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The Rab GTPase RabA4d Regulates Pollen Tube Tip Growth in *Arabidopsis thaliana* [W]

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During reproduction in flowering plants, pollen grains form a tube that grows in a polarized fashion through the female tissues to eventually fertilize the egg cell. These highly polarized pollen tubes have a rapid rate of growth that is supported by a tip-focused delivery of membrane and cell wall components. To gain a better understanding of how this growth is regulated, we investigated the function *RABA4D*, a member of the *Arabidopsis thaliana* RabA4 subfamily of Rab GTPase proteins. Here, we show that *RABA4D* was expressed in a pollen-specific manner and that enhanced yellow fluorescent protein (EYFP)-RabA4d-labeled membrane compartments localized to the tips of growing pollen tubes. Mutant pollen in which the *RABA4D* gene was disrupted displayed bulged pollen tubes with a reduced rate of growth in vitro and displayed altered deposition of some cell wall components. Expression of EYFP-RabA4d restored wild-type phenotypes to the *raba4d* mutant pollen tubes, while expression of EYFP-RabA4b did not rescue the *raba4d* phenotype. In vivo, disruption of *RABA4D* resulted in a male-specific transmission defect with mutant *raba4d* pollen tubes displaying aberrant growth in the ovary and reduced guidance at the micropyle. We propose that RabA4d plays an important role in the regulation of pollen tube tip growth.

INTRODUCTION

In flowering plants, fertilization is mediated by pollen tubes. These specialized cells undergo a highly polarized form of cell growth termed "tip growth" in which cell expansion occurs only at the very apex of the cell. They exhibit a rapid rate of growth, while at the same time maintaining both their shape and structural integrity. Pollen tube growth is supported by a rapid trafficking of vesicles to deliver membrane and cell wall components to the tips by exocytosis; this is balanced by the retrieval of excess membrane by endocytosis (Picton and Steer, 1983). Both of these processes are regulated by GTPase proteins (reviewed in Krichevsky et al., 2007).

The Rab family of small GTPase proteins are key regulators of membrane trafficking. Their regulatory function is based on a GTP hydrolysis cycle; Rab GTPases are active when bound to GTP and inactive when bound to GDP (Stenmark et al., 1994). In the active form, Rab GTPases are membrane associated and perform their trafficking functions through their interactions with effector proteins (Zerial and McBride, 2001). They are inactivated by the hydrolysis of GTP to GDP and are recycled back to the active form by the exchange of GTP for GDP. Rab GTPases act to regulate vesicle formation, motility, and tethering (Molendijk et al., 2004).

Arabidopsis thaliana has 57 Rab GTPase proteins grouped into eight families based on phylogenic analysis, with members of each family predicted to localize to distinct intracellular compartments (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003). Our work has focused on the RabA family of Rab GTPases, which are predicted to function during membrane trafficking steps associated with the trans-Golgi network (Vernoud et al., 2003).

In *Arabidopsis*, root hairs and pollen tubes expand by tip growth (reviewed in Campanoni and Blatt, 2007). Despite having different origins and functions, both of these cells share a common morphology, elongate by tip growth, and have growth requirements in common, such as a tip-focused calcium gradient and an actin cytoskeleton (for reviews, see Hepler et al., 2001; Samaj et al., 2005; Cole and Fowler, 2006). Additionally, mutants, such as *tip growth defective1* and *kinky pollen*, have defects in both root hairs and pollen tubes (Schiefelbein et al., 1993; Procissi et al., 2003). This suggests the presence of similarities in the method of growth and that at least some components are shared in both cell types. Previously, we found that enhanced yellow fluorescent protein (EYFP)-RabA4b-labeled membrane compartments are tip localized in growing root hair cells and are mislocalized in the root hairs of mutants with defects in root hair tip growth (Preuss et al., 2004; Thole et al., 2008). This suggested that RabA4b-labeled membrane compartments are playing important roles during the tip growth of root hairs. Because pollen tubes, like root hairs, expand by tip growth, we were interested to discover if RabA4b, or other closely related RabA GTPase proteins, were expressed in pollen and were important for the regulation of pollen tube tip growth.

The Rab family of GTPase proteins is known to be important for pollen tube tip growth. In tobacco (*Nicotiana tabacum*), overexpression of active or inactive forms of Rab11b, a tobacco homolog of the *Arabidopsis* RabA1 subfamily, inhibits pollen tube elongation and affects the directionality of pollen tube

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growth (de Graaf et al., 2005). Similarly, overexpression of inactive Rab2, a tobacco homolog of the RabB family in *Arabidopsis*, inhibits the growth of pollen tubes, indicating the importance of multiple Rab GTPase proteins in pollen tube tip growth, presumably due to regulation of distinct stages of membrane trafficking in these cells (Cheung et al., 2002). While previous studies have focused on determining Rab function by overexpressing mutant forms of the protein to induce phenotypic changes, we chose to use a mutant line with disrupted expression of *RABA4D*. Here, we report our findings regarding RabA4d, which is essential for the proper regulation of pollen tube tip growth in *Arabidopsis*. *RABA4D* is expressed specifically in pollen, and EYFP-RabA4d-labeled membrane compartments localize to the tips of growing pollen tubes. The loss of *RABA4D* leads to a disruption of polar growth and altered cell wall patterning in vitro, while in vivo, the loss of *RABA4D* causes a competitive disadvantage for pollen tubes and reduced pollen tube guidance at the micropyle. Together, this indicates that RabA4d plays an important role in the regulation of pollen tube tip growth.

RESULTS

RABA4D Is Expressed in a Pollen-Specific Manner in *Arabidopsis*

In *Arabidopsis*, 26 of the 57 total Rab GTPases are members of the RabA family (Pereira-Leal and Seabra, 2001; Rutherford and Moore, 2002; Vernoud et al., 2003). This represents a large expansion of this class of Rab GTPases when compared with yeast and human Rab GTPase complements, and the increased number of RabA GTPases is conserved across diverse plant species (Pereira-Leal and Seabra, 2001; Zhang et al., 2007). While we previously showed that RabA4b-labeled membrane compartments localized to the tips of expanding root hair cells and recruited effector proteins that play important roles in proper root hair growth (Preuss et al., 2004, 2006), the functions of the vast majority of the other 25 *Arabidopsis* RabA GTPases remain unknown. We were therefore interested in investigating the function of some of the other members of the RabA family of Rab GTPases. In *Arabidopsis*, the RabA4 subfamily consists of *RABA4B* and four other closely related genes. Because *RABA4B* is widely expressed in different *Arabidopsis* tissues and organs, we were interested to discover if the other RabA4 subfamily members had more specific expression and whether these expression patterns may highlight the function of other members of the RabA GTPase family in *Arabidopsis* (Preuss et al., 2004).

The expression pattern of each RabA4 subfamily member was evaluated by RT-PCR. Total RNA was extracted from the roots, leaves, stems, flowers, and mature pollen of 4-week-old wild-type plants. Following cDNA synthesis, the relative expression level of each RabA4 subfamily member in each sample was determined by RT-PCR with gene-specific primers. Amplification of *UBIQUITIN* served as a loading control (Figure 1A). As previously determined, *RABA4B* transcripts were present in all tissues and organs tested with the exception of pollen. Expression of *RABA4A* and *RABA4C* genes were similar to that of *RABA4B*, being expressed in roots, leaves, stems, and flowers,

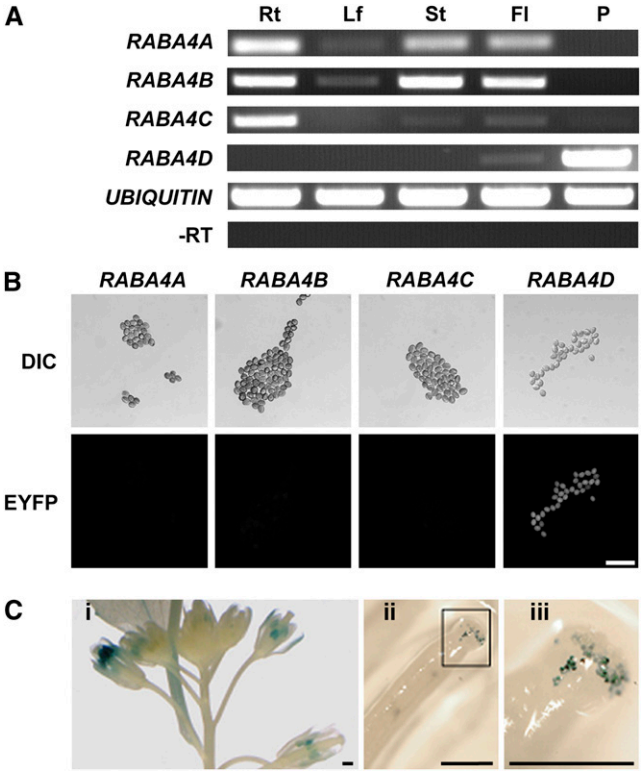

Figure 1. *RABA4D* Is Expressed Specifically in Pollen.

(A) Roots (Rt), leaves (Lf), stems (St), flowers (Fl), and pollen (P) of 4-week-old wild-type plants were collected separately and used for total RNA isolation. RT-PCR analysis of RabA4 subfamily expression was performed with primers specific to *RABA4A*, *RABA4B*, *RABA4C*, and *RABA4D*, with primers specific to *UBIQUITIN* as a loading control. Reactions lacking reverse transcriptase (−RT) were used to detect the presence of genomic DNA. Similar results were obtained in two separate experiments.

(B) Pollen of 4-week-old wild-type plants transformed with promoter:EYFP constructs of RabA4 subfamily members was observed using a Nikon Eclipse using either differential interference contrast (DIC) (top panels) or fluorescence (bottom panels) with appropriate EYFP filters. Bar = 100 μm.

(C) Flowers and inflorescences of 4-week-old wild-type plants expressing $RABA4D_{pro}$:GUS were collected and stained for GUS activity. Images were collected using an Olympus SZX12 (Japan) with a ×1 lens and transmitted light. (i) Inflorescences, (ii) mature flowers, and (iii) pollinated pistils were observed. Panel (iii) shows a close-up view of the pollinated pistil from (ii) and the small black box shows the area of interest. Bars = 500 μm.

although *RABA4C* was expressed much more highly in roots compared with the other tissues and organs. *RABA4D* was expressed only in flowers and in pollen, with the highest expression in pollen. Promoter:EYFP fusions were made for each RabA4 subfamily member and evaluated for their expression in pollen. Approximately two-kilobase regions of promoter sequence of each RabA4 subfamily member were amplified by PCR and cloned into an EYFP fusion vector for expression in wild-type *Arabidopsis*. Pollen from 4-week-old stable transformants

was observed for expression of EYFP. Fluorescence was observed only in the pollen of plants expressing the *RABA4D*$_{pro}$:EYFP fusion construct (Figure 1B).

While the *RABA4D* promoter was expressed in pollen, this does not exclude the possibility that this gene was expressed in other floral tissues in addition to pollen. Due to high levels of autofluorescence, it was difficult to assess the extent of *RABA4D* promoter activity in other floral tissues, so the *RABA4D* promoter was cloned into a promoter:β-glucuronidase (GUS) fusion vector. Inflorescences from 4-week-old stable transformants were stained for GUS reporter gene activity. As GUS activity was only detected in mature stamens and mature pollen grains (Figure 1C), we concluded that *RABA4D* was expressed in a pollen-specific manner.

