

# Subcellular Localization and Functional Analysis of the Arabidopsis GTPase RabE<sup>1[W][OA]</sup>

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Membrane trafficking plays a fundamental role in eukaryotic cell biology. Of the numerous known or predicted protein components of the plant cell trafficking system, only a relatively small subset have been characterized with respect to their biological roles in plant growth, development, and response to stresses. In this study, we investigated the subcellular localization and function of an Arabidopsis (*Arabidopsis thaliana*) small GTPase belonging to the RabE family. RabE proteins are phylogenetically related to well-characterized regulators of polarized vesicle transport from the Golgi apparatus to the plasma membrane in animal and yeast cells. The RabE family of GTPases has also been proposed to be a putative host target of AvrPto, an effector protein produced by the plant pathogen *Pseudomonas syringae*, based on yeast two-hybrid analysis. We generated transgenic Arabidopsis plants that constitutively expressed one of the five RabE proteins (RabE1d) fused to green fluorescent protein (GFP). GFP-RabE1d and endogenous RabE proteins were found to be associated with the Golgi apparatus and the plasma membrane in Arabidopsis leaf cells. RabE down-regulation, due to cosuppression in transgenic plants, resulted in drastically altered leaf morphology and reduced plant size, providing experimental evidence for an important role of RabE GTPases in regulating plant growth. RabE down-regulation did not affect plant susceptibility to pathogenic *P. syringae* bacteria; conversely, expression of the constitutively active RabE1d-Q74L enhanced plant defenses, conferring resistance to *P. syringae* infection.

Eukaryotic cells are compartmentalized by membranes that surround organelles having specific functions. Communication and transport between these membrane-bound compartments are vital to the cell and are accomplished through complex and tightly regulated pathways. Trafficking pathways and their players have been extensively described in yeast and mammalian cells but are still poorly characterized in plants. The main effectors and regulators of these pathways appear to be shared between all eukaryotes. Among these, Rabs are a group of small monomeric GTPases that act as molecular switches to mediate vesicle transport between membrane-bound cellular compartments (Segev, 2001). Rab GTPases participate in vesicle budding from a donor compartment, transport along the cytoskeleton toward a target compart-

ment, and, eventually, tethering and fusion of the vesicles with the target membrane. Like other small GTPases, Rabs alternate between a GTP-bound active form and a GDP-bound inactive form. Their functional specificity is determined, in part, by their unique subcellular distribution (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). The Arabidopsis (*Arabidopsis thaliana*) genome encodes 57 Rab proteins, divided into eight subfamilies (RabA to RabH) based on sequence similarities (Rutherford and Moore, 2002; Vernoud et al., 2003). Recent studies have indicated that members of various plant Rab subfamilies participate in secretion and recycling of cell wall components (Nielsen et al., 2008), apical growth of pollen tubes (de Graaf et al., 2005; Cheung and de Vries, 2008; Cheung and Wu, 2008) and root hair (Preuss et al., 2004, 2006), formation of the phragmoplast in dividing cells (Chow et al., 2008), endocytosis (Ueda et al., 2004), and potentially osmotic stress tolerance (Mazel et al., 2004).

Fungal and bacterial infections in plants are often associated with the activation (or suppression) of extracellular defense responses, including secretion of defense-related proteins and antimicrobial phytoalexins into the apoplast, and the formation of callose-rich cell wall appositions, known as papillae (Snyder and Nicholson, 1990; Snyder et al., 1991; Brown et al., 1995; Soylyu et al., 2005; Field et al., 2006). Deployment of such defenses is expected to be dependent on vesicle trafficking and secretion (Huckelhoven, 2007; Lipka et al., 2007; Robatzek, 2007). Delivery of innate immune receptors to the plasma membrane and ligand-induced immune receptor endocytosis also play a

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role in plant response to pathogen-derived elicitors (Robatzek et al., 2006). Activation and integrity of the secretory pathway have been shown to be critical for resistance against potential pathogens (Collins et al., 2003; Assaad et al., 2004; Wang et al., 2005; Kalde et al., 2007). However, successful pathogens have evolved virulence effectors that inactivate vesicle traffic-associated regulators (Nomura et al., 2006; Robatzek, 2007). Detailed molecular mechanisms underlying vesicle trafficking leading to defense, and the specific cargos transported by these vesicles, have yet to be elucidated.

