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The *Arabidopsis* GNOM ARF-GEF Mediates Endosomal Recycling, Auxin Transport, and Auxin-Dependent Plant Growth

Niko Geldner,[1] Nadine Anders,[1] Hanno Wolters,[1] Jutta Keicher,[1] Wolfgang Kornberger,[1] Philippe Muller,[2] Alain Delbarre,[2] Takashi Ueda,[3] Akihiko Nakano,[3] and Gerd Jürgens[1,*]

[1]ZMBP, Entwicklungsgenetik
Universität Tübingen
Auf der Morgenstelle 3
D-72076 Tübingen
Germany

[2]Institut des Sciences Vegetales
CNRS, Batiment 23
Avenue de la Terrasse
91198 Gif-sur-Yvette
France

[3]Molecular Membrane Biology Laboratory
RIKEN, 2-1 Hirosawa, Wako
Saitama 351-0198
Japan

Summary

Exchange factors for ARF GTPases (ARF-GEFs) regulate vesicle trafficking in a variety of organisms. The *Arabidopsis* protein GNOM is a brefeldin A (BFA) sensitive ARF-GEF that is required for the proper polar localization of PIN1, a candidate transporter of the plant hormone auxin. Mutations in GNOM lead to developmental defects that resemble those caused by interfering with auxin transport. Both PIN1 localization and auxin transport are also sensitive to BFA. In this paper, we show that GNOM localizes to endosomes and is required for their structural integrity. We engineered a BFA-resistant version of GNOM. In plants harboring this fully functional GNOM variant, PIN1 localization and auxin transport are no longer sensitive to BFA, while trafficking of other proteins is still affected by the drug. Our results demonstrate that GNOM is required for the recycling of auxin transport components and suggest that ARF-GEFs regulate specific endosomal trafficking pathways.

Introduction

Intracellular vesicle trafficking maintains the compartmentalized organization of the eukaryotic cell and mediates exchange of information with its environment. Polarization of cells, the correct transduction of extracellular signals, or the establishment of morphogen gradients during development all depend on highly regulated intracellular vesicle trafficking events. Establishment and maintenance of epithelial cell polarity is dependent on directed vesicle transport to distinct plasma membrane subdomains (Mostov et al., 2000). Receptor levels at the surface are controlled by ligand-induced endocytosis and subsequent degradation or recycling (Haj et al., 2002). Some receptors even need to be endocytosed in order to signal through downstream modules that can only be recruited on endomembranes (Defea et al., 2000). Also, interference with endocytosis in *Drosophila* wing disks alters distribution of morphogenic signals, such as dpp (which is thought to be taken up and to be transcytosed during migration through the tissue) (Teleman et al., 2001). In contrast to these examples from the animal literature, virtually nothing is known in higher plants about links between vesicle transport and the establishment of polarity, growth factor distribution, or signal transduction pathways.

A focus of interest in plant research has been to elucidate the transport mechanism for the plant growth regulator auxin. The active, polar transport of auxin through the plant is thought to arise from the coordinated polar distribution of carriers in the plasma membrane and is held responsible for a plethora of developmental processes (Friml and Palme, 2002). Candidates for auxin carriers were identified in the past few years and have indeed been shown to localize in a strictly polar fashion (Gälweiler et al., 1998; Müller et al., 1998). One of these candidates, the multispan membrane protein PIN1, continuously recycles through endomembrane compartments (Geldner et al., 2001), suggesting that its polar localization is highly dependent on directed vesicle trafficking.

The *GNOM(GN)* gene (also called *EMB30*) of *Arabidopsis thaliana* has been defined through loss-of-function mutants displaying striking embryonic phenotypes (Mayer et al., 1993). Mutant embryos show a loss of cell-to-cell alignment along the embryonic axis, lack the embryonic root, and display variable fusion or deletion of cotyledons and hypocotyl. In severe cases, a complete loss of the apical-basal axis was observed. These genetic defects have been mimicked by treating in vitro cultured *Brassica juncea* embryos with high doses of auxin or with inhibitors of its polar transport (Hadfi et al., 1998), suggesting a link between GNOM function and auxin transport or perception. *GNOM* encodes a *G*DP/GTP *e*xchange *f*actor for small G proteins of the ARF class (ARF-GEF) (Busch et al., 1996; Shevell et al., 1994; Steinmann et al., 1999). ARF proteins play a role in the recruitment of vesicle coats necessary for vesicle budding and cargo selection (Donaldson and Jackson, 2000). Thus, GNOM acts as a regulator of intracellular vesicle trafficking. However, it was not at all clear how this cellular activity relates to auxin and to the embryonic axis formation defects of *gnom* mutants.

By several criteria, *gnom* embryos lack any apparent phenotype of a secretory mutant. *gnom* seedlings do not become necrotic after germination, and *gnom* cell suspension cultures are easily established and maintained. Moreover, *gnom* mutants are neither affected in pollen tube growth nor in cytokinesis, two processes that require very active, regulated vesicle trafficking (Bednarek and Falbel, 2002; Parton et al., 2001) and which are often defective in knockout mutants of central components of the vesicle transport machinery (Lukowitz et al., 1996; Sanderfoot et al., 2001). Finally, a number

*Correspondence: gerd.juergens@zmbp.uni-tuebingen.de

of subcellular markers were investigated and found to localize essentially normally in *gnom* (Shevell et al., 2000; Steinmann et al., 1999). The only abnormality observed was the localization of the putative auxin efflux carrier PIN1 during embryogenesis. Its strictly coordinated polar localization in the adult plant arises gradually during embryogenesis from an initially nonpolar distribution. This process is disrupted in *gnom* embryos, which show a largely randomized polarity of cells with respect to each other (Steinmann et al., 1999). This led to the hypothesis that GNOM might be involved in trafficking of auxin efflux carriers. In this view, their disorganization or improper functioning in *gnom* mutants would abolish polar auxin transport, which could account for the observed phenotypes.

PIN1 is rapidly internalized in response to brefeldin A (BFA; Geldner et al., 2001), which has been widely used as a reversible inhibitor of vesicle trafficking in yeast, mammalian, and plant cells. A subclass of large ARF-GEFs is the primary molecular target of BFA. They are defined by a number of critical residues in their central catalytic Sec7 domain (Peyroche et al., 1999; Sata et al., 1999). GNOM carries these hallmarks of BFA sensitivity, and its activity has indeed been shown to be inhibited by the drug (Steinmann et al., 1999). BFA traps sensitive ARF-GEFs in an abortive complex, preventing ARF effector activation necessary for vesicle budding and cargo selection (Peyroche et al., 1999; Robineau et al., 2000). The structural changes of the endomembrane system in a given cell upon BFA treatment are thus contingent on the differential contributions of BFA-sensitive versus BFA-resistant ARF-GEFs to the overall network of membrane traffic.

