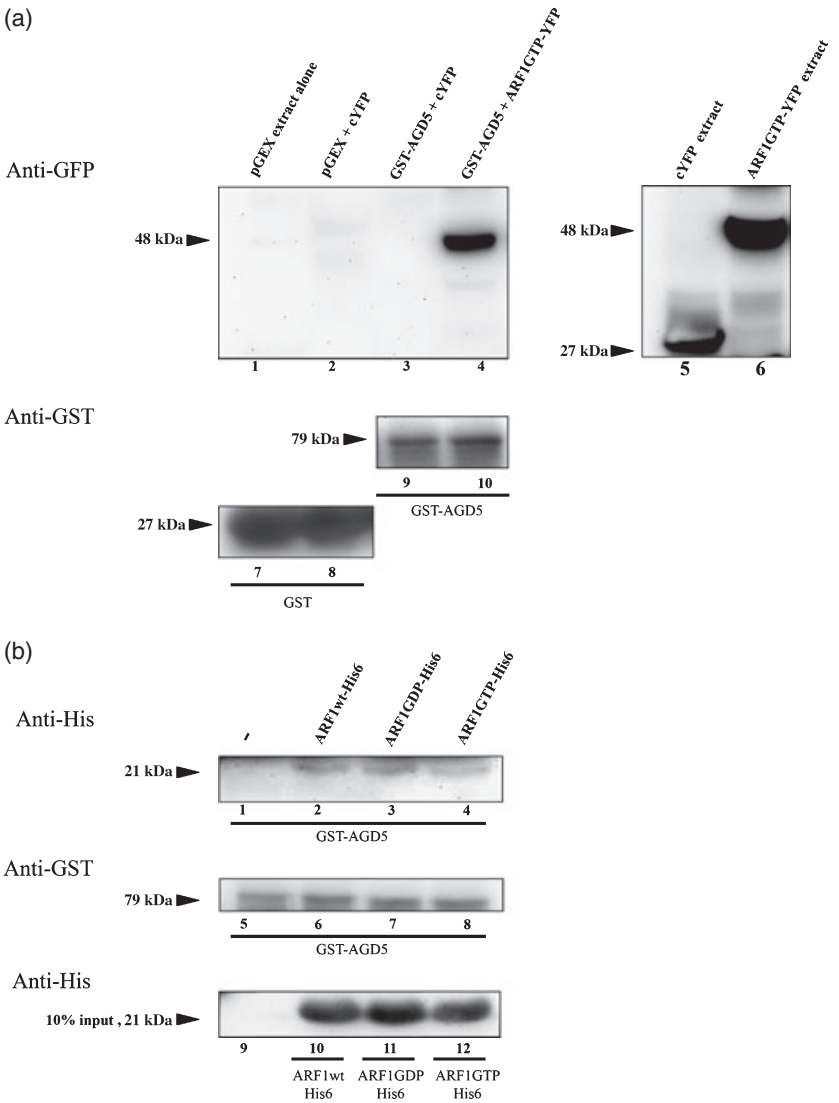

Figure 4. AGD5 interacts with ARF1.
(a) Interaction between GST–AGD5 and ARF1GTP–YFP protein. Western blot probed with an anti-GFP antibody. ARF1GTP–YFP (lane 4) was retained by GST-AGD5. Negative controls comprising extracts of pGEX and pGEX loaded with cytosolic YFP were used to test the specificity of the resin (lanes 1 and 2). In a further negative control, GST–AGD5 did not retain cytosolic YFP (lane 3). Western blot analysis with anti-GFP antibody of extracts of leaves expressing cYFP (lane 5) or ARF1GTP-YFP (lane 6). Lanes 7–10: Western blot probed with anti-GST antibody showing the amount of GST (lanes 7 and 8) or GST–AGD5 (lanes 9 and 10) bound to agarose beads.
(b) Interaction between GST–AGD5 and ARF1 proteins produced in heterologous *E. coli* cells. Lanes 1–4: Western blot probed with anti-His6 antibody. Extracts of *E. coli* at identical total protein concentration, expressing His6-tagged ARF1GDP (lane 2) and ARF1GTP mutant proteins (lane 3), were loaded onto GST–AGD5 beads. Eluates of these columns were subject to immunoblot analysis. Negative control: extracts from *E. coli* expressing His6 tag alone (lane 1). Lanes 5–8: Western blot probed with anti-GST antibody showing that the amount of GST–AGD5 bound to agarose beads was similar for all samples. Lanes 9–12: Western blot with His6 antibody on a fraction of *E. coli* extracts loaded onto the GST columns, showing that comparable amounts of wild-type ARF1 (lane 10), ARF1GDP (lane 11) and ARF1GTP (lane 12) were used for the experiment. Negative control: extracts from *E. coli* expressing His6 tag alone (lane 9). Note that the volume of loaded extracts from the eluted columns was identical for the Western blots with anti-GST or anti-His6 antibody. The ARF signal was revealed with the anti-His6 serum is proportional to the amount of GST–AGD5.

loaded extracts of *Escherichia coli* expressing a GST–AGD5 fusion onto columns, and washed them to eliminate unbound proteins. Then, we extracted total proteins from tobacco leaves expressing an *ARF1GTP–YFP* fusion. The extracts were then loaded onto the pre-loaded GST–AGD5 columns (Figure 4a). Eluates of the columns were analysed by Western blotting using anti-GFP serum. The Western blot showed that ARF1GTP–YFP fusion proteins from leaf extracts can interact with recombinant GST–AGD5 *in vitro* (Figure 4a, lane 4), suggesting that AGD5 may indeed be the ARF-GAP on the TGN.