*Pseudomonas syringae* pv *tomato* strain DC3000 (*Pst* DC3000) is a Gram-negative bacterium that causes bacterial speck of tomato (*Solanum lycopersicum*) and Arabidopsis. Like other Gram-negative bacterial pathogens of plants and animals, *Pst* DC3000 delivers virulence effector proteins directly into the host cell via the type III secretion system (TTSS; Buttner and Bonas, 2003; Alfano and Collmer, 2004; He et al., 2004). These proteins, collectively called TTSS effectors, alter host cellular processes to ultimately favor pathogen growth and promote disease. To carry out virulence functions, bacterial effectors of plant and animal pathogens interact with and often biochemically modify key regulatory components of basic host cellular functions. A major virulence activity of TTSS effectors of *P. syringae* seems to be suppression of host defenses (Nomura et al., 2005; Abramovitch et al., 2006; Desveaux et al., 2006; Bray Speth et al., 2007; Block et al., 2008). For example, the *Pst* DC3000 TTSS effector AvrPto compromises Arabidopsis basal defenses and mitogen-activated protein kinase signaling and promotes Arabidopsis susceptibility to nonpathogenic bacteria such as TTSS-defective mutants and to nonhost pathogens such as *P. syringae* pv *phaseolicola* (Hauck et al., 2003; He et al., 2006). Subsequent studies show that AvrPto physically interacts with the flagellin receptor FLS2 (Xiang et al., 2008) and with its signaling partner BAK1 (Chinchilla et al., 2007; Shan et al., 2008). These results suggest that AvrPto could directly interfere with microbe-associated molecular pattern signaling, thereby blocking downstream plant basal defense responses.

A previous yeast two-hybrid (Y2H) screening of a tomato cDNA library for AvrPto-interacting proteins yielded two small GTPases, named Api2 and Api3 (Bogdanove and Martin, 2000), which are similar to the mammalian protein Rab8 (Huber et al., 1993). Rab8 and its yeast orthologues, Ypt2 of *Schizosaccharomyces pombe* (Craighead et al., 1993) and Sec4p of *Saccharomyces cerevisiae* (Goud et al., 1988), are extensively characterized regulators of polarized vesicle transport from the trans-Golgi network to specific regions of the plasma membrane (PM). The closest homologues of Rab8 in Arabidopsis are the five members of the RabE family of GTPases (Rutherford and Moore, 2002; Vernoud et al., 2003; Supplemental Fig. S1). As described in "Results," we independently isolated a RabE GTPase in a Y2H screen for AvrPto-interacting Arabidopsis proteins.

The identification of Rab GTPases as AvrPto interactors in both tomato and Arabidopsis suggested that, as part of its virulence mechanism, this effector may perturb intracellular vesicle trafficking in the plant (Bogdanove and Martin, 2000). Interestingly, small GTPases regulating cytoskeleton dynamics and membrane trafficking are among the most common host targets of TTSS effectors produced by bacterial pathogens of animals (Harrison et al., 2004; Machner and Isberg, 2006; Murata et al., 2006; Rzomp et al., 2006; Smith et al., 2007). The biological roles of tomato Api2 and Api3 and Arabidopsis RabE GTPases in plant development and defense are unknown. Localization and function of RabE proteins have only recently begun to be investigated. Arabidopsis yellow fluorescent protein-RabE1d, transiently expressed in heterologous tobacco (*Nicotiana tabacum*) epidermal cells, was detected in the Golgi apparatus and in the cytoplasm, and functional analyses indicated that this protein participates in promoting post-Golgi secretory trafficking (Zheng et al., 2005). To gain further insights into the RabE GTPase subcellular localization in native Arabidopsis cells and their function in growth, development, and/or defense at the whole plant level, we generated transgenic plants constitutively expressing GFP-fused wild-type RabE1d and RabE1d-Q74L (predicted to be preferentially GTP bound and therefore in an active state) and examined these plants for morphological and developmental phenotypes and response to *P. syringae* infection.

## RESULTS

### Identification of Arabidopsis RabE Proteins as Y2H Interactors of AvrPto

We conducted a Y2H screening of two separate Arabidopsis cDNA libraries, using AvrPto as bait. Several AvrPto-interacting Arabidopsis proteins were identified, including a member of the RabE family of small GTPases (At5g59840), a putative cytoplasmic kinase (At4g11890), an auxin signaling repressor, IAA7 (At3g23050), two hypothetical proteins (At3g26600 and At5g16840), and several putatively chloroplast- or mitochondria-targeted proteins. The interaction with the small GTPase RabE was particularly interesting because RabE was predicted to be membrane localized, as is AvrPto (Shan et al., 2000; He et al., 2006), and, as mentioned above, because screening of a tomato cDNA library had also yielded two AvrPto-interacting proteins homologous to Rab8 (Bogdanove and Martin, 2000), the mammalian counterpart of Arabidopsis RabE.