In mammals, the sites of BFA action often do not correlate with the subcellular localization of BFA-sensitive ARF-GEFs. For example, the only two clearly BFA-sensitive ARF-GEFs involved in Golgi trafficking do not localize to the *cis*-Golgi, which is strongly sensitive to the drug (Zhao et al., 2002). The same is true for endosomal trafficking. Endosomes have been known for a long time to be structurally affected by BFA, and transcytosis was shown to be inhibited by this drug (Hunziker et al., 1991; Lippincott-Schwartz et al., 1991; Wood et al., 1991). However, the only ARF-GEFs known to localize to endosomes are animal specific BFA-resistant exchange factors such as EFA6, ARNO, or ARF-GEP$_{100}$ (Franco et al., 1999; Frank et al., 1998; Someya et al., 2001). These ARF-GEFs act on animal-specific ARF6, which is involved in endocytosis and endosomal trafficking (D'Souza-Schorey et al., 1998). ARFs often localize to a wide range of membrane compartments, regulating distinct trafficking processes, and ARF-GEFs are thought to confer specificity to ARF action (Donaldson and Jackson, 2000). Although three different exchange factors are known for ARF6, their relative contributions to specific ARF6-regulated transport events remain to be demonstrated.

In comparison to mammals, *Arabidopsis* has a higher number of predominantly BFA-sensitive, large ARF-GEFs. By sequence analysis, only three out of the eight *Arabidopsis* ARF-GEFs can be predicted to be BFA resistant (Figure 1A), and except for ARF1, no clear orthologs of mammalian ARFs, including ARF6, can be identified (Jürgens and Geldner, 2002). Thus, differences in the response to BFA are to be expected between the plant and mammalian endomembrane systems. The internalization of PIN1 into perinuclear compartments upon BFA treatment is an example for such a difference because rapid and strong internalization of plasma membrane markers in response to BFA has not been observed in mammals. BFA-induced intracellular accumulation of PIN1 can be explained by continued endocytosis in the presence of blocked resecretion (Figure 1B). However, since BFA is predicted to inhibit at least 5 ARF-GEFs simultaneously, it cannot be deduced which and how many ARF-GEFs might be involved in this recycling process.

To examine whether GNOM is the ARF-GEF responsible for PIN1 recycling, we analyzed PIN1 localization in transgenic plants expressing an engineered BFA-resistant GNOM variant. Although other ARF-GEFs remained BFA sensitive, BFA-resistant GNOM was sufficient for rendering PIN1 recycling to the plasma membrane insensitive to BFA and, in addition, led to BFA-resistance of auxin transport and auxin-mediated growth responses. Double localization experiments of tagged GNOM variants with a number of markers both in the presence and absence of BFA revealed that GNOM localizes to endosomes and *gnom* loss-of-function mutants display altered endosome morphology.

Results

An Engineered BFA-Resistant GNOM ARF-GEF Rescues *gnom* Mutant Plants

Brefeldin A (BFA) inhibits activation of ARF proteins by binding to an ARF-GDP/ARF-GEF complex (Peyroche et al., 1999; Robineau et al., 2000). Naturally occurring BFA-resistant or BFA-sensitive ARF-GEF variants differ in a number of amino acid residues consistently conserved in the exchange domain. Some of these residues were shown to be critical for BFA resistance of yeast and mammalian ARF-GEFs (Figure 1A). Exchanging those residues renders BFA-resistant ARF-GEFs sensitive and vice versa, both in biochemical assays and in vivo (Peyroche et al., 1999; Sata et al., 1999). We reasoned that if the BFA-sensitive GNOM ARF-GEF of *Arabidopsis* was also rendered BFA-resistant by introducing homologous amino acid exchanges into its catalytic domain, we could determine which BFA-sensitive trafficking pathways are specifically regulated by GNOM.

A 7.5 kb genomic fragment of the *GNOM* gene containing 2.1 kb upstream of the ATG and about 0.6 kb downstream of the stop codon complemented the *gnom* mutant phenotype (data not shown). This fragment was used to insert coding sequences for a 3× myc tag or GFP at the 3′ end of the open reading frame (Figure 1C). A BFA-resistant variant was derived from the myc-tagged construct by introducing an M to L mutation at amino acid position 696 in the Sec7 domain (Figure 1C). We chose this mutation amongst a number of possible ones because it was shown to confer good, albeit not complete, BFA resistance to yeast Gea1p without affecting its ability to complement the *gea1* knockout (Peyroche et al., 1999). Furthermore, the closest homolog of GNOM, GNOM-LIKE 1 (GNL1), carries L instead of M at this position (Figure 1A), suggesting that an M to L

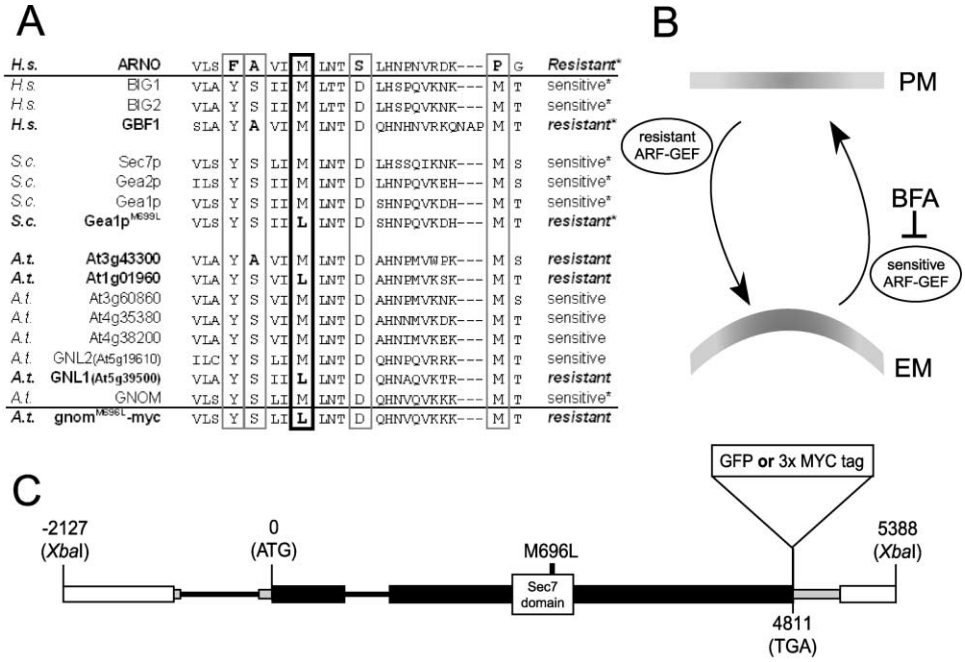

Figure 1. Engineering of a BFA-Resistant GNOM Variant

(A) Sequence alignment of the region determining BFA resistance of ARF-GEFs. All large ARF-GEFs from human (H.s.), yeast (S.c.), and *Arabidopsis* (A.t.) are shown. At the top, ARNO, as an example of a BFA-resistant, small, mammalian ARF-GEF. Residues known to be involved in BFA sensitivity are boxed. The ones determining resistance are written in bold. Asterisks indicate that BFA sensitivity/resistance was determined experimentally, and unmarked ones are predictions inferred from this data. The residue boxed in black indicates the amino acid exchange chosen to be introduced into GNOM (bottom line).

(B) Schematic model to explain internalization of PM markers upon BFA treatment. BFA is thought to block a sensitive ARF-GEF responsible for recycling, while ongoing endocytosis might be mediated by a resistant ARF-GEF or, alternatively, by ARF-independent endocytosis.