To establish whether the interaction of ARF1 with AGD5 was direct and did not require a co-factor or an adaptor protein to bind the GTPase, we tested the interaction of ARF1 and AGD5 in an acellular system using recombinant proteins. For this, equal amounts of purified ARF1 protein and its mutants were used for a binding assay. In each glutathione column, an equal amount of bacterial lysate expressing GST–AGD5 was loaded. Subsequently, unbound proteins were washed from the glutathione columns. Then, bacterial lysates of strains expressing His6-tagged wild-type ARF1, ARF1GDP or ARF1GTP were applied to the GST–AGD5-loaded columns. The bound protein was eluted, loaded on an SDS gel, and transferred to nitrocellulose membrane. Western blotting was performed using anti-His6 serum (Figure 4b, lanes 1–4), and showed that AGD5 was capable of direct interaction with ARF1. To show that GST–AGD5 was equally loaded on each column, a Western blot was performed and analysed with anti-GST serum as a control, confirming that the columns contained similar quantities of the GST fusion (Figure 4b, lanes 5–8). Similarly, a Western blot on a fraction of the total protein extract from *E. coli* expressing His6-tagged ARF1 protein showed that similar quantities of wild-type ARF1, ARF1GDP and ARF1GTP were

loaded on the GST–AGD5 columns (Figure 4b, lanes 10–12), excluding the possibility that the result could be due to a different quantity of proteins being applied to the columns. The Western blots with anti-His6 and anti-GST sera showed that the detectable ARF1 protein signals were proportional to the amount of GST–AGD5 in the columns. Taken together, these results show that the association of ARF1 with AGD5 occurs with all three ARF1 forms, suggesting the interaction with AGD5 occurs regardless of the activation state of ARF1 (Figure 4b, lanes 2–4). This experiment supports our previous results demonstrating an interaction between ARF1 and AGD5 using plant cell extracts and glutathione–agarose beads (Figure 4a). In addition, a direct association between active ARF1 and AGD5 was found.