EYFP-RabA4d Localized to the Tips of Growing Pollen Tubes

Pollen tubes, like root hairs, expand by tip growth. Since the closely related RabA4b localizes to the tips of growing root hairs, we were interested to determine if RabA4d is localized to the tips of pollen tubes. While another RabA family member in tobacco, Rab11b, is present in the tips of pollen tubes, this gene is most similar to the *Arabidopsis* RabA1 subfamily; as a result, we wanted to examine the distribution of RabA4d in pollen tubes (de Graaf et al., 2005). To detect RabA4d localization in pollen tubes, *RABA4D* was PCR amplified and cloned into the *RABA4D*$_{pro}$:EYFP fusion construct and transformed into wild-type *Arabidopsis*. Pollen from 4-week-old stable transformants was germinated in vitro, and pollen tubes were observed with a wide-field fluorescence microscope. EYFP-RabA4d-labeled membrane compartments were tip localized in pollen tubes (Figure 2A). In contrast with the tip localization of EYFP-RabA4d, EYFP alone was diffuse throughout the pollen tube with no distinct structures present (Figure 2A). To determine if the EYFP-RabA4d compartments were associated with the tip region during pollen tube elongation, we used spinning-disk confocal time-lapse microscopy to observe the dynamics of EYFP-RabA4d in growing pollen tubes (Figure 2B; see Supplemental Movie 1 online). EYFP-RabA4d-labeled membrane compartments were tip localized in growing pollen tubes and were present as small punctate structures in the growing pollen tube. While the degree of tip enrichment of the EYFP-RabA4d compartments appeared somewhat reduced versus images collected by conventional fluorescence microscopy, this is likely due to the fact that fluorescence was only collected from a medial optical section in the tips of these pollen tubes rather than the entire apical volume. However, quantification of the percentage of tip-localized EYFP-RabA4d in growing pollen tubes demonstrated that EYFP-RabA4d membrane compartments were indeed enriched in the tips of these pollen tubes and that this localization was maintained during elongation (Figure 2C).

The Disruption of *RABA4D* Expression Leads to Defects in Pollen Tube Tip Growth

If *RABA4D* is important for pollen tube tip growth, then pollen with disrupted expression of *RABA4D* may display tip growth defects. GABI-Kat line 096DO9 contains a T-DNA insertion in *RABA4D* (*raba4d-1*, Figure 3A). We performed RT-PCR analysis on floral cDNA from *raba4d*/*raba4d* and wild-type plants. *RABA4D* transcript was detected in wild-type plants, but not in *raba4d*/*raba4d* flowers, indicating that expression of the *RABA4D* gene was disrupted in this T-DNA insertional mutant (Figure 3B). Mutant plants appeared wild-type in overall appearance, and these plants had full siliques, indicating that the loss of *RABA4D* did not lead to male sterility. While this implied that loss of *RABA4D* does not abolish pollen tube growth, it remained possible that *raba4d* pollen might display defects in development or tube growth. To determine if the morphology or dynamics of *raba4d* pollen tubes were affected, we observed in vitro germinated pollen after 24 h. Wild-type pollen formed relatively long pollen tubes with a regular diameter. By contrast, *raba4d* pollen tubes were shorter than wild-type pollen tubes and frequently exhibited bulging in the tip region. Quantification of the lengths and widths of wild-type and *raba4d* pollen tubes showed significant differences in their morphology (Figures 3C and 3D). Since the loss of *RABA4D* did not abolish pollen tube growth but instead caused the formation of bulged pollen tubes, we were interested to observe the development of this phenotype over time. When wild-type and *raba4d* pollen grains were germinated for increasing intervals of time, the *raba4d* pollen tubes exhibited larger bulges; representative images are shown (Figure 3E). Analysis of the growth dynamics of wild-type (Figure 3F) and *raba4d* pollen tubes (Figure 3G; see Supplemental Movies 2 and 3 online) by time-lapse video microscopy revealed that *raba4d* pollen tubes grew significantly slower than wild-type pollen tubes (Figure 3H). From this we concluded that while loss of *RABA4D* did not lead to abolished pollen tube growth, it did result in pollen tube growth defects.

Expression of EYFP-RabA4d Rescues the *raba4d* Phenotype

While we previously showed that the T-DNA insertion in *RABA4D* disrupted gene expression (Figure 3), it remained a possibility that the observed pollen tube growth defects might be due to secondary mutations. Therefore, we stably transformed homozygous *raba4d* mutant plants with the *RABA4D*$_{pro}$:EYFP-*RABA4D* construct. EYFP-Rab GTPase fusions have been widely used to demonstrate the in vivo distributions of endogenous Rab GTPases, and in general these fusion proteins have been demonstrated to correctly reproduce the subcellular localization of their untagged Rab GTPase counterparts (Chen et al., 1998; Nielsen et al., 1999; Ueda et al., 2001; Preuss et al., 2004). However, to our knowledge, whether these EYFP-Rab fusions can functionally replace endogenous Rab GTPases has not been tested in plants. As a result, we examined the ability of a *RABA4D*$_{pro}$:EYFP-*RABA4D* construct to rescue the *raba4d* mutant phenotype. In homozygous *raba4d* mutant plants heterozygous for expression of EYFP-RabA4d, half of the pollen grains express EYFP-RabA4d and half do not express EYFP-RabA4d. This allowed for a side-by-side comparison of the mutant pollen tubes with the mutant pollen tubes expressing the EYFP-*RABA4D* rescue construct. As shown previously, the *raba4d* pollen (without expression of EYFP-RabA4d) formed short, bulged

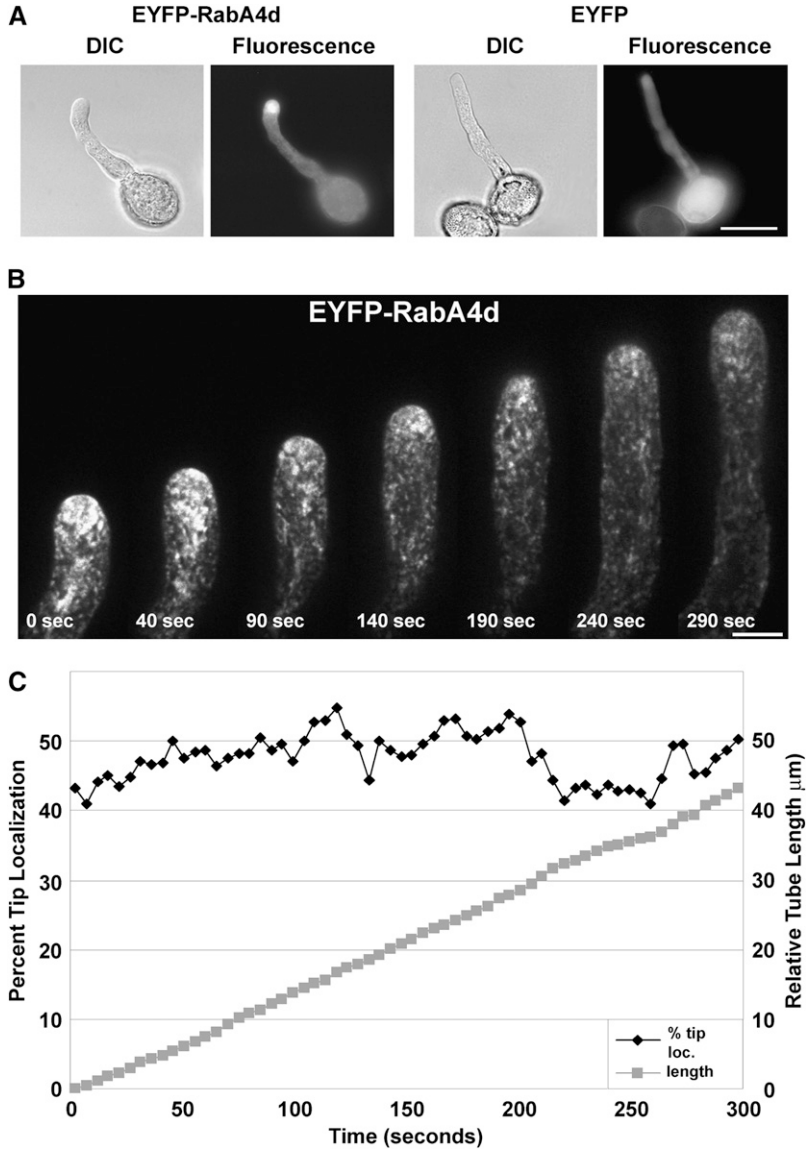

Figure 2. RabA4d Localizes to the Tips of Growing Pollen Tubes.

(A) Germinated pollen of wild-type plants expressing EYFP-RabA4d or free EYFP were observed with a Nikon Eclipse wide-field microscope using DIC or fluorescence with appropriate EYFP filters. Bar = 50 μm.

(B) Time-lapse images of pollen tubes expressing EYFP-RabA4d were collected every 5 s at ×100 (1.46 numerical aperture [NA]) using a Zeiss Observer.A1 confocal microscope, and representative images are shown. Bar = 10 μm.

(C) The percentage of tip-localized EYFP-RabA4d and the relative length of the growing pollen tube were measured. The percentage of tip localization of EYFP-RabA4d was calculated as the percentage of fluorescence signal in the distal 30% of the pollen tube compared with the signal in the entire pollen tube. Fluorescence signal was measured as the sum of the pixel intensities in the area of interest.

pollen tubes (Figure 4A). By contrast, the *raba4d* pollen expressing EYFP-RabA4d formed long pollen tubes of a uniform diameter with tip-localized EYFP-RabA4d (Figure 4A). These pollen tubes were similar in appearance to wild-type pollen tubes. We therefore concluded that the pollen tube defects observed in *raba4d* pollen were due to the loss of *RABA4D* and that EYFP-RabA4d could functionally replace the endogenous RabA4d protein.

Since RabA4b is known to be important for the growth of root hairs, another tip-growing cell type, we investigated whether RabA4b could compensate for the loss of RabA4d in pollen tubes (Preuss et al., 2004). *RABA4B* was PCR amplified and cloned into the $RABA4D_{pro}$:EYFP fusion construct and stably transformed into both wild-type and *raba4d*/*raba4d* plants. We found that EYFP-RabA4b was tip localized in wild-type pollen tubes (Figure 4B). This localization was similar in appearance to that of

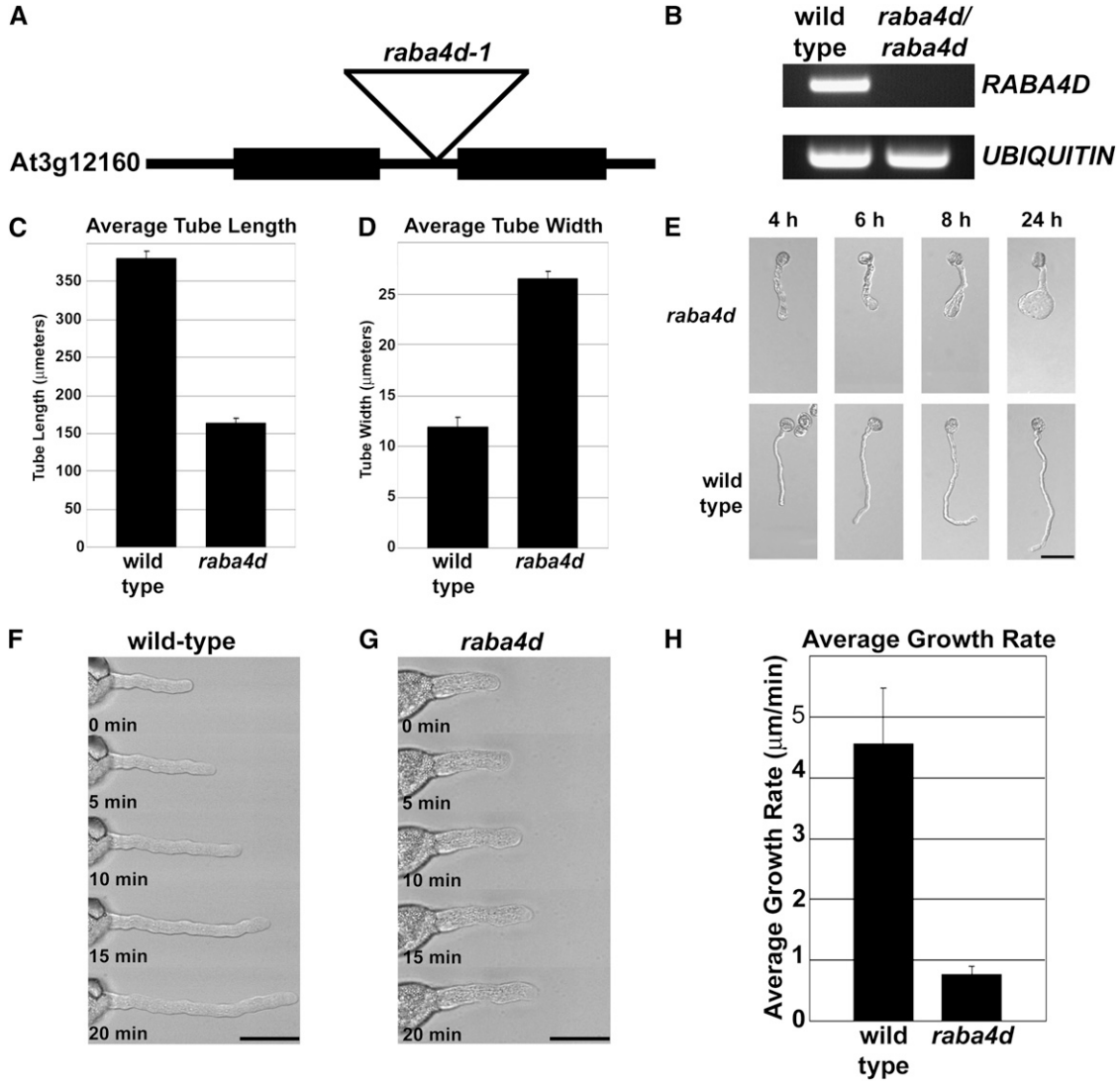

Figure 3. Disruption of *RABA4D* Leads to Abnormal Pollen Tubes in Vitro.

