Ectopic Expression of an Activated RAC in *Arabidopsis* Disrupts Membrane Cycling

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Rho GTPases regulate the actin cytoskeleton, exocytosis, endocytosis, and other signaling cascades. Rhos are subdivided into four subfamilies designated Rho, Rac, Cdc42, and a plant-specific group designated RACs/Rops. This research demonstrates that ectopic expression of a constitutive active *Arabidopsis* RAC, AtRAC10, disrupts actin cytoskeleton organization and membrane cycling. We created transgenic plants expressing either wild-type or constitutive active AtRAC10 fused to the green fluorescent protein. The activated AtRAC10 induced deformation of root hairs and leaf epidermal cells and was primarily localized in Triton X-100-insoluble fractions of the plasma membrane. Actin cytoskeleton reorganization was revealed by creating double transgenic plants expressing activated AtRAC10 and the actin marker YFP-Talin. Plants were further analyzed by membrane staining with *N*-[3-triethylammoniumpropyl]-4-[*p*-diethylamino-phenyl]hexatrienyl pyridinium dibromide (FM4-64) under different treatments, including the protein trafficking inhibitor brefeldin A or the actin-depolymerizing agents latrunculin-B (Lat-B) and cytochalasin-D (CD). After drug treatments, activated AtRAC10 did not accumulate in brefeldin A compartments, but rather reduced their number and colocalized with FM4-64-labeled membranes in large intracellular vesicles. Furthermore, endocytosis was compromised in root hairs of activated AtRAC10 transgenic plants. FM4-64 was endocytosed in nontransgenic root hairs treated with the actin-stabilizing drug jasplakinolide. These findings suggest complex regulation of membrane cycling by plant RACs.

INTRODUCTION

Rho GTPases are molecular switches best known for regulating actin organization (Hall, 1998). Rhos are subdivided into four subfamilies designated Rho, Rac, Cdc42, and a plant-specific group designated RACs or Rops (Hall, 1998; Winge *et al.*, 2000; Yang, 2002). In animal cells, Rhos, Racs, and Cdc42 differentially regulate the actin cytoskeleton (Hall, 1998). Similarly, plant RACs are shown to regulate actin organization (Fu *et al.*, 2001, 2002; Molendijk *et al.*, 2001; Jones *et al.*, 2002; Yang, 2002; Chen *et al.*, 2003; Cheung *et al.*, 2003).

Rhos seem to regulate exocytosis and endocytosis events such as pinocytosis, endocytosis of clathrin-coated pits, and localization of the multiprotein vesicle-tethering complex, the exocyst (Ridley *et al.*, 1992; Lamaze *et al.*, 1996; Di Cesare *et al.*, 2000; Donaldson and Jackson, 2000; Malecz *et al.*, 2000; Guo *et al.*, 2001; Etienne-Manneville and Hall, 2002). Vesicle transport can be divided into five major steps: budding from a source membrane, targeting of the vesicle to specific regions, priming, docking at the target membrane, and fusion of the vesicles with the target membrane (Pfeffer, 1994, 2001; Jurgens and Geldner, 2002). In yeast, Cdc42 and Rho1 have been shown to regulate homotypic vesicle docking during

vacuole formation in an actin-dependent manner (Eitzen *et al.*, 2001, 2002; Muller *et al.*, 2001; Eitzen, 2003), whereas Rho3 and Cdc42 regulate vesicle docking late in exocytosis during polar growth in budding yeast independent of their role in actin polarization (Adamo *et al.*, 1999, 2001). In addition, it is well established that actin cytoskeleton function is crucial for endocytosis (Engqvist-Goldstein and Drubin, 2003).

Like other members of the Ras superfamily of small GTPases, Rhos exist in either GTP-bound active state or GDP-bound inactive state. Rhos have an intrinsic GTPase activity that is enhanced via interaction with GTPase-activating proteins. Activation of the Rhos occurs via interaction with GDP/GTP exchange factors (GEFs). Conserved dominant mutations abolishing the GTPase activity render Rhos constitutive active. Other conserved mutations preventing the GDP/GTP exchange are thought to cause irreversible interactions between the mutant Rhos and GEFs, converting the former dominant negative mutants (Hall, 1998; Winge *et al.*, 2000; Yang, 2002).

The plant-specific Rho subfamily, designated either RACs or Rops, is subdivided into two major subgroups called type-I and type-II (Winge *et al.*, 2000; Yang, 2002; Christensen *et al.*, 2003). All type-I RACs are putatively prenylated, whereas the type-II are palmitoylated but not prenylated (Lavy *et al.*, 2002). Cell shape in plants is regulated by polar growth in one direction or diffused growth in different directions, and RACs participate in the regulation of this process. Expression of constitutive active RACs induced reorganization of actin, implicated in changes in polar growth patterns, such as swelling of root hairs and pollen tubes and the misshaping of leaf epidermis cells concomi-

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It is yet unknown whether expression of activated forms of RACs in plants influence membrane trafficking. Plant RACs studied to date act at the plasma membrane and may be required to regulate membrane-trafficking processes at this site during cell growth. Continuous cycling of some membrane proteins and sterols has recently been shown crucial for proper growth and development of plants (Geldner *et al.*, 2001, 2003; Grebe *et al.*, 2003). The existence of embryonic, together with mature, fully differentiated cells in the same organism through its life cycle, make plants a unique model system to study interaction of cellular processes with growth and differentiation. In the present research, novel evidence for the involvement of ATRAC10 in membrane trafficking in *Arabidopsis* is demonstrated.

MATERIALS AND METHODS

Plasmid Construction and Plant Transformation

Wild-type Col-0 *Arabidopsis* plants were transformed using the floral dip method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* strain GV3101/pMp90. Analysis was carried out on several independent transgenic lines that displayed the same phenotypes.

Creation and Analysis of YFP-Tn GFP-Atrac10^{CA} Plants

Several independent YFP-Tn transgenic lines obtained as described above were carefully screened to verify that the YFP-Tn transgene did not induce any new phenotypes. Three independent YFP-Tn transformants were selected and introduced into the GFP-Atrac10^{CA} background by crosses. Plants that cosegregated for the GFP and YFP markers were selected for further analysis.

Plant Growth Conditions

Seeds of wild-type and transgenic *Arabidopsis* plants were grown on soil under long-day conditions (16-h light/8-h dark cycle) as described previously (Lavy *et al.*, 2002). To facilitate staining with *N*-[3-triethylammoniumpropyl]-4-[*p*-diethylaminophenyl]hexatrienyl pyridinium dibromide (FM4-64) and drug treatments, plants were grown under high-humidity conditions by maintaining the plants under transparent cover. To analyze root hairs and young leaves, seeds were germinated on 0.8% agar plates composed of 1/2× Murashige & Skoog salt mixture (Invitrogen, Carlsbad, CA). After 48 h of vernalization at 4°C, the plates were transferred to a growth chamber and grown vertically under long-day conditions (16-h light/8-h dark cycle) at 23°C for 7 d. The light intensity was 100 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Pharmacological Agents and FM4-64 Staining

For all treatments, 0.5-cm² pieces of fully expanded leaves (>3–4 cm in length), or whole seedling, were used. The plant material was submerged for

2 h at room temperature in distilled water supplemented with one of the following drugs diluted in dimethyl sulfoxide (DMSO): 90 μ M brefeldin A (BFA) (Fluka, Buchs, Switzerland), 25 μ M latrunculin B (Calbiochem, San Diego, CA), 39.4 μ M cytochalasin D (Sigma-Aldrich, St. Louis, MO), and 4 μ M FM4-64 dye (Molecular Probes). For control treatment, plant material was submerged in 0.25–0.5% DMSO solution containing 4 μ M FM4-64 dye. Depolymerization of actin filaments was examined by submerging transgenic seedlings expressing fusion protein of GFP and the actin binding domain of mouse talin (GFP-Tn) (Kost *et al.*, 1998) in 25 μ M latrunculin-B, 39.4 μ M cytochalasin-D, or 0.25% DMSO solution for 2 h at room temperature. For jasplakinolide treatments, seedlings were submerged in 1 μ M jasplakinolide solution for up to 1 h before staining with FM4-64. Control plants were submerged in 0.1% methanol. Seedlings were kept in the jasplakinolide solution throughout the experiment.

Light, Fluorescence, and Confocal Microscopy

Wide-field fluorescence imaging was performed with an Axioplan-2 Imaging fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axio-Cam, cooled charge-coupled device camera by using either 20 $\times$ dry or 63 $\times$ water immersion objectives with numerical aperture (NA) values of 0.5 and 1.2, respectively. Confocal imaging was performed using either a Zeiss R510 or Leica TCS-SL confocal laser scanning microscope with 20 $\times$ dry, 20 $\times$ multi-immersion, 40 $\times$ and 63 $\times$ water objectives with NAs of 0.5, 0.7, and 1.2, respectively.

Endocytosis and Time-Lapse Microscopy. Time-lapse experiments were performed on 7-d-old seedlings that were grown on plates as described above. To minimize sample movement and maintain focus through the experiment, tissues were embedded in 1% low melting agarose supplemented with 7 μ M FM4-64. Double stick tape was used as spacer, to avoid crashing of root hairs. Images were taken from young and growing root hairs at closest location to the root tips.