We next wished to explore whether AGD5 could also interact with other ARF-GTPases outside its cellular environment. To do so, we performed another pull-down experiment using a plasma membrane-localised ARF-GTPase, ARFB (Matheson *et al.*, 2008). To do this, extracts of tobacco leaves expressing ARFBGTP–YFP were loaded onto a column containing recombinant GST–AGD5, in an analogous manner to the pull-down experiment performed with ARF1–YFP (see above). The experiment also revealed an interaction with ARFB (Figure S4). Because ARFB is localised at the plasma membrane in live cells but AGD5 is present at the TGN, these results strongly suggest that the specificity of ARF-GAPs for target ARF-GTPases is spatially regulated.

AGD5 functions as a TGN-localised ARF-GAP *in vivo*

The ability of AGDs to stimulate GTP hydrolysis has been demonstrated *in vitro* using recombinant heterologous ARF substrates (Koizumi *et al.*, 2005; Liljegren *et al.*, 2009). We wished to test the ability of AGD5 to function as an ARF-GAP *in vivo*, and opted for a site-directed mutagenesis approach coupled with advanced live-cell imaging. In mammalian cells, GAP protein stimulation of GTP hydrolysis on GTPases has been shown to require an arginine residue within the GAP domain. This amino acid fits into the GTP-binding pocket of the GTPase, catalysing GTP hydrolysis (Szafer *et al.*, 2000). Consistent with this, replacement of Arg50 by alanine, lysine or glutamine abolished GAP activity on the GTPase ARF1 (Szafer *et al.*, 2000). We used the conserved arginine residue of AGD5 to test whether AGD5 could work as an ARF-GAP in live cells, and generated an AGD5 mutant by substituting Arg59 with a glutamine residue, producing the AGD5^{R59Q} mutant. We reasoned that AGD5^{R59Q} would compete with wild-type AGD5 and affect the GTP/GDP cycle of ARF1, causing ARF1 to be available mainly in the GTP form. Earlier fluorescence recovery after photobleaching (FRAP) experiments demonstrated that an ARF1–GTP mutant resided on the membranes longer than wild-type ARF1 did (Stefano *et al.*, 2006). Therefore, if AGD5 functions as an ARF-GAP in plant cells, an ARF1–GFP fusion would reside longer on the membranes of cells co-expressing AGD5^{R59Q} compared to cells not expressing this mutant. To test this, we performed a FRAP experiment to compare the duration of residence of ARF1 on membranes in cells either expressing ARF1–GFP alone or together with the YFP–AGD5^{R59Q} mutant. Before this, however, we tested whether the R59Q mutation would affect the distribution of AGD5 at the TGN. Confocal microscopy analysis of cells co-expressing GFP–SYP61 and YFP–AGD5^{R59Q} revealed that this mutant was localised at the TGN (Figure 5a–c), indicating that the R59Q substitution did not interfere with the ability of AGD5 to associate with the TGN.

Photobleaching of the ARF1–GFP fluorescence alone or when co-expressed with YFP–AGD5 indicated similar half-time recovery values for ARF1–GFP fluorescence of 9.56 $\pm$ 1.47 and 9.30 $\pm$ 1.51 sec, respectively (n = 11; P > 0.05). In the presence of YFP–AGD5^{R59Q}, the half-time recovery values for ARF1–GFP fluorescence were significantly lower than those of ARF1–GFP alone (12.53 $\pm$ 1.13 sec) (n = 11, P < 0.05) (Figure 5d), indicating that the presence of the AGD5^{R59Q} affected the duration of residence of ARF1 on the membranes. To exclude the possibility that the YFP signal could contribute to measurements of the GFP signal during the recovery phase, we photobleached ARF1–GFP in cells co-expressing YFP–GDAP1, a coiled-coil protein that is known to interact with ARF1 on post-Golgi structures (Matheson *et al.*, 2007). Under these conditions, the half-time recovery of ARF1–GFP (9.05 $\pm$ 2.09 sec) was not significantly different from the values for ARF1–GFP expressed alone or co-expressed with YFP–AGD5 (n = 11, P > 0.05), indicating that the presence of YFP did not influence the recovery values of ARF1–GFP. These data not only show that ARF1 moves on and off of the membranes as described previously (Stefano *et al.*, 2006), but are also consistent with our hypothesis that the AGD5^{R59Q} mutation affects this dynamic cycling of ARF1 on and off membranes by inducing a longer duration of residence of the GTPase at the TGN compared to cells that do not express the mutant. These data indicate that AGD5 is an ARF-GAP at the TGN *in vivo*.