(A) Sequencing of both sides of the *raba4d-1* insertion site confirmed the position of the T-DNA insert within *RABA4D* in intron 1.
(B) Total RNA was extracted from flowers of plants homozygous for an insert in *RABA4D* (*raba4d*/*raba4d*) and from the flowers of wild-type plants and used for cDNA synthesis followed by RT-PCR. *UBIQUITIN* was amplified as a loading control. *RABA4D* transcript was not detected in the cDNA from the flowers of homozygous mutant plants and was detected in the cDNA from the flowers of wild-type plants. Similar results were obtained in three separate experiments. Wild-type and *raba4d* pollen was germinated on slides under high humidity for 24 h. The resulting pollen tubes were imaged with a Nikon Eclipse using DIC optics. Bar = 100 μm.
(C) and **(D)** The lengths **(C)** and widths **(D)** of 120 wild-type and 120 *raba4d* pollen tubes were measured. Bars show SE.
(E) Wild-type and *raba4d* pollen grains were germinated for increasing intervals of time and the resulting pollen tubes imaged. Representative images are shown. Bar = 50 μm.
(F) and **(G)** Time-lapse images of wild-type **(F)** and *raba4d* **(G)** pollen tubes were collected every 10 s. Bars = 50 μm.
(H) The average growth rates of seven wild-type and eight *raba4d* pollen tubes were measured. The *raba4d* pollen tubes had a significantly slower rate of growth than wild-type pollen tubes (P = 0.002). Bars show SE.

EYFP-RabA4d (Figure 2A); however, the average length of mutant pollen tubes expressing EYFP-RabA4b was significantly shorter than that of the wild type (Figure 4C). The widths of *raba4d* pollen tubes expressing EYFP-RabA4d or EYFP-RabA4b were intermediate between that of wild-type and *raba4d* pollen (Figure 4D). Similar data were obtained for three independent YFP-RabA4d and EYFP-RabA4b lines that were chosen for having similar levels of EYFP-Rab expression. We also used time-lapse microscopy to observe the dynamics of the EYFP-RabA4d-complemented pollen tubes and to quantify their growth rates.

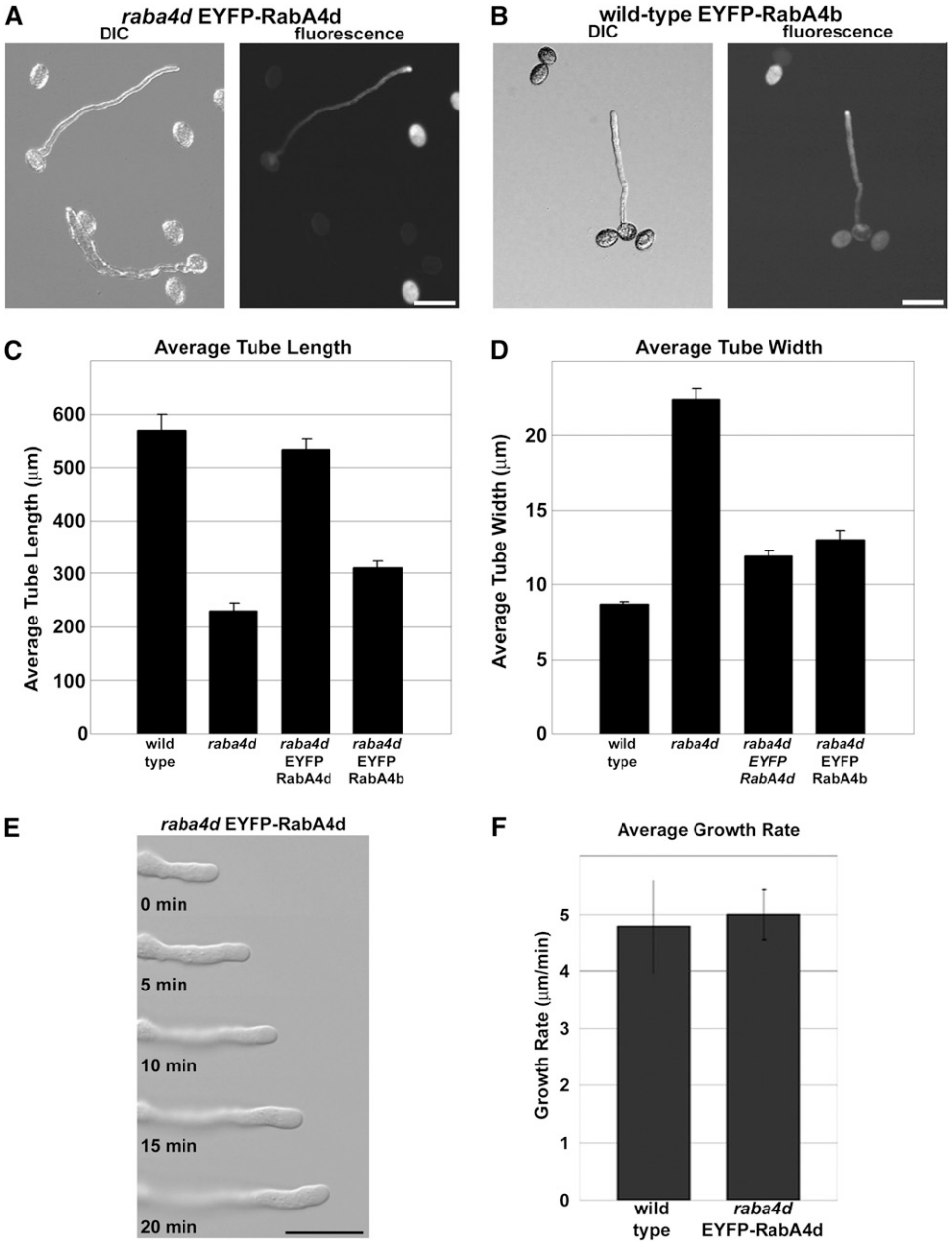

Figure 4. EYFP-RabA4d Complements the *raba4d* Phenotype.

(A) and **(B)** Pollen from homozygous mutant plants heterozygous for expression of EYFP-RabA4d **(A)** or EYFP-RabA4b **(B)** was germinated for 8 h and the resulting pollen tubes imaged using a Nikon Eclipse wide-field microscope using DIC and fluorescence optics. Bars = 50 μm.

(C) and **(D)** Wild-type and *raba4d raba4d* pollen homozygous for expression of EYFP-RabA4d and *raba4d* pollen homozygous for the expression of EYFP-RabA4b was germinated for 24 h and the lengths **(C)** and widths **(D)** of 100 of each kind of pollen tube were measured. Bars show SE.

(E) Time-lapse microscopy was used to observe the dynamics and measure the growth rates of *raba4d* pollen tubes expressing EYFP-RabA4d. Images were collected every 10 s. Bar = 50 μm.

(F) The average rate of growth for seven wild-type pollen tubes and nine *raba4d* EYFP-RabA4d-complemented pollen tubes was measured. The average growth rate of the complemented pollen tubes was 4.98 μm/min, which is not significantly different from that of wild-type pollen tubes ($P > 0.5$). Bars show SE.

We found that the average growth rate of the EYFP-RabA4d-complemented pollen tubes was not significantly different from that of wild-type pollen tubes (Figures 4E and 4F; see Supplemental Movie 4 online). The most likely explanation for the partial rescue of *raba4d* pollen growth defects by EYFP-RabA4b is that there is some functional overlap between RabA4b and RabA4d function during pollen tube tip growth.

The RabA4 Effector Protein PI4Kβ1 Is Required for Normal Pollen Tube Growth

Expression of EYFP-RabA4b resulted in partial rescue of the *raba4d* phenotype. One possible explanation was that RabA4b and RabA4d may have common effector proteins that are important for the tip growth of both pollen tubes and root hairs. The RabA4b effector protein PI4Kβ1 is important for polarized root hair growth as the loss of this gene, and its close relative PI4Kβ2, leads to the formation of abnormal root hairs (Preuss et al., 2006). We wanted to determine if these kinase proteins also had important roles in pollen tube growth. As an initial test, we used a yeast two-hybrid approach to determine if there is an interaction between RabA4d and PI4Kβ1. Previously we showed using both yeast two-hybrid and biochemical methods that the novel homology (NH) domain of PI4Kβ1 interacts with RabA4b in a GTP-dependant manner and that these two proteins colocalize in growing root hairs (Preuss et al., 2006). Active (GTP-bound) and inactive (GDP-bound) forms of RabA4d were used as bait to determine if RabA4d could interact with the NH domain of PI-4Kβ1 by yeast two-hybrid analysis. The NH domain of PI4Kβ1 also associated with RabA4d in a GTP-dependant manner (Figure 5A). To determine if PI4Kβ1 and PI4Kβ2 are important for pollen tube tip growth, pollen from plants homozygous for an insertion in each gene (*b1*/*b1 b2*/*b2*, referred to as *b1 b2*) were germinated in vitro and examined for morphological defects. We found that *b1 b2* mutant pollen tubes were significantly shorter than wild-type pollen tubes (Figures 5B and 5C) and often had a noticeably wavy growth pattern, while the wild-type pollen tubes were relatively straight. To quantify this difference, we measured the angle formed by the initial direction of pollen tube growth and the current direction of the growing tip of the pollen tube (Figure 5D). Quantification of this net angle of pollen tube growth showed that most of the wild-type pollen tubes grew at an angle of 0 to 30 degrees with an average net angle of 30.5 degrees. By contrast, the *b1 b2* mutant pollen tubes curved at an average net angle of 64.5 degrees, which was significantly different from wild-type pollen tubes (Figure 5E). Time-lapse microscopy showed that the *b1 b2* pollen tubes exhibited changes in the directionality of tube growth and had a significantly slower rate of growth than wild-type pollen tubes (Figures 5F and 5G; see Supplemental Movie 5 online). From this we concluded that RabA4b and RabA4d share a common effector that is important for proper regulation of polar tip growth in both root hairs and pollen tubes.