(C) Overview of the BFA-resistant GN^{M696L}-myc construct used. Positions indicated are relative to the translational start. Black boxes indicate translated regions, light gray boxes UTRs, lines indicate introns, and white boxes intergenic regions (promoter). The box marked "Sec7 domain" indicates the region of the central catalytic domain. A 3× myc-tag was translationally fused to the 3′ end of the ORF of the complementing genomic *Xba*I fragment. The resulting construct was then mutated.

mutation will not interfere with the exchange activity of plant ARF-GEFs. The three constructs GN^{M696L}-myc, GN^{wt}-myc, and GN-GFP were transformed into segregating populations of *gnom* heterozygous *Arabidopsis* plants. For each construct, *gnom* mutant plants from several independent lines were shown to be rescued. Thus, GN^{M696L}-myc, GN^{wt}-myc, and GN-GFP fusion proteins appeared to be fully functional variants of the GNOM protein.

PIN1 Recycling Is BFA Resistant in GN^{M696L}-myc Transgenic Seedlings

Root tips of *GNOM^{wt}-myc* seedlings displayed polar localization of PIN1, which strongly accumulated in internal compartments upon BFA treatment for 60 min (Figures 2A and 2B), as previously described for wild-type (Geldner et al., 2001). By contrast, root tips of *GNOM^{M696L}-myc* seedlings showed nearly complete resistance to BFA-induced intracellular accumulation, with no or only very few intracellular patches visible after 60 min (Figure 2C).

Immunolocalization of untreated GN^{wt}-myc seedlings with monoclonal myc-antibody revealed strong cytosolic "background" staining and a number of small intracellular patches (Figure 2D). Upon BFA treatment, cytosolic GN^{wt}-myc signal was decreased, with a concomitant strong increase in apparent membrane signal that coalesced into large perinuclear aggregates (Figure 2E). These observations were consistent with earlier differential centrifugation experiments of cell extracts, which gave a predominantly cytosolic and a minor membrane-bound form of GNOM, with the latter increasing upon BFA treatment (Steinmann et al., 1999). We sometimes also observed a weak plasma membrane label appearing upon BFA treatment, which was not observed in untreated tissue (Figures 2D and 2E). Neither the increase in membrane label nor aggregation into patches was seen for the GN^{M696L}-myc protein, illustrating its BFA resistance (Figure 2F).

In untreated roots of GN^{wt}-myc seedlings, no colocalization of GNOM and PIN1 was observed (Figure 2G). However, double labeling of GN^{wt}-myc after BFA treatment revealed a complete colocalization of PIN1 and GNOM in perinuclear aggregates (Figure 2H), previously described as "BFA compartments" (Geldner et al., 2001; Satiat-Jeunemaitre and Hawes, 1992; Steinmann et al., 1999). Double labeling of GNOM and PIN1 in GN^{M696L}-myc lines after BFA treatment revealed no colocalization of the two proteins, with GNOM remaining mainly cytosolic and PIN1 staying at the plasma membrane (Figure 2I). The BFA insensitivity of PIN1 accumulation at the plasma membrane in GN^{M696L}-myc lines, together with

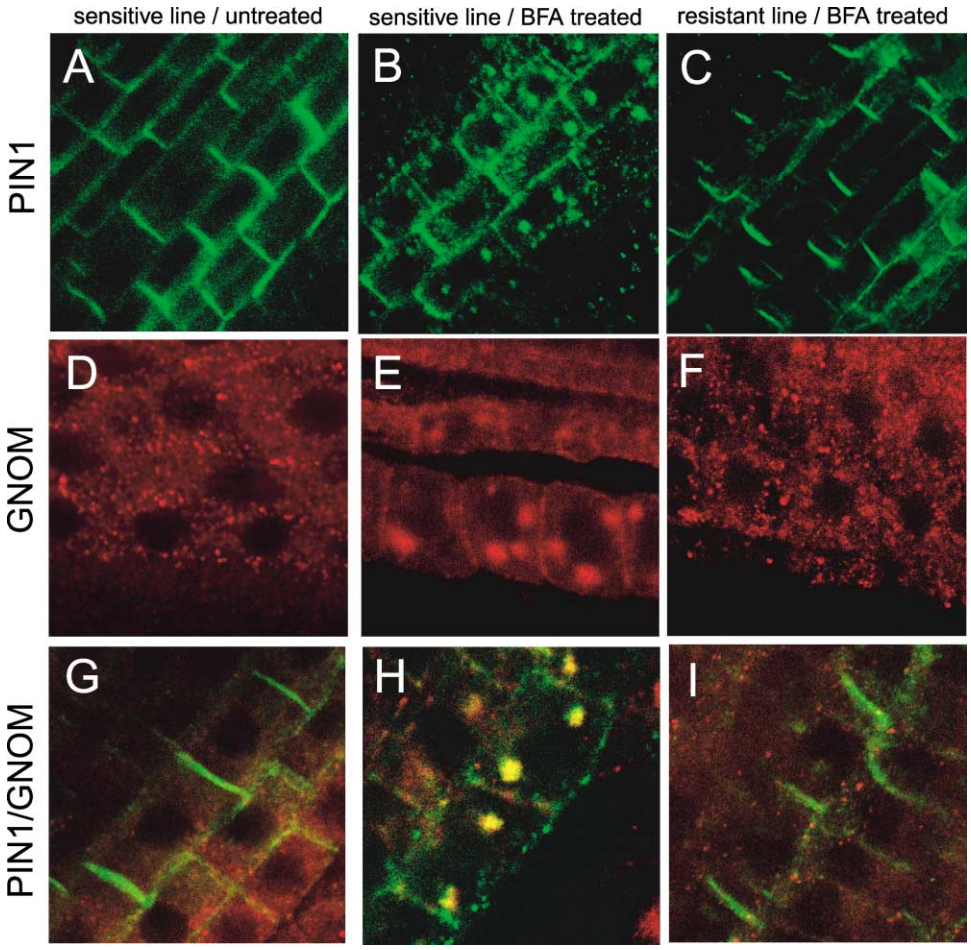

Figure 2. Brefeldin A Responses of PIN1 and GNOM Are Altered in Engineered *GNOM* Lines

Confocal images of seedling root tips stained with PIN1 antibody (green) and monoclonal myc antibody (red) on GNOM-myc transgenic lines. (A–C) PIN1, (D–F) GNOM-myc, and (G–I) PIN1 and GNOM-myc. (A, D, and G) Control treatment on *GN^{wt}-myc* line, (B, E, and H) BFA 50 μM for 60 min on *GN^{wt}-myc* line, and (C, F, and I) BFA 50 μM 60 min on *GN^{M696L}-myc* line. Note yellow intracellular dots in (H), indicating colocalization of PIN1 and GNOM.

the colocalization of PIN1 and BFA-sensitive GNOM to the same endomembranes after BFA treatment, strongly suggests that GNOM ARF-GEF is responsible for recycling PIN1 through an intracellular compartment.

The GNOM ARF-GEF Localizes to Endosomes

To identify the intracellular site of GNOM action, we performed double-labeling experiments of GNOM with available markers for endomembrane compartments. Although a number of specific antibodies are available for plants, very few have been shown to function in confocal microscopy imaging of *Arabidopsis* seedling root tips. The AtSEC12 antiserum detects an ER resident, transmembrane protein (Bar-Peled and Raikhel, 1997). This antiserum labeled membrane compartments resembling previous descriptions of ER membranes (Figure 3A). GN^{wt}-myc did not colocalize with AtSEC12 (Figure 3A). Upon BFA treatment, the ER network appeared unchanged, and the AtSEC12 signal did not colocalize with the GNOM-labeled patches (Figure 3B). Thus, GNOM-positive membranes were different from the ER network.