Visualization of Fluorescent Markers. GFP was visualized by excitation with an argon laser at 488 nm. Emission was detected with a 505- to 530-nm band-pass filter (Zeiss R510) or spectral detector set between 505 and 530 nm (Leica TCS-SL). FM4-64 was visualized by excitation with an argon laser set to 514 nm. Emission was detected with either a 530- to 600-nm band-pass filter (Zeiss R510) or spectral detector set between 530 and 560 nm (Leica TCS-SL). YFP was visualized by excitation with an argon laser at 514 nm. Emission was detected with spectral detector set between 525 and 570 nm (Leica TCS-SL). GFP/YFP double imaging was visualized using Leica TCS-SL confocal microscope. Excitation was with an argon laser at 488 nm. GFP emission was detected by setting the spectral detector between 500 and 525 nm. YFP emission was detected by setting the spectral detector between 530 and 570 nm. A channel dye separation process (Leica TCS) was performed to separate between the GFP and YFP signals. Alternatively, GFP and YFP signals were separated by a λ scan between 500 and 570 nm, at 5-nm increments, followed by spectral dye separation procedure (Leica TCS). Both procedures yielded similar results.

Actin Imaging. To obtain maximal signal-to-noise ratios and minimize bleaching, the argon laser was set to ~30% and the acousto optical tunable filter in all scans did not exceed 16%. All scans were carried at 512 $\times$ 512 pixel resolution with repeated scanning of two lines and two frames. Typically, 70–100 (pending on cell size) 0.4- μ m-thick sections were taken. Maximum intensity projections and nearest point three-dimensional images of multiple confocal scans were used to view actin.

Image Analysis. Image analysis was performed with Zeiss AxioVision, Zeiss confocal laser microscopy (CLSM)-5, Leica LCS, Photoshop 7.0 (Adobe Systems, Mountain View, CA) and ImageJ. Quantifications of signal intensities were performed with Zeiss CLSM-5 or Leica LCS in the following manner. In 8 bit/pixel scans, the potential (signal intensity) on the photomultiplier tubes is converted by the computer to a value on a dimensionless 0–256 scale (2^8), where 0 represents no signal and 256 maximal intensity.

Plasmolysis Experiments

To induce plasmolysis, leaves or whole seedlings were submerged in 400 mM mannitol solution for several minutes and then mounted on microscope slides in the same solution.

Cell Fractionation and Protein Identification

Proteins from leaves of *GFP-Atrac10^{CA}* and *GFP-AtAAT1* transgenic plants were extracted in 10 mM HEPES-KOH, pH 7.5, 10% glycerol, 5 mM β -mercaptoethanol, and plant protease inhibitor mix (Sigma-Aldrich) and incubated for 20 min on ice. To precipitate insoluble material, extracts were centrifuged at 14,000 $\times$ g for 15 min at 4°C. The resulting supernatants were collected and centrifuged again at 125,000 $\times$ g for 1.5 h. The insoluble pellet was incubated on ice for 20 min in 50 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 1% Triton X-100, 5 mM β -mercaptoethanol, and protease inhibitor mix. The samples

were centrifuged at 14,000 $\times$ g and for 20 min and then at 125,000 $\times$ g for 1.5 h to remove the Triton X-100-insoluble material. The Triton X-100-insoluble pellet from *GFP-Atrac10^{CA}* plants was resuspended in 50 mM HEPES-KOH, pH 7.5, 50 mM NaCl, 1% Triton X-100, 1.5% SDS, 5 mM β -mercaptoethanol, and protease inhibitor mix. The SDS soluble, Triton X-100-insoluble samples were centrifuged at 14,000 $\times$ g for 20 min, and the resulting supernatants were further centrifuged at 125,000 $\times$ g for 1.5 h. *GFP-Atrac10^{CA}* and *GFP-AtAAT1* fusion proteins were detected by immunoblots decorated with mouse α -GFP monoclonal antibodies (StressGen Biotechnologies, San Diego, CA), and goat α -mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Blotting grade; Bio-Rad, Hercules, CA). Immunoblots were developed with EZ-ECL detection kit (Biological Industries, Bet Haemek, Israel)

Membrane Flotation Centrifugation

The experiments were performed as described previously (Ono and Freed, 1999) with the following modifications. Tissues were frozen with liquid N₂ and ground to powder by using pestle and mortar. Protein were extracted by adding 1 ml of extraction buffer (50 mM HEPES-KOH, pH 7.5, 10 mM KCl, 5 mM EDTA, 5 mM EGTA, 10% sucrose, 1 mg/ml phenylmethylsulfonyl fluoride, and protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN) per 100 mg of tissue and incubated for 20 min on ice. The extract was filtered through 80- μ m nylon mesh and precipitated at 3000 $\times$ g for 10 min. The pellet was discarded and the supernatant was collected for further analysis. Next, 250 μ l of the supernatant was mixed with 1.25 ml of 85% sucrose in TE buffer (10 mM Tris, pH 8.0, and 1 mM EDTA, pH 8.0). This mixture was placed at the bottom of a 13-ml 10, 65, and 75% sucrose density step gradient as described previously (Ono and Freed, 1999). Centrifugal separation was performed with SW41 swinging bucket rotor (Beckman Coulter, Fullerton, CA) at 100,000 $\times$ g for 18 h. At the end of centrifugation, 1.25-ml samples were collected from the top of the gradient. To precipitate proteins, 0.5-ml samples were mixed with 125 μ l of 50% trichloroacetic acid and 1 μ g of bovine serum albumin and then incubated at –20°C for 10 min. Proteins were precipitated by centrifugation at 10,000 $\times$ g for 20 min and in turn the precipitated protein pellets were dried in SpeedVac. Dried protein pellets were dissolved in SDS-PAGE sample buffer and resolved on gels SDS-gels as described above. Protein immunoblots were performed as described above. All centrifugation steps were carried out at 4°C.

Tissue Sectioning and Staining

Tissues were fixed with formaldehyde acetic acid alcohol, dehydrated in graded ethanol series, and embedded in paraffin blocks by using standard procedures (Ruzin, 1999). Embedded tissues were sectioned with a rotary microtome (Shandon Scientific, Cheshire, England). To view cells, tissues were stained with safranin/fast-green according to standard procedures (Ruzin, 1999).

RESULTS

Activated AtRAC10 Induced Changes in Organ and Cell Morphology

Ectopic expression of constitutive active *GFP-AtRAC10* fusion protein (*GFP-Atrac10^{CA}*) induced morphological changes in leaf shape (Figure 1). Leaves of *Atrac10^{CA}* plants were narrower than wild-type (WT) control and folded downward (Figure 1, B and C). The structure of leaves expressing a WT *GFP-AtRAC10* was similar to that of WT untransformed plants (our unpublished data).

Next, we examined whether the changes in leaf structure reflected altered cell morphogenesis. Cells of *GFP-Atrac10^{CA}* transgenic plants had abnormal structures (Figure 2). Epidermal cells lost their lobing (Figure 2F). The palisade mesophyll cells became isomorphic rather than elongated along the adaxial-abaxial axis, and air spaces were smaller in the spongy mesophyll (Figure 2B). These changes made it difficult to discriminate between the palisade and spongy mesophyll in cross sections (Figure 2, A and B). Epidermal cells from plants expressing a wild-type *GFP-AtRAC10* (*GFP-AtRAC10*), a dominant negative *GFP-AtRAC10* (*GFP-AtRac10^{DN}*), or nonexpressing cells from *GFP-Atrac10^{CA}* partially silenced plants, in which only a subset of the cells expressed the recombinant protein, were similar to cells of wild-type plants (Figure 2, E and G–L). The analysis of the partially silenced plants also showed that the loss of lobing in the epidermal cells was directly correlated to the level of *GFP-Atrac10^{CA}* fluorescence in the cells (Figure 2, K and L). The

Figure 1. Altered morphogenesis of GFP-Atrac10^{CA}-expressing plants. Images of 25-d-old WT (A) and GFP-Atrac10^{CA} transgenic plants (B). The GFP-Atrac10^{CA} leaves were narrower, folded downward at their distal end, and had longer and twisted petioles (B). (C) Abaxial surface of WT and GFP-Atrac10^{CA} leaves.

data presented in Figure 2 indicated that the changes in cell morphology were induced by the expression of the constitutive active form of AtRAC10. To further study the function of AtRAC10, its subcellular localization was investigated.

Plasma Membrane Localization and Function of AtRAC10

Previously, we have shown that AtRAC10 is attached to the plasma membrane by virtue of its palmitoylation on two C-terminal cysteines (Lavy *et al.*, 2002). When palmitoylation was inhibited, AtRAC10 accumulated in the nucleus due to a polybasic domain located proximal to its C-terminal end. Unlike other type-II RACs, a fraction of AtRAC10 was often located in the nucleus (Lavy *et al.*, 2002).

When plant tissues are incubated in high-concentration osmoticum solutions, such as 30% glycerol or mannitol, water exits cells due to differences in water potential. In this process, designated plasmolysis, the plasma membrane is detached from the cell wall and the cytoplasm and vacuole shrink, but cell walls stay intact, maintaining overall cell structure. After plasmolysis, fluorescently labeled plasma membrane is detected as thin lines that are detached from the cell wall and the membrane becomes visible using Nomarsky differential interference contrast (DIC) (Figure 3K). After plasmolysis of either leaf epidermal cells or swollen root hairs, GFP-Atrac10^{CA} remained attached to the plasma membrane, and no perinuclear and transvacuolar cytoplasmic strands that would have indicated vacuolar membrane localization were detected (Figure 3, C, D, G, and H). For comparison, after expression of free GFP, the protein was detected as a thick, patchy line circumventing the cell, in cytoplasmic strands and nuclei (Figure S1, A–C). After plasmolysis, fluorescence accumulated in larger patches (Figure S1, D–F). This contrasts the membrane-bound GFP-Atrac10^{CA}, which showed as a sharp fluorescing line before and after plasmolysis. Occasionally, elongated root hairs expressing GFP-Atrac10^{CA} had swellings that were associated with accumulation of GFP-Atrac10^{CA} protein (Figure 3, I and J).