RabA4d Localization Partially Overlaps With RHD4 in Pollen Tubes

We wanted a method to observe the RabA4d compartment in both wild-type and *raba4d* pollen tubes. Since expression of either EYFP-RabA4d or EYFP-RabA4b led to full or partial rescue of the *raba4d* phenotype, this meant that neither of these fusions could serve as suitable markers for tip-localized compartments in *raba4d* pollen. We chose to test if the protein ROOT HAIR DEFECTIVE4 (RHD4) could serve as potential a marker for the RabA4 compartment. RHD4 colocalizes with RabA4b in root hair cells and is important for the control of polarized membrane trafficking in these cells (Thole et al., 2008). To determine if RabA4d and RHD4 colocalize in pollen tubes, we transiently expressed cyan fluorescent protein CFP-RabA4d and EYFP-RHD4 in tobacco pollen using biolistic transformation. Expression of free green fluorescent protein (GFP) alone under the control of the pollen promoter LAT52 was used as a control (Twell et al., 1990). Free GFP alone was diffuse in transformed tobacco pollen tubes (Figure 6A), whereas EYFP-RHD4 (shown in green) and CFP-RabA4d (shown in red) were present on tip-localized compartments and displayed some overlap (Figure 6B). Intriguingly, while both compartments displayed enrichment in the tips of pollen tubes, we did not observe complete overlap of these markers. While ECFP-RabA4d displayed a clear enrichment in tips of tobacco pollen tubes, EYFP-RHD4 displayed less-focused tip localization and was absent from the extreme apex of the growing pollen tube. While RabA4d appeared to display slightly more prominent tip localization in the tobacco pollen tube than previously observed in *Arabidopsis*, this was likely due to increased levels of transient expression. From this we concluded that while RHD4 and RabA4d both display tip localization in pollen, they do not completely colocalize, raising the possibility that there may be distinct subpopulations of tip-localized membrane compartments. However, despite incomplete overlap with RabA4d membranes, the EYFP-RHD4 fusion could be used to monitor whether localization or dynamics of these tip-localized membranes are affected in *raba4d* pollen. EYFP-RHD4 is tip localized in both wild-type and *raba4d* pollen tubes (Figures 6C and 6D; see Supplemental Movies 6 and 7 online). Quantification of the tip localization showed that YFP-RHD4 remained at the tips of growing wild-type and *raba4d* pollen tubes, indicating that localization of these membranes was not significantly different in *raba4d* pollen (Figure 6E).

To investigate whether endocytosis was affected in *raba4d* pollen, we chose to use the dye FM4-64, which is widely used to study endocytosis and vesicle trafficking (reviewed in Bolte et al., 2004). For this experiment, pollen from wild-type plant expressing EYFP-RabA4d was germinated in liquid medium, FM4-64 was added, and the pollen tubes were observed for the localization of EYFP-RabA4d and FM4-64. Surprisingly, we found that EYFP-RabA4d- and FM4-64-labeled compartments did not significantly colocalize (Figure 7A). While we did not observe significant overlap between FM4-64 and EYFP-RabA4d at the tips of pollen tubes, we often observed that FM4-64-labeled compartments occurred in more distal regions of the pollen tube, and these were often closely associated with EYFP-RabA4d (Figure 7A, arrows). However, importantly, this close localization did not overlap, suggesting that while these compartments may be associated with one another, they are likely distinct. In these experiments, FM4-64 did not label tip-localized compartments as has been previously shown in tobacco and lily (*Lilium longiflorum*) pollen (Parton et al., 2001; de Graaf et al., 2005).

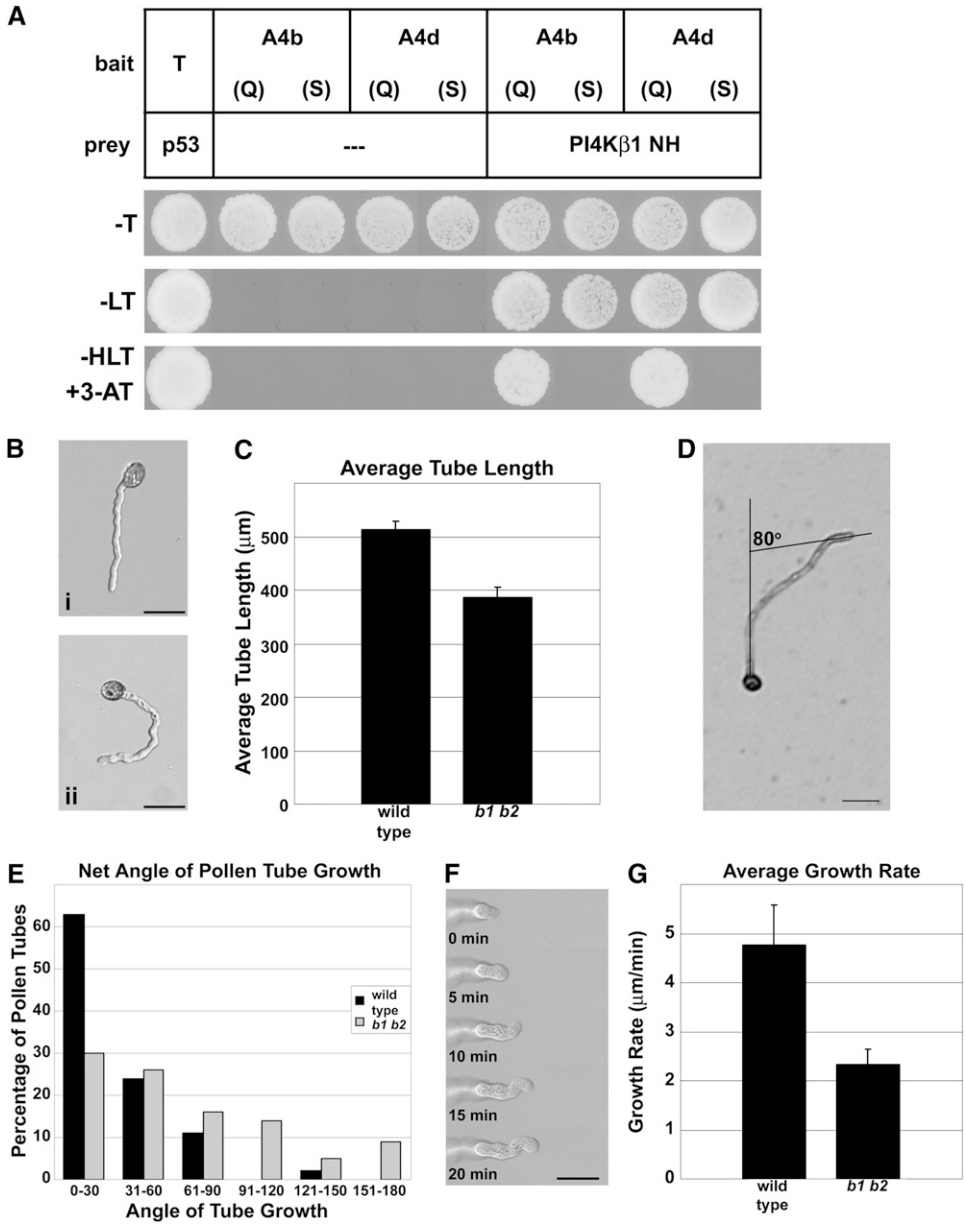

Figure 5. RabA4d Interacts with PI4Kβ1 in Vitro, and the Loss of PI4Kβ1 and PI4Kβ2 Causes Abnormal Pollen Tube Growth in Vivo.

(A) A yeast two-hybrid assay was performed to test the interaction between RabA4d and the NH domain of PI4Kβ1. The interaction between RabA4d and the NH domain was specific for the active form of RabA4d. Interaction with the active form of RabA4b served as a positive control.

(B) Wild-type (i) and *b1 b2* mutant (ii) pollen was germinated for 4 h and observed at ×10 using a Nikon Eclipse with DIC optics. Similar results were obtained for a large number (>50) of wild-type and *b1 b2* pollen tubes. Representative images are shown.

(C) After 24 h of germination time, the lengths of 100 wild-type and 100 *b1 b2* mutant pollen tubes were measured. The *b1 b2* mutant pollen tubes were significantly shorter than wild-type pollen tubes and were noticeably curved in appearance. Bars show SE.

(D) The net angle of growth was measured as the angle formed by the initial direction of pollen tube growth and the direction of the tip of the pollen tube. A sample image is shown.

(E) Quantification of the net angles of pollen tube growth of 100 wild-type and 100 *b1 b2* mutant pollen tubes after 24 h of germination time was measured. The *b1 b2* pollen tubes grew at an average net angle of 64.5 degrees, which was significantly different from the wild-type average of 30.5 degrees.

(F) Time-lapse images of *b1 b2* mutant pollen tubes were collected every 10 s, and the growth rate over time was calculated. Bars = 50 μm.

(G) The growth rates of seven wild-type and four *b1 b2* pollen tubes were measured. The *b1 b2* mutant pollen tubes exhibited a growth rate of 2.3 μm per minute, which was significantly slower than wild-type pollen tubes. Bars show SE (P = 0.05).

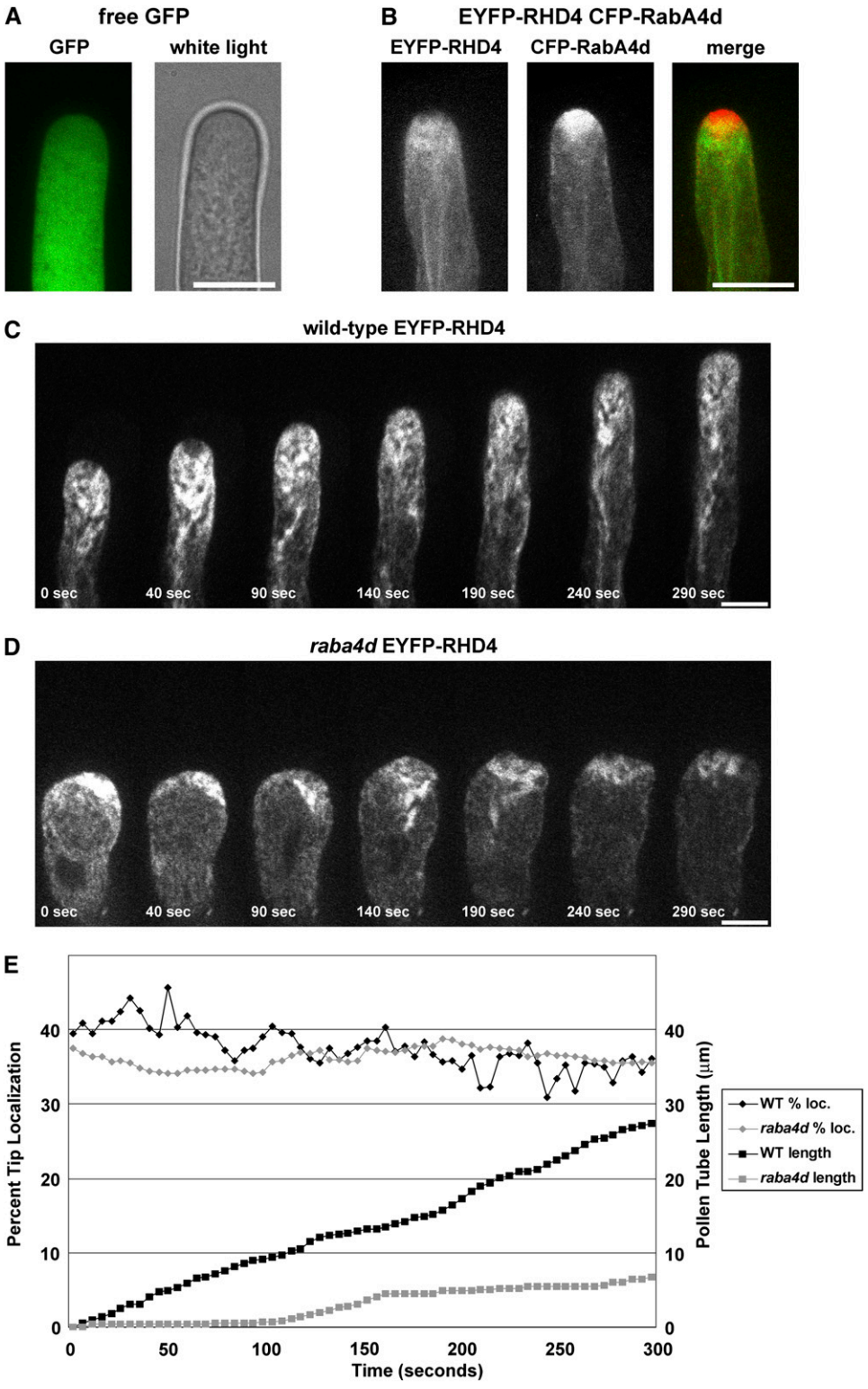

Figure 6. RabA4d Localization Partially Overlaps with RHD4 in Pollen Tubes.