DISCUSSION

Although the function of several ARF-GAPs in membrane trafficking has been defined in detail in mammalian and yeast cells (Goldberg, 1999; Yanagisawa *et al.*, 2002; Bigay *et al.*, 2003; Liu *et al.*, 2005; Natsume *et al.*, 2006), the role of most of the putative ARF-GAP proteins in plants is as yet uncharacterized. Here, we report on the subcellular localization and the biological role of AGD5, one of the 15 putative ARF-GAPs in Arabidopsis. We identified this protein via similarity searches with two well-known GAPs: yeast Age2p and human SMAP1/2, both of which function in post-Golgi trafficking (Poon *et al.*, 2001; Jurgens and Geldner, 2002; Natsume *et al.*, 2006). We demonstrated that the subcellular

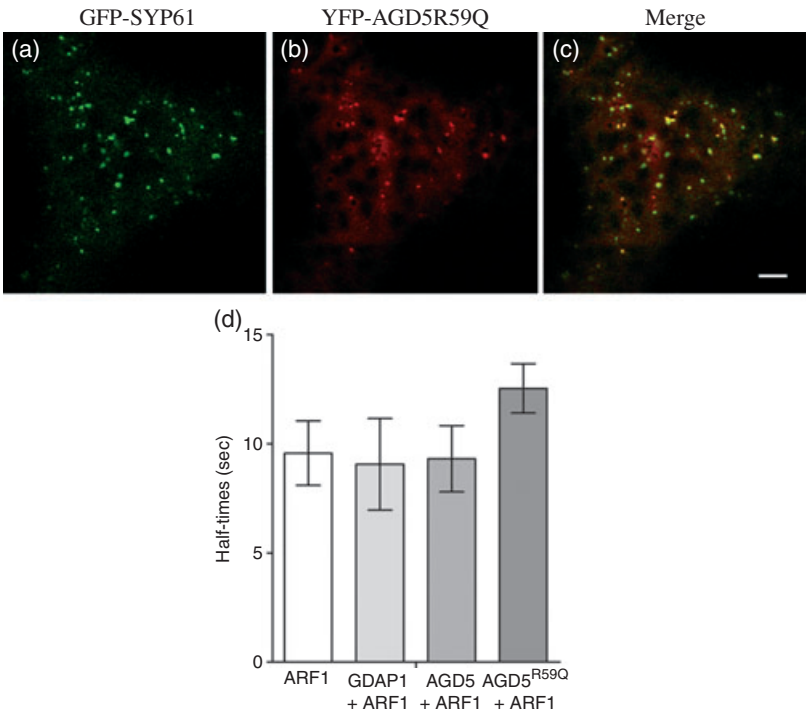

Figure 5. Subcellular distribution of the AGD5 mutant protein.
Confocal images of tobacco leaf epidermal cells 2 days after *A. tumefaciens* infiltration. Images of YFP–AGD5^{R59Q} (b) with TGN marker GFP–SYP61 (a), showing fluorescence distributed as punctate structures and in the cytosol. (c) Merged image of (a) and (b). Scale bars = 5 μm. (d) Histogram of the half-time recovery values for GFP fluorescence after photobleaching in cells expressing ARF1–GFP alone (n = 11) or in the presence of YFP–GDAP1 (n = 11), YFP–AGD5 (n = 11) or YFP–AGD^{R59Q} (n = 11). The difference between the groups was significant at $P < 0.05$ (Student's t-test).

distribution of AGD5 overlaps with that of ARF1 at the TGN. Live-cell imaging analyses also showed that expression of an AGD5 mutant with an amino acid substitution in a conserved region necessary for GAP activity influenced the duration of residence of ARF1 on the TGN membranes. Together, these data provide *in vivo* evidence that AGD5 is an ARF-GAP at the TGN.

