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Rab2 GTPase Regulates Vesicle Trafficking between the Endoplasmic Reticulum and the Golgi Bodies and Is Important to Pollen Tube Growth[W]

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Pollen tube elongation depends on the secretion of large amounts of membrane and cell wall materials at the pollen tube tip to sustain rapid growth. A large family of RAS-related small GTPases, Rabs or Ypts, is known to regulate both anterograde and retrograde trafficking of transport vesicles between different endomembrane compartments and the plasma membrane in mammalian and yeast cells. Studies on the functional roles of analogous plant proteins are emerging. We report here that a tobacco pollen-predominant Rab2, NtRab2, functions in the secretory pathway between the endoplasmic reticulum and the Golgi in elongating pollen tubes. Green fluorescent protein–NtRab2 fusion protein localized to the Golgi bodies in elongating pollen tubes. Dominant-negative mutations in NtRab2 proteins inhibited their Golgi localization, blocked the delivery of Golgi-resident as well as plasmalemma and secreted proteins to their normal locations, and inhibited pollen tube growth. On the other hand, when green fluorescent protein–NtRab2 was over-expressed in transiently transformed leaf protoplasts and epidermal cells, in which NtRab2 mRNA have not been observed to accumulate to detectable levels, these proteins did not target efficiently to Golgi bodies. Together, these observations indicate that NtRab2 is important for trafficking between the endoplasmic reticulum and the Golgi bodies in pollen tubes and may be specialized to optimally support the high secretory demands in these tip growth cells.

INTRODUCTION

In the pistils of flowering plants, pollen tubes elongate directionally from the stigma to the ovules to deliver the male gametes to the egg cells for fertilization (Lord, 2000; Palanivelu and Preuss, 2000; Cheung and Wu, 2001). Pollen tubes elongate by tip growth, whereby new membrane components are incorporated into the plasmalemma and copious amounts of cell wall materials are secreted at the tip to support growth that can occur as rapidly as 1 cm/hr in some species. A highly efficient vesicular transport system mobilized largely by the actin cytoskeleton is believed to underlie this rapid tip growth process (reviewed by Hepler et al., 2001). Rapid cytoplasmic streaming delivers large numbers of secretory vesicles to the apical region of elongating pollen tubes. These vesicles accumulate to a high density in an inverse conical region at the extreme apex of the tube, ready for fusion with the cell membrane. These vesicles are packed with complex polysaccharides and glycoproteins for the pollen tube wall (Roy et al., 1998). They also deliver to the pollen tube surface a wide variety of membrane, cell wall, and secretory proteins, including possible signaling molecules and hydrolytic enzymes that could be important for pollen tube growth. The presence of clathrin heavy chains, clathrin-coated pits, and vesicles near the pollen tube tip (Derksen et al., 1995; Blackbourn and Jackson, 1996), the internalization of labeled dextran and FM4-64 from the growth medium into pollen tube vacuoles (O'Driscoll et al., 1993; Parton et al., 2001), and the uptake of pistil tissue–secreted proteins into pollen tubes (Luu et al., 2000) suggest that active endocytosis also occurs in these tip growth cells.

Vesicular transport in mammalian cells and yeast is known to be regulated by a large family of RAS-related GTPases known as Rab and Ypt proteins, respectively (Novick and Zerial, 1997; Schimmoller et al., 1998; Chavrier and Goud, 1999; Der and Balch, 2000; Zerial and McBride, 2001). Genetic and biochemical analyses have shown that these small GTPases are essential for delivering transport vesicles to specific target endomembrane compartments or

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to the plasma membrane. Some Rabs are known to regulate the endocytic pathway. Like other RAS-related small GTPases, Rabs act as molecular switches to regulate the activity of downstream effector molecules, and they cycle between the active GTP-bound form and the inactive GDP-bound form. In addition, several regulatory proteins are critical for this cycling process. The current model for Rab function suggests that active, GTP-bound Rabs are prenylated and recruited to membranes via interactions with specific surface proteins on budding or newly budded transport vesicles. They are delivered to target membranes via interactions with specific effectors to activate vesicle and target membrane fusion. In the process, the intrinsic GTPase activity converts the GTP-bound Rabs to the inactive GDP-bound form, which are then released from membranes and recycled to the cytosol. Subsequent interactions between vesicle and target SNARE proteins result in membrane fusion and the delivery of cargo molecules to the target compartment or to the cell surface (Sanderfoot and Raikhel, 1999; Chen and Scheller, 2001). The mechanism underlying the target specificity of individual Rab proteins remains unclear.

Although there is considerable similarity between the endomembrane system of plant cells and that of other eukaryotic cells, there are notable differences (Battey et al., 1999; Blatt et al., 1999; Hawes et al., 1999; Sanderfoot and Raikhel, 1999; Vitale and Denecke, 1999; Andreeva et al., 2000). For example, the Golgi apparatus in plant cells is composed of numerous dictyosomes that are translocated along actin cables and are in close association with the endoplasmic reticulum (ER) (Boevink et al., 1998; Nebenfuhr et al., 1999). This differs from the microtubule-mobilized mammalian Golgi apparatus, which is composed of a single interconnected cluster of membranes around the perinuclear region of the cell (Presley et al., 1997; Featherstone, 1998; Pelham, 1998; Lippincott-Schwartz et al., 2001). Furthermore, a structure analogous to the pre-Golgi "intermediate compartment" in mammalian cells has not been observed in plant cells (Crofts et al., 1999). However, sialyl transferase, a mammalian enzyme not found in plants, can be expressed and targeted properly to and retained in the Golgi of plant cells (Boevink et al., 1998), suggesting considerable functional analogy between animal and plant early secretory pathways.

A large number of plant genes/cDNAs homologous with Rab/Ypt GTPases have been identified (Borg et al., 1997; Palme, 1997; Uchimiya et al., 1998; Arabidopsis Genome Initiative, 2000). Their functions in vesicular transport have been inferred largely by sequence homology with Rabs/Ypts or by localization studies based on cytoimmunodetection or fusion with green fluorescent protein (GFP) (Sano et al., 1994; Moore et al., 1997; Kang et al., 2001). To date, a functional role in membrane flow between the ER and the Golgi in plant cells has been demonstrated only for an Arabidopsis Rab1, AtRab1b (Batoko et al., 2000). However, overexpression or suppression of a few of the plant Rabs in transgenic plants has resulted in developmental and morphological phenotypes (Kamada et al., 1992; Sano et al., 1994; Ueda et al., 1996; Kang et al., 2001; Lu et al., 2001), emphasizing the importance of these small G-proteins to plant growth and development. Here, we show that a pollen-predominant Rab2 from tobacco, NtRab2, functions in trafficking between the ER and the Golgi bodies and is important for pollen tube growth. Dominant-negative mutations in NtRab2, which maintain these small G-proteins in the inactive GDP-bound form, inhibited the transport of pollen proteins that enter the secretory pathway and suppressed pollen tube elongation.

RESULTS

NtRab2 Is Expressed Predominantly in Pollen

The Arabidopsis *AtRab2* cDNA (Moore et al., 1997) was used as a probe to screen for its homologs in a tobacco pollen grain cDNA library. The isolated tobacco cDNA is referred to as *NtRab2*. The deduced amino acid sequence of NtRab2 shows identity and similarity levels up to 98 and 99%, respectively, with sequences of AtRab2 and other plant Rab2s and differs from them only in five or six amino acid positions. NtRab2 also is very similar to mammalian Rab2s, although the C-terminal one-third of plant and animal Rab2s is more diverged than the rest of the molecules. NtRab2 terminates in GGCCS, and GGCC is a motif for isoprenylation typical of many characterized Rabs (Chavrier et al., 1991; Zerial and Huber, 1995). His-tagged NtRab2 produced by *Escherichia coli* has been observed to bind γ-^{32}P-GTP in in vitro binding assays (data not shown).

RNA gel blot hybridization performed under stringent conditions showed that *NtRab2* mRNA was expressed predominantly in pollen (Figure 1A, top). As in many highly expressed pollen mRNAs (Taylor and Hepler, 1997), the level of *NtRab2* mRNAs declined during pollen tube elongation. Leaves from 8- to 10-week-old plants and immature pistil tissues showed almost undetectable levels of NtRab2 mRNA. On the other hand, young seedlings, roots, mature but unpollinated pistil tissues, developing fruit, and germinating seed accumulated detectable levels of NtRab2 mRNAs (Figure 1A, bottom). Forty-eight hours of auxin treatment of liquid root cultures enhanced the level of NtRab2 mRNAs (Figure 1A, bottom, lane Lr_{au}), although auxin treatment of seedlings grown under regular tissue culture conditions (Figure 1A, bottom, lane C_{au}) did not affect Rab2 mRNA accumulation appreciably in cotyledons and emerging leaves.

GFP-NtRab2 Fusion Protein Associates with Golgi Bodies in Elongating Pollen Tubes

The pollen-specific Lat52 promoter (Twell et al., 1989) was used to express N-terminal GFP-tagged NtRab2 (GPF-NtRab2)

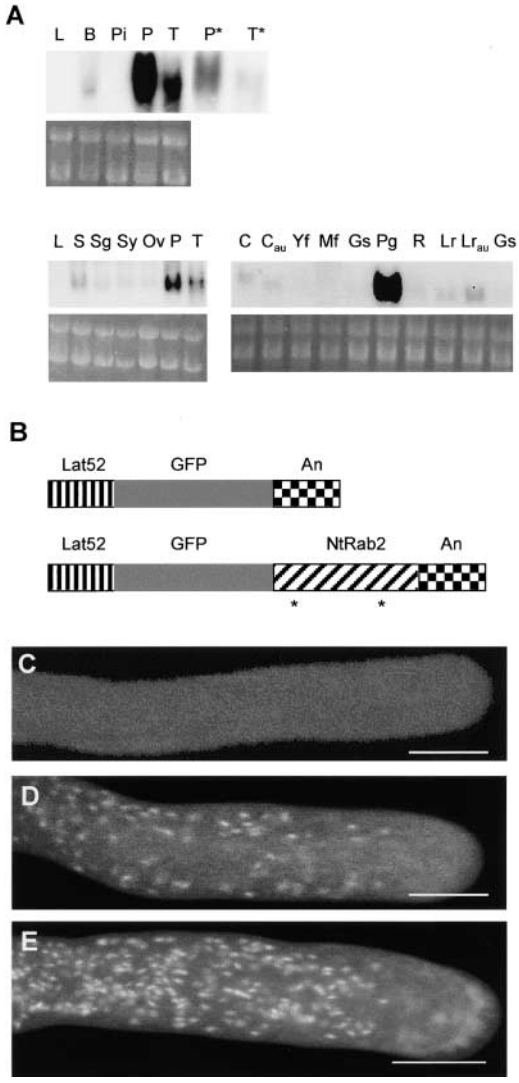

Figure 1. RNA Gel Blots Showing Pollen-Predominant Expression of NtRab2 mRNA.