To characterize the specificity of the AvrPto-RabE interaction, we investigated whether AvrPto interacts with other members of the RabE family and with other Rab proteins. Of the other Arabidopsis Rab protein families (i.e. RabA to -D, -F, and -G), we cloned and expressed in yeast *RabA1a*, *-B1b*, *-C1*, *-D2a*, *-F2a*, and

-G3a; none of these representatives interacted with AvrPto in the Y2H system (Fig. 1A). Of the five *RabE* genes, all but *RabE1c* were successfully cloned and expressed in yeast. All four RabE proteins tested (RabE1a, -b, -d, and -e) interacted with AvrPto (Fig. 1B). Thus, it appears that AvrPto interacts specifically with the Arabidopsis RabE family of GTPases.

Small GTPases normally cycle between the active GTP-bound and the inactive GDP-bound states. Mutation of highly conserved residues can be used to alter the nucleotide binding and hydrolysis activities of Rab proteins (Nielsen et al., 2008; Supplemental Fig. S1). The mutant forms RabE1d-S29N (predicted to be GDP bound) and RabE1d-Q74L (predicted to be GTP bound) were engineered. In the Y2H system, AvrPto interacted only with wild-type RabE1d or with GTP-restricted RabE1d-Q74L but not with GDP-restricted RabE1d-S29N (Fig. 1C), suggesting that AvrPto preferentially binds to the active form.

### RabE Gene Expression in Arabidopsis Leaves

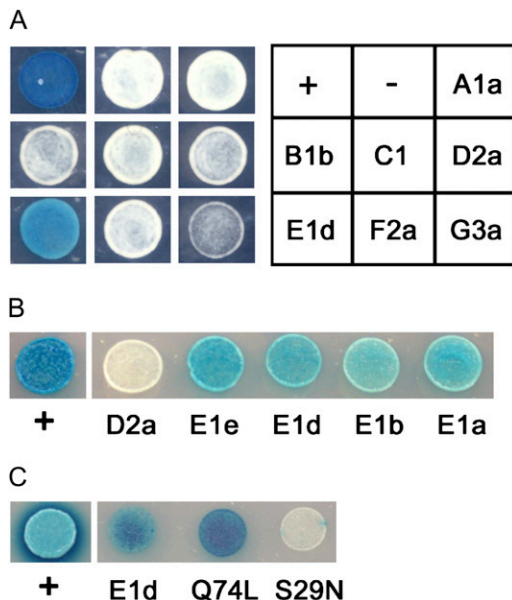
Analysis of the Arabidopsis genome showed that 44 of the 57 *Rab* genes reside in duplicated regions. Of the

five *RabE* genes, *RabE1d* and *-E1e* appear to be derived from a major duplication event between chromosomes III and V, and the same holds true for RabE1b and *-E1c* (Rutherford and Moore, 2002; Fig. 2A). The high degree of sequence identity among the five RabE proteins, equal to or higher than 86%, suggests functional redundancy. However, members of a gene family could be preferentially expressed in different tissues, at specific developmental stages, or in response to stresses. We used the Genevestigator Gene Atlas Tool (Zimmermann et al., 2004) to investigate whether this is the case with the five *RabE* genes. The in silico analysis revealed that all five genes are expressed in all Arabidopsis tissues and developmental stages. *RabE1d* and *-E1e* (encoding 94% identical proteins) are the only two gene family members whose expression is much lower in pollen than in all other tissues. Based on publicly available microarray data (Genevestigator; Zimmermann et al., 2004), *RabE1d* appeared to be the most highly expressed *RabE* gene in Arabidopsis ecotype Columbia (Col-0) rosette leaves, followed by *RabE1c*. The *RabE1a*, *-E1e*, and *-E1b* genes had the lowest expression levels (Fig. 2B). We conducted reverse transcription (RT)-PCR analysis on rosette leaf total RNA and confirmed a high expression level for *RabE1d* (Fig. 2C).