AGD5 is a *bona fide* GAP *in vivo*

To date, GAP activity has been shown only for VAN3 and AGD5 using heterologous substrates *in vitro* (Koizumi *et al.*, 2005; Liljegren *et al.*, 2009), but the activity of the other putative AGDs remains unexplored. Given that not all proteins containing a GAP domain have a regulatory activity on ARF-GTPases in mammalian cells (Spang *et al.*, 2010), it is still to be proven that all AGDs may be functional GAPs, especially in an *in vivo* context. Our *in vitro* pull-down assays have shown that AGD5 is capable of an interaction with ARF1, and that such interaction is direct. The results of these experiments suggested a method to test whether AGD5 functions as a GAP *in vivo*. Thus, we devised an experiment based on expression of a fluorescent fusion of AGD5 bearing a mutation (AGD5^{R59Q}) in a conserved residue that is known to be important for the GAP activity of homologous proteins, followed by photobleaching analyses. Previous photobleaching experiments in live mammalian and plant cells showed that the duration of residence of a GTP-locked ARF1 mutant on the membranes is longer than that of wild-type ARF1 (Presley *et al.*, 1997; Matheson *et al.*, 2007). We expected that AGD5^{R59Q} would compete with the endogenous GAP, and anticipated that, if AGD5 is indeed a GAP, ARF1 release from the membranes would be slower in the presence of the GAP mutant compared to control cells. Our reasoning was that the GAP mutant would interfere with the activity of the endogenous GAP to activate hydrolysis of GTP on ARF1. Such activity would block ARF1 in the active state and result in greater stability of the GTPase on the membrane compared to cells that did not express the GAP mutant. Consistent with our hypothesis, we found that the duration of residence of ARF1 on the membranes was longer in the presence of the AGD5 mutant compared to control cells. Therefore, our approach allowed us to establish *in vivo* that AGD5 is a functional GAP at the TGN.

The specificity of ARF-GAPs is spatially regulated

The evidence that ARF1 and AGD5 co-localise at the TGN and that they can interact suggests that AGD5 may be a GAP of ARF1 at the TGN. Although this is plausible, it is also possible that ARF1 interacts with AGD5 due to overexpression of the two proteins. Therefore, our results do not exclude the possibility that, at endogenous expression levels, AGD5 might function as a GAP of other GTPases at the TGN.

We have shown that AGD5 can interact with ARF1 and also with plasma membrane-localised ARFB *in vitro*. In line with these results, recombinant plant AGDs can act on human and yeast ARF substrates *in vitro* (Koizumi *et al.*, 2005; Liljegren *et al.*, 2009). Together, these findings confirm that the specificity of an AGD/ARF interaction depends on factors present on target membranes. On the other hand,

it is also tempting to speculate that ARFs may naturally interact with multiple AGDs (i.e. that AGDs are not necessarily specific for a single ARF protein). Thus, the subcellular distribution of the ARF and AGD proteins may be a key factor that specifies the ARF interaction with a sub-group of AGDs distributed on the same target membranes.

ARF1 is localised at the TGN

In plants, the small GTPase ARF1 was known to localise not only on the Golgi apparatus, but also on extra-Golgi structures of unknown nature (Xu and Scheres, 2005; Stefano *et al.*, 2006). These structures partially overlap with structures labelled by the endocytic dye FM4-64 (Matheson *et al.*, 2007). Our co-localization experiments have shown that a pool of ARF1–GFP localises at the TGN, which is at the intersection between the exocytic and the endocytic pathways (Dettmer *et al.*, 2006). The results identify the post-Golgi structures as the TGN, and lend experimental support to the hypothesis that ARF1 may perform a number of functions in plants in addition to retrograde Golgi/ER protein transport, including roles in post-Golgi traffic and the endocytic pathway (Aniento *et al.*, 1996; Gu *et al.*, 1997; Yahara *et al.*, 2001; Pimpl *et al.*, 2003; Matheson *et al.*, 2007).