(A) and **(B)** Tobacco pollen was transiently transformed with free GFP **(A)** or EYFP-*RHD4* and CFP-*RABA4D* **(B)**. Images were collected at ×100 (1.46 NA) using a Zeiss Observer.A1 confocal microscope. Medial sections are shown. Bars = 25 μm.

(C) and **(D)** Time-lapse images of stably transformed *Arabidopsis* expressing EYFP-RHD4 in wild-type **(C)** and *raba4d* **(D)** pollen tubes were collected every 5 s at ×100. Representative images are shown. Bars = 10 μm.

(E) The percentage of tip-localized EYFP-RHD4 and the relative length of the growing pollen tubes were measured. The percentage of tip localization of EYFP-RHD4 was calculated as the sum of the pixel intensities in the distal 30% of the pollen tube compared with the sum of the pixel intensities in the entire pollen tube.

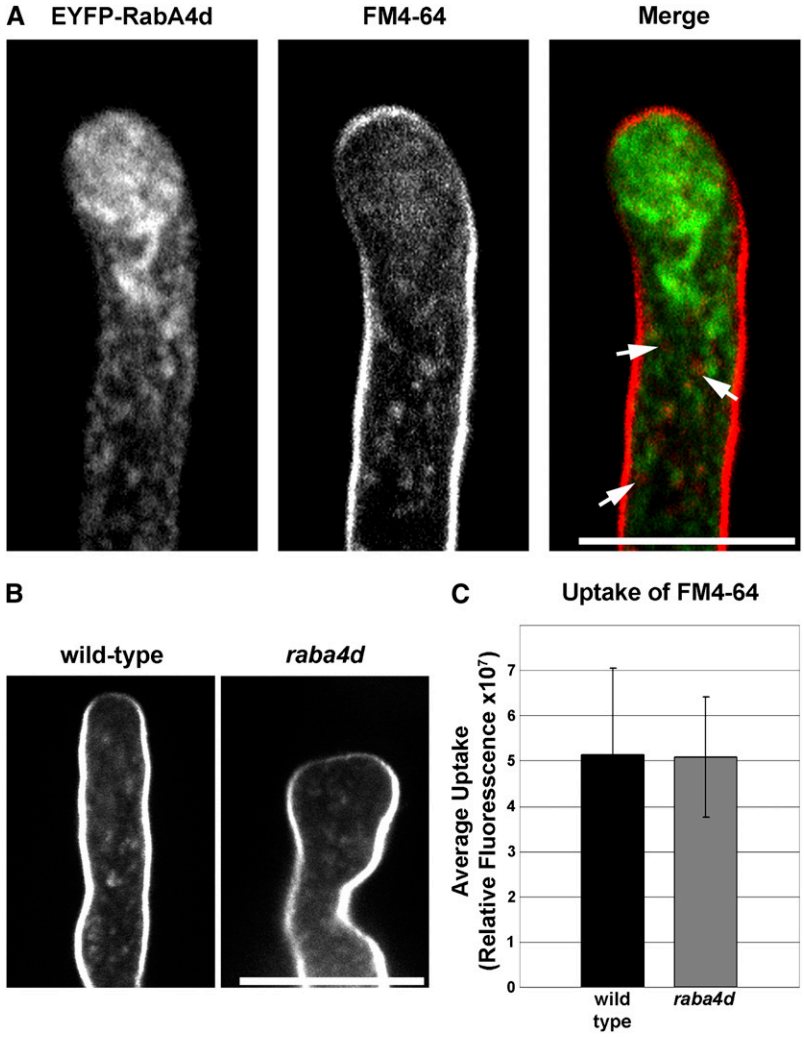

Figure 7. RabA4d Does Not Localize to a Primarily Endocytic Compartment.

(A) Wild-type pollen expressing EYFP-RabA4d was stained with 4 μM FM4-64 and imaged using a Zeiss Observer.A1 confocal microscope. Small arrows show FM4-64–labeled compartments. Bar = 25 μm.
(B) Wild-type and *raba4d* pollen tubes were stained with FM4-64 for visualization of dye uptake. Representative images are shown. Bar = 25 μm.
(C) The average amount of dye internalized in 5 min by eight wild-type and six *raba4d* pollen tubes was measured. Bars show SE. There was no significant difference in dye uptake.

However, we believe this is due to differences in growth between *Arabidopsis* pollen and these other systems because tip-focused labeling of FM4-64 could be observed in tobacco pollen tubes in identical experimental conditions (see Supplemental Figure 1 online). We then observed the uptake of FM4-64 in wild-type and *raba4d* pollen tubes over time. We found that both wild-type and *raba4d* pollen tubes internalized FM4-64 to similar levels (Figures 7B and 7C). Quantification of the amount of internalized dye showed that FM4-64 uptake was time dependent, and we observed no significant difference in the dye uptake of wild-type and *raba4d* pollen tubes (Figure 7D). Taken together, this suggests that, at least in *Arabidopsis* pollen, RabA4d is not present on a compartment that is primarily endocytic in nature.

The Loss of *RABA4D* Leads to Alterations in Deposition of Pollen Tube Cell Wall Components

Since the loss of *RABA4D* led to nonpolar cell expansion rather than a cessation of growth, this implied that the process of cell expansion was misregulated, rather than inhibited. Since polarized pollen tube expansion relies on the deposition of new cell wall materials at the tip of the pollen tube, we decided to observe the composition of the cell wall in wild-type and *raba4d* pollen tubes. One possibility for the bulges observed in the *raba4d* pollen tubes is that the normal pattern of cell wall secretion and modification is altered. To investigate this possibility, we used the carbohydrate antibody JIM7 to localize pectins with a high

level of methylesterification (Clausen et al., 2003). We found that in wild-type pollen tubes, the JIM7 antibody labeled a small crescent at the tip of the pollen tube (Figure 8A), a distribution similar to previous JIM7 labeling results in tobacco pollen tubes (Bosch and Hepler, 2005). In *raba4d* pollen tubes, JIM7 labeling was present on a much larger region of the apex of the pollen tube and also appeared to label more distal regions of the pollen wall (Figure 8B). This labeling was specific, as only weak background fluorescence was observed in wild-type pollen tubes incubated with the secondary antibody alone (Figure 8C). To confirm these findings, we used ruthenium red to stain for pectin in both wild-type and *raba4d* pollen tubes. Ruthenium red stains pectin with a broad range of methylesterification and is used as a general stain for the presence of pectin (Hou et al., 1999). We found that in wild-type pollen tubes ruthenium red primarily stained a small region of the tip of the pollen tube (Figure 8D, arrow). This was similar to our labeling with JIM7. By contrast, *raba4d* pollen tubes exhibited staining in both the tip region (arrows) and in regions distal to the tip (Figures 8E and 8F, arrowheads). This distal staining was present in bulged regions of the pollen tube (Figure 8E) as well as in patches (Figure 8F). These results show that the *raba4d* pollen tubes have an altered localization of pectin compared with wild-type pollen tubes.

While the tip of the pollen tube is primarily formed from pectin, the distal regions of the pollen tube have an additional inner wall composed of callose (Steer and Steer, 1989; Ferguson et al., 1998; Li et al., 2002). We used the callose stain aniline blue to visualize callose distribution in wild-type and *raba4d* pollen tubes (Coleman and Goff, 1985). We found that in both wild-type and *raba4d* pollen tubes, callose was present along the flanks of the tube and was excluded from the apex of the pollen tube (Figures 8G and 8H). From this we concluded that proper trafficking of pectin and/or modification of pectin was altered during cell wall deposition in *raba4d* mutant pollen but that the deposition of callose in the flanks of these pollen tubes was unaffected.

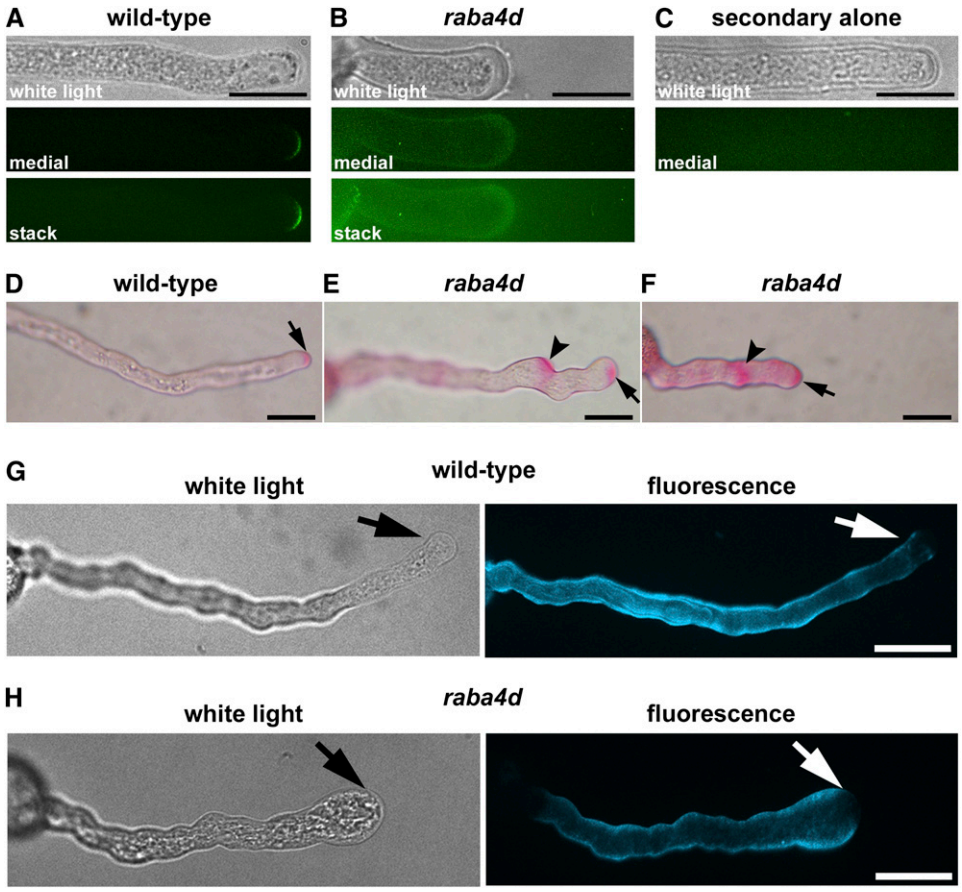

Figure 8. The Loss of *RABA4D* Leads to Alterations in Pollen Tube Cell Wall Patterning.

(A) and **(B)** Wild-type **(A)** and *raba4d* **(B)** pollen was germinated for 4 h, fixed, and labeled with the monoclonal antibody JIM7.
(C) Only background fluorescence was observed in wild-type pollen tubes labeled with the secondary antibody alone. Pollen tubes were observed using a Zeiss Observer.A1 confocal microscope.
(D) to **(F)** Wild-type **(D)** and *raba4d* (**[E]** and **[F]**) pollen tubes were stained with ruthenium red and observed for red staining. Arrows indicate staining at the tip of the pollen tubes, and arrowheads indicate staining in distal regions of the tubes.
(G) and **(H)** Wild-type **(G)** and *raba4d* **(H)** pollen tubes were stained with aniline blue. Arrows indicate the tip region. Bars = 25 μm.