To substantiate GFP-Atrac10^{CA} localization at the plasma membrane and obtain further insight into its possible functions, total protein extracts were analyzed by membrane flotation centrifugation assays (Figure 4A). In these assays, the protein extract is placed at the bottom of a sucrose density gradient. After centrifugal separation, membranes float and the soluble fraction remains at the bottom of the gradient (Ono and Freed, 1999). In membrane flotation centrifugation assays, GFP-Atrac10^{CA} was detected in the third and fourth fractions from the top of the gradient (Figure 4A). This localization in the gradient, which corresponds to the border between 10 and 65% sucrose, was expected for membrane-attached proteins (Ono and Freed, 1999). The soluble protein fraction, which in plants is characterized by a major 50-kDa band of the large subunit of ribulose biphosphate carboxylase oxygenase enzyme complex, remained at the bottom of the gradient (our unpublished data). Further experiments were carried out to verify whether AtRAC10 could be extracted out of the membranes by Triton X-100.

Protein extracts from GFP-Atrac10^{CA} plants were fractionated into soluble and insoluble fractions. The insoluble fraction was fractionated into Triton X-100-soluble and -insoluble fractions. In turn, the Triton X-100-insoluble fraction was solubilized with SDS. The results (Figure 4B) show that the majority of the GFP-Atrac10^{CA} protein existed in the insoluble fraction with most protein in the Triton X-100-insoluble fraction. Control experiments with a known membrane protein were used to verify that the inability to extract GFP-Atrac10^{CA} by Triton X-100 did not result from the experimental system. AtAAT1 is a highly hydrophobic plasma membrane-localized amino acid transporter containing 14 putative transmembrane domains (Frommer *et al.*, 1995). Protein extracts prepared from AtAAT1 transgenic plants were fractionated into soluble and insoluble fractions. In turn, the insoluble fraction was solubilized with Triton X-100. Although no GFP-AtAAT1 protein was detected in the soluble fraction, the fusion protein was extracted from the membrane by Triton X-100 (Figure 4C). The same expression system and buffers were used to express and extract GFP-Atrac10^{CA} and GFP-AtAAT1, indicating that the inability to solubilize GFP-Atrac10^{CA} with TritonX-100 was not a result from the experimental conditions. The partitioning of GFP-AtRAC10^{CA} between Triton X-100-soluble and -insoluble membranes suggested that this protein might be fractionated into specific membrane microdomains (Simons and Toomre, 2000). More experiments were carried out to study the function of AtRAC10 at the plasma membrane.

AtRAC10 and Actin Organization

Changes in plant cell morphology induced by activated RACs were previously associated with actin reorganization (Fu *et al.*, 2001, 2002; Molendijk *et al.*, 2001; Jones *et al.*, 2002; Yang, 2002; Chen *et al.*, 2003; Cheung *et al.*, 2003). To determine whether expression of GFP-Atrac10^{CA} induced reorganization in actin cytoskeleton, transgenic *Arabidopsis* lines expressing the F-actin binding domain of mouse Tn (Kost *et al.*, 1998) fused to YFP (YFP-Tn) were created. The YFP-Tn transgenic lines were carefully selected to ensure that the expression of the transgene did not induce new phenotypes.

Three independent YFP-Tn lines were crossed into GFP-Atrac10^{CA} background to obtain double transgenic plants. In root hairs of Col-0 YFP-Tn plants, the actin bundles were organized parallel to long axis of the cell (Figure 5A). Fine mesh but no actin bundles were observed under the tip of growing root hairs. More actin bundles were observed in swollen root hairs of GFP-Atrac10^{CA} YFP-Tn plants. These actin filaments were organized at different angles, often

Figure 2. GFP-Atrac10^{CA}-induced cell deformation. (A and B) Safranin/fast-green stained cross sections of rosette leaves of WT (A) and GFP-Atrac10^{CA} (B) plants. Note the lack of clear distinction in the mesophyll between palisade and spongy tissues. (C) DIC image of a root of WT plant with elongated root hairs. (D) DIC image of a root of GFP-Atrac10^{CA} plant with swollen root hairs. (E and F) DIC images of cleared leaf epidermal cells of WT (E) and GFP-Atrac10^{CA} (F) plants. Note the reduced lobing of the mutant cells. (G and H) Epidermal cells of GFP-Atrac10^{DN} plant expressing

arranging as a band oriented at a right angle to the original elongation axis of the root hair (Figure 5, B and C). The arrangement of the actin bundles, circumventing the swollen root hair, became clear by a 120° angle tilt of a three-dimensional projection of the image (Figure 5C).

To view both YFP-Tn and GFP-Atrac10^{CA} markers, samples were excited with argon laser at 488 nm, and the GFP and YFP signals were separated using a channel dye separation algorithm (Leica TCS) (Figure 5D). Although some of the YFP signal was lost during this procedure, the GFP signal (green label) was detected at the cell surface and the YFP signal (red label) was detected mainly in the cortical region. Similar results were obtained when the GFP and YFP signals were resolved using a spectral dye separation algorithm (Leica TCS), after a λ -scan with the spectral detector of Leica TCS-SL confocal microscope (our unpublished data).

In yeast and animal systems, Rho GTPases have been reported to affect endocytosis and exocytosis by actin-dependent and -independent mechanisms. Plant RACs have recently been suggested to play critical roles in regulation of vesicle trafficking, but the mechanisms of their action are still unknown (Molendijk *et al.*, 2004). Moreover, the effects of plant RACs on membrane dynamics have not been addressed.

Vesicle Cycling and GFP-Atrac10^{CA} Function

As in axonal growth, root hair elongation requires the polar movement of vesicles that add the necessary substances and plasma membrane components by fusing at the cell's growing tip. RACs regulate membrane vesicle movement in two major ways: first, vesicle movement depends on F-actin, the dynamic of which is regulated by RACs. Second, RACs may regulate vesicle movement by using mechanisms not directly related with the cytoskeleton. To examine whether RACs influence vesicular transport, membranes were visualized with the lipophilic dye FM4-64, a common probe used for detection of membrane endocytosis (Parton *et al.*, 2001; Santos *et al.*, 2001; Heese-Peck *et al.*, 2002; Jochum *et al.*, 2002).

When WT seedlings were submerged in FM4-64 solution, the dye was internalized immediately (Figure 6, A and B). Movies prepared from a time-lapse series of the same cell, consisting of 60 scans taken every 10 s for 10 min, depicted the cytoplasmic streaming and the internalization of FM4-64 into the root hair (Movies 1-1 and 1-2).

The majority (80–90%) of root hairs of the WT RAC GFP-Atrac10 transgenic plants were similar to root hairs of untransformed plants (Figure 6, C–E). In these root hairs, FM4-64 was internalized like in untransformed plants (Figure 6, D and E). Movies prepared from time-lapse series of similar root hairs showed the cytoplasmic streaming and endocytosis of FM4-64 (Movies 2-1 and 2-2). Approximately

a dominant negative AtRAC10. DIC image (G) and GFP fluorescent image (H). The GFP-Atrac10^{DN} leaf epidermal cells are similar to those of wild-type cells. (I and J) Leaf epidermal cells of plant expressing a wild-type GFP-AtRAC10. DIC image (I) and GFP fluorescent image (J). Lobing of the GFP-AtRAC10 epidermal cells is similar to that of wild-type. (K and L) Leaf epidermal cells of GFP-Atrac10^{CA} partially silenced plant. The cell morphology is directly related to the level of expression of GFP-Atrac10^{CA} (arrow). DIC image (K) and DIC/GFP overlay (L). (A–F) Images were prepared with a microscope equipped with cooled charged device (CCD) camera. (G–L) Images were prepared with confocal laser scanning microscope (CLSM). Green indicates GFP fluorescence. Bars, 20 μ m.

Figure 3. Membrane localization of GFP-Atrac10^{CA} in leaf epidermis and root hairs of GFP-Atrac10^{CA} transgenic plants. The subcellular localization of GFP-Atrac10^{CA} was examined in leaf epidermis and root hairs of GFP-Atrac10^{CA} seedlings. (A–D) Leaf epidermal cells were visualized by Nomarsky DIC (A) or GFP fluorescence (B and C) or DIC/GFP overlay (D). Plasmolysis of the cells indicated that the GFP-Atrac10^{CA} protein was localized at the plasma membrane (C and D). (E–H and K) Root hair cells of GFP-Atrac10^{CA} seedlings. Cells were visualized by DIC (E and K), GFP (F and G), or DIC/GFP overlay (H). Plasmolysis of the cells indicated that in swollen root hairs, GFP-Atrac10^{CA} was localized at the plasma membrane (G, H, and K). Arrow in G denotes contact points between plasma membrane and cell wall detected after plasmolysis. Arrow in K denoted the detached plasma membrane. Local swelling on elongated GFP-Atrac10^{CA} root hairs of was associated with accumulation of GFP-Atrac10^{CA} (arrow) (I and J). The root hairs were visualized by Nomarsky DIC (I) and GFP fluorescence (J) on an epifluorescence microscope. Bars, 20 μ m.