(A) Comparable amounts of total RNA (10 μg) were loaded in each lane, as confirmed by ethidium bromide staining (below the autoradiograms). The RNA gel blots were hybridized with ^{32}P-labeled *NtRab2* cDNA. Top, L, leaves from 8- to 10-week-old plants grown in vitro; B, young floral buds (<1.5 cm); Pi, developing pistils (stigmas and styles) from 2- to 3-cm floral buds; P, pollen grains; T, 6-hr-old pollen tubes; P* and T*, one-tenth exposure relative to the P and T lanes. Bottom left, S, 11-day-old seedlings grown in vitro; Sg, Sy, and Ov, stigma, style, and ovary tissues, respectively, from mature but unpollinated pistils. (For floral developmental stages, see Kultonow et al. [1990].) Bottom right, C and C_{au}, cotyledons and emerging true leave of 10-day-old seedlings without or with auxin treatment, respectively, for 2 days before tissue collection; Yf, Mf, and Gs, young fruit, mature fruit, and germinating seed, respectively; Pg, pollen grains; R, roots from 8- to 10-week-old tissue culture–grown plants; Lr and Lr_{au}, 10-day-old liquid root cultures without and with auxin treatment, respectively, for the last 2 days.

(Figure 1B). In pollen tubes transformed by microprojectile bombardment or those that germinated from pollen grains from *Lat52-GFP-NtRab2*–transformed plants, green fluorescence was associated with numerous highly motile organelles throughout the cytosol, except at the most apical region (Figures 1D and 1E). The apical region of elongating pollen tubes is referred to as the "clear zone," because large organelles are excluded from this region and the pollen tube cytoplasm appears to have a smooth texture (Hepler et al., 2001). The GFP-NtRab2–labeled organelles appeared similar to those labeled with two C-terminal GFP-tagged Golgi marker proteins, the soybean mannosidase I–GFP (GmManI-GFP) (Nebenfuhr et al., 1999) (Figure 2A, top; see Figure 4A for the gene construct) and the AtErd2-GFP (Boevink et al., 1998; Takeuchi et al., 2000) (Figure 2A, bottom; see Figure 4A for the gene construct). The movement of GFP-NtRab2–labeled organelles showed the reverse-fountain streaming pattern characteristic of cytoplasmic streaming in elongating pollen tubes (see Figure 1D for a link to a time-lapse movie). The addition of cytochalasin D, an actin filament–disrupting drug, arrested the motility of GFP-NtRab2–labeled organelles (cf. Figures 2B and 2C). This observation suggests that the motility of these organelles relies on an

(B) Fusion gene constructs *Lat52-GFP* (top) and *Lat52-GFP-NtRab2* (bottom). "An" indicates a fragment with the polyadenylation signal from the T-DNA nopaline synthase gene. Asterisks indicate approximate locations for the dominant-negative S20N and N119I mutations.

(C) to **(E)** Confocal images showing the GFP-NtRab2 association with highly motile organelles in elongating pollen tubes.

(C) Elongating *Lat52-GFP*–transformed pollen tube in which the fluorescence signal was observed throughout the cytosol.

(D) Single optical section at the median plane of a *Lat52-GFP-NtRab2*–transformed elongating pollen tube. A time series of this section that shows the motility of the GFP-labeled organelles and pollen tube growth can be viewed in the supplemental data online. These organelles streamed tipward along the cortical region of the tube, reversed direction at the base of the clear zone, and streamed backward in the center of the tube in what is known as a "reverse-fountain" pattern.

(E) Projection of 10 optical sections obtained by the fast-scan mode in 1-μm steps through an entire elongating pollen tube expressing GFP-NtRab2.

The apical region of the tubes shown in **(D)** and **(E)** in which fluorescent organelles were absent is the clear zone. Pollen tubes shown in **(D)** and **(E)** were transformed by microprojectile bombardment. Tubes that developed from pollen grains isolated from *Lat52-GFP-NtRab2*–transformed plants showed GFP labeling patterns indistinguishable from those shown in **(D)** and **(E)** (data not shown). Tobacco pollen tubes expressing GFP-AtRab2 also showed labeling patterns indistinguishable from those shown in **(D)** and **(E)** (data not shown). Bars = 10 μm.

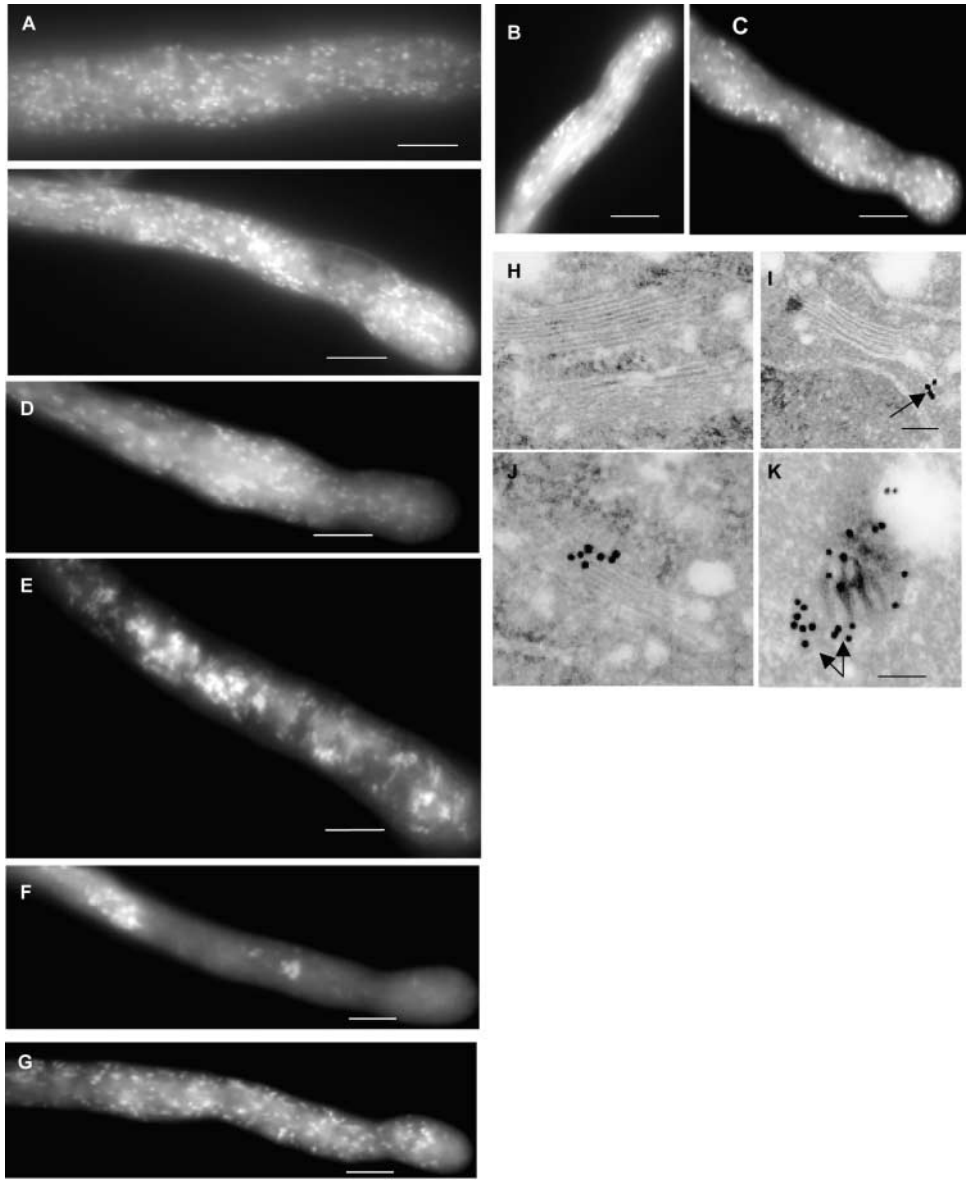

Figure 2. GFP-NtRab2 Is Targeted to Golgi Bodies.

(A) Segment of a transformed pollen tube expressing the Golgi marker protein ManI-GFP (top) and another tube expressing AtErd2-GFP (bottom). Both pollen tubes were transformed by microprojectile bombardment.

(B) and **(C)** Five-second exposures of a pollen tube expressing NtRab2-GFP **(B)** and of a similar tube that had been treated with 3.9 μM cytochalasin D for 5 min **(C)**. The arrest of organelle motility by the actin filament–disrupting drug is reflected by the discrete organelles shown in **(C)** relative to the extensive tailing observed in **(B)**, which reflects the motility of the fluorescent organelles.

(D) to **(G)** GFP-NtRab2–labeled organelles in transformed pollen tubes are dissociated by BFA treatment. Elongating *Lat52-GFP-NtRab2*–transformed pollen tubes were treated with 1 μg/mL BFA for 1, 5, and 10 min in **(D)**, **(E)**, and **(F)**, respectively, before imaging. **(G)** shows a similar pollen tube after a 20-min treatment with BFA and a 15-min incubation in BFA-free medium, demonstrating recovery of the GFP-labeled structures from the BFA treatment. Normal growth resumed in these tubes (data not shown) and was reflected in part by the reappearance of the clear zone at the tube apex.