ARF GTPases recruit COPI coats as well as clathrin coats to membranes for vesicle budding (Boman, 2001, and references therein). Whereas COPI coats mediate intra-Golgi transport and recycling to the ER, clathrin coats have been implicated in the formation of endocytic vesicles and vesicle budding from the trans-Golgi network (for review, see Holstein, 2002). An early observation regarding BFA action was the inhibition of COPI recruitment to membranes (Orci et al., 1991). A similar effect on the γ-COP subunit has recently been described in tobacco BY-2 cells (Ritzenthaler et al., 2002). We reasoned that if GNOM were involved in the recruitment of COPI coats, γCOP and GNOM should colocalize and γ-COP localization should become BFA resistant in *GN^{M696L}-myc* roots. In untreated cells, γ-COP appeared to be associated with Golgi stacks, as judged from the number, size, and characteristic shape of the signal (Figure 3C), which is consistent with previous descriptions of γ-COP label in tobacco (Ritzenthaler et al., 2002). However, upon BFA treatment, the reported abolishment of γ-COP membrane association was not observed in our system. Instead, γ-COP stayed at the membrane,

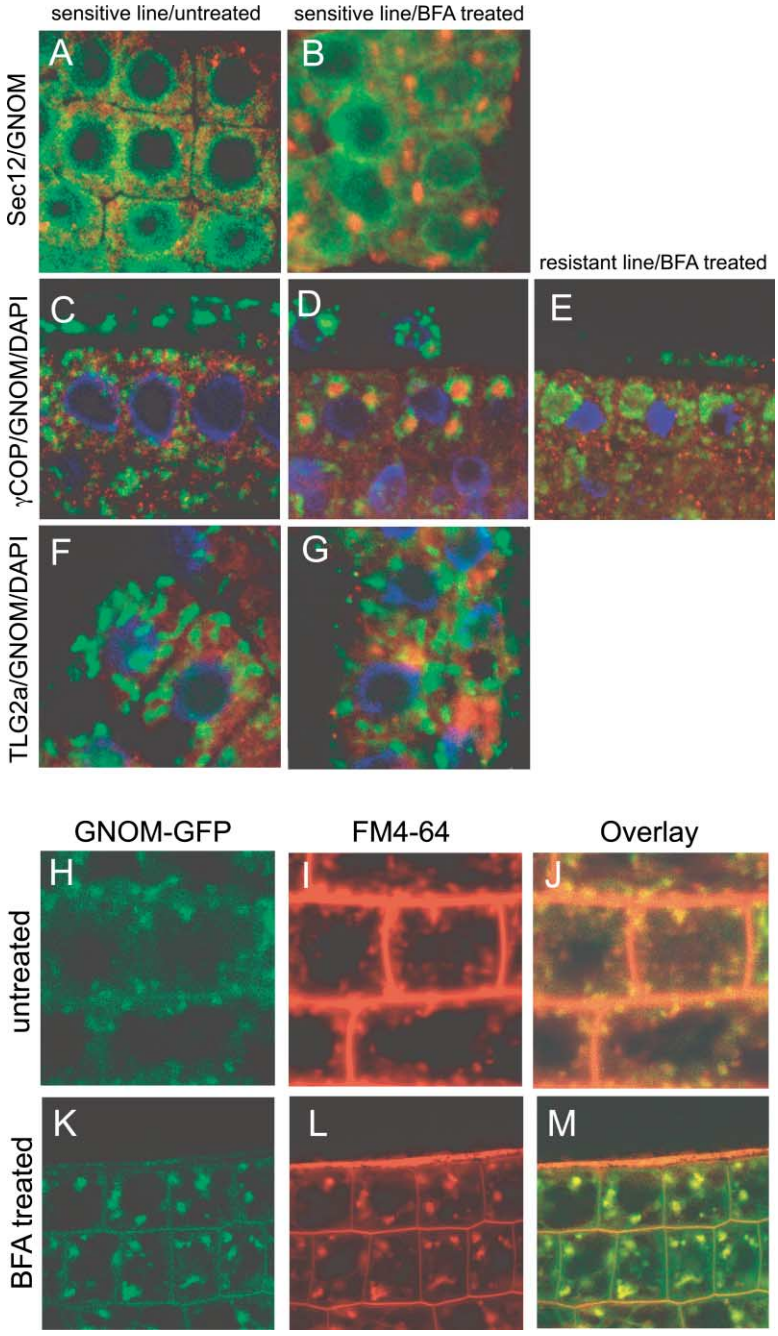

Figure 3. Colocalization with Intracellular Markers Define the GNOM Compartment as Endosomal

Confocal pictures of seedling root tips with either myc antibody (red) and Sec12p, γCOP, TLG2a antibodies (green) or GN-GFP (green) and FM4-64 (red), DAPI staining of nuclei (blue). (A and B) AtSec12 and GN^{wt}-myc, (C and D) γCOP and GN^{wt}-myc, (F–G) TLG2a and GN^{wt}-myc, and (H–M) GN-GFP and FM4-64. (A, C, and F) Control treatment on *$GNOM^{wt}$-myc* line, (B and D) BFA 50 μM for 60 min teated GN^{wt}-myc line, (G) BFA 200 μM for 4 hr, and (E) BFA 50 μM for 60 min on *GN^{M696L}-myc* line. (H–J) untreated cells after 30 min of FM4-64 uptake, (H) and (I) single channels, (J) overlay, (K–M) BFA 50 μM for 45 min with equal time of FM4-64 uptake, (K) and (L) single channels, and (M) overlay. Note tightly associated, sometimes overlapping green and red dots in (J) and completely overlapping intracellular dots in (M).

but the individual Golgi stacks aggregated into larger clusters, without completely losing their integrity (Figure 3D). Such discrepancies have previously been reported between different mammalian cell lines (Hunziker et al., 1991). GN^{wt}-myc did not appear to colocalize with γ-COP in untreated cells (Figure 3C). Upon BFA-treatment, γ-COP-labeled clusters surrounded a GNOM-positive central patch, without showing any obvious colocalization (Figure 3D). In BFA-resistant GNOM lines, γ-COP label still aggregated to enclose a "core" which, however, was GNOM negative (Figure 3E). Thus, γ-COP and GNOM did not colocalize nor did γ-COP compartments become resistant in BFA-resistant GNOM lines. We conclude that GNOM does not regulate budding of Golgi-associated, COPI-coated vesicles.

The trans-Golgi network (TGN) can be viewed as the station of the secretory pathway at which COPI-dependent membrane trafficking is taken over by non-COPI coats, such as clathrin (Kirchhausen, 2000). Antibodies raised against TGN-localized AtTLG2a (SYP41) (Bassham et al., 2000) showed strong staining of elongated, often sickle-shaped structures (Figure 3F) that appeared to be different from structures labeled with Golgi stack markers (Wee et al., 1998). Again, no colocalization was observed between TLG2a and GN^{wt}-myc (Figure 3F). In contrast to GNOM, TLG2a label did not change upon

treatment with BFA, even when high doses were applied for prolonged time periods, and GN^{wt}-myc did not colocalize with TLG2a (Figure 3G). We conclude that GNOM-positive endomembranes are also different from the TGN and that GNOM probably does not act along the secretory pathway.