10–20% of root hairs of GFP-AtRAC10 plants were partially swollen (Figure 6, F–H). In these root hairs, internalization of FM4-64 was barely detectable, and most of the dye stayed on the membrane (Figure 6, G and H). Movies prepared from a time-lapse series of 60 scans taken every 10 s for 10 min depicted the cytoplasmic streaming and steady but slow internalization of FM4-64 into the root hair (Movies 3-1 and 3-2).

FM4-64 was not internalized in swollen root hairs expressing GFP-Atrac10^{CA}, remaining instead on the membrane (Figure 6, I–K). Most of the dye remained in the plasma membrane even after 90-min incubation at room temperature (Figure 6, L–N). Movies prepared from a time-lapse series of 60 scans taken every 10 s for 10 min showed that although cytoplasmic streaming continued, only very little FM4-64 was internalized, and most of the dye remained on the plasma membrane (Movies 4-1 and 4-2). This result suggested that the inhibition of FM4-64 internalization was not associated with cessation of cytoplasmic streaming.

To illustrate the distribution of GFP and FM4-64 markers in the WT Col-0 and transgenic plants, signal intensities were quantified (Figure 7). The plots represent distribution

of signal intensity per micrometer in GFP and FM4-64 channels in the line defined in the inset of each graph. The insets in A, B, C, and D correspond to Figure 6, B, E, H, and N, respectively. The plots provide quantitative illustration to internalization of FM4-64 and colocalization of the two signals. Collectively, these data suggested a correlation between the localization of activated RAC at the plasma membrane, root hair swelling, and inhibition of vesicle internalization.

Recent results indicate that actin turnover is required for root hair and plant cell growth (Ketelaar *et al.*, 2003, 2004). Atrac10^{CA} induced actin reorganization (Figure 5), and RACs have been shown to inhibit ADF/cofilin that is required for actin turnover (Chen *et al.*, 2002; Chen *et al.*, 2003). To examine whether actin stabilization inhibited endocytosis of FM4-64, seedling were treated with the actin-stabilizing drug jasplakinolide (Figure 8). Jasplakinolide stabilizes actin by preventing filament disassembly both in vitro (Bubb *et al.*, 1994) and in vivo (McGrath *et al.*, 1998; Cramer, 1999) and induces actin polymerization in a concentration-dependent manner (Bubb *et al.*, 1994; Lee *et al.*, 1998; Shurety *et al.*, 1998) by increasing actin nucleation (Bubb *et al.*, 2000). Ad-

Figure 4. Partitioning of GFP-Atrac10^{CA} in the membrane. Immunoblots of protein extracts prepared from GFP-Atrac10^{CA} transgenic plants (A and B). (A) Membrane flotation experiment. Total protein extracts were adjusted to 73% sucrose solution and placed at the bottom of a 10–73% sucrose density gradient. After centrifugal separation, typical of membrane bound proteins, GFP-Atrac10^{CA} accumulated at fractions 3 and 4, which form the boundary between the 10 and 65% sucrose phases. The numbers in A correspond to gradient fraction from top to bottom. (B) After fractionation into soluble and insoluble fractions, the insoluble membrane fractions were fractionated into Triton X-100 (Triton)-soluble and -insoluble fraction. Triton X-100-insoluble material was solubilized with SDS. The majority of GFP-Atrac10^{CA} precipitated in the insoluble fraction and partitioned into the Triton X-100-insoluble, SDS-soluble fraction. The apparent molecular mass of the GFP-Atrac10^{CA} fusion protein is ~50 kDa (50). (C) Immunoblots of protein extracts prepared from GFP-AtAAT1 transgenic plants. AtAAT1 is a highly hydrophobic membrane localized amino acid transporter (Frommer *et al.*, 1995). In contrast to GFP-AtRAC10^{CA}, GFP-AtAAT1 was solubilized from the membrane with Triton X-100. The apparent molecular mass of the GFP-AtAAT1 fusion protein is ~80 kDa.

ditionally, jasplakinolide has been shown to be highly cell permeate in both animal (Cramer, 1999) and plant cells (Samaj *et al.*, 2002). Aggregation and increase in thickness of actin bundles were detected in jasplakinolide-treated YFT-Tn root hairs (Figure 8A), whereas no effect was detected in control plants incubated in 0.1% methanol. FM4-64 was internalized into jasplakinolide-treated root hairs and accumulated in intracellular bodies (Figure 8C), reflecting the perturbation to the actin cytoskeleton. Thus, unlike the effects of Atrac10^{CA}, which inhibited internalization of FM4-64, jasplakinolide interfered with intracellular vesicle cycling but not with FM4-64 internalization from the plasma membrane.

Next we sought to determine whether Atrac10^{CA} activity altered vesicle cycling directly rather than indirectly, by processes such as growth inhibition. To this end, cells of wild-type and Atrac10^{CA} plants were visualized after disruption of the balance between endocytosis and exocytosis.

Inhibition of Exocytosis and AtRAC10-induced Vesicle Fusion

Unless exocytosis is inhibited, vesicle movement between the plasma membrane and endosomes is often invisible due to its speed. To inhibit exocytosis and render movement visible, cells were treated BFA. BFA, a specific inhibitor of ADP ribosylation factor (ARF) GEF, arrests vesicle trafficking at various points along the secretory pathway (Peyroche *et al.*, 1999; Nebenfuhr *et al.*, 2002; Ritzenthaler *et al.*, 2002; Geldner *et al.*, 2003). BFA treatments induce intracellular accumulation of different plasma membrane proteins, sterols, and of cell wall components (Geldner *et al.*, 2001, 2003; Baluska *et al.*, 2002; Friml *et al.*, 2002; Grebe *et al.*, 2002, 2003).

Figure 5. Actin organization in GFP-Atrac10^{CA} root hairs. (A) Longitudinal-oriented actin bundles and perinuclear mesh were detected in a developing root hair of YFP-Tn-expressing Col-0 (WT) plant. (B) Transverse-oriented actin bundles in YFP-Tn GFP-Atrac10^{CA} double transgenic plants. (C) The same image as in B after three-dimensional (3D) projection and 120° tilting to a top to bottom view. Transverse bundles circumventing the cell were seen. (D) A YFP-Tn (labeled red) GFP-Atrac10^{CA} (labeled green) overlay image. The image was produced by a channel dye separation after excitation with argon laser at 488 nm. (A–C) Images were produced after excitation with argon laser at 514 nm to minimize GFP fluorescence. (A, B, and D) Maximum projection images of multiple confocal scans. Bars, 10 μ m.

On treatment of wild-type roots with BFA, FM4-64-stained membranes accumulated in intracellular bodies, known as BFA bodies/compartments (Nebenfuhr *et al.*, 2002; Ritzenthaler *et al.*, 2002; Geldner *et al.*, 2003; Grebe *et al.*, 2003). Similarly, under the experimental condition used in this study, FM4-64-labeled membranes accumulated in BFA compartments after treatments of roots from wild-type seedlings with BFA (Figure 9A). Similar numbers of BFA bodies formed in root cells of BFA-treated GFP-AtRAC10 plants (Figure 9, C, E, and F). Much fewer BFA bodies were visible in BFA-treated GFP-Atrac10^{CA} roots (Figure 9, C, H, and I). Moreover, the BFA bodies were only stained by FM4-64 and not with GFP (Figure 9, G and L). Quantitative analysis of BFA body numbers in 10 WT and GFP-AtRAC10 and eight GFP-Atrac10^{CA} seedlings confirmed that their number was significantly lower ($p \leq 0.001$) in the GFP-Atrac10^{CA} roots (Figure 9C). Quantification of signal intensities/pixel of the BFA bodies indicated that almost all the fluorescence came from the FM4-64 but not the GFP (Figure 9L). The maximum intensity projection stack image (Figure 9J) revealed that the labeling of BFA bodies with FM4-64 and not with GFP did not result from imaging of certain focal planes.

There were several interpretations to the results. GFP-Atrac10^{CA} might have resided in membrane compartments that did not accumulate in BFA bodies. Alternatively, the ectopic expression of the activated RAC-disrupted vesicle accumulation in BFA bodies. The latter explanation was supported by data showing that BFA causes intracellular accumulation of different proteins and membrane components (Geldner *et al.*, 2001, 2003; Friml *et al.*, 2002; Grebe *et al.*, 2002, 2003). More experiments were carried out to clarify this point.

When root hairs were examined a slightly different picture emerged (Figure 10). In wild-type root hairs, FM4-64 stain-

Figure 6. Membrane internalization in wild-type, *GFP-AtRAC10*, and *GFP-Atrac10^{CA}* root hairs. Immediate internalization of membranes was detected in root hairs labeled with the membrane and endocytosis marker FM4-64 (A and B). (A) Entire root hair. (B) Magnification of the same root hair as in A. (C–E) Wild-type-like root hair of *GFP-AtRAC10* plant. Most of the FM4-64 was internalized within minutes of its application and was not colocalized with GFP-AtRAC10. (F–H) Most of the FM4-64 was not internalized in partially swollen root hairs of *GFP-AtRAC10* plants remaining on the plasma membrane colocalized with GFP-AtRAC10 (H, yellow label). Some of the GFP-AtRAC10^{CA} protein accumulated in the nucleus (F and H). (I–N) FM4-64 was not internalized into balloon-shaped swollen root hairs of *GFP-Atrac10^{CA}* plants. Images were taken either 3 (I–K) or 90 min after dye application (L–N). Green indicates GFP, red indicates FM4-64, and yellow indicates GFP/FM4-64 overlay. (A, B, D, G, J, and M) FM4-64 channel. (C, F, I, and L) GFP channel. (E, H, K, and N) GFP/FM4-64 overlay. Bars, 20 μ m (A), 5 μ m (B), and 10 μ m (C–N). Movies depicting endocytosis of FM4-64 in the same or similar root hairs are shown in the supplemental online material.

ing disappeared from the plasma membrane, and many intracellular bodies were visible after BFA application (Figure 10A). A similar picture emerged after BFA treatments of plants expressing the WT *GFP-AtRAC10* where intracellular bodies stained with both FM4-64 and GFP occurred (Figure 10, C–E). In the *GFP-Atrac10^{CA}* root hairs, FM4-64-stained bodies were visible, but in addition large vesicles measuring up to 20 μ m in diameter, emanating from the plasma membrane, formed (Figure 10, G–I). In contrast to the BFA bodies in which only FM4-64 fluorescence was visible, the large vesicles were positive for FM4-64 and GFP signals. These, unique large vesicles, suggested that *Atrac10^{CA}* disrupted vesicle cycling and inhibited the formation of BFA bodies.