The Loss of *RABA4D* Leads to a Male-Specific Transmission Defect

While we found that *raba4d* pollen tubes had defects in polarized growth in vitro, mutations that disrupt pollen tube growth in vitro do not always have severe phenotypes in vivo (Tian et al., 2006). Therefore, we wanted to investigate if the disruption of *RABA4D* caused defects in pollen tube growth in vivo. To test if the disruption of *RABA4D* caused a transmission defect, the homozygous *raba4d* mutant line was crossed into the *quartet1* (*qrt1*) background, and plants heterozygous for the insert in *RABA4D* and homozygous for the *qrt1* mutation were identified. We used the *qrt1* mutation, in which the meiotic progeny during pollen formation remain associated as tetrads (Preuss et al., 1994; Francis et al., 2006), to ensure that an equal number of *raba4d* mutant and wild-type pollen grains were used for this experiment. This prevents any transmission bias that could arise due to unequal amounts of pollen. When pollen from heterozygous *RABA4D*, homozygous *qrt1* plants was germinated for 8 h, single tetrads produced two pollen tubes that had a wild-type appearance and two pollen tubes that had a *raba4d* mutant appearance (Figure 9A). This indicated that the *raba4d* phenotype was present in the *qrt1* background and that presence of the *qrt1* mutation did not significantly alter *raba4d* mutant phenotypes in vitro. Pollen from heterozygous *RABA4D*, homozygous *qrt1* plants was used to pollinate wild-type stigmas and the resulting cross progeny genotyped. The percentage of wild-type to heterozygous mutant offspring was significantly reduced versus wild-type (Figure 9B). To determine if the observed transmission defect was pollen specific, wild-type pollen was used to pollinate heterozygous *RABA4D*, homozygous *qrt1* stigmas. When the progeny resulting from this cross were genotyped, the percentage of wild-type to mutant offspring was not significantly different from 50 to 50, consistent with the reduced transmission of the *raba4d* mutation being due to defects in the ability of mutant pollen to fertilize the female gametophyte.

Because the loss of *RABA4D* caused the formation of short pollen tubes in vitro and caused a male-specific transmission defect in vivo, we examined the distribution of wild-type and mutant seeds in wild-type siliques fertilized with the pollen of heterozygous *RABA4D*, homozygous *qrt1* plants. When the resulting siliques were mature, but prior to their shattering, the seeds were collected and planted in the order in which they were arranged in the silique. The seeds that formed from ovules closest to the stigma were 45% mutant and 55% wild-type (P = 0.507), while the seeds from ovules farthest from the stigma were 19% mutant and 81% wild-type (P = 0.0002; Figure 9C). The overall percentage of wild-type and mutant *raba4d* seeds was not significantly different from what we observed in the initial competition assay (Figure 9B). From this we concluded that

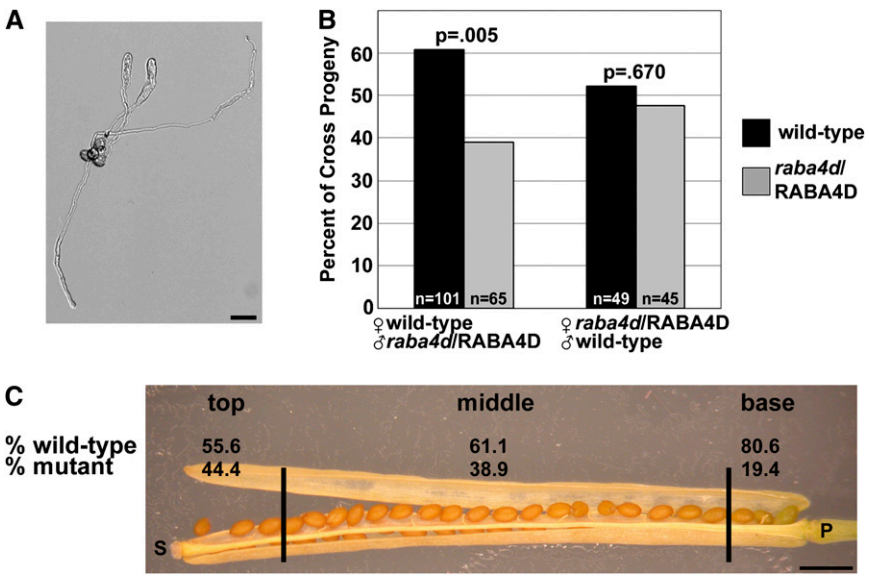

Figure 9. Disruption of *RABA4D* Leads to a Pollen-Specific Transmission Defect.

(A) Plants heterozygous for an insertion in *RABA4D* and homozygous for the *qrt1* mutation were identified and their pollen germinated for 8 h. Bar = 50 μm.

(B) Pollen from heterozygous *RABA4D*, homozygous *qrt1* plants was used to pollinate wild-type stigmas and the resulting cross progeny genotyped. The percentage of wild-type (*RABA4D*/*RABA4D*) to mutant (*raba4d*/*RABA4D*) offspring was significantly different from 50 to 50% (P = 0.005). Pollen from wild-type plants was used to pollinate heterozygous *RABA4D*, homozygous *qrt1* stigmas and the resulting cross progeny genotyped. The percentage of wild-type to mutant offspring was not significantly different from 50 to 50% (P = 0.670).

(C) The arrangement of wild-type and mutant seeds from five crosses between pollen from a heterozygous *RABA4D*, homozygous *qrt1* plant and a wild-type pistil was determined by collecting and planting seeds in the arrangement in which they formed in the silique and genotyping the resulting plants. The overall percentages of wild-type and mutant seeds in the top, middle, and base of a silique are shown above each area. Black bars indicate the divisions between areas. The stigma (S) and pedicle (P) are labeled. Bar = 1 mm.

wild-type and heterozygous seeds are not evenly distributed in the silique, which indicated that *raba4d* pollen tubes have growth defects in vivo and in vitro.

The Loss of *RABA4D* Leads to Abnormal Pollen Tube Growth in Vivo

Because *raba4d* pollen tubes have defects in polarized growth in vitro and have a competitive defect that is indicative of pollen tube growth defects in vivo, we were interested in observing the length and morphology of wild-type and *raba4d* pollen tubes in vivo. Wild-type pistils were pollinated with either wild-type pollen or *raba4d* pollen, fixed and stained with aniline blue, and observed with confocal laser scanning microscopy. We found that 2 h after pollination both wild-type and *raba4d* pollen produced pollen tubes that penetrated the stigmatic tissue and entered the transmitting tract (Figures 10A and 10B). At this point, no significant differences were observed in the maximum length or morphology of wild-type and *raba4d* pollen tubes. The large amount of pollen used in these experiments made it difficult to asses the percentage of pollen grains that germinated; however, both wild-type and *raba4d* pollen produced a mass of pollen tubes that appear similar in overall appearance. Sixteen hours after pollination, both wild-type and *raba4d* pollen tubes had exited the transmitting tract and were observed growing toward ovules (Figures 10C and 10D). Wild-type pollen tubes entered 83% of the ovules, while *raba4d* pollen tubes only entered 47% of the ovules, indicating that the mutant pollen was impaired in this step of fertilization. We classified pollen tubes growing on the funiculus (Figure 10G) and toward the ovule without extensive overgrowth of the pollen tube, such as growing across the surface of the ovule, as having entered that ovule. At this longer incubation time, we also observed occasional bulging at the tip of the *raba4d* pollen tubes (Figure 10E) or aberrant pollen tube growth of the *raba4d* pollen tubes, such as extensive wandering of the pollen tube on the surface of ovules (Figure 10F, arrows). Neither of these defects was observed in pistils pollinated with wild-type pollen. From this we concluded that while *raba4d* pollen tubes are capable of growing to a length equivalent to that of wild-type pollen tubes in vivo, they display abnormal pollen tube morphology and reduced micropyle targeting in vivo.

DISCUSSION

Rab GTPase proteins are important regulators of membrane trafficking. In tip-growing cells, such as pollen tubes and root hairs, certain members of the Rab family of GTPase proteins have been shown to be involved in regulating the polarized growth that occurs in these cells. While members of different Rab families, including several members of the RabA family, and tobacco homologs of the RabA and RabB families, are expressed specifically in pollen, *RABA4D* is the only member of the *Arabidopsis* RabA4 subfamily to be expressed in pollen (Cheung et al., 2002; de Graaf et al., 2005; Pina et al., 2005). It has been proposed that the expansion of the RabA family reflects the complex and often polarized cell wall deposition that occurs in plants (Vernoud et al., 2003), but whether or not members have distinct roles in this process is not yet known. The specific expression of *RABA4D* in pollen and the observation that it is the only RabA4 subfamily member expressed in this cell type provided an opportunity to examine whether this particular RabA GTPase family member fulfilled a nonredundant function in membrane trafficking in pollen tube growth.

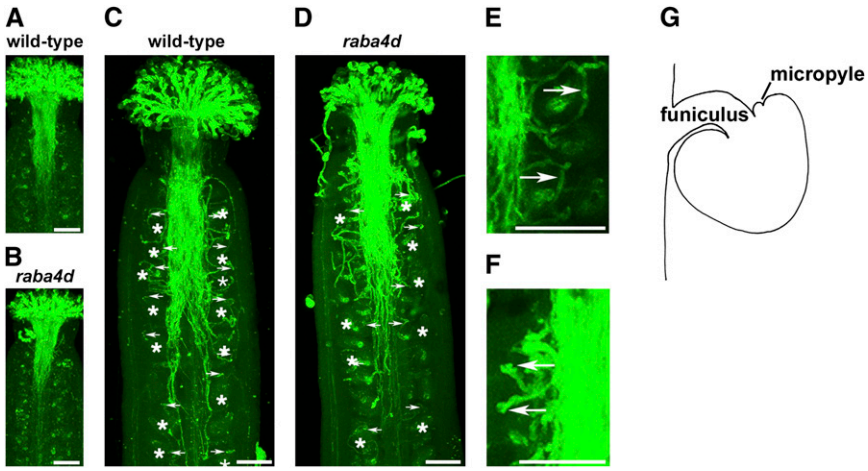

Figure 10. The *raba4d* Pollen Tubes Exhibit Reduced Ovule Targeting and Abnormal Growth in Vivo.

(A) to **(D)** Wild-type pistils were pollinated with wild-type (**[A]** and **[C]**) or *raba4d* (**[B]** and **[D]**) pollen and incubated for 2 h (**[A]** and **[B]**) or for 16 h (**[C]** and **[D]**) before fixation and staining with aniline blue. Asterisks indicate ovules where a pollen tube has entered the micropyle; small arrows indicate the pollen tube of interest. Bars = 100 μm.

(E) and **(F)** The *raba4d* pollen tubes often displayed abnormal growth, such as growing on the surface of ovules **(E)** and forming bulges **(F)**. Bars = 100 μm.

(G) A diagram of ovule structure showing the location of the funiculus and micropyle.

In this study, we demonstrate that RabA4d-labeled membrane compartments are tip localized in growing pollen tubes (Figure 2). Two other Rab GTPase proteins, tobacco Rab11 and *Arabidopsis* RabA4b, localized to the tips of growing pollen tubes and root hairs, respectively, and are important for regulating the polar expansion of these cells (Preuss et al., 2004; de Graaf et al., 2005). Rab11 from tobacco is similar to the RabA1 subfamily from *Arabidopsis*, which is predicted to regulate trafficking in post-Golgi compartments, and colocalizes with an endocytic marker in tobacco pollen tubes, while RabA4b is a close relative of RabA4d and was found to be present on tip-localized compartments in expanding root hairs (Pereira-Leal and Seabra, 2001; Preuss et al., 2004; de Graaf et al., 2005; Chow et al., 2008). Tobacco Rab11 was shown to play a role in the targeting of transport vesicles at the tip of the pollen tube, as overexpression of constitutively active or dominant-negative forms of Rab11 lead to short, twisted pollen tubes (de Graaf et al., 2005). Since RabA4d was present at the tips of pollen tubes (Figure 2B), it is likely that it also plays a role in regulating the tip growth of these cells.

Previously, overexpression of mutant forms of Rab GTPase proteins has been used to study the role of this class of proteins in membrane trafficking, as these mutant proteins can produce phenotypic changes by perturbing wild-type protein function (Stenmark et al., 1994; Kost et al., 1999; Li et al., 1999; de Graaf et al., 2005). However, one potential limitation of this technique is that it can be unclear whether the phenotypic changes observed are due to essential functions provided by these Rab GTPases or if they are a result of novel phenotypes induced by overexpression of the mutant protein. Previous attempts to study Rab GTPase function by isolating insertional mutants in genes of interest have not revealed any observable phenotypes, probably due to a large degree of redundancy in the plant Rab GTPase family (Preuss et al., 2004). In this case, our observation that *RABA4D* was the only member of the RabA4 subfamily expressed in pollen raised the possibility that potential gene redundancy issues previously observed in Rab GTPase knockout plants might not apply to this particular case (Preuss et al., 2004). Indeed, we found that the loss of *RABA4D* led to bulged pollen tubes with a reduced rate of growth in vitro (Figure 3).

