Overexpression of Arabidopsis AGD7 Causes Relocation of Golgi-Localized Proteins to the Endoplasmic Reticulum and Inhibits Protein Trafficking in Plant Cells1[C][OA]

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ADP ribosylation factor (Arf) GTPase-activating proteins (GAPs) promote the hydrolysis of GTP bound to Arfs to GDP, which plays a pivotal role in regulating Arfs by converting the active GTP-bound forms of these proteins into their inactive GDP-bound forms. Here, we investigated the biological role of AGD7, an Arf GAP homolog, in Arabidopsis (Arabidopsis thaliana). We show that AGD7 bears a highly conserved N-terminal region and a unique C-terminal region, interacts with Arf1 both in vitro and in vivo, and stimulates Arf1 GTPase activity in a phosphatidic acid-dependent manner in vitro. In plant cells, AGD7 localized to the Golgi complex, where its overexpression was found to inhibit the Golgi localization of γ-subunit of coat proteins and promote the relocation of Golgi proteins into the endoplasmic reticulum in both protoplasts and transgenic plants. Furthermore, overexpression of AGD7 inhibited anterograde trafficking of proteins from the endoplasmic reticulum. We propose that AGD7 functions as a GAP for Arf1 in the Golgi complex and plays a critical role in protein trafficking by controlling Arf1 activity.

GTPase-activating proteins (GAPs) are a family of proteins that promote hydrolysis of GTP bound to small GTP-binding proteins to GDP, thereby playing a pivotal regulatory role in their function by inducing an appropriately timed deactivation of them. GAPs can be divided into several groups based on their substrate proteins, such as ADP ribosylation factor (Arf) GAPs, Rab GAPs, and Rho GAPs. Arf GAPs are specific to Arfs, which function as small GTP-binding proteins. The GTPase activity of Arf is so low that GAPs are needed to ensure the efficient hydrolysis of the bound GTP molecule (Cukierman et al., 1995; Moss and Vaughan, 1998; Goldberg, 1999; Donaldson and Jackson, 2000).

Numerous Arf-specific GAPs have been identified, including ACAPs, ARF1GAP, ASAP1, Glo3p, GITs, and Gsc1p (Cukierman et al., 1995; Dogic et al., 1999; Goldberg, 1999; Poon et al., 1999; Jackson et al., 2000; Premont et al., 2000). The detailed mechanism by which Arf GAPs activate the GTPase activity of Arf has been revealed by structural analyses of Arf GAPs and Arf1 (Goldberg, 1999). Arf GAPs consist of a large number of diverse proteins (Donaldson and Jackson, 2000; Jensen et al., 2000) that share a common N-terminal GAP homology domain bearing a Cys4 zinc-finger motif (Cukierman et al., 1995). This domain activates the GTPase activity of Arf by interacting with Arf effector domains. Unlike their N-terminal domains, the C-terminal regions of GAPs vary and contain conserved regions, such as the pleckstrin homology (PH), ankyrin repeat, and Ca2+-binding domains (Donaldson and Jackson, 2000; Jensen et al., 2000). The PH or Ca2+-binding domains probably target Arf GAPs to specific membranes. GAPs play an important role in Arf activity during intracellular trafficking as shown in yeast and animal cells (Poon et al., 1999; Szafer et al., 2000; Lanoix et al., 2001; Yahara et al., 2001). Mutant yeast cells that simultaneously lack the two Arf GAPs Glo3p and Gsc1p are unable to proliferate, exhibit a dramatic accumulation of proteins in the endoplasmic reticulum (ER), and are defective for protein transport between the ER and the Golgi (Poon et al., 1999). In animal cells, overexpression of Arf GAP disrupts retrograde trafficking (Aoe et al., 1997), which is similar to that seen in cells treated with brefeldin A (BFA), a retrograde trafficking inhibitor (Misumi et al., 1986; Driouich et al., 1993). This suggests that Arf GAP is a critical regulator of Arf during Golgi to ER transport.

Originally, Arf GAPs were identified as a group of proteins that stimulate the intrinsic GTPase activity of
Arf. However, the biological roles of Arf GAPs now supersede their role as a GTPase-activating protein. It has been suggested that Arf GAP functions as a subunit of coat proteins and directly contributes to vesicle formation (Nie and Randazzo, 2006). Furthermore, it has recently been shown that AGAPI and AGAP2 interact with adaptor protein (AP) complexes AP-3 and AP-1, respectively (Nie and Randazzo, 2006), demonstrating that Arf GAPs are involved in protein trafficking from the Golgi apparatus to the lysosomes.

Additionally, it has been shown that Arf1 plays a critical role in protein trafficking (Pimpl et al., 2000, 2003; Couchy et al., 2003; for review, see Memon, 2004). As in animal cells, a dominant negative mutant of Arf1 or BFA treatment strongly inhibits intracellular trafficking in plant cells and causes the relocation of Golgi-specific markers to the ER (Driouich et al., 1993; Kim et al., 2001; Lee et al., 2002; Nebenführ et al., 2002). As observed in animal and yeast cells (Balch et al., 1992; Moss and Vaughan, 1998), Arf in plant cells may also be regulated by a large number of proteins. Supporting this is that numerous proteins with GAP and sec7 domains have been identified by sequence analysis of the entire Arabidopsis (Arabidopsis thaliana) genome (Vernoud et al., 2003; Memon, 2004). However, of these large numbers of proteins, only a few have been studied in detail (Steinmann et al., 1999; Jensen et al., 2000; Koizumi et al., 2005; Zhuang et al., 2005; Sieburth et al., 2006). Arabidopsis ZAC, a protein that bears homology to the GAP domain of Arf GAPs, can activate the GTPase activity of Arf1 in vitro. In addition, several mutants have been identified that have mutations in the genes encoding Arabidopsis GAPs. These mutants displayed various phenotypes, such as a defect in auxin efflux and vein patterning, indicating that Arf GAPs play a role in these functions (Koizumi et al., 2005; Zhuang et al., 2005; Sieburth et al., 2006).