EXPERIMENTAL PROCEDURES

Molecular cloning

Standard molecular biological techniques were used for sub-cloning. The fluorescent proteins used in this study were based on fusions with either mGFP5 (Haseloff *et al.*, 1997), enhanced cyan fluorescent protein (ECFP) or enhanced yellow fluorescent protein (EYFP) (Clontech Inc., http://www.clontech.com/). The spectral properties of mGFP5 allow efficient spectral separation from YFP (Brandizzi *et al.*, 2002b). The coding sequence for AGD5 (AGI number At5g54310) was obtained from RIKEN (http://www.brc.riken.jp/lab/epd/catalog/cdnaclone.html) and amplified by PCR using primers containing *Bam*HI and *Sac*I sites for sub-cloning downstream of YFP in the binary vector pVKH18-En6 (Batoko *et al.*, 2000). To generate fluorescent fusions of ARF1 proteins (Pimpl *et al.*, 2003), we used cDNA provided by RIKEN (AGI number At1g47740), which was amplified by PCR using primers containing *Xba*I and *Sal*I sites for sub-cloning upstream of GFP in the binary vector pVKH18-En6.

The ERD2–CFP construct was provided by Professor Chris Hawes (School of Life Sciences, Oxford Brookes University, UK). For the SYP61 construct, a cDNA sequence provided by Professor Masa Sato (Kyoto Prefectural University, Graduate School of Human and Environmental Studies, Japan) was amplified by PCR and sub-cloned into pVKH18-En6 upstream of YFP or GFP sequence using *Bam*HI and *Sac*I sites of the binary vector. Mutant sequences of $AGD5^{R59Q}$ were generated by site-directed mutagenesis using specific primers. For His6 and GST tagging, cDNA sequences of wild-type and mutant proteins were sub-cloned in recombinant *E. coli* expression vectors pET-28b(+) (Novagen, http://www.emdchemicals.com/life-science-research/novagen) or pGEX-4T1 (Amersham, http://www5.amershambiosciences.com/), respectively. The His6 and GST tags were positioned downstream of ARF1 or upstream of AGD5, respectively. The primer sequences used for the sub-cloning and mutagenesis were 5′-CAGGACGTCTAGATGGGGTTGTCATTCGGAAAGTTGTTCAGC-3′ and 5′-CATGACCGTCGACTTTGCCTTGCTTGCGATGTTGTTGGAG-3′ for ARF1, 5′-GGTGCTGGATCCGGTGCCATGTCTTCAGCTCAAGATCCATTC-3′ and 5′-GCGCCGGAGCTCTTAGGTCAAGAAGACAAGAATGAATAGGAT-3′ for SYP61, and 5′-GGTGCTGGATCCGGTGCCATGAACGAGAAAGCCAACGTCTCT-3′ and 5′-GCGCCGGAGCTCTCAATGTTTTGTGAACATTCCATCCATC-3′ for AGD5. Constructs and mutations were confirmed by sequencing.

Plant material and transient expression systems

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 tagged versions of ARF1, ARFB, GDAP1 and SYP61, 0.1 for AGD5 and 0.2 for ERD2 constructs.

Sampling, imaging and fluorescence recovery after photobleaching (FRAP) analysis

Transformed leaves were analysed 48 h after infection of the lower epidermis. Imaging was performed using an upright Leica SP5 laser scanning confocal microscope (http://www.leica.com/), and a 63× oil immersion objective. To image expression of either GFP constructs or YFP constructs, or both, we used the imaging setting as described by Brandizzi *et al.* (2002a). Time-lapse scanning was acquired with the Leica imaging system software. Comparison of the various levels of expression between cells expressing tagged ARF1 mutants was performed by visualising the cells using the imaging settings present on the confocal microscope (i.e. laser intensity, pinhole diameter and imaging detector settings). FRAP experiments were performed using an inverted Zeiss LSM510 META laser scanning confocal microscope (Zeiss, http://www.zeiss.com/) and a 63× water immersion objective. Fluorochrome photobleaching and calculation of half-time recovery values were performed as described by Brandizzi *et al.* (2002a).