The fluorescent styryl dye FM4-64 is used as an endocytic tracer in yeast and mammalian cells. It fluoresces upon insertion into the plasma membrane and is taken up by the cell exclusively through membrane trafficking in yeast (Vida and Emr, 1995). This eventually leads to labeling of all the compartments along the endocytic pathway down to the vacuole. Recently, there have been a number of reports about the use of FM dyes as endocytic markers in plants (Emans et al., 2002; Parton et al., 2001; Ueda et al., 2001). Because FM4-64 can only be used for labeling of live cells, we performed double labeling with GN-GFP (Figures 3H–3M). Although weak, GN-GFP labeling of live cells resembled GN-myc immunofluorescence labeling of fixed cells (Figure 3H; compare with Figure 2D). Upon uptake of FM4-64 for 30 min, GNOM-GFP appeared to be closely associated, and partially overlapping with, the dye-labeled compartments (Figures 3I and 3J). In the presence of BFA, FM4-64 accumulated in larger patches and, in this case, GN-GFP completely colocalized with the FM4-64 label to those patches (Figures 3K–3M). At this time point, vacuoles were not yet labeled by FM4-64. These data strongly suggest that GNOM acts at an endosomal compartment that partially overlaps with, but is not entirely identical to, the FM4-64-labeled compartment.

Endosomes Are Abnormal in *gnom* Mutant Cells

If GNOM acts at an endosomal compartment, *gnom* mutant cells might show some abnormality in endosomal structure or function. To test this idea, *gnom* mutant cell cultures were established and transfected with ARA7-GFP constructs for transient expression. By sequence similarity, ARA7 is a member of the Rab5 family of endosomal Rab GTPases (Ueda et al., 2001). ARA7 was shown to localize to structures very similar to GNOM-positive compartments and to also colocalize with compartments of early FM4-64 accumulation (Ueda et al., 2001, and T.U. and A.N., unpublished data). Upon transient expression in cultured cells, ARA7-positive compartments appeared as larger patches in *gnom* than in wild-type cells (Figures 4A and 4B). In addition, ARA7 compartments were often ring shaped, which was only rarely observed in wild-type cells. Similar alterations of ARA7 compartments were observed when treating wild-type cell cultures with BFA (T.U. and A.N., unpublished data). These observations support the subcellular localization data and strongly suggest that GNOM ARF-GEF plays a role in regulating endosome structure and function.

Recycling of Other Plasma-Membrane Proteins in BFA-Resistant GNOM Seedlings

BFA has been shown to induce intracellular accumulation of PIN1 and to alter the distribution of other plasma membrane localized proteins, including KNOLLE (SYP111), AtSNAP-33, PM-ATPase, the auxin influx carrier AUX1, the PIN1 homolog PIN3, or even cell-wall components

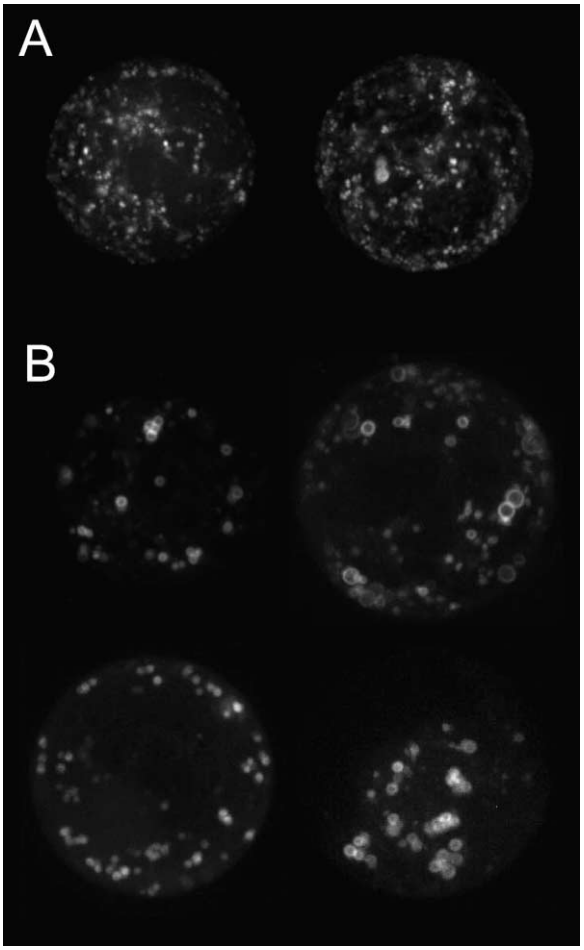

Figure 4. Endosomal ARA7-GFP Labeling in Wild-Type and *gnom* *Arabidopsis* Protoplasts

Projections of confocal sections. Representative examples of ARA7-GFP label in (A) *wild-type* and (B) *gnom* mutant cells.

such as pectins (Baluska et al., 2002; Friml et al., 2002; Geldner et al., 2001; Grebe et al., 2002; Heese et al., 2001). To examine whether GNOM is specifically involved in PIN1 trafficking, we analyzed the effects of BFA treatment on the localization of KNOLLE, PM-ATPase, and the PIN1 homolog PIN2 in our lines.

The cytokinesis-specific KNOLLE syntaxin localizes to intracellular patches and the newly forming cell plate, destined to become a new plasma membrane (Lauber et al., 1997). Upon BFA treatment, KNOLLE coalesced into large patches, similar to the described BFA compartments (Figures 5A and 5B; Geldner et al., 2001). BFA-induced accumulation was also observed for PM-ATPase and PIN2 (Figures 5D and 5E, and 5G and 5H, respectively). GNOM colocalized with all three proteins upon BFA treatment (data not shown). However, in the GN^{M696L}-myc line, KNOLLE aggregation into large patches still occurred (Figure 5C), suggesting that KNOLLE trafficking in cytokinetic cells is dependent on other BFA-sensitive GEFs. For PM-ATPase and PIN2 localization, the situation was more complex. First of all, not all PM-ATPase or PIN2-labeled cells responded to BFA, and the variability between individual roots was higher than