In this study, we investigated the biological role of an Arabidopsis Arf GAP in protein trafficking in plant cells. We show that AGD7 interacts with Arf1 and activates Arf1 GTPase in a phosphatidic acid (PA)-dependent manner. Furthermore, we demonstrate that AGD7 localizes to the Golgi apparatus, and its overexpression inhibits membrane association of γ-subunit of coat proteins (γ-COP) and anterograde trafficking of cargo proteins in plant cells.

**RESULTS**

**AGD7 Interacts with Arf1 and Stimulates Arf1 GTPase Activity in Vitro**

The Arabidopsis genome encodes a large number of proteins that exhibit a high degree of amino acid sequence homology to Arf GAPs (Fig. 1; Kumar et al., 2001; Vernoud et al., 2003). Although these proteins are diverse, they share a common GAP domain. In previous studies, we and others have demonstrated that Arf1 plays a critical role in protein trafficking from the Golgi complex to the ER (Lee et al., 2002; Takeuchi et al., 2002). To study protein trafficking between the Golgi complex and ER, we attempted to identify a GAP that regulates Arf1 activity in the Golgi complex. Among many GAP homologs, phylogenetic analysis revealed that AGD7 is most closely related to human Arf GAP1 (Cukierman et al., 1995), a GAP specific for Arf1 in the Golgi complex (Fig. 1). AGD7 contains an N-terminal Arf GAP domain (7–122 amino acids) that exhibits 54.8% amino acid sequence identity to N-terminal human Arf GAP1 domain (10–125 amino acids), strongly suggesting that it may also function as an Arf GAP in the Golgi complex. To investigate this possibility, we examined whether AGD7 interacts with Arabidopsis Arf1. AGD7 was tagged with the small epitope hemagglutinin (HA) at its N terminus and expressed transiently in protoplasts after polyethylene glycol-mediated transformation (Jin et al., 2001; Kim et al., 2001). As a control, the unrelated peroxisomal protein import receptor Arabidopsis PEX5 (Brickner et al., 1998) was expressed transiently in protoplasts. This protein was also tagged with HA at its N terminus. Protein extracts were obtained from transformed protoplasts, and immunoprecipitates generated using anti-HA antibody were probed with an anti-Arf1 antibody that had been raised against Arabidopsis Arf1 (Pimpl et al., 2000). Endogenous Arf1 was detected in the pellet fraction obtained from protoplasts expressing HA:AGD7 but not in pellets from protoplasts expressing HA:PEX5 (Fig. 2A, left). These results suggest that in plant cells, HA:AGD7 interacts specifically with endogenous Arf1. To confirm the specificity of this interaction, HA:AGD7 was introduced...
into protoplasts together with Arf1:GFP or GFP (Lee et al., 2002). Protein extracts prepared from transformed protoplasts were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were probed with anti-GFP antibody. Arf1:GFP, but not GFP, was detected in the immunoprecipitates (Fig. 2B, left), indicating that Arf1:GFP interacts with HA:AGD7 in plant cells. Western-blot analysis of total protein extracts confirmed that the introduced genes were expressed equally well in the protoplasts (Fig. 2, A and B, right). To exclude the possibility that the interaction was due to overexpression of HA:AGD7 in the protoplasts, we generated transgenic plants harboring HA:AGD7 under control of the dexamethasone (dex)-inducible promoter (Aoyama and Chua, 1997), and protein extracts from transgenic plants expressing HA:AGD7 were used for coimmunoprecipitation experiments. Wild-type and transgenic plants harboring ST:GFP (Lee et al., 2002) were transformed with Dex-HA:AGD7. Initially, we examined expression of HA:AGD7 in transgenic plants. Transgenic plants harboring Dex-HA:AGD7 alone (AGD7) or both ST:GFP and Dex-HA:AGD7 (ST/AGD7) were treated with dex, and the expression of HA:AGD7 was detected at various time points by western-blot analysis using an anti-HA antibody. In both types of transgenic plants, HA:AGD7 levels were barely detectable without dex treatment, confirming that HA:AGD7 was expressed at very low levels without induction. However, HA:AGD7 levels were increased by approximately 20-fold 12 h after dex treatment (Fig. 2C). To examine the interaction between endogenous Arf1 and HA:AGD7 in transgenic plants, protein extracts prepared from AGD7 transgenic plants that had been treated with dex for 0 and 4 h were used for coimmunoprecipitation experiments with anti-HA antibody. As a negative control, we included protein extracts of wild-type plants. As observed in protoplasts, endogenous Arf1 was coimmunoprecipitated with HA:AGD7 by the anti-HA antibody (Fig. 2D). The level of HA:AGD7 required to coimmunoprecipitate endogenous Arf1 in the transgenic plants was as low as the uninduced basal level. In contrast, Arf1 was not detected in the immunoprecipitates obtained from wild-type extracts. These results further confirm that AGD7 interacts specifically with Arf1 in plants.

To investigate further the biological activity of AGD7, we examined whether AGD7 can stimulate intrinsic Arf1 GTPase activity. The N-terminal GAP domains (133 amino acids) of AGD7 and full-length Arf1 were expressed in Escherichia coli as glutathione S-transferase (GST):AGD7N and Arf1:His, respectively (Fig. 3A). GST:AGD7N was incubated with GTP or GTPγS-bound Arf1:His. GST alone was used as a negative control. GTPase activity of Arf1 was quantified using the change in intrinsic Arf1 Trp fluorescence during the transition from GTP- to GDP-bound states (Antonny et al., 1997a). Within 500 s of incubation with GST:AGD7N, Arf fluorescence signals were reduced to 40% of the initial value, whereas in the presence of GTPγ(S), this signal was reduced to 75% of the initial value (Fig. 3B), indicating that GTPγ(S) strongly suppressed the reduction of fluorescence signal. GST alone (control) caused no significant reduction in the intensity of fluorescence. These results clearly demonstrate that AGD7 functions as an Arf GAP.