(H) to **(K)** Cytoimmunodetection localized GFP-NtRab2 to the Golgi stacks. Immunogold labeling of ultrathin sections prepared from in vitro–grown nontransformed **(H)** or *Lat52-GFP-NtRab2*–transformed (**[I]** to **[K]**) pollen tubes. Tissue sections were reacted with primary antibodies against GFP and then with colloidal gold–labeled secondary antibodies. Arrows point to the periphery regions of Golgi stacks.

(A) to **(G)** show fluorescence micrographs, and **(H)** to **(K)** show transmission electron micrographs. Samples shown in **(B)** to **(K)** were from pollen tubes developed from grains isolated from *Lat52-GFP-NtRab2*–transformed plants. Observations indistinguishable from those shown here also were been made with transformed tobacco pollen tubes expressing GFP-AtRab2 (data not shown). Bars in **(A)** to **(G)** = 10 μm; bars in **(I)** and **(K)** = 60 nm for **(H)** to **(K)**.

active actin cytoskeleton system, as has been shown previously for plant Golgi body motility (Boevink et al., 1998; Nebenfuhr et al., 1999) and for pollen tube cytoplasmic streaming (Hepler et al., 2001).

Brefeldin A (BFA) is known to affect the structural integrity of the Golgi network, inhibit secretion, and cause a redistribution of the Golgi membrane and components of the ER in animal and plant cells (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Boevink et al., 1998; Batoko et al., 2000; Pagny et al., 2000). BFA also has been shown to be a potent inhibitor of pollen tube growth and to induce a rapid dissociation and disappearance of Golgi bodies (Rutten and Knuiman, 1993). When GFP-NtRab2–expressing pollen tubes were treated with BFA, GFP-NtRab2–labeled organelles disappeared and pollen tube growth ceased, although cytoplasmic streaming was not arrested immediately. Instead, green fluorescent signals began to aggregate (Figure 2E). These patches were similar to what have been referred to previously as "BFA compartments," which are formed by the clustering of Golgi stacks in BFA-treated plant cells (Satiat-Jeunemaitre and Hawes, 1992, 1993; Driouich et al., 1993). Green fluorescent signal eventually became diffused throughout the tube cytosol (Figure 2F). Discrete GFP labeling of highly motile organelles indistinguishable from untreated pollen tubes reappeared ~10 min after incubation in BFA-free medium (Figure 2G). An inverse-fountain streaming pattern of GFP-NtRab2–labeled organelles and pollen tube growth resumed. The total reversibility of the effect of BFA on GFP-NtRab2 localization pattern and pollen tube growth was similar to the effect of BFA on other cell types, including pollen tubes (Satiat-Jeunemaitre and Hawes, 1992; Rutten and Knuiman, 1993). All of these observations suggest that NtRab2-GFP associated predominantly with pollen tube Golgi bodies.

Immunoblot analysis using antibodies against GFP of total proteins from tubes that developed from pollen grains isolated from *Lat52-GFP-NtRab2*–transformed plants showed a single GFP antibody reactive protein species of ~60 kD, consistent with the size expected for the GFP-NtRab2 fusion protein (data not shown). Immunostaining of ultrathin sections of these transformed pollen tubes revealed an association of GFP antibodies with Golgi stacks (Figures 2I to 2K). Immunogold labeling was most predominant on the cisternae; occasional labeling on the Golgi periphery also was observed. These observations provided cytological evidence that GFP-NtRab2 localized to Golgi bodies in pollen tubes. In contrast to mammalian Rab2, GFP-NtRab2 has not been observed in vesicular compartments that are similar to the pre-Golgi intermediate compartment in which the mammalian protein and its functions have been localized (Chavrier et al., 1990; Tisdale and Jackson, 1998; Tisdale, 1999). This finding is consistent with previous reports that plant cells do not harbor a pre-Golgi compartment (Crofts et al., 1999). Observations indistinguishable from those shown in Figures 2B to 2G have been made using transformed tobacco pollen tubes expressing GFP-AtRab2 (data not shown), consistent with the high level of structural homology between the tobacco and Arabidopsis Rab2 proteins.

Dominant-Negative Forms of NtRab2 Inhibit Pollen Tube Growth

Mutations in small G-proteins that maintain these molecules predominantly in the GDP-bound inactive form, either by inhibiting the GDP-to-GTP exchange or by enhancing GTP hydrolysis, are referred to as dominant-negative mutations. When expressed in transformed cells, these GDP-bound inactive GTPases compete with endogenous regulators and effectors and interfere with the endogenous wild-type GTPase function (Novick and Zerial, 1997; Schimmoller et al., 1998; Chavrier and Goud, 1999; Der and Balch, 2000; Zerial and McBride, 2001). To perturb the wild-type NtRab2 function, we constructed two NtRab2 mutations, S20N and N119I, which were analogous to previously determined dominant-negative mutations in a number of other RAS-related proteins, including mammalian Rab2 (Tisdale et al., 1992) and Arabidopsis AtRab1b (Batoko et al., 2000). Microspore and pollen grain development involve highly secretory processes (Bedinger, 1992), and perturbation of such processes in stable transformed lines by various suppression strategies often resulted in the arrest in pollen grain development, precluding studies in elongating pollen tubes (unpublished observations). Therefore, we have focused our studies on using micro-projectile bombardment transformed pollen (Twell et al., 1989).

Lat52-GFP-NtRab2(S20N)– and *Lat52-GFP-NtRab2(N119I)*–transformed pollen grains germinated more slowly than control GFP-NtRab2–expressing pollen grains. Approximately 3 hr after pollination, control pollen tubes had elongated for distances of approximately two to five pollen grain diameters, whereas GFP-NtRab2(S20N)– and GFP-NtRab2(N119I)–expressing transformed pollen tubes were too short to be scored (data not shown). Differences in pollen tube lengths attained by control and GFP-NtRab2(S20N)– and GFP-NtRab2(N119I)–expressing tubes continued to increase over time. The inhibitory effect of these dominant-negative GFP-NtRab2s on pollen tube growth is shown in Figure 3B. Similarly, pollen tubes cotransformed by *Lat52-GFP* and unlabeled wild-type genes or either of the two dominant-negative NtRab2 mutant genes showed pollen tube growth characteristics qualitatively similar to those shown in Figure 3B (data not shown; see also Figure 4O). This finding demonstrated that GFP fusion to wild-type or mutant NtRab2s did not affect their activities appreciably.

In contrast to GFP-NtRab2, GFP-NtRab2(S20N) and GFP-NtRab2(N119I) did not associate with Golgi bodies when examined by epifluorescence or confocal microscopy. Instead, green fluorescence was detected throughout the pollen tube cytosol (Figure 3A), consistent with the idea that GDP-bound Rab proteins remain cytosolic and cannot be recruited onto membranes (Novick and Zerial, 1997; Alory and Balch, 2000).

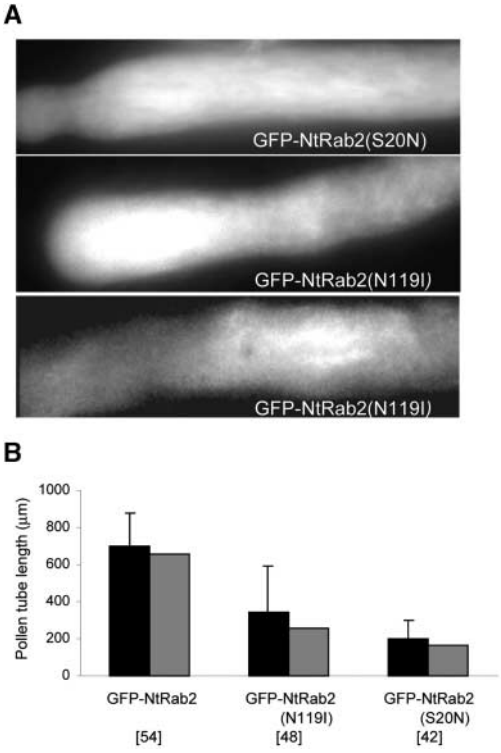

Figure 3. Dominant-Negative Mutants of NtRab2 Do Not Target Golgi Bodies and Inhibit Pollen Tube Elongation.

(A) Fluorescence images (top and middle) taken from cultures with dominant-negative GFP-NtRab2–expressing tubes that provided the data shown in **(B)**, and confocal image (bottom; a projection of three optical sections at 1-μm steps in the median region) of a pollen tube expressing GFP-NtRab2(N119I). Similar confocal images also were observed for pollen tubes expressing GFP-NtRab2(S20N) (data not shown). Pollen tubes shown here were transformed by microprojectile bombardment. The transgenic proteins expressed in these transformed pollen tubes are indicated.

(B) Length comparison of pollen tubes transformed by *Lat52-GFP-NtRab2*, *Lat52-GFP-NtRab2(S20N)*, or *Lat52-GFP-NtRab2(N119N)* (10 μg of each) after 6 hr of growth. Average (black bars) and median (gray bars) tube lengths are shown. The number in brackets below each histogram indicates the number of tubes scored for the experiment shown. The median tube length in pollen tube cultures more closely reflects the visual observation that most of the GFP-NtRab2(S20N)– and GFP-NtRab2(N119I)–expressing tubes extended considerably shorter distances than GFP-NtRab2–expressing control tubes. Data shown are from one of three replicated experiments that gave qualitatively similar results. Pollen tubes cotransformed by *Lat52-GFP* and unlabeled *Lat52-NtRab2*, *Lat52-NtRab2(S20N)*, or *Lat52-NtRab2(N119I)* gave qualitatively similar results as those shown in **(B)**.