As shown in Figure 2, like root hairs, leaf epidermal cells of *GFP-Atrac10^{CA}* plants had distinct morphology. It was interesting to examine whether the expression of activated the RAC affected membrane dynamics in a similar manner in both root hairs and leaf epidermal cells (Figure 11).

On treatment of wild-type leaves with BFA, FM4-64 accumulated inside the cells in two ways: either cone-shaped structures formed in cellular growth lobes (Figure 11, A and B) or

distinct BFA bodies were detected (Figure 11C). Inward movement of FM4-64-labeled membranes in the cellular growth lobes was revealed by time-lapse microscopy (Movie 5). Similar results were obtained after BFA treatments of leaves taken from transgenic plants expressing the WT *GFP-AtRAC10* (our unpublished data). In some *GFP-Atrac10^{CA}* cells, BFA induced formation of BFA bodies. Similar to roots, these BFA bodies mostly contained FM4-64 and no GFP signal, indicating that *GFP-Atrac10^{CA}*-containing membranes did not cycle into the BFA bodies (Figure 11, E–G). In other *GFP-Atrac10^{CA}* epidermal cells, large vesicles emanated from the plasma membrane, measuring up to 40 μ m in diameter (Figure 11, I–K, and the lower cell E–G). Similar to the root hair cells, these large vesicles were stained by both FM4-64 and GFP, implying colocalization of the two dyes (Figure 11K, yellow label). Time-lapse microscopy revealed small vesicles budding from the plasma membrane and fusing into larger vesicles and fission of large vesicles from the membrane (Movie 6). The results from BFA treatments of either root hairs or leaf epidermal cells suggested that the activated RAC disrupted vesicle cycling, inhibiting

Figure 7. Quantification signal intensities at defined cellular locations. Signal intensities in the GFP and FM4-64 channels along the marked lines in the insets of each panel. Insets in A–D correspond to Figure 6, B, E, H, and N, respectively. The drop in signal intensities in the FM4-64 channel highlights its distribution in the cell. The computer generated scale corresponds to $2^8 = 256$, in an 8 bit/pixel scan mode.

their accumulation in BFA bodies, and instead promoting formation of large membrane vesicles.

Depolymerization of Actin and Changes in Membrane Dynamics

It has been shown that vesicle recycling between plasma membrane and endosomes could be inhibited by the actin-

depolymerizing agents CD and Lat-B (Geldner *et al.*, 2001; Grebe *et al.*, 2003). It was therefore interesting to examine whether the effect of actin-depolymerizing agents would be similar to that of BFA and would provide an additional control for the BFA experiments.

To verify the effectiveness of Lat-B and CD in our system, transgenic plants expressing the actin marker GFP-Tn were treated with either drug. Treatment with either Lat-B or CD induced complete depolymerization of both actin bundles and fine meshwork (Figure S2).

Leaves taken from wild-type and *GFP-Atrac10^{CA}* transgenic plants were treated with Lat-B or CD (Figure 12). In wild-type leaves, either Lat-B or CD treatment induced intracellular accumulation of FM4-64, which was confined to growth lobes (Figure 12, A and E), as in the case of BFA treatments (Figure 11, A and B). In *GFP-Atrac10^{CA}* transgenic leaves, Lat-B treatment induced the formation of vesicles, emanating from the plasma membrane, reaching up to 40 μm in diameter (Figure 12, B–D). Similar effects were obtained when *GFP-Atrac10^{CA}* leaves were treated with CD (Figure 12, F–H).

The results in Figures 11 and 12 provide evidence that disruption of membrane cycling by *Atrac10^{CA}* could be revealed by both BFA treatment, which inhibits the exocytic pathway, and by the disassembly of actin, which may block vesicular movement.

DISCUSSION

In this study, transgenic plants expressing an activated *GFP-Atrac10^{CA}* as well as wild-type (*GFP-Atrac10*) and inhibitory (*GFP-Atrac10^{DN}*) fusion proteins were analyzed. The activated RAC protein induced deformation of organs, loss of lobing of leaf epidermal cells, and isotropic growth of the mesophyll cells. The effects of constitutively active *Atrac10* on the morphology of leaf epidermis and mesophyll cells, its effect on root hair cells (Figure 2), and the reduced phenotype of partially silenced plants (Figure 2, K and L) imply close association between expression of the activated RAC and changes in cell morphology. Reorganization of actin in the *Atrac10^{CA}*-expressing plants were visualized with

Figure 8. YFP-Tn and FM4-64 stained jasplakinolide-treated root hairs. Jasplakinolide treated (1 μM) (A and C) and control (0.1% methanol) (B and D) root hairs. The jasplakinolide-induced aggregation of actin bundles (A) but did not inhibit internalization of FM4-64 (C). YFP-Tn is labeled in white and FM4-64 in red. Bars, 10 μm (A and B) and 5 μm (C and D).

Figure 9. Effects of BFA on wild-type, *GFP-AtRAC10*, and *GFP-Atrac10^{CA}* root cells. (A and B) FM4-64 fluorescent images of WT roots treated with BFA (A) and DMSO (B). BFA bodies were detected after the BFA treatments. (D–F) Roots of *GFP-AtRAC10* plant treated with BFA. BFA bodies were detected in most of the cells (E). (G–I) Roots of *GFP-Atrac10^{CA}* plants were treated with BFA. The number of cells with BFA bodies was reduced (I). (C) Percentage of cells with BFA bodies was significantly reduced in *GFP-Atrac10^{CA}* plants ($p \leq 0.001$, t test). One hundred cells were scored in each sample. Counts were repeated with 10 seedlings of WT and *GFP-AtRAC10* lines and eight seedlings of *GFP-Atrac10^{CA}* lines, without significant deviation in the results. Error bars represent SE. (J and K) Enlargement of the region specified by the rectangle in I. (J) Maximum intensity projection stack to verify that labeling of BFA bodies was not dependent on specific focal planes. (L) Quantification of signal intensity per pixel (see Figure 7) in the FM4-64 and GFP channels of a representative BFA body specified by circle on K. The distribution of points on the graph indicated the almost complete absence of GFP signal. Cells were visualized with CLSM. Green indicates GFP, red indicates FM4-64, and yellow indicates colocalization of GFP and FM4-64 in overlay images. Bars, 20 μ m.

YFP-Tn actin marker. Changes of membrane dynamics in *GFP-Atrac10^{CA}* plants were visualized in experiments with the membrane and endocytosis probe FM4-64, the exocytosis inhibitor BFA or the actin-depolymerizing agents Lat-B and CD. We demonstrated that activated AtRAC10 disrupted membrane cycling.

AtRAC10 and Membrane Cycling

BFA induces intracellular accumulation of different membrane proteins, such as PIN1 and PIN3 putative auxin transporters, plasma membrane H^+ -ATPase, the auxin influx carrier AUX1, and the cytokinesis-specific syntaxin KNOLLE, suggesting that

Figure 10. Effects of BFA on wild-type Col-0, *GFP-AtRAC10* and *GFP-Atrac10^{CA}* root hair membranes. FM4-64 fluorescent images of root hairs of wild-type plants treated with BFA (A) or DMSO (B). (C–F) Root hairs of *GFP-AtRAC10* plants treated with BFA (C–E) or DMSO (F). (G–J) Root hairs of *GFP-Atrac10^{CA}* plants treated with BFA (G–I) or DMSO (J). BFA treatments induced formation of vesicles measuring up to 20 μm (I, arrow) labeled with both GFP and FM4-64. Cells were visualized with CLSM. Green indicates GFP, red indicates FM4-64, and yellow indicates colocalization of GFP and FM4-64 in overlay images. Bars, 10 μm .

protein accumulation induced by the drug is nonspecific (Geldner *et al.*, 2001, 2003; Friml *et al.*, 2002; Grebe *et al.*, 2002). Polar auxin transport has been shown to depend on continuous cycling of PIN1 between the PM and endosomes requiring the activity of the ARF-GEF GNOM (Geldner *et al.*, 2001, 2003). Either BFA or the actin-destabilizing agents CD or Lat-B arrested cycling of PIN1, inhibiting polar auxin transport. The absence of *GFP-Atrac10^{CA}* from BFA bodies (Figures 9 and 11), the reduction in numbers of BFA bodies (Figure 9), and the colocalization of *GFP-Atrac10^{CA}* with FM4-64-labeled membrane in plasma membrane emanating, large vesicles after treatments with either BFA, Lat-B, or CD (Figures 10–12) indicated that the activated RAC disrupted cycling of membrane vesicles.