Previous studies have indicated that GAP activity is stimulated by phospholipids (Makler et al., 1995; Antony et al., 1997b; Connolly and Engebrecht, 2006). Accordingly, we investigated the effect of phospholipids on Arf GAP activity of AGD7. Initially, we examined whether AGD7 binds to phospholipids by far western-blot analysis (Stevenson et al., 1998). A membrane strip containing a variety of phospholipid spots was incubated with GST:AGD7 or GST alone, and anti-GST antibody was used for immunodetection of bound proteins. AGD7 displayed strong binding to PA and weak binding to phosphatidylinositol 5-phosphate (Fig. 3C). No binding was observed with GST alone (control), confirming the lipid-binding specificity of AGD7. We then examined GAP activity of AGD7 using GST:AGD7N and GTP-loaded Arf1 in the presence of various concentrations of PA. PA (10%) strongly stimulated AGD7 GAP activity. However, at lower concentrations, the stimulatory effect was not noticeable (Fig. 3B). These results suggest that PA activation occurs only above a threshold concentration. Addition of other phosphoinositides, such as phosphatidylinositol 3-phosphate or phosphatidylinositol 4-phosphate, had only a marginal influence on PA stimulation of GAP activity (data not shown). These results demonstrate clearly that AGD7 exhibits PA-dependent Arf GAP activity.

**AGD7 Localizes to the Golgi Complex**

To get an insight into the biological role of AGD7, we examined its localization in Arabidopsis protoplasts. Protoplasts were transformed with HA:AGD7, and its localization was detected by immunostaining with anti-HA antibody (Frigerio et al., 2001; Park et al., 2004). HA:AGD7 exhibited a punctate staining pattern (Fig. 4A, c), indicating that AGD7 may associate with a specific organelle. Protein extracts were prepared from HA:AGD7-transformed protoplasts, separated into soluble and membrane fractions, and examined by western-blot analysis using anti-HA antibody. Approximately 50% of the HA:AGD7 protein was detected in the pellet fraction (Fig. 4B), indicating that AGD7 exists as both soluble and membrane-associated forms. As controls for fractionation of soluble and membrane proteins, we used antibodies specific for Arabidopsis aleurain-like protein (AALP) and AtPEP12p, which localize to the lumen of the central vacuole and the prevacuolar compartment, respectively (da Silva Conceicao et al., 1997; Ahmed et al., 2000). As expected, AALP and AtPEP12p were detected in the soluble and pellet fractions, respectively.

To examine HA:AGD7 localization, AGD7 plants were treated with dex for 4 h (a condition for a low expression level of HA:AGD7), and cryosections of...
various tissues were immunostained with anti-γ-COP, monoclonal JIM84, and anti-HA antibodies. Anti-γ-COP recognizes γ-COP, which is present in COPI vesicles that localize to the Golgi complex (Pimpl et al., 2000), and the monoclonal antibody JIM84 recognizes Lewis a-containing N-glycans in the trans-Golgi (Evans et al., 1997; Fitchette et al., 1999). HA:AGD7 produced a punctate staining pattern in cryosections of transgenic plant tissues (Fig. 5A, a) as observed in protoplasts. The anti-HA antibody did not produce any signal in wild-type plants, confirming the specificity of this antibody (data not shown). Furthermore, HA:AGD7-positive punctate stains overlapped closely with both γ-COP- and JIM84-immunostained compartments (Fig. 5A), suggesting that HA:AGD7 localizes to the Golgi complex.

Next, we compared the immunostaining patterns of HA:AGD7 to those of Rab homologs Ara6 and Ara7, which localize to different types of FM4-64-stained endosomes (Ueda et al., 2001). Cryosections of dextreated AGD7 plants were immunostained with both anti-Ara6 and anti-HA antibodies. Although the anti-Ara6 antibody produced a punctate staining pattern (Fig. 5B, a; Ueda et al., 2001), it did not overlap with the HA:AGD7-positive stains (Fig. 5B, a–c), consistent with the suggestion that AGD7 localizes to the Golgi complex. Next, we compared the immunostaining patterns of HA:AGD7 and Ara7. Ara7 and GFP-tagged Ara7 are thought to localize in the prevacuolar compartment (Lee et al., 2004). We introduced GFP:Ara7 and HA:AGD7 into protoplasts and then immuno-stained with anti-HA antibody. GFP:Ara7 was observed directly. Although GFP:Ara7 produced a punctate staining pattern (Fig. 5B, e; Ueda et al., 2001; Sohn et al., 2003; Lee et al., 2004), it did not colocalize with that of HA:AGD7 (Fig. 5B, e–g), indicating that AGD7 does not localize to the Ara7 compartment. These results are again consistent with the suggestion that AGD7 localizes specifically to the Golgi complex.

**Overexpression of HA:AGD7 Inhibits Anterograde Trafficking of Soluble Cargo Proteins**

AGD7 may regulate Arf1 in the Golgi complex in a manner similar to that observed in animal and yeast cells (Poon et al., 1999; Szafer et al., 2000; Lanoix et al., 2001; Yahara et al., 2001). To investigate its biological function, we examined the effect of HA:AGD7 overexpression on protein trafficking in protoplasts. Overexpression of Arf1 GAP has been shown to inhibit anterograde trafficking in animal cells (Aoe et al., 1997). This was thought to be due to premature activation of the intrinsic Arf1 GTPase activity, leading to premature hydrolysis of bound GTP and halting Arf1-mediated recruitment of COPI components to the Golgi complex. In protoplasts, we transiently expressed cargo proteins sporamin:GFP and phaseolin, together with HA:AGD7. Sporamin:GFP is a chimeric vacuolar protein that consists of a vacuolar storage protein in the tuber of sweet potato (*Ipomoea batatas*).
and GFP (Kim et al., 2001), while phaseolin is a storage protein in the common bean (*Phaseolus vulgaris*; Frigerio et al., 1998). Normally, sporamin:GFP and phaseolin are transported to the central vacuole and the protein storage vacuole (PSV)-like organelle in leaf protoplasts, respectively (Jin et al., 2001; Kim et al., 2001; Park et al., 2004, 2005). When sporamin:GFP is transported to the central vacuole, it is processed proteolytically into a smaller form (Jin et al., 2001; Kim et al., 2001). In Arabidopsis leaf protoplasts, phaseolin molecules that are transported to the PSV-like organelle also undergo proteolytic processing (Park et al., 2004). Thus, the degree to which these proteins are processed can indicate the efficiency with which they are trafficked to their final destination. The introduction of increasing amounts of HA:AGD7 resulted in reduced levels of the smaller sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A). We also examined the effect of HA:AGD7 overexpression on trafficking of invertase:GFP, a chimeric secretory protein comprising the full-length secretory invertase and GFP (Sohn et al., 2003). In the presence of coexpressed HA:AGD7, the majority (70%–80%) of the protoplasts transformed with sporamin:GFP and phaseolin fragments (approximately 31 and 25 kD, respectively; Fig. 6A).
In Transgenic Plants, Higher HA:AGD7 Levels Lead to Solubilization of γ-COP and Disruption of the Golgi Complex