Dominant-Negative NtRab2 Mutations Block Trafficking between the ER and the Golgi

The localization of GFP-NtRab2 to Golgi bodies (Figures 1 and 2) and information available on mammalian Rab2 functions (Tisdale et al., 1992; Tisdale and Balch, 1996; Tisdale and Jackson, 1998; Tisdale, 1999) suggest a functional role for this plant Rab2 in the secretory pathway between the ER and the Golgi in pollen tubes. To determine if NtRab2 is in fact functionally significant in pollen tube vesicular transport between the ER and the Golgi, we adopted the use of two previously characterized Golgi marker proteins, the K/HDEL receptor Erd2 from Arabidopsis, AtErd2 (Lee et al., 1993; Boevink et al., 1998; Takeuchi et al., 2000), and mannosidase I from soybean, GmManI (Nebenfuhr et al., 1999). Yeast and animal Erd proteins localize preferentially to the *cis*-Golgi bodies and the intermediate compartment, bind the K/HDEL peptide on escaped ER proteins, and recycle them back to the ER via retrograde trafficking on Golgi-derived vesicles (Townsley et al., 1993).

Transformed pollen tubes expressing AtErd2-GFP showed predominantly GFP-labeled Golgi bodies (Figure 2A, bottom; Figures 4B, 4C, and 4G), similar to those labeled by GFP-NtRab2 (Figures 1 and 2). In 15-hr-old AtErd2-GFP–expressing control tubes in which cytoplasmic streaming has slowed, GFP-labeled ER-like membrane fragments (see Figures 6 and 7) were captured by confocal microscopy along with strong fluorescence associated with Golgi bodies (Figure 4G). When pollen tubes were cotransformed with *LAT52-AtErd2-GFP* and *LAT52-NtRab2(S20N)* or *LAT52-NtRab2(N119I)*, the Golgi localization of AtErd2-GFP was reduced significantly, sometimes even to undetectable levels, with a concomitant increase in cytosolic fluorescent signal and signal on membrane structures that are similar to the ER (Figures 4D, 4E, and 4H).

Many cotransformed pollen grains did not germinate, and GFP signal was observed throughout the grain and associated with membrane structures that could be ER fragments (Figure 4K). This pattern differed from the AtErd2-GFF–labeled Golgi body pattern seen in control transformed pollen grains expressing this marker protein and the unlabeled wild-type NtRab2 (Figure 4J). As in transformed pollen tubes expressing only the dominant-negative GFP-NtRab2 proteins, pollen tube elongation was inhibited partially in these AtErd2-GFP– and unlabeled dominant-negative NtRab2–coexpressing pollen tubes (Figure 4O).

When GmManI-GFP (Nebenfuhr et al., 1999) (Figure 4A) was used as a Golgi marker protein for ER-to-Golgi vesicle trafficking in combination with the dominant-negative NtRab2s, its Golgi localization (Figures 2A, top, and 4L) also was reduced to unobservable levels (Figures 4M and 4N). Instead, GFP signal was observed throughout the tube. Growth also was retarded in these GmManI-GFP– and unlabeled dominant-negative NtRab2-coexpressing pollen tubes (data not shown).

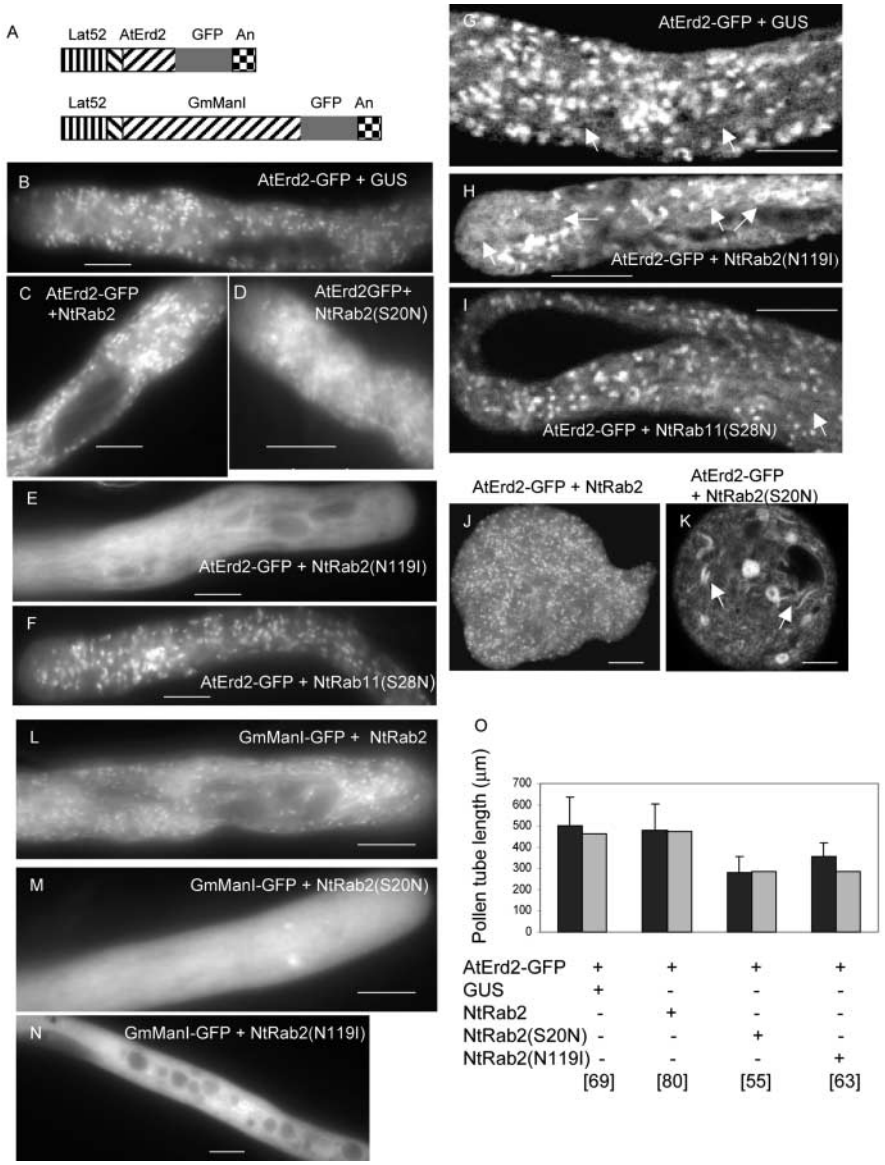

Figure 4. Localization of AtErd2-GFP and GmManI-GFP to Golgi Bodies Is Inhibited by the Coexpression of Dominant-Negative NtRab2(S20N) or NtRab2(N119I).

(A) Fusion gene constructs *Lat52-AtErd2-GFP* (top) and *Lat52-GmManI-GFP* (bottom) for Golgi marker proteins. The diagonally striped region indicates the signal peptide for each of the proteins. "An" indicates the polyadenylation signal.

(B) to **(E)** Pollen tubes from cultures that provided the growth comparison data shown in **(O)**.

(G) to **(K)** Projections of three optical sections at 1-μm steps near the median region of the pollen tubes or grains. Arrows in **(G)** to **(I)** and **(K)** point to ER membrane fragments.

Pollen tubes shown in the light micrographs in **(B)** to **(F)** and **(L)** to **(N)** were from 6-hr growth cultures; those shown in the confocal micrographs in **(G)** to **(I)** were from 15-hr cultures when growth and cytoplasmic streaming had slowed. The transgenic proteins expressed by these pollen tubes are indicated in each micrograph. The marker proteins were tagged with GFP; the various forms of Rab2 proteins were not tagged. **(B)** to **(F)** and **(L)** to **(N)** show fluorescence micrographs; and **(G)** to **(K)** show confocal images. Cotransformation with *Lat52-GUS* or *Lat52-NtRab2* (**[B]**, **[C]**, **[G]**, **[J]**, and **[O]**) served as a control to ensure that the amounts of DNA introduced into the transformed pollen tubes did not affect pollen tube growth and marker protein localization in these comparative studies. Bars = 10 μm.

(O) Length comparison of microprojectile-transformed pollen tubes expressing the proteins indicated after 6 hr of growth. Black bars indicate average length, and gray bars indicate median length. In all of the pollen tubes used, 5 μg of the marker gene *LAT52-AtErd2-GFP* DNA and 15 μg of the other non-GFP–tagged chimeric DNA were used for transformation. Transgenic proteins expressed by each transformed class of pollen tubes are indicated at the bottom of the histograms. The number in brackets below each histogram indicates the number of tubes scored in the experiment shown. Data shown are from one of three replicated experiments that gave qualitatively similar results.

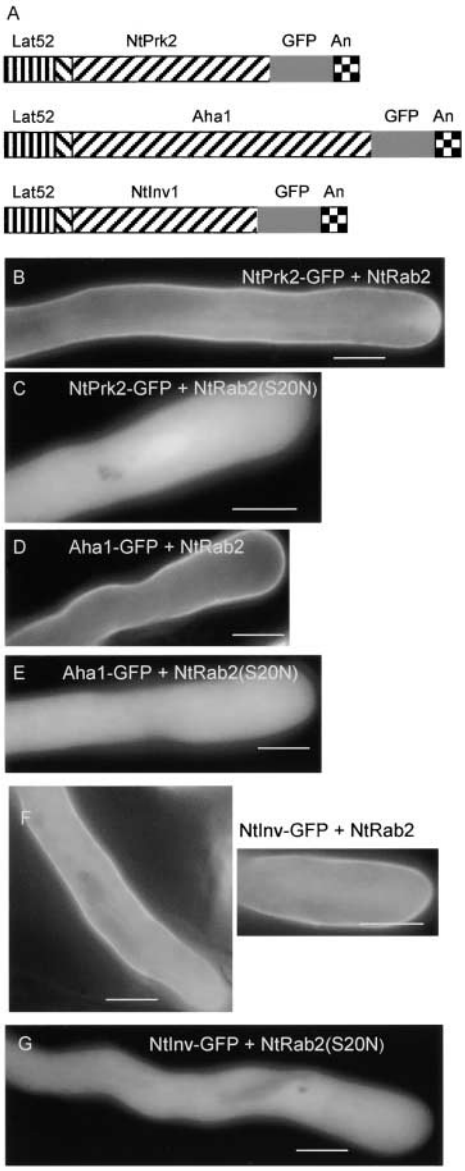

Figure 5. Dominant-Negative NtRab2s Inhibit the Transport of Pollen Tube Cell Membrane and Secreted Proteins.