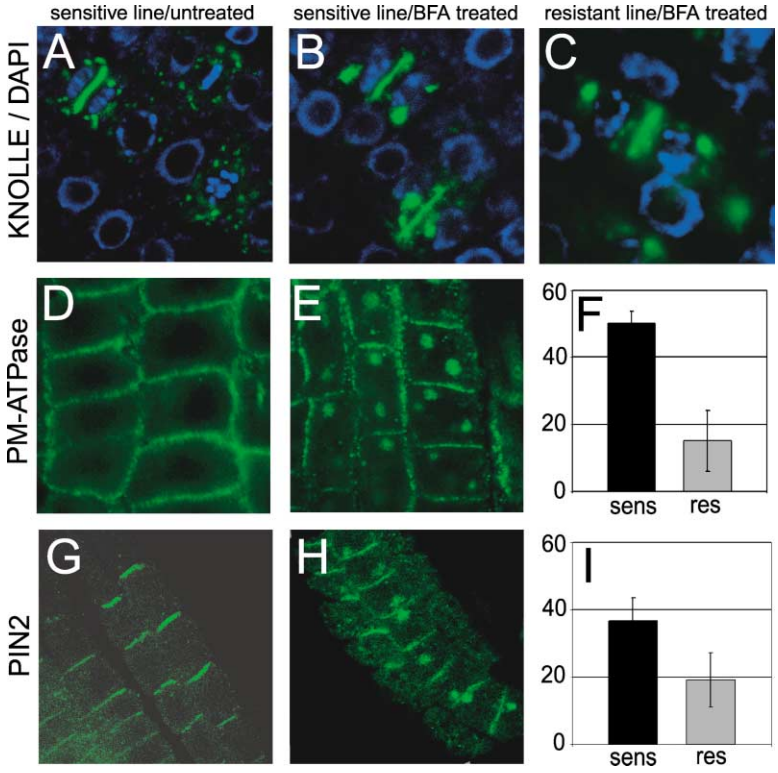

Figure 5. BFA Resistance of Other Plasma Membrane Markers in BFA-Resistant Lines

(A–C) KNOLLE, (D and E) PM-ATPase, and (G and H) PIN2. (A, D, and G) Untreated GN^{wt}-myc line, (B, E, and H) BFA 50 μM for 60 min GN^{wt}-myc, and (C) BFA 50 μM for 60 min GN^{M696L}-myc line. (F and I) Percentage of cells showing intracellular accumulation of label after BFA treatment in sensitive versus resistant lines. "Sens" is GN^{wt}-myc line (black) and "res" is GN^{M696L}-myc (gray). Each bar is an average of five root tips, representing about 1000 cells in total.

in the case of PIN1. This might be due to differences in recycling rates or transport routes used between individual cells and roots. When comparing effects of BFA in *GN^{M696L}-myc* lines, a similar variability was observed, ranging from almost complete to very weak increases in resistance compared to *$GNOM^{wt}$-myc* lines. In order to determine if there is a significant partial resistance despite strong variability, we counted several thousand cells from z axis scans of a number of root tips. This revealed a partial BFA-resistance of PIN2 and PM-ATPase in BFA-resistant lines (Figures 5F and 5I). Thus, GNOM action does not mediate trafficking of all plasma membrane proteins to the same extent.

GN^{M696L} Conferred BFA Resistance of Auxin Transport and Auxin-Mediated Physiological Responses

The observation that cycling of PIN proteins became BFA-resistant in GN^{M696L}-myc lines led us to investigate if this would be reflected in BFA insensitivity of polar auxin transport. Reliable measurements of polar transport in *Arabidopsis* can be done on inflorescence stems. Exposing the upper end of inflorescence stems of GN^{wt}-myc lines to a pulse of radioactively labeled auxin led to a peak of radioactive auxin at a distance of about 13 mm after 90 min of transport. Upon concomitant BFA treatment, this peak was nearly abolished (Figure 6A, left). By contrast, GN^{M696L}-myc lines showed no difference of auxin peak intensity with or without BFA treatment (Figure 6A, right). Thus, BFA-resistant GNOM confers BFA-resistance of both PIN1 cycling and polar auxin transport activity in planta.

High concentrations of BFA lead to growth arrest of cells, probably due to the severe block of intracellular trafficking, which inhibits fundamental processes such as cell-plate expansion during cytokinesis (Yasuhara and Shibaoka, 2000). In contrast, low concentrations of BFA have surprisingly specific effects on auxin transport-related processes such as root hair cell polarity, gravitropism, or initiation of lateral root primordia (Geldner et al., 2001; Grebe et al., 2002). We tested whether GNOM is the ARF-GEF responsible for the inhibition of these processes by BFA. We exposed seedlings to a change in gravity vector and recorded their realignment after 36 hr. Treatment with 5 μM BFA strongly interfered with the gravitropic response of GN^{wt}-myc lines (Figures 6B and 6E). GN^{M696L}-myc seedlings, in contrast, showed absolutely no difference in their gravitropic response in the absence or presence of BFA (Figures 6B and 6E). The same was true for lateral root formation. Treatment with 10 μM BFA led to complete inhibition of lateral root initiation in BFA-sensitive lines whereas, resistant lines showed no decrease in the number of lateral roots formed (Figure 6C). Additionally, this concentration of BFA significantly reduced primary root elongation, which was also less affected in BFA-resistant GNOM lines (Figure 6D). Thus, the sole alteration of GNOM ARF-GEF rendered auxin-related developmental processes insensitive to inhibition by low concentrations of BFA.

Discussion

GNOM Is a Large ARF-GEF that Localizes to Endosomes and Regulates Their Structure and Function

Our results indicate that GNOM is an ARF-GEF involved in recycling of plasma membrane proteins from endosomes. This was surprising because GNOM is a member

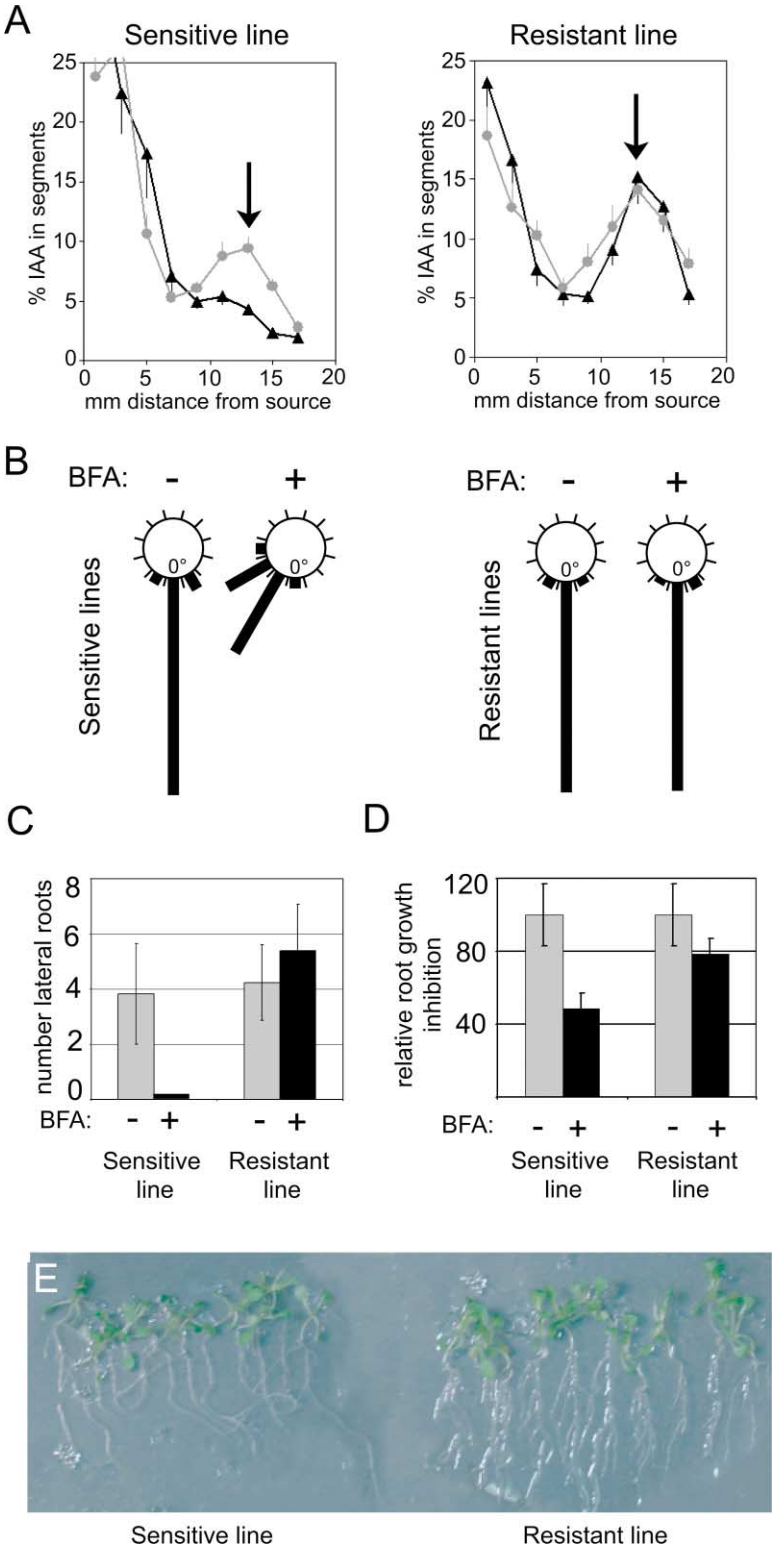

Figure 6. BFA Resistance of Auxin Transport and Auxin-Dependent Growth Responses in BFA-Resistant Lines