Interestingly, loss of RabA4d function resulted in pollen tube growth defects that were distinct from those previously obtained by overexpression of mutant forms of Rab11 from tobacco, in which pollen tube directionality and elongation were inhibited without alterations in width (de Graaf et al., 2005). This supports the possibility that *Arabidopsis* RabA4d may play a distinct role from that of tobacco Rab11 during pollen tube growth. We interpret bulge formation in the *raba4d* pollen tubes as an indication of loss of proper regulation of tip growth rather than overall inhibition of cell expansion in these cells. If the loss of RabA4d activity simply reduced cell expansion, then one would expect overall impairment of pollen tube elongation. Instead, we observed the formation of bulges in pollen tubes, which must be accompanied by delivery of membranes and secretory cargo to the plasma membrane. In the *raba4d* pollen, this delivery appears to occur in an unregulated fashion rather than being restricted to the polarized tip of the elongating pollen tube. Intriguingly, the bulged phenotype we observe in the *raba4d* pollen tubes was similar to that observed in tobacco pollen overexpressing the GTPase protein Rop1 and is also similar to the bulged root hairs of the *root hair defective4* (*rhd4*) mutant, two other instances in which the proper regulation of tip expansion seems to be disrupted (Li et al., 1999; Thole et al., 2008).

Rop1 is normally localized to the plasma membrane at the apex of the pollen tube and Rop1 activity is thought to act as a spatial cue for cell expansion at the apex (Li et al., 1999). In the case of overexpression of Rop1, the bulge is thought to be due to the delocalization of Rop1 activity due to over accumulation of active Rop1 at the tip of the pollen tube (Li et al., 1999; Gu et al., 2003). In the case of *rhd4*, bulge formation is thought to be due to an increase in accumulation of vesicles at the tip of the root hair, which then fuse with the membrane (Thole et al., 2008). Since RabA4d was present on internal tip-localized compartments and the loss of *RABA4D* led to bulge formation, which is indicative of unregulated vesicle delivery, we propose that RabA4d may be involved in regulating vesicle targeting or delivery at the tip of the pollen tube. It may be that the presence of RabA4d on tip-localized compartments helps to direct vesicle fusion to this tip region. In the absence of RabA4d, vesicle fusion is not properly regulated, and this leads to bulge formation.

Expression of EYFP-RabA4d restored wild-type morphology and a wild-type growth rate to *raba4d* pollen tubes (Figure 4). From this we concluded that the observed pollen tube defects are due to the disruption of *RABA4D* and that the EYFP-RabA4d fusion protein is able to act as a functional Rab GTPase protein. We thus showed that an EYFP-Rab fusion can functionally replace the endogenous Rab GTPase protein in plants. We also showed that the closely related RabA4 family member, RabA4b, also localized to the tips of pollen tubes when driven by the *RABA4D* promoter and could partially restore wild-type phenotypes to *raba4d* pollen tubes (Figure 4). Therefore, even though both RabA4b and RabA4d localize to membranes in the tips of pollen tubes, RabA4b function cannot completely replace RabA4d function. At this point we cannot exclude the possibility that other Rab GTPases outside of the RabA family may be capable of compensating for the loss of RabA4d as well. However, the simplest explanation for our results is that RabA4d and RabA4b share some common functions but that they are not completely functionally redundant. We have also demonstrated that RabA4d-labeled membranes display partial overlap with RHD4 in transiently transformed tobacco pollen tubes. However, RHD4, which is necessary for the polar growth of root hairs and colocalizes with RabA4b in these cells (Figure 6; Thole et al., 2008) displays much more significant overlap in the tips of root hairs (Thole et al., 2008). This difference in localization of RabA4d and RabA4b with respect to RHD4 serves to support the idea that these RabA family members may not have completely identical functions.

Rab GTPase proteins carry out their membrane trafficking functions through their interactions with specific downstream effectors (reviewed in Grosshans et al., 2006). The partial rescue of the *raba4d* phenotype by RabA4b led us to investigate whether RabA4d and RabA4d may have some effectors in common that are important for tip growth in both root hairs and pollen tubes. We found that the RabA4b effector, PI4Kβ1, also interacted with RabA4d (Figure 5A). Consistent with this, the loss of PI4Kβ1 and its close relative PI4Kβ2 led to the formation of short, wavy pollen tubes (Figure 5), indicating that as in root hairs,

PI4Kβ activity plays important roles in pollen tube growth, presumably through interactions with RabA4d.

Previously, the RabA GTPase from tobacco, Rab11, was shown to be tip localized in growing pollen tubes and was involved in both vesicle delivery and endocytosis in tobacco pollen tubes (de Graaf et al., 2005). In our experiments, FM4-64 did not label tip-localized compartments as has been previously shown in tobacco and lily pollen (Parton et al., 2001; de Graaf et al., 2005), and did not significantly colocalize with compartments labeled by EYFP-RabA4d in *Arabidopsis* pollen (Figure 7A). However, we believe this is due to differences in growth between *Arabidopsis* pollen and these other systems because we did, in fact, observe tip-focused labeling of FM4-64 in tobacco pollen tubes under identical experimental conditions (see Supplemental Figure 1 online). Tobacco pollen tubes are larger than those of *Arabidopsis* and have a faster rate of growth; therefore, there may be different requirements for the amount and rate of membrane turnover in the apex of these cells.

The walls of pollen tubes have a characteristic organization, with the growing tip composed primarily of pectin and the flanks containing a layer of callose (reviewed in Steer and Steer, 1989). We found that in *raba4d* pollen tubes the normal distribution of pectin was altered such that it was no longer present exclusively in the growing tip (Figures 8A to 8F). We found that callose was present along the flanks of both wild-type and *raba4d* pollen tubes and was excluded from the apical portion of the pollen tubes (Figures 8G and 8H). This suggests that callose and pectin have distinct mechanisms of delivery and that the loss of RabA4d has more dramatic effects on the trafficking of pectin than callose. The loss of detectible callose does not prohibit the formation of pollen tubes with a normal appearance, while the disruption of a single pectin methyl esterase leads to severe pollen tube defects (Nishikawa et al., 2005; Tian et al., 2006). Therefore, our finding that pectin was associated with the bulges observed in *raba4d* mutant pollen tubes fits in with the established importance of pectin in pollen tube growth.

Since the loss of *RABA4D* led to alterations in pollen tube morphology and growth rate in vitro, we wanted to determine if these changes resulted in differences in vivo. We found that the loss of *RABA4D* led to a transmission defect and a competitive disadvantage. These in vivo defects together with the in vitro pollen tube growth rates imply that a reduced rate of growth is an important factor for reduced transmission through the *raba4d* pollen. However, there was no appreciable difference in the length of mutant and wild-type pollen tubes in vivo (Figure 10), implying that within floral tissues, *raba4d* pollen tubes have a similar rate of growth as those of the wild type. Furthermore, examination of seed position data showed *raba4d* pollen tubes were capable of reaching the base of the silique (Figure 9C). The fact that the in vitro phenotype of *raba4d* pollen tubes was not identical to the in vivo phenotype was not completely unexpected. Other *Arabidopsis* pollen mutants, such as *shaker pollen inward k^+ channel* and *pollen-specific pectin methylesterase1*, show more dramatic pollen phenotypes in vitro than in vivo (Mouline et al., 2002; Tian et al., 2006). There are some important differences between these growth conditions that may account for phenotypic variation between in vitro and in vivo pollen tube growth. Pollen tubes in the pistil must grow between maternal tissues and adhere to both maternal cells and their fellow pollen tubes, while pollen tubes grown in culture are not subject to any spatial constraint (Lord, 2000). Thus, the dramatic bulging present in *raba4d* pollen tubes grown in vitro may be due in part to the absence or presence of surrounding cells and by the physical anchoring of the pollen tube to surrounding cells in vivo.

We suggest that, in vivo, any pollen tube elongation defects are exacerbated by the associated guidance defects we observed. Interestingly, the main defects we observed during *raba4d* pollination in vivo were in the ability of *raba4d* pollen tubes to effectively target the micropyle once the tube had reached an ovule (Figure 10). These defects were quite similar to those previously characterized in guidance mutants, which elongate to normal lengths but exhibit chaotic growth within the ovary and do not target the micropyle as effectively as wild-type pollen tubes (Johnson et al., 2004).

Taken together, all these observations suggest that RabA4d function is important for polarized membrane trafficking during tip growth, which is consistent with our earlier observations for RabA4b in root hairs (Preuss et al., 2004). However, based on our observations of defects in the cell wall composition and pollen tube guidance in the *raba4d* mutant pollen, we suggest that, in pollen, RabA4d functions may include roles in pollen tube guidance as well as in selective delivery of cargo to the pollen tube tip.

METHODS

RT-PCR

Five flats of wild-type plants (~150 plants per flat, ecotype Columbia) were grown at 25°C in soil with 16 h of light per day. Roots, leaves, stems, and flowers were collected separately and frozen in liquid nitrogen. Twenty milligrams of pollen was harvested using a vacuum collection system as described by Johnson-Brousseau and McCormick (2004) and frozen in liquid nitrogen. Total RNA was extracted from each sample using the Qiagen RNeasy Kit. cDNA was synthesized from each RNA sample using the Invitrogen SuperScript III first-strand synthesis system for RT-PCR using the oligo(dT) primer according to the kit's instructions. Reactions with primers specific to *UBIQUITIN10* (5′-GATCTTTGCCGGAAAACAATTGGAGGATGGT-3′; 5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′) were used to equalize the amounts of cDNA from the different sources. PCR was performed with GoTaq Green (Promega) using primers specific to *RABA4A* (5′-CTGCGAGTGAAGATCAAGAGA-3′; 5′-GGTTTGTTATACTTGGATTCAAC-3′), *RABA4B* (5′-GGGGTACCATGGCCGGAGGAGGCGGATACG-3′; 5′-CGGGATCCTCAAGAAGAAGTACAACAAGTG-3′), *RABA4C* (5′-CGGGGTACCATGTCAAAATTTCAGAGCAAT-3′; 5′-CGCGGATCCCTATGATGTTCCACAACAACC-3′), and *RABA4D* (5′-CGGGGTACCATGTCTAATTTGTATGGAGAT-3′; 5′-CGCGGATCCTTACGATTTGCCGCAACATCC-3′). Reactions without reverse transcriptase (−RT) were used to detect the presence of contaminating genomic DNA. The amplification program consisted of 30 s at 94°C, 30 s at 58°C, and 1 min at 68°C for 30 cycles, followed by a 10-min extension at 68°C. PCR products were visualized on an agarose gel using ethidium bromide.

Characterization of the *raba4d* T-DNA Insertion Line

The *raba4d-1* T-DNA insertion mutant (line 096DO9) was obtained from the GABI-Kat collection (Rosso et al., 2003). The T-DNA mutant was generated in the context of the GABI-Kat program and provided by Bernd

Weisshaar (MPI for Plant Breeding Research; Cologne, Germany). The T-DNA insertion site in the first intron of *RABA4D* at base 422 of the genomic sequence was confirmed by sequencing both sides of the insertion site using primers specific to the left border of the T-DNA (5′-CCCATTTG-GACGTGAATGTAGACAC-3′) and specific to *RABA4D* (5′-CCGGGAT-CCTCATCCACCTCTTTTCCCACT-3′ and 5′-CCGGGATCCTCATCCAC-CTCTTTTCCCACT-3′). The insertion site consists of a head-to-head insertion of two copies of the T-DNA.