(A) Fusion gene constructs *Lat52-NtPrk2-GFP* (top), *Lat52-Aha1-GFP* (middle) for cell membrane–associated marker proteins, and *Lat52-NtInv-GFP* (bottom) for a cell wall marker protein. The diagonally striped region indicates the signal peptide for each protein. "An" indicates the polyadenylation signal.

(B) to **(G)** Fluorescence micrographs of 6-hr-old pollen tubes. Transgenic proteins expressed by the pollen tubes are indicated; the cargo marker proteins were tagged with GFP, but the various forms of Rab2 proteins were not tagged. **(F)** shows the shank (left) and the tip (right) regions of two different pollen tubes expressing the same cargo protein, NtInv-GFP. The effect of NtRab2(N119I) was similar to that of the S20N mutant (data not shown). The highly fluorescent reverse conical region at the apex of the tube shown in **(B)** reflects the region packed with secretory vesicles in elongating pollen tubes. Bars = 10 μm.

To ascertain that the dominant-negative effect of the NtRab2 mutations resulted from specific interactions with Nt-Rab2–interacting proteins, the effect of a dominant-negative form of another tobacco pollen Rab protein that functions in post-Golgi trafficking, NtRab11(S28N), on AtErd2-GFP localization was examined. The S28N conversion was similar to mutations created in analogous positions in a phosphoryl binding site of other Rabs, including Rab11s, and has reproducibly exerted dominant-negative activities (Zerial and Huber, 1995). In plants, the analogous mutation, T34N, in a pea Rab11 homolog, Pra2, also showed dominant-negative activity (Kang et al., 2001). The dominant-negative activity of NtRab11(S28N) has been observed to affect its localization level and its ability to inhibit cargo molecule delivery and pollen tube growth (unpublished results). Coexpression of NtRab11(S28N) did not affect the AtErd2-GFP labeling pattern in cotransformed tubes (Figures 4F and 4I), although growth was inhibited (data not shown) similar to that induced by the dominant-negative NtRab2s.

NtRab2 Is Important for Transporting Cell Membrane and Secretory Proteins

Post-Golgi vesicle trafficking delivers cell membrane and secreted proteins to their destinations. Whether NtRab2 acts on the transport of pollen tube plasmalemma and secreted proteins also has been examined. A tobacco pollen-expressed receptor kinase (NtPrk2) and an Arabidopsis proton-ATPase (Aha1) (Harper et al., 1989) were fused C terminally to GFP (Figure 5A) and used as cell membrane marker proteins. A chimeric gene composed of the coding region of a tobacco pollen-expressed invertase fused C terminally to GFP, NtInv-GFP, was used as a cell wall and secretory marker protein (Figure 5A). Both NtPrk2-GFP and Aha1-GFP showed a strong association with the pollen surface (Figures 5B and 5D), consistent with a cell membrane localization, as has been shown for similar receptor kinases in tomato (Muschietti et al., 1998), Aha1, and other proton ATPases (DeWitt and Sussman, 1995; DeWitt et al., 1996).

Similar to what is known for the secretory property of invertases (Segev et al., 1988; Schaewen et al., 1990; Sturm and Tang, 1999), transformed pollen tubes expressing NtInv-GFP showed strong cell surface fluorescence (Figure 5F). A cell wall localization of Inv-GFP was especially evident in plasmolyzed pollen tubes (data not shown). Despite the strong cell surface association of these three marker proteins in transformed pollen tubes, a weak fluorescent signal always was observed throughout the pollen tubes, presumably reflecting GFP-labeled proteins in transit within the vesicular transport system. Most of the pollen surface-associated NtInv-GFP probably represented molecules that were in transit to the extracellular milieu, although fluorescence in the medium was too low to be detected. A highly fluorescent inverted cone region often was observed in pol-

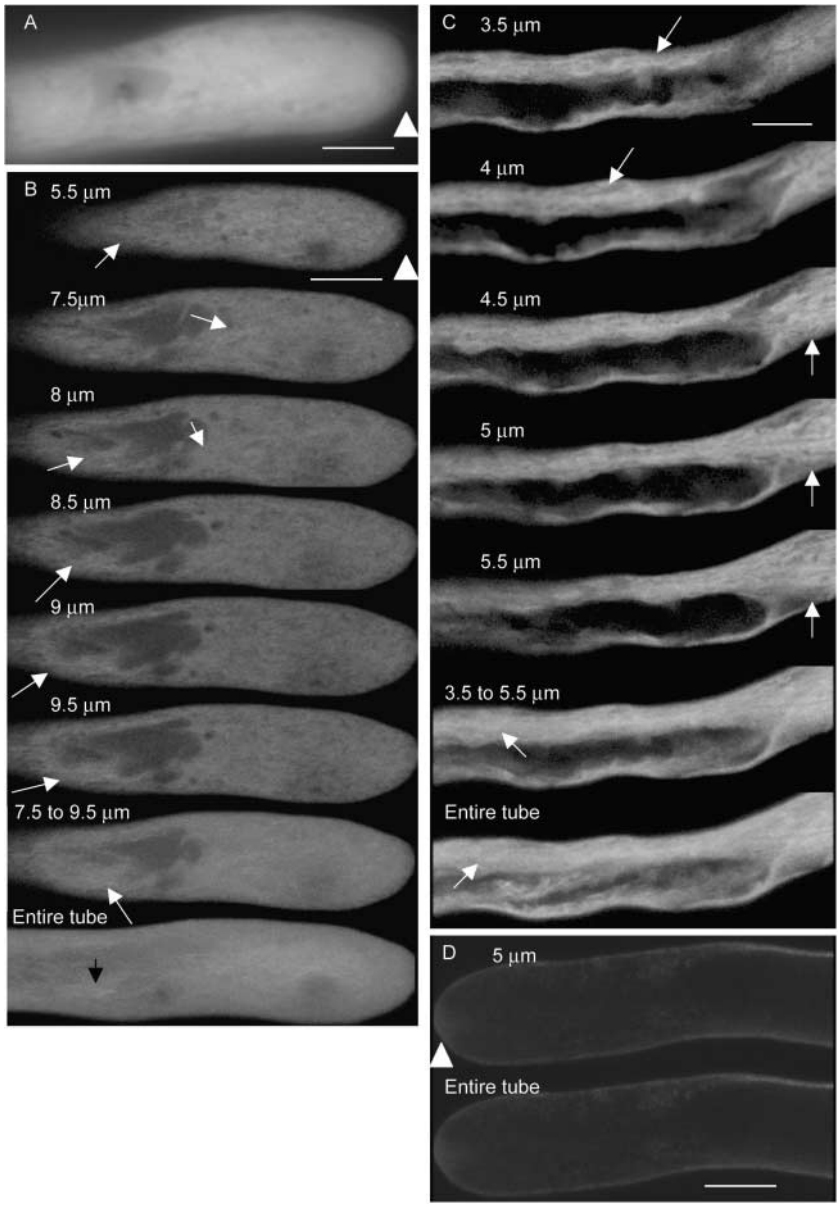

Figure 6. Dominant-Negative NtRab2 Induced the Retention of Cell Membrane–Associated Marker Proteins in the ER.

(A) Fluorescence micrograph of an NtPrk2-GFP– and NtRab2(S20N)-coexpressing pollen tube showing a GFP labeling pattern suggestive of extensive internal membrane structure. (Compare with Figures 6A and 7C.)

(B) Top six images show single optical sections taken at 0.5-μm intervals of the tip region of an NtPrk2-GFP– and NtRab2(S20N)-coexpressing transformed pollen tube similar to that shown in **(A)**. The seventh image shows a projection of the second to the sixth consecutive images. The bottom image shows a projection of 0.5-μm sections across the entire pollen tube.

(C) Top five images show single optical sections taken at 0.5-μm intervals of the shank region, where large vacuoles began to accumulate, of an Aha1-GFP– and NtRab2(S20N)-coexpressing transformed pollen tube. The bottom two images show projections of the top five images and of images taken across the entire tube, respectively.

(D) Top image shows a single optical section at the tip region of an NtPrk2-GFP–expressing control transformed pollen tube showing a predominant cell membrane association for this fusion protein; bottom image shows a projection of all images across the entire tube.

All pollen tubes were from 15-hr-old cultures. The numbers at the top left corner of each image represent the distance from the bottom of the tube. All pollen tubes shown were transformed by microprojectile bombardment. Triangles indicate the pollen tube tips. Arrows point to the more prominent ER membrane fragments. Bars = 10 μm.

len tubes expressing these GFP-tagged membrane and secreted proteins (Figure 5B), probably reflecting the accumulation of secretory vesicles loaded with these proteins at the tube apex.

The cell surface association of NtPrk2-GFP, Aha1-GFP, and NtInv-GFP was reduced significantly in pollen tubes that coexpressed the unlabeled dominant-negative NtRab2s, sometimes even reaching undetectable levels (Figures 5C, 5E, and 5G). Instead, epifluorescence microscopy revealed strong fluorescence throughout the tubes, but an association with resolvable organelle or discernible membrane structures was difficult to observe. Similar to their effects on pollen tube growth reported above (Figures 3 and 4), NtRab2(S20N) and NtRab2(N119I) also suppressed elongation of the six classes of transformed pollen tubes, each expressing a labeled marker protein and a dominant-negative NtRab2 (data not shown).