Next, we investigated the effect of overexpressing HA:AGD7 (12 h induction of HA:AGD7 expression) on γ-COP localization. GAPs induce hydrolysis of GTP bound to Arf1 and thus cause dissociation of Arf1 from the Golgi membrane (Cukierman et al., 1995; Goldberg, 1999). In animal and yeast cells, Arf1 recruits COPI components to the Golgi complex (Donaldson et al., 1992; Palmer et al., 1993). Thus, if AGD7 functions as a GAP for Arf1 localized to the Golgi complex, its overexpression may result in the aberrant localization of γ-COP. In AGD7 plant tissues, we examined endogenous γ-COP localization with or without dex treatment. Cryosections of plant tissues were immunostained with anti-γ-COP antibody. At 12 h after dex treatment, a condition that results in strong expression of HA:AGD7, both HA:AGD7 and γ-COP exhibited strong diffuse patterns in the same cells (Fig. 7A, g, f, o, and n). In contrast, γ-COP produced a punctate staining pattern in dex-untreated cells (Fig. 7A, b and j), indicating that γ-COP does not localize to the Golgi complex at high HA:AGD7 levels. The diffuse γ-COP pattern observed at high levels of HA:AGD7 may be caused by either dissociation of γ-COP from the Golgi complex or disruption of the Golgi complex itself.

To distinguish between these possibilities, we examined the localization of a fusion reporter protein ST:GFP, an integral membrane protein localized to the Golgi complex. Transgenic ST/AGD7 plants were treated with dex for 12 h, and cryosections of various tissues were immunostained with anti-HA antibody. ST:GFP was observed directly. Again, cells in plants treated with dex for 12 h produced diffuse patterns of ST:GFP staining (Fig. 7A, u–w), whereas cells in uninduced control plants exhibited punctate staining patterns (Fig. 7A, r). These results strongly suggest that in the presence of high levels of HA:AGD7, the Golgi complex is disassembled, and Golgi proteins such as ST:GFP are relocated to the ER. However, at high levels of HA:AGD7, the staining pattern of ST:GFP did not exactly resemble that of the ER network. This may be due to the technical difficulty inherent in preserving such a fine network pattern of the ER in fixed cells. Thus, to obtain a better image of ST:GFP localization in the presence of high levels of HA:AGD7, we used protoplasts. Protoplasts from transgenic plants expressing ST:GFP (ST plants) were transformed with HA:AGD7, and localization of ST:GFP was examined in live protoplasts. In untransformed protoplasts from ST plants (Lee et al., 2002), ST:GFP exhibited the typical punctate staining pattern (Fig. 7B, a). However, in HA:AGD7-transformed protoplasts, some of them (approximately 30%) produced a network pattern (Fig. 7B, c) similar to the ER network. Considering that the transformation efficiency of protoplasts is usually 20% to 40% (Lee et al., 2002), these results strongly suggest that the expression of HA:AGD7 causes relocation of Golgi-localized ST:GFP to the ER, similar to what has been observed with ArfGAP1 in animal cells (Aoe et al., 1997).

To confirm this finding at the biochemical level, we analyzed the N-glycans of proteins located to the ER in the presence of high levels of HA:AGD7. The N-glycans of ER proteins are sensitive to endo H, whereas those of Golgi proteins are resistant (Kornfeld and Kornfeld, 1985; Rabouille et al., 1995; Crofts et al., 1999). Upon disassembly of the Golgi complex, Golgi-localized N-glycan-modifying enzymes relocate to the ER, which triggers the conversion of endo H-sensitive N-glycans on ER proteins into the endo H-resistant Golgi form. This effect has been observed previously in the presence of...
BFA and dominant negative mutant of Arf1 (Driouch et al., 1993; Lee et al., 2002). To determine whether HA:AGD7 exerts a similar effect on N-glycans of ER-localized proteins, we generated an artificial ER marker protein (GH). GH contains a leader sequence from the luminal-binding protein BiP, an N glycosylation site comprising three amino acid residues (NLT), the GFP coding region, and an ER retention motif that consists of four amino acid residues (HDEL; Fig. 8A; Schweizer et al., 1993; Crofts et al., 1999). Protoplasts were transformed with GH and an ER marker BiP:RFP, and localization of these proteins was examined (Jin et al., 2001). GH exhibited a network pattern (Fig. 8B, b), and its green fluorescent signals overlapped closely with the red fluorescent signal of BiP:RFP (Fig. 8B, a), confirming its localization to the ER. Next, we determined whether GH was N-glycosylated. Protoplasts were transformed with GH and an ER marker BiP:RFP, and localization of these proteins was examined (Jin et al., 2001). GH exhibited a network pattern (Fig. 8B, b), and its green fluorescent signals overlapped closely with the red fluorescent signal of BiP:RFP (Fig. 8B, a), confirming its localization to the ER. Next, we determined whether GH was N-glycosylated. Protoplasts were transformed with GH and incubated in the presence and absence of tunicamycin, which inhibits N-glycosylation (Leavitt et al., 1977). The GH band detected using the anti-GFP antibody migrated faster in the presence of tunicamycin (Fig. 8C, a), implying that GH is N-glycosylated. Moreover, we demonstrated that these GH glycans are sensitive to endo H treatment (Fig. 8C, b), confirming its localization to the ER. To examine whether overexpressed HA:AGD7 causes the Golgi-localized proteins to relocate to the ER, we examined the endo H sensitivity of the GH glycans in protein extracts prepared from protoplasts cotransformed with HA:AGD7 and GH. In addition, we examined the endo H sensitivity of the GH glycans in protein extracts prepared from protoplasts cotransformed with Arf1[T31N] and GH. Arf1[T31N] has been shown to induce the Golgi complex to disassemble and to cause the relocation of the Golgi-localized proteins to the ER (Lee et al., 2002). In both cases, the majority of GH was resistant to endo H (Fig. 8C, b). However, Arf1[T31N] was more efficient in generating the endo H-resistant form of the GH glycans than HA:AGD7. This may be due to either to differential effects of Arf1[T31N]:HA and HA:AGD7 on the distribution of Golgi proteins or to differences in their levels of expression. In fact, Arf1[T31N]:HA was expressed at higher levels than HA:AGD7. Furthermore, the levels of the endo H-resistant GH were proportional to the amount of HA:AGD7 introduced into the protoplasts (Fig. 8C, c).