Comparison of root hair structure and vesicle internalization between *GFP-AtRAC10* and the activated *GFP-Atrac10^{CA}* plants suggested a correlation between RAC activation, root hair swelling, and inhibition of endocytosis. Some *GFP-AtRAC10* root hairs were more swollen than in WT Col-0 plants but not as swollen as in *GFP-Atrac10^{CA}* plants. Concomitantly, vesicle internalization in partially swollen *GFP-AtRAC10* root hairs was reduced compared with WT cell but more pronounced compared with *GFP-Atrac10^{CA}* cells (Figures 6 and 7 and Movies 1–1–4–2).

Ectopic expression of different RACs has been shown to alter auxin-induced gene expression. It has therefore been proposed that RACs regulate auxin response (Tao *et al.*, 2002). Careful

evaluation should be carried out to distinguish between the effects of activated RACs on auxin-dependent gene expression and transport and internalization of auxin.

BFA treatments of *Atrac10^{CA}* plants had different effects in roots, root hairs, and leaf epidermal cells. In young roots, only BFA bodies were detected, whereas in both root hairs and leaf epidermal cells large vesicles formed (Figures 9–12). It is possible that the penetration of BFA into root hairs and leaf epidermal cells was greater than into other root tissues. Root hairs are adapted to absorbed materials from the environment. Leaves used in this study were taken from plants that were grown under 100% humidity, either eliminating or substantially reducing the amount of the waxy cuticle on their surface and thereby facilitating uptake of hydrophobic compounds such as BFA. Furthermore, penetration of BFA through the stomata pores could have enhanced its uptake into leaves. Thus, the actual concentrations of BFA to which different cells were exposed might have been variable, causing phenotypic differences. Alternatively, there could be inherent differences between different cell types and tissues, causing specific phenotypes.

Membrane Localization of *AtRAC10*

The partitioning of *AtRAC10* into Triton X-100-insoluble membrane may indicate its existence in sterol-rich membrane domains. Sterol trafficking in plants was shown to be

Figure 11. Effects of BFA on wild-type and *GFP-Atrac10^{CA}* leaf epidermal cells. FM4-64 fluorescence images of WT leaf epidermal cells treated with BFA (A–C) or DMSO (D). Endocytotic vesicles forming funnel-like structures are seen in growth lobes (rectangle) of BFA-treated cells. (B) Enlargement of the rectangle specified area in A. (C) BFA-bodies (arrows). (E–K) *GFP-Atrac10^{CA}* leaf epidermal cells treated with BFA (E–G and I–K) or DMSO (H). The BFA treatments induced formation of BFA bodies labeled only with FM4-64 (E and G, arrow) and of vesicles reaching diameters of $\sim 40\ \mu\text{m}$, displaying colocalization of FM4-64 and *GFP-Atrac10^{CA}* (E–G [arrowheads] and I–K [arrow]). Cells were visualized with CLSM. Green indicates GFP (F and J), red indicates FM4-64 (A–D, E, and I), and yellow indicates colocalization of GFP and FM4-64 in overlay images (G, H, and K). Bars, $20\ \mu\text{m}$.

actin dependent and BFA sensitive, and the PIN2 putative auxin transporter was colocalized with sterols after BFA treatments (Grebe *et al.*, 2003). Consistently, PIN1 localization was altered in sterol methyltransferase1 mutants (Willemssen *et al.*, 2003), and several sterol biosynthetic *Arabidopsis* mutants were shown to be disturbed in cell and tissue polarity (Betts and Moore, 2003). The ectopic expression of mutant forms of AtRAC10 in *Arabidopsis* affected plant morphology, leaf mesophyll cell shape (Figures 1 and 2), vascular differentiation, and auxin response (our unpublished data). Future analysis of the function of RACs in endocytic and exocytic events, their localization to specific membrane compartments, and their effect on membrane docking and fusion would be crucial for understanding the diversity of the function of the RACs. It is tempting to speculate that the localized and temporal activation of AtRAC10 and possibly other RACs leads to changes in membrane dynamics, promoting cell growth.

RACs, Membrane Cycling, and the Actin Cytoskeleton

In yeast, Cdc42 and Rho promote homotypic vesicle docking during vacuole formation in an actin-dependent manner. Although Cdc42 and Rho1 promoted vesicle docking under

in vitro conditions, it was suspected that some actin bundles were not dissociated from membranes during their separation and could have mediated vesicle fusion (Eitzen *et al.*, 2001, 2002; Muller *et al.*, 2001; Eitzen, 2003). Furthermore, the function of actin cytoskeleton in endocytosis is well established (Engqvist-Goldstein and Drubin, 2003).

In a more parallel situation to the one shown here, it has been shown that Cdc42 and Rho3 promote vesicle docking late in exocytosis during polar growth in yeast (Adamo *et al.*, 1999, 2001). Yeast Rho1 and Cdc42 interact with the exocyst complex subunits Sec3 (Novick and Guo, 2002). It was proposed that these interactions are required for placement of the exocyst complex and the establishment of polar growth (Adamo *et al.*, 1999; Guo *et al.*, 2001). AtRAC10 was localized at the plasma membrane (Figures 3 and 4) and likely disrupted vesicle cycling at this location. Genes encoding putative components of the exocyst were identified in the *Arabidopsis* and other plant genomes (Jurgens and Geldner, 2002; Elias *et al.*, 2003), and preliminary results suggest that GFP-AtEXO70 fusion protein was localized close to the plasma membrane after its transient expression in protoplasts (Elias *et al.*, 2003). Future examination of the interactions between plant RACs and exocyst complex or other

Figure 12. Effects of actin-depolymerizing agents Lat-B and CD treatments on epidermal cells of wild-type and *GFP-Atrac10^{CA}* leaves. FM4-64 fluorescence images of leaf epidermal cells of WT plants treated with Lat-B (A) or CD (E). Endocytotic vesicles forming funnel-like structures are seen in growth lobes (arrows). (B–D) *GFP-Atrac10^{CA}* leaf epidermal cells treated with Lat-B. The Lat-B treatments induced formation of vesicles reaching diameters of $\sim 40\ \mu\text{m}$, displaying colocalization of FM4-64 and *GFP-Atrac10^{CA}* (arrow). (F–H) *GFP-Atrac10^{CA}* leaf epidermis cells treated with CD. Similar to Lat-B, CD treatments induced formation of vesicles at diameters of $\sim 40\ \mu\text{m}$; large endocytotic vesicles labeled with both FM4-64 and *GFP-Atrac10^{CA}*. Cells were visualized with CLSM. Green indicates GFP (C and D), red indicates FM4-64 (A, B, E, and F), and yellow indicates colocalization of GFP and FM4-64 in overlay images (D and H). Bars, $20\ \mu\text{m}$.

components of the secretory pathway would be required to elucidate the molecular mechanisms by which RACs regulate vesicle trafficking.

Similar to other plant RACs (Fu *et al.*, 2001, 2002; Molendijk *et al.*, 2001; Jones *et al.*, 2002; Yang, 2002; Chen *et al.*, 2003; Cheung *et al.*, 2003), activated AtRAC10 induced reorganization of actin (Figure 5). In tobacco pollen tubes, expression of activated NtRAC1 induced swelling and increased phosphorylation of actin-depolymerizing factors (ADFs)/cofilins, inhibiting their interaction with actin (Chen *et al.*, 2003). ADFs/cofilins enhance depolymerization of microfilaments by phosphorylation-dependent interaction with slow-growing minus ends. Overexpression of tobacco NtADF1 in growing tobacco pollen tubes resulted in growth inhibition and *GFP-NtADF1* fusion protein was associated with subapical fine mesh and with long actin cables (Chen *et al.*, 2002). It has been demonstrated that actin turnover is required for cell growth in plants (Ketelaar *et al.*, 2003, 2004). Collectively, these data correlated between RAC function, actin organization and polar cell growth. The *Arabidopsis* homologue of the Arp2/3-regulating WAVE complex subunit AtSRA1 interacted with AtRAC4 in yeast. AtSRA1 is encoded by the distorted group gene *PIROGI* that affect actin organization and cell structure (Basu *et al.*, 2004). Other WAVE and Arp2/3 *Arabidopsis* mutants have altered actin cytoskeleton organization and cell shape (Hulskamp *et al.*, 1994; Schwab *et al.*, 2003; Brembu *et al.*, 2004; Zimmermann *et al.*, 2004). These data indicate that RACs regulate plant actin organization by evolutionarily conserved pathways. RAC-induced actin reorganization likely disrupted vesicle trafficking and was associated with changes in cell structure.

The involvement of RACs in actin organization makes it difficult to dissect between actin dependent and independent mechanisms by which they regulate trafficking. Inter-

estingly, actin stabilization by jasplakinolide did not inhibit internalization of FM4-64 (Figure 8), suggesting that additional processes were activated by the RAC.

RAC Activation of NADPH Oxidase and Cell Growth

Several lines of evidence suggest that RAC-activated NADPH oxidase is required for polar cell growth. Activation of NADPH oxidase by RACs was suggested to play important functions in plant defense against pathogens (Agrawal *et al.*, 2003). Compromised NADPH oxidase function inhibited Ca^{2+} uptake and cell growth in roots and root hairs (Foreman *et al.*, 2003). Expression of activated AtRAC4 and AtRAC6 disrupted Ca^{2+} gradients in swollen root hairs (Molendijk *et al.*, 2001). It is possible that RACs generate Ca^{2+} uptake at the tip of the growing root hair (and possibly other cells) by activating NADPH oxidase, which in turn activates Ca^{2+} channels in the plasma membrane. This suggests that localization of the NADPH enzyme complex at the tip of growing root hairs (and possibly other cells) is crucial for their growth. The disruption of membrane dynamics by activated RACs could have interfered with the localization of the NADPH oxidase complex, inducing changes in cell growth patterns.