In fluorescence micrographs, the arrested cargo molecules in some of the pollen tubes coexpressing the dominant-negative NtRab2s showed fluorescent signals that were not as smooth as that shown by cytosolic GFP in *Lat52-GFP*–transformed pollen tubes (cf. Figures 6A and 7C). Individual optical sections from confocal scanning of these pollen tubes showed a GFP labeling pattern that was distinct from that observed in GFP-expressing pollen tubes (cf. Figures 6B and 7D). Instead, along with a diffused signal throughout the tube, the GFP-labeled cargo molecules associated with a dense network of tubular membranous structures when single optical sections were examined (Figure 6B). Elongated membranous structures similar to ER tubules were more obvious in single optical sections of the shank region of 15-hr-old dominant-negative NtRab2 and the GFP-labeled secretory cargo molecule coexpressing pollen tubes when growth had slowed considerably (Figure 6C). Projections of several optical sections or of the entire tube significantly blurred these membranous structures, especially at the more cytosolic tip region (Figure 6B; see also Figure 7B, bottom two panels).

To confirm that the structures revealed by the retained GFP-labeled secretory pathway cargo molecules were ER membranes, an ER-targeted yellow fluorescent protein (YFP; a GFP spectral variant [Miyawaki et al., 1997]) modified by the C-terminal addition of the ER-retention peptide KDEL was expressed under the control of Lat52 promoter in microprojectile bombardment–transformed pollen. Figure 7E shows the classic ER pattern revealed by this YFP in a transformed guard cell. The fluorescent signals in single optical sections of these elongating pollen tubes also associated with a network of tubular structure (Figure 7B), similar to but denser than those observed in the pollen tubes shown in Figure 6B, in which the secretory cargo molecules were retained and growth was inhibited. These membranous structures were no longer obvious when projections of several optical sections or of the entire tubes (Figure 7B, bottom two panels) were observed. The faster growth rate and higher cytoplasmic streaming dynamics in these ER-targeted YFP-expressing pollen tubes that were not influenced by any secretion and growth-inhibitory activity probably contributed to the difference in density and dynamics of the ER network.

GFP-NtRab2 Does Not Associate Efficiently with Golgi Bodies in Leaf Cells

We also examined whether NtRab2 acts in vegetative cells like it does in pollen tubes. Figure 8 shows tobacco leaf protoplasts and epidermal cells that were transformed transiently by either the constitutively expressed 35S promoter of *Cauliflower mosaic virus* (CaMV35S)-GFP-NtRab2 or, as a control, a CaMV35S-GFP-NtRab1b (Figure 8A). NtRab1b is most homologous with the Arabidopsis AtRab1b, which has been shown to regulate trafficking between the ER and the Golgi bodies in tobacco epidermal cells (Batoko et al., 2000). GFP-NtRab1b has been observed to localize to a reticulate network and associated punctate structures in these transiently transformed leaf cells (Figures 8B and 8E), as did GFP-AtRab1b (data not shown). This labeling pattern was indistinguishable from the ER and Golgi labeling pattern shown by GFP-labeled marker proteins that associate with epidermal cell and protoplast ER and Golgi (Boevink et al., 1998; Takeuchi et al., 2000). The localization pattern of GFP-NtRab1b (Figures 8B and 8E, top) is consistent with a functional role for Rab1 in ER-to-Golgi trafficking, as demonstrated previously for AtRab1b (Batoko et al., 2000). On the other hand, GFP-NtRab2 was distributed mostly in the cytosol or in the nucleus (Figures 8C, 8D, and 8F) of transiently transformed leaf cells, similar to constructs expressing GFP alone (Grebenok et al., 1997). Occasionally, a few punctate structures, possibly Golgi bodies, labeled by GFP-NtRab2 were observed (Figure 8F, top).

DISCUSSION

It is well known that in mammals and yeast, Rab GTPases play an important role in membrane trafficking. Except for a functional role in ER-to-Golgi trafficking demonstrated recently for a Rab1 from Arabidopsis (Batoko et al., 2000), our understanding of these small GTPases in higher plants, based on functional studies in the secretory process, is not yet well developed. The amplified membrane-trafficking activities in elongating pollen tubes relative to other plant cell types and the dependence on these activities for cell growth make pollen tubes ideal for functional studies in vesicular transport. In the present study, we performed localization studies of GFP-NtRab2 and functional assays based on the inhibitory effects of dominant-negative NtRab2 mutations on the delivery of secretory pathway cargo molecules and on pollen tube growth. Together, our results show that NtRab2

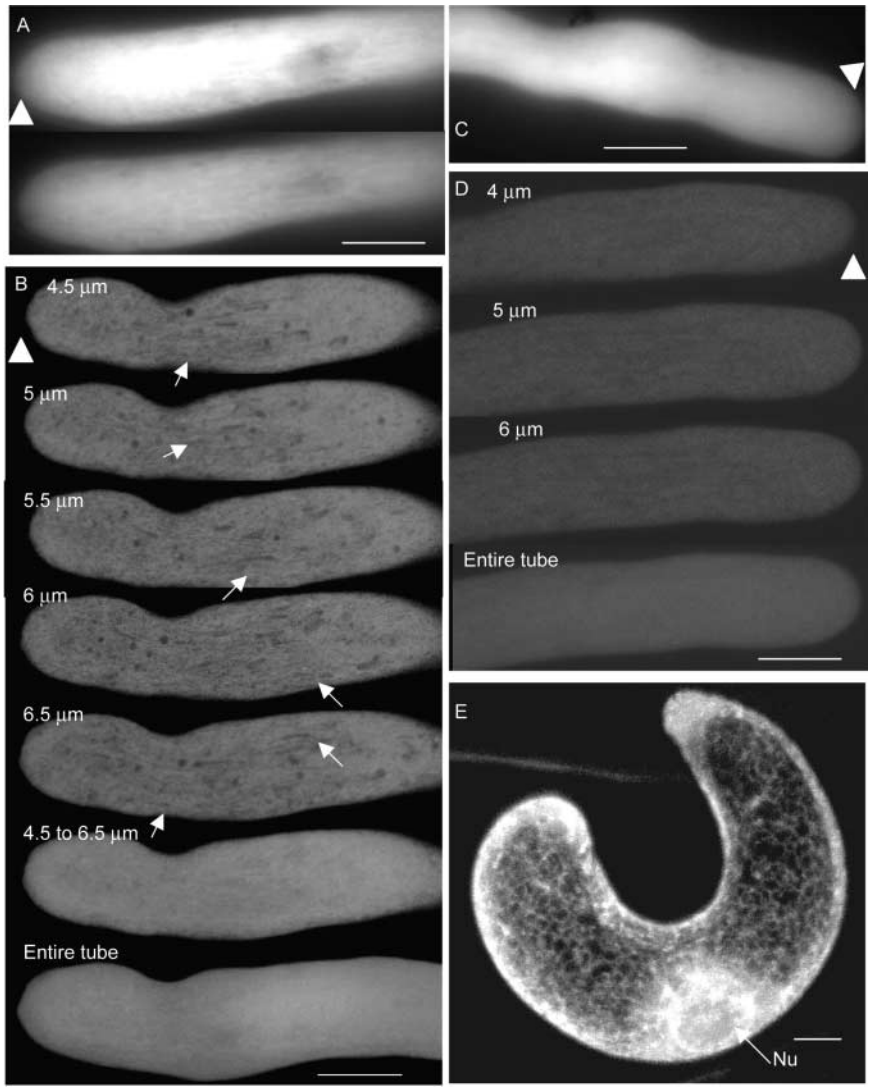

Figure 7. Imaging of the Pollen Tube ER Network Using an ER-Targeted YFP.

(A) Fluorescence micrographs of a pollen tube expressing the ER-targeted YFP. Top and bottom images show a longer and a shorter exposure, respectively, of the same tube to demonstrate a labeling pattern that was not as smooth as that shown by transformed pollen tubes expressing GFP alone (as shown in **[C]**).

(B) Top five images show consecutive optical sections taken at 0.5-μm steps and demonstrate a dense network of ER tubules in the tip region of an ER-targeted YFP-expressing transformed tube (as shown in **[A]**). Bottom two images show projections of the top five optical sections and of the entire tube, respectively. Arrows point to the more prominent tubular structures, which are blurred when multiple images are projected together.

(C) Fluorescence micrograph of a GFP-expressing transformed pollen tube.

(D) Top three images show single optical sections of a GFP-expressing pollen tube. Bottom image shows a projection of sections across the entire tube.

(E) Microprojectile bombardment–transformed guard cell expressing the same ER-targeted YFP from a CaMV 35S promoter. The labeled ER network confirmed proper ER targeting of the fusion protein used.

All pollen tubes shown were transformed by microprojectile bombardment. Triangles point to the pollen tube tips. Nu, nucleus. Bars = 10 μm.

is a major participant in ER-to-Golgi membrane trafficking in these tip growth cells. Understanding the components of exocytosis and endocytosis, both of which are critical for pollen tube growth, and how they function will be important to our understanding of the cellular mechanisms that underlie a major event in the reproductive process.

Protein Secretion in Pollen Tubes Relies on Rab2 GTPase Functions

It is known that two distinct Rabs, Rab1 and Rab2, regulate ER-to-Golgi trafficking in mammalian cells (Plutner et al., 1991; Tisdale et al., 1992; Nuoffer et al., 1994; Tisdale and Balch, 1996; Tisdale and Jackson, 1998; Tisdale, 1999; Allan et al., 2000; Moyer et al., 2001). Both Rab1 and Rab2 have been reported in a number of plant species (Loraine et al., 1996; Borg et al., 1997; Moore et al., 1997; Uchimiya et al., 1998; O'Mahony and Oliver, 1999; Arabidopsis Genome Initiative, 2000; Batoko et al., 2000). Here, we show that dominant-negative mutations (S20N and N119I) of the tobacco pollen-predominant NtRab2 inhibited the normal localization patterns of different classes of marker proteins that entered the pollen tube protein secretory pathway, including Golgi resident proteins, cell membrane, and secreted proteins (Figures 4 to 6). The inhibitory effect of these dominant-negative NtRab2s on the localization of several marker proteins in transformed pollen tubes presumably resulted from the interference of the interactions between the endogenous wild-type NtRab2 and their effectors. The observation that a dominant-negative mutant form of NtRab11 (unpublished results) had no effect on AtErd2-GFP localization (Figures 4F and 4I) is consistent with this hypothesis because different classes of Rabs differ in their effector-interacting domains (Zerial and Huber, 1995; Zerial and McBride, 2001).