Next, we examined whether other Arabidopsis GAP proteins also have a similar effect on the Golgi complex. ZAC(AGD12), a membrane-associated Arabidopsis protein with ArfGAP zinc finger and a C2 domain, has
been isolated previously from Arabidopsis as a GAP of Arf (Jensen et al., 2000). Protoplasts were transformed with the constructs indicated. Protein extracts were prepared and subjected to western-blot analysis using anti-GFP, anti-phaseolin, and anti-HA antibodies. Additionally, proteins were collected from the medium (M) for protoplasts transformed with invertase:GFP. The amount of HA:AGD7 DNA used for each transformation is indicated. Pre, Precursor forms; Pro, processed forms. B. Localization pattern of cargo proteins in the presence of HA:AGD7. Protoplasts were transformed as described in A. Images are representative of protoplasts transformed with 20 μg of HA:AGD7. Protoplasts transformed with phaseolin were immunostained with anti-phaseolin antibody, and those transformed with sporamin:GFP and invertase:GFP were observed directly. Note that images show the pattern observed in the majority (70%–80%) of transformed protoplasts. Control, No HA: AGD7; CH, chloroplasts. Bar = 10 μm.

A large number of Arf GAP proteins have been identified in a variety of eukaryotic cells. Arf GAPs are divided into three groups on the basis of primary structure, specifically, ArfGAP1, GIT, and AZAP (Randazzo and Hirsch, 2004; Nie and Randazzo, 2006). Members of the ArfGAP1 subfamily display a simple structure with a conserved N-terminal GAP domain and a short divergent C-terminal region. In contrast, members of the GIT and AZAP subfamilies contain multiple domains involved in protein-protein or protein-lipid interactions, such as ankyrin, PH, paxillin-binding sequence, src homolog 3 and bin, amphiphysin, and Rvs domains, in addition to the GAP domain. As observed in other eukaryotic cells, the Arabidopsis genome also encodes a large number of Arf GAPs that belong to all three subfamilies (Vernoud et al., 2003). Of these, AGD7 belongs to the ArfGAP1 subfamily. In animal cells, ArfGAP1, the first Arf1 GAP to be cloned, plays a role in protein trafficking in the Golgi complex through regulation of Arf1 activity (Cukierman et al., 1995). The structural similarity of AGD7 to Golgi-localized ArfGAP1 in animal cells and Gcs1p and Glo3p in yeast cells (Cukierman et al., 1995; Makler et al., 1995; Poon et al., 1996, 1999) is in strong support for a role for AGD7 in protein trafficking in the Golgi complex.

The biological activity of AGD7 was addressed using several different approaches. Initially, we examined whether AGD7 displays GAP activity toward plant Arf proteins. Previously, we and others showed that Arf1 plays a critical role in protein trafficking in plant cells (Lee et al., 2002; Takeuchi et al., 2002; Pimpl et al., 2003). Indeed, AGD7 displays GAP activity toward Arf1 of Arabidopsis in vitro. The AGD7 GAP activity was strongly stimulated by PA, similar to other ArfGAP members, such as Gcs1p in yeast cells (Connolly and Engebrecht, 2006), but distinct from ArfGAP1 in animal cells in that the protein was activated by diacylglycerol and phosphatidylinositol 4,5-bisphosphate (Makler et al., 1995; Antonny et al., 1997b). However, in contrast to Gcs1p, which is additionally activated by phosphatidylinositol 4,5-bisphosphate supplemented...
to PA-containing liposomes, the GAP activity of AGD7 was not further stimulated (data not shown). PA is generated from phosphatidylcholine by phospholipase D (PLD). In animal cells, PLD is activated by Arf1 in the Golgi complex (Brown et al., 1993), and PA generated through Arf1-mediated activation is a critical regulator of vesicle trafficking in the early secretory pathway (Cockcroft et al., 1994; Ktistakis et al., 1996). Currently, it is not clear whether PA is produced by a PLD in the membrane of the Golgi complex in plant cells and whether Arf1 is involved in PA production through PLD activation. However, the finding that AGD7 displays specific binding to PA and that its GAP activity is stimulated by PA strongly suggests that a mechanism analogous to that in animal cells operates in the early secretory pathways in plant cells.