RACs and Rabs in Regulation of Membrane Trafficking

Members of the Rab family of small GTP binding proteins are known to regulate trafficking along the secretory pathway in eukaryotes (Pfeffer, 1994, 2001; Seabra *et al.*, 2002). Plants have a conserved but more diverse family of Rabs (Nebenfuhr, 2002; Rutherford and Moore, 2002). During *in vitro* homotypic yeast vacuole fusion, Rho1 and Cdc42 were shown act downstream of Rabs (Eitzen *et al.*, 2001). Rho1, Rho3, Cdc42, and Rabs were implicated in the regulation of the exocyst complex (Lipschutz and Mostov, 2002; Novick

and Guo, 2002). Rabs are required for polar growth of pollen tubes (Cheung *et al.*, 2002), and RabA4b was detected at the tip of elongating root hairs and was implicated in regulation of trafficking during cell elongation (Preuss *et al.*, 2004). It will be important in the future to determine the functional relations between Rabs and RACs in the regulation of membrane trafficking and cycling. The identification of new Rabs and their subcellular localization would likely facilitate this study (Molendijk *et al.*, 2004).

The present study opens the door to further elucidate the role of RAC GTPases in membrane cycling events in polar growth and differentiation of plant cells. The possible effects of RACs on membrane cycling should now be considered when examining their function in signaling pathways. The reiterative nature of plant development and the existence of cells at different developmental stages throughout the life cycle make plants a unique system allowing to link cell growth and differentiation to cellular processes.

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REFERENCES

- Adamo, J. E., Moskow, J. J., Gladfelter, A. S., Viterbo, D., Lew, D. J., and Brennwald, P. J. (2001). Yeast Cdc42 functions at a late step in exocytosis, specifically during polarized growth of the emerging bud. *J. Cell Biol.* 155, 581–592.
- Adamo, J. E., Rossi, G., and Brennwald, P. (1999). The Rho GTPase Rho3 has a direct role in exocytosis that is distinct from its role in actin polarity. *Mol. Biol. Cell* 10, 4121–4133.
- Agrawal, G. K., Iwahashi, H., and Rakwal, R. (2003). Small GTPase 'Rop': molecular switch for plant defense responses. *FEBS Lett.* 546, 173–180.
- Baluska, F., Hlavacka, A., Samaj, J., Palme, K., Robinson, D. G., Matoh, T., McCurdy, D. W., Menzel, D., and Volkmann, D. (2002). F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. *Plant Physiol.* 130, 422–431.
- Basu, D., El-Assal Sel, D., Le, J., Mallery, E. L., and Szymanski, D. B. (2004). Interchangeable functions of *Arabidopsis* PIROGI and the human WAVE complex subunit SRA1 during leaf epidermal development. *Development* 131, 4345–4355.
- Betts, H., and Moore, I. (2003). Plant cell polarity: the ins-and-outs of sterol transport. *Curr. Biol.* 13, R781–R783.
- Brembu, T., Winge, P., Seem, M., and Bones, A. M. (2004). NAPP and PIRP encode subunits of a putative wave regulatory protein complex involved in plant cell morphogenesis. *Plant Cell* 16, 2335–2349.
- Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L., and Korn, E. D. (1994). Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* 269, 14869–14871.
- Bubb, M. R., Spector, I., Beyer, B. B., and Fosen, K. M. (2000). Effects of jasplakinolide on the kinetics of actin polymerization. An explanation for certain *in vivo* observations. *J. Biol. Chem.* 275, 5163–5170.
- Chen, C. Y., Cheung, A. Y., and Wu, H. M. (2003). Actin-depolymerizing factor mediates Rac/Rop GTPase-regulated pollen tube growth. *Plant Cell* 15, 237–249.
- Chen, C. Y., Wong, E. I., Vidali, L., Estavillo, A., Hepler, P. K., Wu, H. M., and Cheung, A. Y. (2002). The regulation of actin organization by actin-depolymerizing factor in elongating pollen tubes. *Plant Cell* 14, 2175–2190.
- Cheung, A. Y., Chen, C. Y., Glaven, R. H., de Graaf, B. H., Vidali, L., Hepler, P. K., and Wu, H. M. (2002). Rab2 GTPase regulates vesicle trafficking between the endoplasmic reticulum and the Golgi bodies and is important to pollen tube growth. *Plant Cell* 14, 945–962.
- Cheung, A. Y., Chen, C. Y., Tao, L. Z., Andreyeva, T., Twell, D., and Wu, H. M. (2003). Regulation of pollen tube growth by Rac-like GTPases. *J. Exp. Bot.* 54, 73–81.
- Christensen, T. M., Vejlupekova, Z., Sharma, Y. K., Arthur, K. M., Spatafora, J. W., Albright, C. A., Meeley, R. B., Duvick, J. P., Quatrano, R. S., and Fowler, J. E. (2003). Conserved subgroups and developmental regulation in the monocot rop gene family. *Plant Physiol.* 133, 1791–1808.
- Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743.
- Cramer, L. P. (1999). Role of actin-filament disassembly in lamellipodium protrusion in motile cells revealed using the drug jasplakinolide. *Curr. Biol.* 9, 1095–1105.
- Di Cesare, A., Paris, S., Albertinazzi, C., Dariozzi, S., Andersen, J., Mann, M., Longhi, R., and de Curtis, I. (2000). p95-APP1 links membrane transport to Rac-mediated reorganization of actin. *Nat. Cell Biol.* 2, 521–530.
- Donaldson, J. G., and Jackson, C. L. (2000). Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* 12, 475–482.
- Eitzen, G. (2003). Actin remodeling to facilitate membrane fusion. *Biochim Biophys Acta* 1641, 175–181.
- Eitzen, G., Thorngren, N., and Wickner, W. (2001). Rho1p and Cdc42p act after Ypt7p to regulate vacuole docking. *EMBO J.* 20, 5650–5656.
- Eitzen, G., Wang, L., Thorngren, N., and Wickner, W. (2002). Remodeling of organelle-bound actin is required for yeast vacuole fusion. *J. Cell Biol.* 158, 669–679.
- Elias, M., Drdova, E., Ziak, D., Bavlínka, B., Hala, M., Cvrckova, F., Soukupova, H., and Zarsky, V. (2003). The exocyst complex in plants. *Cell Biol. Int.* 27, 199–201.
- Engqvist-Goldstein, A. E., and Drubin, D. G. (2003). Actin assembly and endocytosis: from yeast to mammals. *Annu. Rev. Cell Dev. Biol.* 19, 287–332.
- Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. *Nature* 420, 629–635.
- Foreman, J., *et al.* (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422, 442–446.
- Friml, J., Wisniewska, J., Benkova, E., Mendgen, K., and Palme, K. (2002). Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* 415, 806–809.
- Frommer, W. B., Hummel, S., Unseld, M., and Ninnemann, O. (1995). Seed and vascular expression of a high-affinity transporter for cationic amino acids in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* 92, 12036–12040.
- Fu, Y., Li, H., and Yang, Z. (2002). The ROP2 GTPase controls the formation of cortical fine F-actin and the early phase of directional cell expansion during *Arabidopsis* organogenesis. *Plant Cell* 14, 777–794.
- Fu, Y., Wu, G., and Yang, Z. (2001). Rop GTPase-dependent dynamics of tip-localized F-actin controls tip growth in pollen tubes. *J. Cell Biol.* 152, 1019–1032.
- Geldner, N., Anders, N., Wolters, H., Keicher, J., Kornberger, W., Müller, P., Delbarre, A., Ueda, T., Nakano, A., and Jurgens, G. (2003). The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* 112, 219–230.
- Geldner, N., Friml, J., Stierhof, Y. D., Jurgens, G., and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 413, 425–428.
- Grebe, M., Friml, J., Swarup, R., Ljung, K., Sandberg, G., Terlou, M., Palme, K., Bennett, M. J., and Scheres, B. (2002). Cell polarity signaling in *Arabidopsis* involves a BFA-sensitive auxin influx pathway. *Curr. Biol.* 12, 329–334.
- Grebe, M., Xu, J., Mobius, W., Ueda, T., Nakano, A., Geuze, H. J., Rook, M. B., and Scheres, B. (2003). *Arabidopsis* sterol endocytosis involves actin-mediated trafficking via ARA6-positive early endosomes. *Curr. Biol.* 13, 1378–1387.
- Guo, W., Tamanoi, F., and Novick, P. (2001). Spatial regulation of the exocyst complex by Rho1 GTPase. *Nat. Cell Biol.* 3, 353–360.
- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514.
- Heese-Peck, A., Pichler, H., Zanolari, B., Watanabe, R., Daum, G., and Riezman, H. (2002). Multiple functions of sterols in yeast endocytosis. *Mol. Biol. Cell* 13, 2664–2680.
- Hulskamp, M., Misra, S., and Jurgens, G. (1994). Genetic dissection of trichome cell development in *Arabidopsis*. *Cell* 76, 555–566.
- Jochum, A., Jackson, D., Schwarz, H., Pipkorn, R., and Singer-Kruger, B. (2002). Yeast Ysl2p, homologous to Sec7 domain guanine nucleotide exchange factors, functions in endocytosis and maintenance of vacuole integrity and interacts with the Arf-Like small GTPase Arl1p. *Mol. Cell. Biol.* 22, 4914–4928.