Mammalian Rab2 has been found to regulate membrane flow from the pre-Golgi intermediate compartment to the Golgi and back to the ER (Tisdale et al., 1992; Tisdale and Jackson, 1998; Tisdale, 1999). The effects of dominant-negative NtRab2s on the Golgi marker protein AtErd2-GFP (Figures 4D, 4E, 4H, and 4K) and GmManI-GFP (Figures 4M and 4N) indicate that normal NtRab2 activity is important for vesicle trafficking between the plant ER and Golgi. The retention of fluorescence signal within the pollen tube protoplasts and the lack of Golgi-associated signal and the presence of ER-associated signal in transformed pollen tubes coexpressing the GFP-labeled cell membrane or secreted cargo molecules and dominant-negative NtRab2s (Figures 5C, 5E, 5G, and 6A to 6C) also support a role for this GTPase in trafficking between the ER and the Golgi.

The retention of GFP-labeled cargo molecules in the ER membranes in dominant-negative NtRab2–coexpressing pollen tubes has been difficult to detect (Figure 6), especially in the more cytosolic tip region of these tubes (cf. Figures 6B and 6C). The highly motile central ER system, the occasional splitting into small vesicles that later fuse to linear membrane structures, and the curvature of the cortical region have been suggested to contribute to the difficulty of observing the dynamic pollen tube ER network (Pierson et al., 1990). For this study, we relied on the labeling pattern observed in pollen tubes that expressed a ER-localized YFP (Figures 7B and 7E) to confirm that the retained GFP cargo molecules were retained at least partially in the ER. However, fluorescence signals that appeared diffuse throughout the tube often were strong and sometimes overwhelmed the imaging of membranous structures. The diffuse signal could have resulted from the accumulation of labeled cargo-loaded transport vesicles that had failed to dock with a recognizable membrane system.

The inhibition of the Golgi association of Golgi marker proteins (Figure 4) and the retention of cell membrane and secreted proteins within the protoplast of pollen tube cells (Figures 5C, 5E, 5G, and 6) together support a functional role for Rab2 GTPase in trafficking between the ER and the Golgi and in secretion. However, whether all of these observations were based on a primary functional role for NtRab2 in anterograde or retrograde trafficking between the ER and the Golgi remains to be determined. Biochemical studies of the processing of cargo molecules in the pollen that coexpress dominant-negative NtRab2, such as studies of the presence or absence of typical Golgi-processed glycans on glycoproteins, and the identification of effector molecules for NtRab2 will help to determine the exact function of this GTPase in membrane exchange between the ER and the Golgi complex.

Rab2 GTPase May Be Specialized to Function in ER-to-Golgi Trafficking in Highly Secretory Cell Types

This and a previous study (Batoko et al., 2000) established the functional contribution by plant Rab1 and Rab2 to vesicle transport between the ER and the Golgi. These two plant Rabs may have functions in discrete steps of the vesicular trafficking pathways between the ER and the Golgi, as has been shown for mammalian Rab1 and Rab2 (Tisdale and Jackson, 1998; Tisdale, 1999; Allan et al., 2000; Moyer et al., 2001). On the other hand, the spatial relationship between plant ER and Golgi bodies is significantly more intimate than that between their animal counterparts (Boevink et al., 1998; Nebenfuhr et al., 1999). Plant Golgi bodies appear to track on the ER, and vesicular trafficking between these two compartments does not appear to rely on intermediate membrane compartments like those observed in mammalian cells (Crofts et al., 1999). Therefore, the extent of functional analogies between animal and plant Rab1 and Rab2 and where in the ER/Golgi transport pathway these two plant Rabs function remain to be determined.

NtRab2 mRNA is extremely predominant in pollen, whereas it is not detectable among mRNAs of full-grown leaves from plants maintained under tissue culture conditions, even after prolonged exposure of the RNA gel blot hybridization

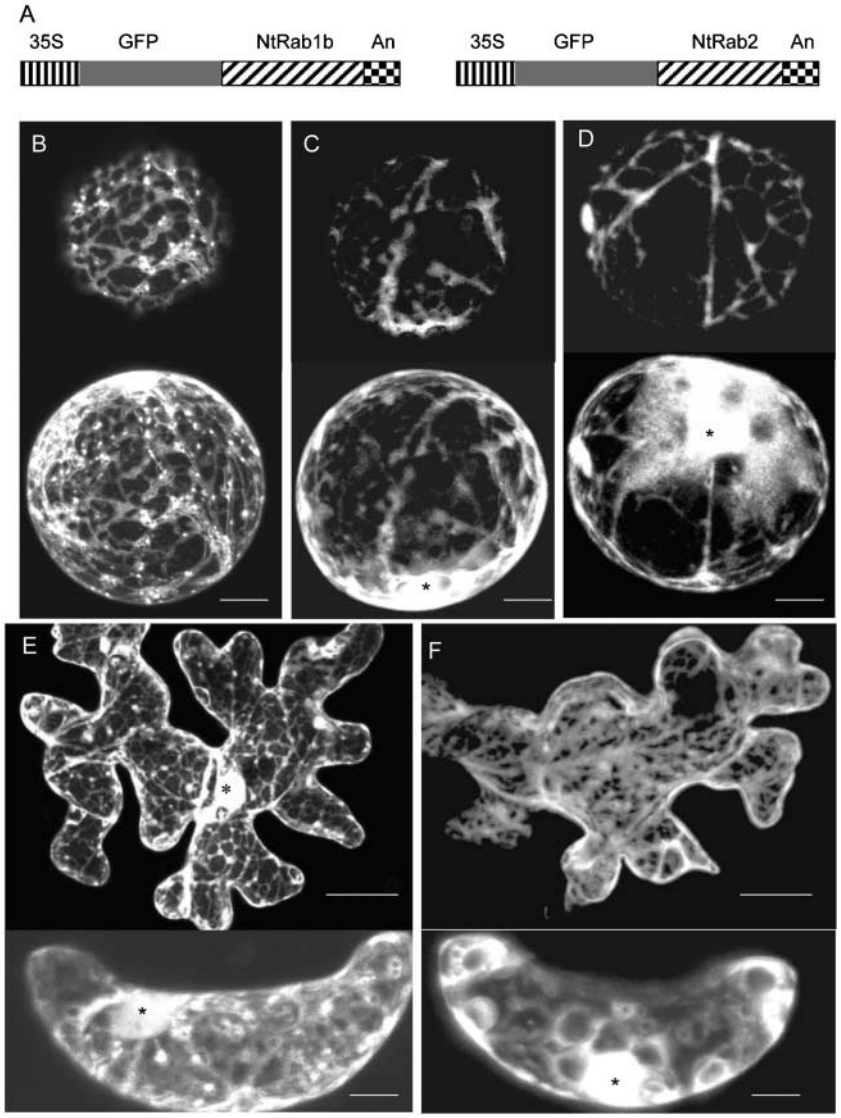

Figure 8. CaMV35S-Expressed GFP-NtRab2 Does Not Target Efficiently to Golgi Bodies of Leaf Cells.

(A) Fusion gene constructs *35S-GFP-NtRab1b* (left) and *35S-GFP-NtRab2* (right). "An" indicates the polyadenylation signal.
(B) Transiently transformed protoplast expressing GFP-NtRab1b.
(C) and **(D)** Transiently transformed protoplasts expressing GFP-NtRab2.
Top and bottom images in **(B)** to **(D)** are from the same protoplast. Top images show projections of four 1-μm sections close to the cortical region of the protoplast. Bottom images show projections of 1-μm sections across the entire protoplast.
(E) Microprojectile bombardment–transformed epidermal cell (top) and guard cell (bottom) expressing GFP-NtRab1b. Transient expression of GFP-AtRab1b in similarly transformed leaf protoplasts and epidermal cells showed patterns indistinguishable from those shown in **(B)** and **(E)** (data not shown).
(F) Microprojectile bombardment–transformed epidermal cell (top) and guard cell (bottom) expressing GFP-NtRab2.
Top and bottom images in **(E)** and bottom image in **(F)** show projections of 1-μm optical sections across the entire cell; top image in **(F)** shows a projection of 1-μm sections across half of the cell. Asterisks indicates the nucleus as visualized by differential interference contrast imaging. Bars in **(B)** to **(D)** and the bottom images in **(E)** and **(F)** = 10 μm; bars in the top images in **(E)** and **(F)** = 50 μm.

membrane (Figure 1A, top). On the other hand, tissues that are composed of cells with higher demands for secretory activities showed detectable levels of NtRab2 mRNA (Figure 1A, bottom). For instance, the tissue culture–grown young seedlings were composed mostly of cotyledons, elongating hypocotyl, emerging true leaves, and developing roots, all of which are tissues with dividing and growing cells. Furthermore, mature pistil tissues, which secrete an enriched extracellular matrix to prepare for pollen germination and tube elongation (Cheung, 1996), accumulate a detectable level of NtRab2 mRNA, whereas young pistils do not (Figure 1A).

Germinating seeds, which need to mobilize cotyledon stored reserves, also showed a detectable level of NtRab2 mRNAs. Root hairs are another example of plant cells that, like pollen tubes, exhibit tip growth. Root hair elongation also relies on active secretion. Roots from 8- to 10-week-old plants grown in agar medium and root cultures maintained in liquid medium and in the dark showed detectable levels of Rab2 mRNA. When auxin was added to stimulate root hair development in the liquid root cultures, the level of NtRab2 mRNA was enhanced (Figure 1A, bottom, right), consistent with a higher demand for secretion when root hairs initiate and develop. However, it is apparent that the level of NtRab2 mRNA in any of the tissues examined did not approach that in pollen. This could be because pollen, by virtue of its extraordinary developmental and growth characteristics, has a significantly greater demand for secretion relative to any other plant cell type. The observed difference also must have been enhanced by the fact that pollen was a homogeneous sample, whereas cells with higher levels of secretory activities constituted only a fraction of the cells in all of the other tissues examined.