Competition Assay

Wild-type plants and heterozygous *RABA4D* mutant plants homozygous for *qrt1* were grown in soil at 22°C with 12 h of light per day. Reciprocal crosses were performed between wild-type and heterozygous *RABA4D* plants, and the resulting cross progeny genotyped using primers specific to *RABA4D* (5′-CCGGAATTCATGTCTAATTTGTATGGAGAT-3′; 5′-CCGGG-ATCCTCATCCACCTCTTTTCCCACT-3′) and to the T-DNA (5′-CCCATTT-GGACGTGAATGTAGACAC-3′; 5′-GTAAACCTTGGCATATTGTTTCGT-3′). Seed position data were obtained by mounting mature siliques on double-sided tape and removing the seeds in the order in which they were formed in the silique. Seeds were planted in this order and the resulting seedlings genotyped as described above. Statistical analysis consisted of a χ^2 test.

Construction of Plasmids and Transformed Plants

The plant transformation vector pCAMBIA (CAMBIA) was used for the generation of transformed plants. A 1.9-kb region of the *RABA4D* promoter was amplified from wild-type genomic DNA using the primers 5′-TCCGTCTCGAATTCGTGGGAGGTTCCATT-3′ and 5′-CATGACATG-TCTCTTCTTCTTCTTATTA-3′. The product was cloned into the pCAMBIA vector and used to control the expression of EYFP, ECFP, or GUS. *RABA4D* was amplified from wild-type floral cDNA using the primers 5′-CGGGGTACCATGTCTAATTTGTATGGAGAT-3′ and 5′-CGCGGAT-CCTTACGATTTGCCGCAACATCC-3′ and cloned into the pCAMBIA-*RABA4D*$_{pro}$:EXFP vector with EYFP or ECFP at the N terminus. *RABA4B* was amplified as described by Preuss et al. (2004) and cloned into the pCAMBIA-*RABA4D*$_{pro}$:EYFP vector with EYFP at the N terminus. Plants were transformed by dipping in *Agrobacterium tumefaciens* (Clough and Bent, 1998).

GUS Staining of Transformed Plants

Wild-type plants stably transformed with the pCAMBIA-*RABA4D*$_{pro}$:GUS fusion construct were stained for GUS activity as follows. Inflorescences were harvested and vacuum infiltrated for 20 min in a staining solution containing 1 mM Xgluc, 500 μM $K_4Fe(CN)_6$, 500 μM $K_3Fe(CN)_6$, 0.5% Triton x-100, 50 mM Na_2HPO_4, 50 mM $Na_2H_2PO_4$, and 1 mM DTT. Following infiltration, the specimens were incubated in the staining solution overnight at 37°C. The staining solution was removed and replaced with SafefixII (Fisher). SafefixII was vacuum infiltrated for 20 min before being removed and replaced with 70% ethanol. Specimens were cleared with 70% ethanol and viewed using an Olympus SZX12 (Japan) with a ×1 lens and transmitted light.

Growth of Pollen Tubes and Quantification of EYFP-RabA4d and EYFP-RHD4 Localization

Plants were grown in 12-h light cycles at 22°C in soil. Pollen was germinated on slides in *Arabidopsis thaliana* pollen medium consisting of 18% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, and 1 mM $MgSO_4$ in a humid chamber. Single images were obtained using a Nikon Eclipse E600 wide-field microscope with a ×60 (1.2 NA) lens with either DIC or epifluorescence illumination with appropriate EYFP filters. Time-lapse images were obtained at ×100 (1.46 NA) using a Zeiss Observer.A1 confocal microscope with a Hamamatsu C9100-50 camera and a 491-nm laser line excitation and EYFP filters. The percentage of tip-localized EYFP-RabA4d or EYFP-RHD4 was measured using the Volocity program (Perkin-Elmer) as the percentage of fluorescence signal in the distal 30% of the pollen tube compared with the signal in the entire pollen tube. The fluorescence signal was calculated as the sum of the pixel intensities in the area of interest.

Complementation of *raba4d* Mutant Plants

Homozygous mutant plants were transformed with the pCAMBIA-*RABA4D*$_{pro}$:EYFP-*RABA4D* construct or the pCAMBIA-*RABA4D*$_{pro}$:EYFP-*RABA4B* construct. Plants heterozygous or homozygous for expression of EYFP-RabA4d or EYFP-RabA4b were identified by observing pollen grains with epifluorescence illumination and appropriate EYFP filters. Pollen was germinated and imaged using a Nikon Eclipse microscope as described above.

Localization of RabA4d and RHD4

Tobacco plants were grown in soil with continuous illumination. Tobacco pollen was transiently transformed with the plasmids pCAMBIA-*RABA4D*$_{pro}$:CFP-*RABA4D* and pCAMBIA-*RABA4D*$_{pro}$:EYFP-*RHD4* or with the control plasmid pUC-*LAT52*$_{pro}$:GFP. These constructs were transiently expressed in tobacco (*Nicotiana tabacum*) pollen using biolistic transformation with a Bio-Rad PDS-1000 gene gun system at a vacuum of 27 inches Hg and 900 p.s.i. of pressure. Following bombardment, the tobacco pollen was germinated for 7 h in tobacco pollen medium consisting of 18% sucrose, 0.01% boric acid, 5 μM $CaCl_2$, 5 μM $Ca(NO_3)_2$, and 1 mM $MgSO_4$, with a final pH of 6.5. Images were taken using a Zeiss Observer.A1 with a Hamamatsu C9100-50 camera with a ×100 objective (1.46 NA), 440-nm laser excitation, and a 480-nm emission filter for ECFP, and 491-nm laser excitation and a 535-nm emission filter for EYFP.

Immunolabeling of Pollen Tubes

A small droplet (10 μL) of 0.5% polyethyleneimine was placed on microscope slides and allowed to dry. Pollen from six flowers was brushed onto each dried spot of PEI and allowed to adhere for 10 min before the addition of 20 μL of germination medium. Pollen was germinated for 4 h in a humid chamber. Pollen was fixed by the addition of 5% paraformaldehyde in germination medium for 30 min. The fixation was quenched for 15 min by the addition of 50 mM ammonium chloride in germination medium. The pollen was washed three times with plain germination medium and blocked for 1 h in 3% milk in germination medium. The carbohydrate antibody JIM7 (CarboSource) was diluted 1:5 in germination medium containing 3% milk. The pollen was incubated for 1 h in the primary antibody, washed three times with 3% milk in germination medium, and incubated for 1 h in 1:100 anti-RatIgG conjugated to FITC (Sigma-Aldrich) diluted in 3% milk in germination medium. The pollen was washed three times with plain germination medium, mounted in MOVIOL, and viewed using a Zeiss Observer.A1 with a Hamamatsu C9100-50 camera with a ×100 objective (1.46 NA), a 491-nm laser excitation, and a 535-nm emission filter. Z stacks (2 μm thickness) were taken through each specimen. Medial sections and maximum projections are shown.

FM4-64 Staining of Pollen Tubes

Tobacco pollen, wild-type *Arabidopsis*, and *raba4d* mutant pollen were germinated for 2 h. FM4-64 (Molecular Probes) was dissolved in tobacco

or *Arabidopsis* pollen germination medium and added to germinated pollen at a final concentration of 4 μM. Time-lapse images of dye uptake were obtained using a Zeiss Observer.A1 with a Hamamatsu C9100-50 camera with a ×100 objective (1.46 NA) and 561-nm laser excitation. Dye uptake was quantified using Volocity software (Perkin-Elmer) as the sum of the pixel intensities in a 100-μm^2 region in the interior of the pollen tube apex. To account for any differences due to photobleaching, the internalized signal was normalized to the signal in a 10-μm region of the membrane.

Aniline Staining of Pollen Tubes in Vivo

Decolorized aniline blue solution (DABS) was prepared by making a 0.1% solution of aniline blue (Acros Organics), adding 1 M NaOH drop-wise, and incubating the solution overnight at 4°C until it became a clear yellow color.

Following pollination, pistils were removed from the plant and incubated in a humid chamber for the desired length of time. Pistils were fixed for 30 min in a solution of 1:3 glacial acetic acid to ethanol and then cleared overnight (16 h) in 1 M NaOH. Pistils were placed into DABS and allowed to stain for 24 h before being mounted on slides and observed using a Leica DM6000 with a ×10 objective (0.4 NA) and 405-nm laser line excitation. Emission spectra were collected from 440 to 612 nm. Z stacks were taken through specimens. Maximum projections are shown.

Aniline Staining of Pollen Tubes in Vitro

DABS was prepared in *Arabidopsis* pollen germination medium. Wild-type and *raba4d* pollen was germinated for 6 h and stained. Images were collected using a Zeiss Observer.A1 with a Hamamatsu C9100-50 camera with a ×40 objective (1.3 NA) and 440-nm laser excitation with a 480-nm emissions filter. Two-micrometer-thick Z stacks were taken through specimens. Maximum projections are shown.

Ruthenium Red Staining of Pollen Tubes

Ruthenium red (Sigma-Aldrich) was dissolved in *Arabidopsis* pollen germination medium and added to germinated pollen at a final concentration of 0.01%. After staining for 10 min, images were collected using a Leitz Laborlux S scope at ×40 (0.70 NA) and a Kodac MDS290 camera.

Construction of Plasmids for Yeast Two-Hybrid Interaction Analysis

Constitutively active (GTP bound) and dominant-negative (GDP bound) forms of RabA4d were generated using PCR-based methods and cloned into the pGBKT7 bait vector (Clontech Laboratories). The GTP-bound form of RabA4d (Q74 → L) was generated using primers 5′-CATCGTCTCGCCTAGAAAGATATAGGGCGGTGA-3′ and 5′-CATCGTCTCCTAGGCCAGCAGTATCCCAAATCT-3′, and the GDP-bound form of RabA4d (T29 → N) was generated using primers 5′-GGCAAAAATCAGCTTCTTGCTC-3′ and 5′-AACGGCAGAATCACCGATC-3′. The GTP-bound and GDP-bound forms of RabA4b were generated and cloned into the pGBKT7 bait vector as described by Preuss et al. (2006). The NH domain of PI4Kβ1 was cloned into the pGADT7 prey vector as described by Preuss et al. (2006).

Yeast Two-Hybrid Interaction Analysis

The yeast strain AH109 (Clontech Laboratories) was used for testing protein interactions. The pGBKT7-RabA4d clones were transformed into yeast containing the pGADT7-NH domain construct using a polyethylene glycol–mediated transformation method (TRAFCO). Two milliliters of liquid cultures, selected for the presence of the plasmid(s) of interest, were grown for 2 d at 30°C at 260 rpm. These were diluted to an OD_{600} of 0.02, and 10-μL droplets were placed onto selective and nonselective media. Plates were scanned after 7 d of growth at 30°C.

Growth of *b1 b2* Mutant Pollen and Quantification of Net Angle of Growth

Pollen from double mutant plants (as described in Preuss et al., 2006) was germinated in liquid medium as described above. Pollen tube lengths were quantified using Image J. The net angle of pollen tube growth (Figure 5E) was measured as the angle formed by the initial direction of pollen tube growth and the current direction of the growing tip of the pollen tube (as diagramed in Figure 5D).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: RabA4a (At5g65270), RabA4b (At4g39990), RabA4c (At5g47960), RabA4d (At3g12160), and RHD4 (At3g51560).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. FM4-64 Is Tip Localized in Tobacco Pollen Tubes.

Supplemental Movie 1. EYFP-RabA4d Is Localized to the Tips of Growing Pollen Tubes.

Supplemental Movie 2. Wild-Type Pollen Tubes Exhibit a Rapid Rate of Growth.

Supplemental Movie 3. The Disruption of *RABA4D* Leads to a Reduction in Pollen Tube Growth Rate.

Supplemental Movie 4. Expression of EYFP-RabA4d Restores a Wild-Type Rate of Growth to *raba4d* Pollen Tubes.

Supplemental Movie 5. The Disruption of PI4Kβ1 and PI4Kβ2 Leads to a Reduction in Pollen Tube Growth Rate and to Alterations in the Directionality of Pollen Tube Growth.

Supplemental Movie 6. EYFP-RHD4 Is Localized to the Tips of Wild-Type Pollen Tubes.

Supplemental Movie 7. EYFP-RHD4 Is Localized to the Tips of *raba4d* Mutant Pollen Tubes.

Supplemental Movie Legends.

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