(A) Polar auxin transport of a sensitive and a resistant line in the absence (gray circles) or presence (black triangles) of 20 μM BFA. Arrows indicate the peak of polarly transported auxin, the slope to the left is diffusive transport of auxin. Note that there is no difference in the radioactive auxin peak upon BFA treatment in the resistant line.

(B) Gravitropic root growth response of BFA sensitive or resistant *GNOM* lines in the absence (−) or presence (+) of 5 μM BFA. 4-day-old seedlings were turned by 135° and grown for another 36 hr. Degree deviation from the gravitropic vector was measured. Each root was assigned one of twelve 30° sectors. About 80 seedlings were counted per histogram. Note that sensitive lines display less gravitropic curvature in the presence of BFA (90° ± 15°) than resistant lines (139.5° ± 8°).

(C) Lateral root numbers of GNOM BFA-sensitive and -resistant lines in the absence (−) or presence (+) of 10 μM BFA. 5-day-old seedlings were treated for 6 days with or without BFA. Lateral root primordia visible under the binocular were counted. About 16 seedlings were counted for each value. Note that lateral root formation is abolished in GNOM BFA-sensitive lines in the presence of BFA.

(D) Primary root elongation in the absence (−) or presence (+) of 10 μM BFA of GNOM BFA-sensitive or resistant lines. 5-day-old seedlings were grown for 4 days. About 20 seedlings were counted for each value.

(E) Example of 11-day-old seedlings grown for 7 days on 5 μM BFA. GNOM BFA-sensitive line (left), GNOM BFA-resistant line (right). Note lack of lateral roots and gravitropic growth in the GNOM BFA-sensitive line.

of the *Gea/GNOM/GBF1* (GGG) subfamily of large ARF-GEFs. GGG-type ARF-GEFs have been implicated in ER-Golgi or intra-Golgi traffic, in yeast as well as in animals (Peyroche et al., 2001; Zhao et al., 2002). This class of ARF-GEFs can be distinguished from other subfamilies, such as the Sec7/BIG class, by overall homology and differences in size and domain structure. None of the large ARF-GEFs, neither the GGG-type nor the Sec7/BIG type, has been shown previously to act on endosomes.

Our conclusion that GNOM is not involved in transport through the secretory pathway is based on two lines of evidence. First it does not colocalize with several markers of the secretory system, neither before nor after BFA treatment. Second, the compartments investigated are

either naturally resistant to BFA treatment and thus cannot be regulated by GNOM or, if sensitive, they do not become resistant in BFA-resistant *GNOM* lines. In contrast to the secretory markers, the endocytic tracer FM4-64 showed significant colocalization with GNOM. While the two signals were closely associated and partially overlapping in the absence of BFA, they completely colocalized in the presence of BFA. Although the organization of plant endosomes is essentially unknown, mammalian endosomes are thought to be largely contiguous membrane compartments that are subdivided through combinatorial action of partially overlapping rab domains (Zerial and McBride, 2001). It is thus conceivable that GNOM localizes to a subdomain of a more or less contiguous endosomal system, which partially breaks down in response to the drug.

In addition to its endosomal localization and role in recycling, GNOM also appears to regulate endosome structure, as evidenced by structural alterations of ARA7 compartments in *gnom* loss-of-function cell lines. Interestingly, the ring-shaped structures observed in *gnom* cells very much resemble endosomes observed in ARF loss-of-function mutants of yeast (Gaynor et al., 1998; Yahara et al., 2001). Our findings indicate that loss of ARF-GEF function has a similar effect on endosomal structure. Although the endosomes observed in *gnom* cells may seem dysfunctional, there is precedence from animal cells that BFA-induced gross morphological alterations of the endosomal system do not prevent continued recycling of markers through this system (Hunziker et al., 1991). This suggests that structural integrity and function can be uncoupled to some degree.

What are the ARF substrates regulated by GNOM? The *Arabidopsis* genome encodes six class I ARF proteins related to ARF1 and three more divergent ARFs that cannot be grouped unambiguously with mammalian or yeast non-ARF1 classes (Jürgens and Geldner, 2002). *Arabidopsis* ARF1 acts in ER/Golgi trafficking in a similar fashion to yeast or mammalian ARF1 (Ritzenthaler et al., 2002; Lee et al., 2002; Takeuchi et al., 2002). Whereas nonplant ARF1 also plays a role in endosomal trafficking (Gaynor et al., 1998; Gu and Gruenberg, 2000), this has not been investigated for *Arabidopsis* ARF1, and the more divergent ARFs are functionally uncharacterized. Thus, the ARF substrate in GNOM-regulated endosomal trafficking remains to be determined.

GNOM might act only on a subset of endosomes, as suggested by the differential effects of BFA on plasma-membrane protein recycling in BFA-resistant GNOM plants. Whereas trafficking of basally localized PIN1 is regulated by GNOM, recycling of other proteins appears to be partially or completely independent of GNOM. The latter proteins include apically localized PIN2, apolarly localized PM-ATPase, and the cytokinesis-specific syntaxin KNOLLE. These findings suggest the existence of additional recycling routes. In animals, distinct recycling pathways have been described (Gruenberg, 2001). Polarized cells, in particular, make use of specialized apical and basolateral recycling routes (Mostov et al., 2000). Plants endosomes are essentially undefined. Yet the impressively high number of putative endosomal rab GTPases in *Arabidopsis* suggests a rather complex endosomal system, which may easily consist of several, functionally independent endosomes (Rutherford and Moore, 2002; Ueda and Nakano, 2002). GNOM-related ARF-GEFs such as GNL1 or GNL2 might be candidates for regulating such alternative, perhaps more general, pathways.

While in yeast, ARF-GEFs for the endosomal system are ill-defined, in animals, a number of specific ARF-GEF classes have evolved for endosomal trafficking (Donaldson and Jackson, 2000). In *Arabidopsis*, there are only large ARF-GEFs, which, however, are more numerous than in yeast and mammals (Jürgens and Geldner, 2002). Thus, increased complexity of vesicle trafficking pathways in plants was achieved by a different functional diversification of ARF-GEFs than in animals. In this view, the subcellular specificity of GNOM action apparently reflects the evolution of plant-specific ways to regulate endosomal trafficking.