The accumulation pattern of NtRab2 mRNA suggests that this GTPase may not be used in some plant cell types to a considerable extent, whereas others, especially those that require greater secretory activities, rely on the function of this GTPase. The inefficient targeting efficiency of GFP-NtRab2 to the ER/Golgi system in transiently transformed leaf cells (Figure 8) suggests that these cells may not have adequate levels of NtRab2-interacting proteins to support the activity of the over-expressed GFP-NtRab2.

Several other reports concerning Rab2 also suggest the likelihood that this GTPase is more actively required under special circumstances, especially when the demand for secretory activities is high. Like *NtRab2*, Arabidopsis *AtRab2* also is highly expressed in pollen and in germinating seedlings, in which higher levels of secretory activities are expected to sustain the dividing and growing cells (Moore et al., 1997). In *Lotus japonicus*, *LjRab2* was isolated repeatedly from a cDNA library made from developing root nodules, which exhibit high levels of secretory activities during their formation (Borg et al., 1997). A correlation with desiccation and rehydration has been reported with the expression of a Rab2, SsRab2, in the grass *Sporobolus stapfianus* (O'Mahony and Oliver, 1999). Both leaves and roots in this plant normally accumulate very low levels of SsRab2 mRNA. Desiccation induced a dramatic increase in the level of SsRab2 mRNA. Rehydration reduced its levels to different extents, depending on whether the species examined was desiccation resistant or sensitive, although a transient increase during the rehydration process also occurred. It was suggested that the desiccation-induced increase in SsRab2 mRNA may correlate with processes induced to repair membrane damage incurred during desiccation and again when cells swell while rehydrating (O'Mahony and Oliver, 1999).

In mammalian cells, Rab2 has been implicated in increasing neurite outgrowth and neuronal adhesion (Ayala et al., 1989, 1990), processes that are associated with high levels of vesicular activities and secretion. Moreover, a Rab2 homolog is absent from both budding and fission yeast, which require secretory activities at levels more akin to those of highly secretory cells only in transient periods of their life cycles (e.g., during budding, mating pair formation, and cell division). Furthermore, several plant *Rab1* cDNAs have been shown to complement the yeast *ypt1* mutant (Park et al., 1994; Kim et al., 1996), but a *Rab2* cDNA failed to do so (Kim et al., 1996). These observations are not inconsistent with the possibility that activities performed by the Rab2 class of plant GTPases may be specialized for cells with a high demand for secretory activities, whereas they are not needed as prominently in cells that do not have such a high demand for secretion.

Our observation that transiently transformed leaf cells could not support the efficient targeting of GFP-NtRab2 also supports the notion that other components required for the normal function of this GTPase may not be present at adequate levels in these cells. It has been suggested recently that a pair of Rab5-related Arabidopsis Rabs, Ara6 and Ara7, both of which have been localized to endosomes in protoplasts, might have different functions in cell types with different levels of secretory activities (Ueda et al., 2001). Genomic blot analysis showed that tobacco has at most two to three NtRab2 genes (data not shown), similar to the Arabidopsis AtRab2 family (Arabidopsis Genome Initiative, 2000). Whether the function of membrane trafficking between the ER and the Golgi performed by NtRab2 in pollen tubes is performed by these other Rab2s in leaf cells remains to be determined.

METHODS

Plant Material

Tobacco (*Nicotiana tabacum* var Petit Havana SR1) grown in vitro was used for *Agrobacterium tumefaciens* Ti plasmid and protoplast transformation and for seedling and leaf RNA preparations. Flowering tobacco plants were maintained under greenhouse conditions. Flower parts, pollen grains, and developing fruit were obtained from freshly dehisced anthers.

Pollen Tube Growth Conditions and Pharmaceutical Treatment

Freshly collected pollen grains were germinated in pollen germination medium (Cheung et al., 1995). Whenever pollen tube growth was maintained in agarose (final concentration, 0.7%), polyethylene glycol was substituted by 7% Suc. Pollen tube growth cultures were maintained at ~26 to 28°C in the dark. Unless indicated otherwise, pollen tubes were observed between 3 and 8 hr after germination. When pollen tubes grown in liquid medium were treated with the pharmaceutical drugs cytochalasin D and brefeldin A (Molecular Probes, Eugene, OR), the appropriate amount of drug was diluted from stock solutions at least 100-fold into the pollen tube cultures. Removal of brefeldin A was accomplished by allowing the pollen tubes to sediment, and the medium was removed and replenished with a 10-fold excess of germination medium. The procedure was repeated twice.

cDNA Isolation and Chimeric Gene Constructions

A pollen grain cDNA library was constructed in λZapII, and plasmids carrying the clones of interest were excised according to the manufacturer's manual (Stratagene). The tobacco pollen-expressed *NtRab2* and *NtPrk2* cDNAs were isolated from this library. The Arabidopsis Rab2 (AtRab2) (Moore et al., 1997), Erd2 (*AtErd2*) (Lee et al., 1993), and proton ATPase (*Aha1*) (Harper et al., 1989) cDNAs were isolated by reverse transcriptase–mediated polymerase chain reaction (RT-PCR) of Arabidopsis floral mRNAs. *NtInv* cDNA was obtained by RT-PCR of tobacco pollen mRNA. Arabidopsis AtRab1b (Batoko et al., 2000) and tobacco *NtRab1b* were isolated by RT-PCR of Arabidopsis and tobacco seedling mRNAs, respectively. All isolated clones have been sequenced to confirm identity and sequence integrity.

A 35S–soybean mannosidase I–green fluorescent protein (GmManI-GFP) construct (Nebenfuhr et al., 1999) was obtained from Drs. A. Staehelin and A. Nebenfuhr (University of Colorado, Boulder). The endoplasmic reticulum–targeted yellow fluorescent protein (a GFP spectral variant fused C terminally to KDEL) was modified from the yellow cameleon-er construct (Miyawaki et al., 1997) and placed behind the Lat52 promoter for pollen expression. Standard recombinant DNA methodology was used in constructing all of the chimeric genes used in this study. Fusion gene constructs are shown in the figures in which their use is described. The Lat52 promoter (Twell et al., 1989) was used to express these chimeric genes in pollen; the 35S promoter of *Cauliflower mosaic virus* was used to express them in leaf cells.

RNA Gel Blot Analysis

RNA isolation for gel blot analyses was performed as described previously (Kawata and Cheung, 1990). Seedlings and leaves were from tobacco plants grown in vitro for 11 days and 8 to 10 weeks, respectively. Floral tissues were from greenhouse-grown tobacco plants. Immature pistil tissues were from 2- to 3-cm floral buds; mature but unpollinated pistils were from open flowers that had been emasculated previously. Young fruit were green expanding fruit ~2 weeks after fertilization, and mature fruit were brown and desiccated, ~45 days after fertilization. Roots were from 8- to 10-week-old plants grown in agar. Liquid root cultures were initiated and maintained in the dark, with shaking, for 10 days in basal liquid B5 medium with 1% sugar.

For auxin treatment to stimulate root hair initiation, 25 μM naphthylacetic acid (NAA) was added on the 8th day, and the culture was grown for another 2 days. Stimulation of root hair emergence was apparent when examined microscopically. Eight-day-old seedlings grown on agar also were treated with 25 μM NAA by the addition of 5 mL of NAA-supplemented liquid medium to the plate. After 2 days, cotyledons and emerging leaves were collected from these cultures for RNA preparations. Pollen tubes were grown for 6 hr under the pollen tube growth conditions described above. RNA gel blot hybridization was performed at 60°C (Kawata and Cheung, 1990).

Cytoimmunodetection Analysis

A glutathione *S*-transferase–tagged GFP-overproducing line of *Escherichia coli* was used as a source of antigen for GFP antibody production. Protein gel blot analysis using plant total proteins revealed that these antibodies are highly specific for GFP (data not shown). Cytoimmunodetection studies of chemically fixed pollen tubes were performed as described previously (Cheung et al., 1995).

Light Microscopy Analysis

Epifluorescence for GFP observation was performed with a Nikon Eclipse E-800 microscope (Tokyo, Japan) using either a GFP or a fluorescein isothiocyanate filter block for detection. Confocal laser scanning microscopy was conducted with either a Bio-Rad MRC-600 system or a Leica TCS system (Wetzlar, Germany) using the 488-nm excitation line of an argon laser and a fluorescein isothiocyanate filter. Observation of pollen tubes was conducted routinely between 3 and 8 hr after germination for observation of elongating pollen tubes that had the best tube morphology. Occasionally, and for the purpose of observing endoplasmic reticulum membranes, pollen tubes were used after a 15-hr culture period, when growth and cytoplasmic streaming have slowed considerably.

Plant, Protoplast, and Pollen Transformation

Leaf disc transformation by Agrobacterium Ti plasmid was performed according to a standard protocol (Delebrese et al., 1986). Pollen grains from transformed plants were used in some experiments. Transient transformation of pollen grains was accomplished with micro-projectile bombardment equipment using a Bio-Rad Biolistic PDS-1000 and according to Twell et al. (1989) and the manufacturer's suggested protocol. Microprojectile bombardment of leaf cells was performed similarly using leaves from 8- to 10-week-old tobacco plants grown in vitro. Rupture discs of 1100 p.s.i. were used for bombardment.

For imaging purposes only, sample was bombarded once. For growth comparison experiments, pollen grain samples were bombarded twice to obtain higher numbers of transformed pollen tubes. Pollen grains were germinated as described above. Bombarded leaves were cultured in basal Murashige and Skoog (1962) medium supplemented with 1% Suc. Observations were made 15 to 20 hr after bombardment. Polyethylene glycol–mediated DNA transformation of protoplasts was performed as described (Chiu et al., 1996) using protoplasts isolated from 8- to 10-week-old tobacco plants grown in vitro and 10 μg of plasmid DNA for each transformation. Transformed protoplasts were cultured for 15 to 20 hr before observation.

Accession Number

The GenBank accession number for *NtRab2* is AF39741.

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