AGD7 localizes to the Golgi complex. Similarly, ArfGAP1, an animal GAP that is most closely related to AGD7, localizes to the Golgi complex. In plant cells, Arf1 plays a critical role in COPI vesicle formation at

Figure 7. High levels of HA:AGD7 cause dissociation of γ-COP and relocation of Golgi-localized ST:GFP to the ER. A and B, Disruption of the Golgi complex in the presence of high levels of HA:AGD7. Various tissues from transgenic plants harboring Dex-HA:AGD7 alone or together with ST:GFP were treated with dex (30 μM) for 12 h (A). In addition, protoplasts from transgenic plants harboring ST:GFP were transformed with 20 μg of HA:AGD7 (B). HA:AGD7 and γ-COP were localized by immunostaining with anti-HA and anti-γ-COP antibodies. ST:GFP was observed directly. Bar = 10 μm.
the Golgi complex (Pimpl et al., 2000; Lee et al., 2002; Takeuchi et al., 2002), similar to other eukaryotic cells (Balch et al., 1992; Moss and Vaughan, 1998). The finding that AGD7 localizes to the Golgi complex raises the possibility that the protein is involved in stimulation of Arf1 GTPase activity in this organelle. The best characterized role of Arf GAPs is its contribution to COPI vesicle formation (Kartberg et al., 2005; Lee et al., 2005). Thus, AGD7 may participate in COPI vesicle formation at the Golgi complex. The finding that HA:AGD7 overexpression causes γ-COP dissociation from the Golgi complex is consistent with this notion.

In animal cells, ArfGAP1 overexpression causes disruption of the Golgi complex (Aoe et al., 1997). In addition, ectopic expression of AGAP1 and AGAP2 at high levels induces dissociation of AP-3 and AP-1 from endosomes, respectively (Nie et al., 2003, 2005), which leads to the conclusion that ArfGAP1 participates in COPI vesicle formation for retrograde trafficking, and AGAPs are involved in protein trafficking from endosomes. The underlying mechanism for these effects on the Golgi complex and AP complexes is the premature hydrolysis of Arf1-bound GTP to GDP by overexpressed Arf GAPs. We employed a similar approach in both protoplasts and transgenic plants. In both systems, ectopic expression of AGD7 induced dissociation of Golgi-localized γ-COP. Golgi-localized proteins are relocated to the ER upon overexpression of AGD7, as disclosed by the localization pattern of ST:GFP in both protoplasts and transgenic plants. Furthermore, the N-glycans of ER-localized proteins were converted to endo H-resistant forms in

Figure 8. In protoplasts, HA:AGD7 overexpression leads to solubilization of γ-COP and relocation of Golgi proteins to the ER. A, Schematic depiction of GH. GH consists of the BiP leader sequence, an N-glycosylation site (NXT), a GFP-coding region, and an ER-retention motif (HDEL). B, Localization of the artificial ER marker. Protoplasts were transformed with GH and BiP-RFP, and localization of these proteins was examined by fluorescence microscopy. Bar = 10 μm. C, Endo H resistance of GH glycans in the presence of HA:AGD7. Protoplasts were transformed with the indicated constructs. The amount of plasmid DNA used was 15 μg unless specified. Transformed protoplasts were incubated in the presence (+) or absence (−) of tunicamycin. Protein extracts were treated with endo H and analyzed by western blotting using anti-GFP and anti-HA antibodies. Control, Empty plasmid, R6. D, Localization pattern of ST:GFP in the presence of ZAC(AGD12):HA. ST:GFP was introduced into protoplasts alone (Control) or together with ZAC:HA or HA:AGD7, followed by localization of ST:GFP. Red and green fluorescent signals represent the autofluorescence of chlorophyll and GFP, respectively. CH, Chloroplasts. Bar = 15 μm. E, Expression of ZAC:HA. Protoplasts were transformed with ZAC:HA or HA:AGD7, and their expression was examined by western blotting using anti-HA antibody. The immunoblot was stained with Coomassie Blue as a leading control. RbCL, Rubisco complex large subunit.
protoplasts, indicating that AGD7 causes relocation of Golgi-localized N-glycan processing enzymes to the ER. Thus, the effect of AGD7 is reminiscent of that of ArfGAP1 in animal cells, strongly suggesting that this protein is involved in COPI vesicle formation during retrograde trafficking at the Golgi complex. However, not all Arf GAPs in plant cells may cause dissociation of γ-COP from the Golgi complex upon overexpression. For instance, ectopic expression of ZAC(AGD12) and AGD8 (another Arabidopsis GAP homolog; M.K. Min and I. Hwang, unpublished data) does not cause relocation of Golgi proteins to the ER, indicating that the effect on the Golgi complex is specific to a subgroup of Arabidopsis Arf GAPs, including AGD7. As suggested previously (Aoe et al., 1997), the effect of AGD7 may be explained by premature hydrolysis of GTP bound to Arf1 to GDP, which may induce dissociation of Arf1 from the Golgi complex to the cytoplasm, resulting in dissociation of γ-COP from the Golgi complex and relocation of Golgi proteins to the ER. However, we cannot rule out other possibilities. Recently, it has been proposed that ArfGAPs function as a component of COPI (Lewis et al., 2004; Nie and Randazzo, 2006). The issue of whether AGD7 is a component of COPI in plant cells is currently unclear.

AGD7 overexpression leads to dissociation of γ-COP from the membrane and relocation of Golgi-localized proteins to the ER, suggesting that intracellular trafficking is affected by HA:AGD7, similar to that observed in the presence of BFA or in the Arf1 dominant-negative mutant (Driouich et al., 1993; Lee et al., 2002; Ritzenhalter et al., 2002; Takeuchi et al., 2002). As expected, trafficking of sporamin:GFP and phaseolin, which are transported to the central vacuole and PSV-like organelle, respectively (Sohn et al., 2003; Park et al., 2004), was strongly inhibited by coexpressing them with HA:AGD7. In addition, the presence of coexpressed HA:AGD7, there was a decrease in the amount of invertase:GFP secreted into the medium and a concomitant increase in its level in the ER. These results collectively suggest that high levels of HA:AGD7 inhibit anterograde trafficking. However, this may not be a direct effect but may rather be the result of disassembly of the Golgi complex as a result of dissociation of Arf1 and γ-COP from the Golgi membrane. The presence of Arf1 at the Golgi membrane is crucial for maintaining the integrity of the Golgi complex, as well as anterograde trafficking (Ward et al., 2001).

In conclusion, we propose that AGD7, one of the Arabidopsis genome, functions as a GAP of Arf1 in the Golgi complex during COPI vesicle formation. MATERIALS AND METHODS

Growth of Plants and Generation of Transgenic Plants

Arabidopsis (Arabidopsis thaliana) ecotype Columbia was grown either on Murashige and Skoog (1962) plates at 22°C in a culture room or on soil in a greenhouse under conditions of 70% relative humidity and a 16-h-light/8-h-dark cycle.