- Jones, M. A., Shen, J.-J., Fu, Y., Li, H., Yang, Z., and Grierson, C. S. (2002). The *Arabidopsis* Rop2 GTPase is a positive regulator of both root hair initiation and tip growth. *Plant Cell* 14, 763–776.
- Jurgens, G., and Geldner, N. (2002). Protein secretion in plants: from the trans-Golgi network to the outer space. *Traffic* 3, 605–613.
- Ketelaar, T., Allwood, E. G., Anthony, R., Voigt, B., Menzel, D., and Hussey, P. J. (2004). The actin-interacting protein AIP1 is essential for actin organization and plant development. *Curr. Biol.* 14, 145–149.
- Ketelaar, T., de Ruijter, N. C., and Emons, A. M. (2003). Unstable F-actin specifies the area and microtubule direction of cell expansion in *Arabidopsis* root hairs. *Plant Cell* 15, 285–292.
- Kost, B., Lemichez, E., Spielhofer, P., Hong, Y., Tolias, K., Carpenter, C., and Chua, N. H. (1999). Rac homologues and compartmentalized phosphatidylinositol 4, 5-bisphosphate act in a common pathway to regulate polar pollen tube growth. *J. Cell Biol.* 145, 317–330.
- Kost, B., Spielhofer, P., and Chua, N. H. (1998). A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes. *Plant J.* 16, 393–401.
- Lamaze, C., Chuang, T. H., Terlecky, L. J., Bokoch, G. M., and Schmid, S. L. (1996). Regulation of receptor-mediated endocytosis by Rho and Rac. *Nature* 382, 177–179.
- Lavy, M., Bracha-Drori, K., Sternberg, H., and Yalovsky, S. (2002). A cell-specific, prenylation-independent mechanism regulates targeting of type II RACs. *Plant Cell* 14, 2431–2450.
- Le, J., El-Assal Sel, D., Basu, D., Saad, M. E., and Szymanski, D. B. (2003). Requirements for *Arabidopsis* ATARP2 and ATARP3 during epidermal development. *Curr. Biol.* 13, 1341–1347.
- Lee, E., Shelden, E. A., and Knecht, D. A. (1998). Formation of F-actin aggregates in cells treated with actin stabilizing drugs. *Cell Motil. Cytoskeleton* 39, 122–133.
- Li, H., Lin, Y., Heath, R. M., Zhu, M. X., and Yang, Z. (1999). Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. *Plant Cell* 11, 1731–1742.
- Lipschutz, J. H., and Mostov, K. E. (2002). Exocytosis: the many masters of the exocyst. *Curr. Biol.* 12, R212–R214.
- Malecz, N., McCabe, P. C., Spaargaren, C., Qiu, R., Chuang, Y., and Symons, M. (2000). Synaptojanin 2, a novel Rac1 effector that regulates clathrin-mediated endocytosis. *Curr. Biol.* 10, 1383–1386.
- McGrath, J. L., Tardy, Y., Dewey, C. F., Jr., Meister, J. J., and Hartwig, J. H. (1998). Simultaneous measurements of actin filament turnover, filament fraction, and monomer diffusion in endothelial cells. *Biophys. J.* 75, 2070–2078.
- Molendijk, A. J., Bischoff, F., Rajendrakumar, C. S., Friml, J., Braun, M., Gilroy, S., and Palme, K. (2001). *Arabidopsis thaliana* Rop GTPases are localized to tips of root hairs and control polar growth. *EMBO J.* 20, 2779–2788.
- Molendijk, A. J., Ruperti, B., and Palme, K. (2004). Small GTPases in vesicle trafficking. *Curr. Opin. Plant Biol.* 7, 694–700.
- Muller, O., Johnson, D. I., and Mayer, A. (2001). Cdc42p functions at the docking stage of yeast vacuole membrane fusion. *EMBO J.* 20, 5657–5665.
- Nebenfuhr, A. (2002). Vesicle traffic in the endomembrane system: a tale of COPs, Rabs and SNAREs. *Curr. Opin. Plant Biol.* 5, 507–512.
- Nebenfuhr, A., Ritzenthaler, C., and Robinson, D. G. (2002). Brefeldin A: deciphering an enigmatic inhibitor of secretion. *Plant Physiol.* 130, 1102–1108.
- Novick, P., and Guo, W. (2002). Ras family therapy: Rab, Rho and Ral talk to the exocyst. *Trends Cell Biol.* 12, 247–249.
- Ono, A., and Freed, E. O. (1999). Binding of human immunodeficiency virus type 1 Gag to membrane: role of the matrix amino terminus. *J. Virol.* 73, 4136–4144.
- Parton, R. M., Fischer-Parton, S., Watahiki, M. K., and Trewavas, A. J. (2001). Dynamics of the apical vesicle accumulation and the rate of growth are related in individual pollen tubes. *J. Cell Sci.* 114, 2685–2695.
- Peyroche, A., Antonny, B., Robineau, S., Acker, J., Cherfils, J., and Jackson, C. L. (1999). Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. *Mol. Cell* 3, 275–285.
- Pfeffer, S. R. (1994). Rab GTPases: master regulators of membrane trafficking. *Curr. Opin. Cell Biol.* 6, 522–526.
- Pfeffer, S. R. (2001). Rab GTPases: specifying and deciphering organelle identity and function. *Trends Cell Biol.* 11, 487–491.
- Preuss, M. L., Serna, J., Falbel, T. G., Bednarek, S. Y., and Nielsen, E. (2004). The *Arabidopsis* Rab GTPase RabA4b localizes to the tips of growing root hair cells. *Plant Cell* 16, 1589–1603.
- Qiu, J. L., Jilk, R., Marks, M. D., and Szymanski, D. B. (2002). The *Arabidopsis* SPIKE1 gene is required for normal cell shape control and tissue development. *Plant Cell* 14, 101–118.
- Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D., and Hall, A. (1992). The small GTP-binding protein Rac regulates growth factor-induced membrane ruffling. *Cell* 70, 401–410.
- Ritzenthaler, C., Nebenfuhr, A., Movafeghi, A., Stussi-Garaud, C., Behnia, L., Pimpl, P., Staehelin, L. A., and Robinson, D. G. (2002). Reevaluation of the effects of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. *Plant Cell* 14, 237–261.
- Rodriguez-Concepcion, M., Yalovsky, S., Zik, M., Fromm, H., and Gruissem, W. (1999). The prenylation status of a novel plant calmodulin directs plasma membrane or nuclear localization of the protein. *EMBO J.* 18, 1996–2007.
- Rutherford, S., and Moore, I. (2002). The *Arabidopsis* Rab GTPase family: another enigma variation. *Curr. Opin. Plant Biol.* 5, 518–528.
- Ruzin, S. E. (1999). *Plant Microtechnique and Microscopy*, New York: Oxford University Press.
- Samaj, J., et al. (2002). Involvement of the mitogen-activated protein kinase SIMK in regulation of root hair tip growth. *EMBO J.* 21, 3296–3306.
- Santos, M. S., Barbosa, J., Jr., Veloso, G. S., Ribeiro, F., Kushmerick, C., Gomez, M. V., Ferguson, S. S., Prado, V. F., and Prado, M. A. (2001). Trafficking of green fluorescent protein tagged-vesicular acetylcholine transporter to varicosities in a cholinergic cell line. *J. Neurochem.* 78, 1104–1113.
- Schwab, B., Mathur, J., Saedler, R., Schwarz, H., Frey, B., Scheidegger, C., and Hulskamp, M. (2003). Regulation of cell expansion by the DISTORTED genes in *Arabidopsis thaliana*: actin controls the spatial organization of microtubules. *Mol. Genet. Genomics* 269, 350–360.
- Seabra, M. C., Mules, E. H., and Hume, A. N. (2002). Rab GTPases, intracellular traffic and disease. *Trends Mol. Med.* 8, 23–30.
- Shurety, W., Stewart, N. L., and Stow, J. L. (1998). Fluid-phase markers in the basolateral endocytic pathway accumulate in response to the actin assembly-promoting drug jasplakinolide. *Mol. Biol. Cell* 9, 957–975.
- Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* 1, 31–39.
- Tao, L. Z., Cheung, A. Y., and Wu, H. M. (2002). Plant Rac-like GTPases are activated by auxin and mediate auxin-responsive gene expression. *Plant Cell* 14, 2745–2760.
- Willemsen, V., Friml, J., Grebe, M., van den Toorn, A., Palme, K., and Scheres, B. (2003). Cell polarity and PIN protein positioning in *Arabidopsis* require sterol methyltransferase1 function. *Plant Cell* 15, 612–625.
- Winge, P., Brembu, T., Kristensen, R., and Bones, A. M. (2000). Genetic structure and evolution of RAC-GTPases in *Arabidopsis thaliana*. *Genetics* 156, 1959–1971.
- Yang, Z. (2002). Small GTPases: versatile signaling switches in plants. *Plant Cell* 14, S375–S388.
- Zheng, Z. L., and Yang, Z. (2000). The Rop GTPase switch turns on polar growth in pollen. *Trends Plant Sci.* 5, 298–303.
- Zimmermann, I., Saedler, R., Mutondo, M., and Hulskamp, M. (2004). The *Arabidopsis* GNARLED gene encodes the NAP125 homolog and controls several actin-based cell shape changes. *Mol. Genet. Genomics* 272, 290–296.