The Importance of GNOM-Regulated Vesicle Trafficking for Polar Auxin Transport

A specific feature of plants is the regulation of diverse developmental processes through the polar flow of the growth regulator auxin, achieved by the coordinated polar distribution of auxin efflux components of the PIN family. The fact that the polar distribution of PIN1 results from a continuous recycling process suggests that regulation of vesicle trafficking is central to establishment and maintenance of polar auxin transport (Geldner et al., 2001).

BFA-induced internal accumulation of PIN1 was postulated to reflect inhibition of exocytosis in the presence of continued endocytosis (Figure 1B), which is consistent with other recent findings (Emans et al., 2002). Here, we have identified the responsible molecular target by demonstrating that a BFA-resistant GNOM ARF-GEF is sufficient to maintain PIN1 cycling dynamics in the presence of BFA. Moreover, this single alteration also leads to BFA-resistant polar auxin transport, which indicates that GNOM is an important in vivo mediator of auxin efflux.

Low concentrations of BFA cause growth and developmental defects that can be interpreted as consequences of auxin transport inhibition, consistent with reported effects of BFA on auxin efflux (Delbarre et al., 1998; Morris and Robinson, 1998). This effect of BFA was abrogated by exclusively rendering the endosome-localized GNOM ARF-GEF resistant to BFA. This is in contrast to the current view that BFA primarily affects Golgi-based secretion (Nebenführ et al., 2002). The apparent specificity of low concentrations of BFA to plant endosomal trafficking is surprising. It is conceivable that endosomal trafficking is more sensitive to BFA inhibition than is Golgi-based secretion. Alternatively, partial inhibition of endosomal trafficking might more readily lead to phenotypic consequences than partial inhibition of the secretory pathway, which might reflect the importance of endosomes in auxin transport and possibly other signaling events.

Engineered BFA Sensitivities of ARF-GEFs Are a New Approach to Dissect Vesicle Transport Pathways

Brefeldin A (BFA) has been extensively used to probe vesicle trafficking pathways in eukaryotic cells, and it has been established that the catalytic domains of ARF GEFs are primary targets of BFA action (Peyroche et

al., 1999; Sata et al., 1999). Since eukaryotic genomes encode several BFA-sensitive ARF-GEFs that may act at different stations within the network of intracellular trafficking, the cellular responses to BFA treatment are manifold, depending on the organism and cell type under study, and are often difficult to interpret. Here, we have taken the converse approach by assessing specific differences in response to BFA in cells that express a single ARF-GEF rendered BFA-resistant on the background of all other sensitive ARF-GEFs. This approach might also prove useful to investigate in vivo activities of mammalian ARF-GEFs and to identify the molecular target for the BFA effects on endosomal trafficking in mammals. The comparative study of BFA effects on cells with only one resistant versus sensitive ARF-GEF allows for a much more precise dissection of vesicle trafficking pathways than was possible before. The same would apply for the reverse experiment of rendering a BFA-resistant ARF-GEF BFA-sensitive. A systematic extension of this approach might be a means to map specificity of ARF-GEF-dependent trafficking pathways in higher eukaryotic cells.

Experimental Procedures

Plant Growth Conditions

Plants on soil or plates were grown in growth chambers under long day conditions at 21°C.

Plasmid Construction, Generation of Transgenic Plants, and Complementation Analysis

An *Avr*II site was introduced at the 3′ end of the GNOM-ORF in the GNOM cDNA Vector c96 (Busch et al., 1996) by primer-extension PCR (Ausubel et al., 2002; Sata et al., 1999). A 3× myc tag or GFP tag was synthesized by oligonucleotide hybridization (myc) or PCR from pSMGFP vector (U70495) and inserted into this site. A *Bpu*10I, *Pac*I fragment encompassing the myc-tag or GFP-tag was then transferred into the complementing 7,5 GNOM genomic fragment (*Col* ecotype) in pBluescript. The resulting pBlue-GN*Xba*I-myc was further modified by introducing a fragment containing the mutagenised Sec7-domain encoding region. Site-directed mutagenesis of the Sec7 domain was done in c96 through primer-extension PCR, introducing an ATG (M) to CTG (L) change into the GNOM ORF, and the mutated region was transferred to pBlue-GN*Xba*I-myc as an *Msc*I-*Bpu*10I fragment. All PCR-derived fragments were sequenced. The resulting *GNXbaI*^{M696L}*-myc, GNXbaI*^{wt}*-myc* and *GNXbaI-GFP* genomic fragments were then transferred as *Xba*I restricted fragments into pBAR A (AJ251013) and transformed into plants of a *gnom* heterozygous population (F2 population of *emb30-1*^{Col}/*wt*^{Ler} F1 hybrids). Complementation analysis was done by PCR screening of T1 plants using a Ler/Col polymorphism in the *GNOM* coding region (Busch et al., 1996). Mutant complementation by the constructs was then confirmed by segregation analysis of seedling phenotypes of homozygous and heterozygous *gnom* mutant lines in the F2. In order to exclude effects in transgenic plants that are not linked to the introduced GNOM locus itself, but to differences in the lines or the genetic background of the hybrids used for transformation, we always compared independent BFA-resistant transgenic lines to independent sensitive ones.

Antibody Staining and Confocal Laser Scanning Microscopy

Immunofluorescence preparations and confocal microscopy were done as described (Lauber et al., 1997). Antibodies and dilutions used were: anti-PIN1 (1:200) and anti-PIN2 (1:600), both kindly provided by K. Palme; anti-KNOLLE (1:4000), 9E10 anti-myc (1:600) (SantaCruz), anti-TLG2a (1:200) (Rosebiotech), anti-AtSec12 (1:50) (Rosebiotech), and anti-AtγCOP (1:1000), kindly provided by D.G. Robinson; and anti-PM-ATPase (1:1000), kindly provided by W. Michalke.

BFA Treatments

BFA incubation of seedlings for immunofluorescence labeling was done in cell-culture dishes containing 1 ml basal medium (BM) (0.5 MS, 1% sucrose [pH 5.8]) and the indicated concentration of BFA (BFA stock solution was 50 mM). Control treatments contained equal amounts of BFA solvent (DMSO/ethanol 1:1). BFA treatments for growth assays were done by transferring seedlings germinated on BM plates onto BFA-containing plates. BFA plates were stored at 4°C for no longer than 2–3 days before use.

Transient Transfection of Protoplasts

Transient expression of GFP-ARA7 in protoplasts of cultured cells were done as described (Ueda et al., 2001), except that suspension cultured cells were incubated in enzyme solution for 2 hr at 30°C and then passed through the nylon mesh twice (125 μm pore at first and then 40 μm pore).

Measurement of Polar Auxin Transport

Polar auxin transport was measured in inflorescence axes of transgenic *Arabidopsis* plants as described (Parry et al., 2001), with the following modifications. A concentration of 2 μM IAA were used for all incubations. BFA treatment was done by immersion for 30 min in a solution containing IAA and either 20 μM BFA or the same volume of DMSO (0.05% v/v). The specific activity of [^{3}H]IAA used was 962 TBq mole^{-1}.

Growth Measurements

Gravitropic growth was assessed by marking the gravity vector on plates. Pictures of plates were taken and angles measured from digital images with Adobe Illustrator.

Lateral root formation was assessed by inspecting the primary root under a binocular for lateral root primordia. In the case of absence of visible primordia, chloral hydrate-cleared preparations of roots were also inspected microscopically for absence of pericycle divisions, indicative of early stages of lateral root development, not visible in the binocular.

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