In vitro expression

Production of GST–AGD5 sub-cloned in the pGEX vector was induced in *E. coli* BL21(DE3) lysogens. Positive clones were selected for low-scale protein production. A single colony was initially inoculated into 5 ml LB medium containing ampicillin (100 μl ml^{-1}), and then further expanded into a 100 ml shaker culture in a 250 ml flask. The cells were incubated with shaking at 30°C until an OD_{600} of approximately 1.0 was reached. Protein production was induced by addition of 1 mM IPTG, and the culture was further incubated for 5 h at 30°C. Cells were then pelleted at 4°C, 500 ***g***, for 5 min, and lysed in glutathione resin columns according to the manufacturer's instructions (BD Biosciences, http://www.bdbiosciences.com) for binding GST-tagged proteins. Protein binding, the removal of endogenous proteins and the elution of GST-tagged proteins were performed according to the manufacturer's instructions.

Glutathione–agarose affinity chromatography of leaf extracts

Samples of leaves (1 g) transformed with ARF1GTP–YFP, ARFBGTP–YFP or cYFP (used as control) were subjected to protein extraction in 1.25 ml NE buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM EDTA, 5 mM $MgCl_2$) with protease inhibitor cocktail for plant cell extracts (Sigma, http://www.sigmaaldrich.com/) in liquid N_2. The resulting suspension was then centrifuged at 4°C, 14 000 ***g***, for 15 min. A 1 ml aliquot of the supernatant was added to 150 μl of a glutathione–agarose bead suspension (see below) [72% of glutathione resin in NS buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$)] that had previously been mixed with bacterial lysates containing GST–AGD5 and then washed to remove the unbound

proteins. The mixture was kept at 4°C for 3 h under gentle rotation. The beads were centrifuged at 4°C, 500 ***g***, for 1 min, and then washed by inverting the tube six times for a total of five washes with NS buffer. Bound proteins were eluted from the beads using 20 μl 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 (as described in QIAexpressionist kit; Qiagen, www.qiagen.com/) in a ratio sample:buffer = 1:0.4, respectively] and run on a 10% SDS-PAGE gel.

Western blot analysis

The proteins in SDS-PAGE gels were transferred onto a nitrocellulose membrane and then blocked with PBS, 0.5% Tween-20 and 5% milk powder for 1 h. The filter was then incubated in blocking buffer with primary antibodies at a dilution of 1:1000 for anti-His6 (Santa Cruz, http://www.scbt.com), anti-GST and anti-GFP serum (AbCam, http://www.abcam.com/) and 1:5000 for the anti-IgG antibody. All of the antisera originated from rabbits. Further steps in the analysis were performed as described by Crofts *et al.* (1999).

Transgenic line

YFP–AGD5 sub-cloned using the binary vector pVKH18-En6 was transformed into *A. tumefaciens* (GV3101) (Koncz and Schell, 1986). Arabidopsis Col-0 plants were stably transformed using the floral-dip method (Clough and Bent, 1998). The transformed plants were selected on Murashige & Skoog plates by addition of hygromycin to the plant culture medium at a concentration of 15 μg ml^{-1} (Calbiochem, http://www.emdchemicals.com/life-science-research/calbiochem).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic tree of AGD1–AGD15 from Arabidopsis and Age2p from yeast.

Figure S2. Multiple sequence alignment of ARF-GAPs by ClustalW.

Figure S3. Subcellular distribution of AGD5 in *A. thaliana* epidermal cells.

Figure S4. Interaction between GST–AGD5, ARF1GTP–YFP and ARFBGTP–YFP proteins.

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