To generate transgenic plants expressing HA:AGD7, wild-type and transgenic plants expressing BGFP were transformed with HA:AGD7 under a dex-inducible promoter (Aoyama and Chua, 1997) in a hygromycin vector, pTA7002, by the floral dip method (Clough and Bent, 1998). Transgenic plants were selected based on hygromycin resistance. Dex treatment (30 μM) was done as described previously (Aoyama and Chua, 1997).

Generation of Constructs

AGD7 (At2g37550) was amplified by PCR using primers 5'-ATGGCGAGCCGCCGAGAGG-3' and 5'-TTAGAGAAAACCTCCACCCGTC-3'. To generate HA:AGD7, Smal and XhoI sites were introduced into the 5' and 3' ends of AGD7 by PCR, respectively, using two primers (5'-GCCCGGGAATGCGCACG-GGCGAGACC-3' and 5'-CCCGGGATGAGAAAACCTCCACCCGTC-3'). The PCR product was subsequently ligated into a vector so that its protein product contained the influenza HA epitope at its N terminus. To construct an expression vector for transgenic plants, HA:AGD7 was amplified using primers 5'-GACCTCGAGATGCTTACCATACCGTC-3' and 5'-GACTGATTAGTAGAGAAAACCTCCACCCGTC-3'. The PCR product digested with XhoI and SpeI was ligated into a binary vector, pTA7002, containing the corresponding restriction sites. ZAC(AGD12) (Aoe et al.) was amplified with primers 5'-ATGAGTATTCGTGAGGCCGAAG-3' and 5'-TTATGGTCTCAAGAGTGATCC-3' and ligated to a C-terminal HA-tagging vector.

To generate an ER reporter construct, CH, an N-glycosylation site, was initially added to the first half region (44 residues) of Arabidopsis BiP (accession no. D84414) by PCR using primers 5'-GATAGCTCATGGATGTTATGCTCATACCTCTATTCTGTATGCTACATCCATG-3' and 5'-TTCCCAAATTTTGCTCGCATGTC-3' and 5'-TGCGCTCGAGTTAGAGAAAACCTCCACCCGTC-3' and 5'-TACGAAATTTTGCTCGCATGTC-3'. The amplified product was fused to the N terminus of the GFP-coding region in a GFP vector to yield BiP:NLT:GFP. Next, the C-terminal region (seven amino acids) of BiP containing the HDEL retrieval motif at the C terminus was added to BiP:NLT:GFP by two sequential PCRs using the BiP:NLT:GFP construct as template. The primer sets for the first and second PCR reactions were 5'-GGGGGCCTCACATGCAGCCATGCATTCATCCATTGTATGCTACATCCATG-3' and 5'-TTCCCGGGATTAGCGCTATGAGACTCATCTATTCTGTATGCTACATCCATG-3'. The amplified products were subcloned into pUC containing the cauliflower mosaic virus 35S promoter, and their sequences were confirmed.

Fractionation of Proteins and Western-Blot Analysis

To prepare cell extracts, transformed protoplasts were subjected to repeated freeze and thaw cycles in lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 3 mM MgCl2, 0.1 mg/mL antipain, 2 mg/mL aprotinin, 0.1 mg/mL E-64, 0.1 mg/mL leupeptin, 10 mg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride), and centrifuged at 7,000g at 4°C for 5 min. Cell extracts were separated into soluble and membrane fractions by ultracentrifugation at 100,000g for 2 h. These fractions were used for western-blot analysis with anti-HA (SFI0; Roche Diagnostics), anti-Arf1 (Pimpl et al., 2000), anti-γ-COP (Pimpl et al., 2000), anti-alburein (Sohn et al., 2003), and anti-PEP12p (Rose Biotechnology) antibodies. Protein blots were developed with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech).

Comminoprecipitation

To perform immunoprecipitation (IP) experiments, protein extracts in IP buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.1 mg/mL antipain, 2 mg/mL aprotinin, 0.1 mg/mL E-64, 0.1 mg/mL leupeptin, 10 mg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride, 5 mM MgCl2, 0.05% NP-40, and 0.1% Triton X-100) were preclaried at 4°C for 2 h with protein A-Sepharose beads. The rat anti-HA antibody (Roche Diagnostics) was incubated with preclaried extracts at 4°C for 2 h on a rotating platform. Immune complexes were isolated using protein A-Sepharose beads. Beads were washed five times with IP buffer after which proteins were released by 5-min incubation in SDS sample buffer at 98°C. Samples were subjected to SDS-PAGE and analyzed by western blotting with the appropriate antibodies, including anti-phaseolin (Frigerio et al., 1998), mouse anti-HA (12CA5; Roche Diagnostics), and anti-GFP (CLONTECH).
Endo H and Tunicamycin Treatment

Protein extracts were prepared from transformed protoplasts, and proteins were denatured in denaturation solution (1% SDS, 2% β-mercaptoethanol) by 10-min incubation at 100°C. Denatured proteins were incubated with 2 mg/mL endo H (Roche Diagnostics) in G5 buffer (50 mM sodium citrate, pH 5.5) at 37°C for 2 h. Transformed protoplasts were incubated with 15 µg/mL tunicamycin. Samples were subjected to SDS-PAGE and analyzed by western blotting with the appropriate antibodies.

In Vivo Localization and Trafficking Assay Using GFP-Fused Proteins in Arabidopsis Protoplasts

Plasmids were purified using Qiagen columns according to the manufacturer’s protocol and introduced by polyethylene glycol-mediated transformation (Jin et al., 2001) into Arabidopsis protoplasts prepared from whole seedlings. The localization of GFP fusion proteins was monitored at various time points after transformation, and images were captured with a cooled CCD camera using a Zeiss Axioskop fluorescence microscope. The filter sets used were XF116 (exciter, 474/420; dichroic, 500DRLP; emitter, 510AFA23), XF33/E (exciter, 535DF35; dichroic, 570DRLP; emitter, 585ALP; Omega) for GFP, red fluorescent protein, and autofluorescence of chlorophyll, respectively. Data were processed using Photoshop software (Adobe) and the images rendered in pseudocolor.

Immunohistochemistry

Transformed protoplasts or cryosections of plant tissues were used for immunohistochemistry. Cryosections were prepared and fixed as described previously (Wick, 1993). Transformed protoplasts were allowed to adhere to poly-L-lys-coated slides (Sigma) for 30 min, fixed in W5 buffer containing 4% paraformaldehyde (EM) for 30 min, and permeabilized in TSW buffer (10 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.25% NaCl, 0.02% SDS, 0.1% Triton X-100) for 10 min. Fixed protoplasts or cryosections were subsequently incubated overnight at 4°C with primary antibodies, such as anti-HA (Roche), anti-γ-COP (Pimpl et al., 2000) or anti-Phaseolin (Frigieri et al., 1998), anti-AraB (Ueda et al., 2001), and monoclonal JFM4 (CarboSource, University of Georgia) antibodies in the same buffer. Cells were washed three times in TSW buffer and incubated for 1 h at room temperature with the secondary antibodies tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit, anti-mouse IgG, fluorescein isothiocyanate-conjugated goat anti-rabbit IgC, or rabbit anti-rat IgM antibody (Zymed Laboratories). Following another three washes in TSW buffer, cells were mounted in Mowiol (Hoechst) containing 0.5% 1,4-diazobicyclo-[2.2.2]-octane (Sigma).

Immunofluorescent labeling of proteins in root cells was performed as described by Wee et al. (1998) and Kircher et al. (1999), with modifications. Four-day-old seedlings were fixed for 60 min in PEX buffer (5 mM MgCl2, 100 mM PIPES, pH 6.9, 5 mM ECTA) containing 4% paraformaldehyde and 5% dimethyl sulfoxide and digested using 1% cellulose (Yukai Honsha) R-10, 2% Macerozyme (Yukai Honsha) R-10 for 30 min at room temperature. Partially digested roots were squashed on poly-L-lys-coated slides. The slides were blocked in TSW buffer containing 0.5% bovine serum albumin overnight at 4°C and subsequently incubated for 2 h at room temperature with primary antibodies, such as anti-HA and anti-γ-COP, in TSW buffer. After washing five times in TSW buffer, cells were incubated for 1 h at room temperature with the secondary antibodies TRITC- and fluorescein isothiocyanate-conjugated anti-rabbit and rabbit IgG. Cells were subjected to another five washes in TSW buffer and mounted in Mowiol. Stained cells were visualized with a Zeiss fluorescence (Axioskop) and laser scanning confocal microscope (Meta System, Zeiss).

Lipid-Binding Assay

A lipid-binding assay with GST-tagged GAP domains of AGD7 was performed, according to Dowler et al. (2002). Phospholipids immobilized on membranes (Echelon) were blocked with 3% fatty acid-free bovine serum albumin in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.1% Tween 20 (TBST) for 1 h at room temperature. Membranes were incubated for 4 h at 4°C in solution containing 2 µg of GST-tagged GAP domain and GST only. After washing three times with TBST, membranes were incubated with anti-GST rabbit antibody (Oncogene) in TBST containing 3% fatty acid-free bovine serum albumin. Blots were washed three times with TBST incubated with the secondary anti-rabbit IgG goat antibody in TBST buffer. Following another three washes with TBST, blots were developed with an enhanced chemiluminescence kit.

GAP Activity Assay Using Recombinant Proteins Expressed in Escherichia coli

To express Arf1::His, Arf1 cDNA was amplified using the primers 5′-CCCATATGGAAGGTCTCAGG-3′ and 5′-GATGCGTGGCTGGGCTTGCAGG-3′. The PCR product was ligated in-frame to an expression vector, PET21a (Novagen). To express GST-tagged AGD7 proteins, AGD7 DNA was ligated to an expression vector, pgEX-5x-2. To construct the GST-tagged N-terminal domain (1–133 amino acid residues) of AGD7, a DNA fragment containing GST-AGD7N was amplified using the following oligonucleotides: 5′-GGCTTCCGAGATCC-3′ and GST sequencing primer 5′-CCAGCAAG-TATATGAGTGCAGC-3′. The PCR product was ligated in-frame to pgEX-5x-2, an expression vector. Constructs were introduced into the E. coli BL21(DE3) strain. Expression of recombinant proteins was induced by isopropylthio-β-galactoside (1 mM) at 37°C for 1 h. Arf1::His and GST-AGD7N were purified using an Ni2+-NTA affinity column and gluthathione agarose beads, respectively.

To assay Arf1-GTPase activity, we performed a fluorometric assay, based on the change in intrinsic Trp fluorescence of Arf during transition from the GTP- to GDP-bound state, as described previously (Antonny et al., 1997a). To prepare liposomes (200 µg), total lipids were extracted from Arabidopsis leaf tissues according to the Folch method (Folch et al., 1957), supplemented with several concentrations of PA (Sigma), reextracted with 2:1 chloroform:methanol, and suspended in 3 mL of reaction buffer (150 mM KCl, 2 mM MgCl2, 1 mM dithiothreitol, 20 mM HEPES, pH 7.4). Arf1 (10 µg) was mixed with GTP (10 µM) or the nonhydrolysable analog, GTPγ(S) (10 µM), in the presence of 4 mM EDTA (Paris et al., 1997). GTP- and GTPγ(S)-loaded Arf1 was stabilized by 4 mM MgCl2 and Arf1-GTPase activity was measured after mixing with GST-AGD7N (4 µg) or GST (4 µg) in the presence and absence of liposomes supplemented with various concentrations of PA. Tryptophan fluorescence was recorded at 340 nm (bandwidth 20 nm) excitation at 297.5 nm (bandwidth 5 nm) with a SHIMADZU RF-5301 PC fluorimeter in a cylindrical cuvette at 37°C with stirring (Krujac-Letunic et al., 2003).

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Role of AGD7 in Protein Trafficking