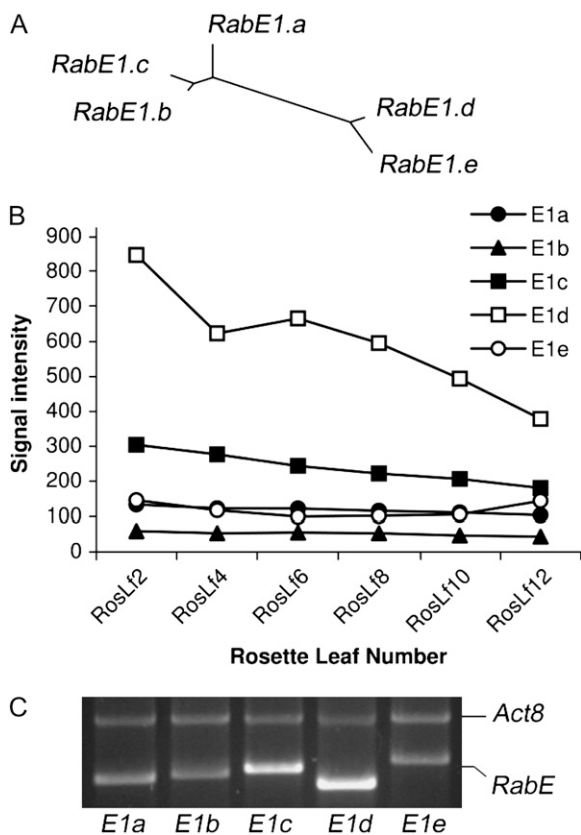
To investigate potential up- or down-regulation of the *RabE* family members in response to pathogens or other stresses, we analyzed the expression patterns of the *RabE* genes with the AtGenExpress Visualization Tool (<http://jsp.weigelworld.org/expviz/expviz.jsp>; Schmid et al., 2005) across several publicly available microarray data sets. None of the five genes appeared to be significantly (more than 2.5-fold) up- or down-regulated in response to pathogens or elicitors. In the absence of any expression-based indication of whether the five RabE proteins have redundant or distinct functions, we chose the highly expressed *RabE1d* (At5g03520) for further study.

### RabE1d Is Associated Not Only with the Golgi Apparatus But Also with the Plasma Membrane in Arabidopsis Leaf Cells

All Rab proteins are normally present in cells in two pools, one cytoplasmic and the other membrane associated (Novick and Brennwald, 1993). Nucleotide-binding state and interaction with accessory proteins determine whether a Rab is in the cytosol or the membrane at any given time. A hallmark feature of Rab proteins is that they localize to the specific membrane compartments in which they function. It was previously reported that Arabidopsis RabE1d, when transiently and heterologously expressed in tobacco epidermal cells as a fusion with yellow fluorescent protein, was detected in the Golgi apparatus and in the cytoplasm (Zheng et al., 2005). To determine RabE localization in native Arabidopsis cells, we created stable transgenic plants that express RabE1d fused with enhanced GFP under the control of the caulic-



**Figure 1.** Arabidopsis RabE proteins interact with *P. syringae* AvrPto in the Y2H system. Interaction is visualized by development of blue color on medium containing X-Gal. Negative control (–) = pNLexA (bait) and pB42AD (prey) vectors; positive control (+) = pLexA-A53 and pB42AD-T. A, AvrPto (in pNLexA) interacts with Arabidopsis RabE1d but not with other members of the Rab superfamily (in pB42AD). B, AvrPto (in pNLexA) interacts with all four tested Arabidopsis RabE proteins (in pB42AD). Yeast expressing AvrPto in pNLexA and RabD2a in pB42AD is shown as a negative control. C, AvrPto (in pB42AD) interacts with RabE1d or RabE1d-Q74L but not with RabE1d-S29N. For this experiment only, wild-type RabE1d and the mutated RabE1d proteins were cloned in the bait vector pGILDA and modified by replacing the two C-terminal conserved Cys residues (sites of geranylgeranylation [Supplemental Fig. S1]) with Gly and Ser to prevent prenylation and membrane association.



**Figure 2.** A, Phylogenetic relationship among RabE family members; *E1d* and *E1e*, as well as *E1b* and *E1c*, are predicted to be the result of genome duplication events. B, E-northern of *RabE* gene expression in Arabidopsis Col-0 rosette leaves at different developmental stages. Data were obtained from AtGenExpress at the Genevestigator site (<https://www.genevestigator.ethz.ch/>); signal intensities were averaged across three biological replicates. C, RT-PCR showing *RabE* gene expression in rosette leaves. RT-PCR products represent the five *RabE* genes, reverse transcribed and amplified from Arabidopsis Col-0 *gl1* rosette leaf total RNA. A single RT reaction was performed, and equal amounts of the resulting cDNA were used as template in PCR. Each PCR sample contained primers for the *Actin8* gene in addition to primers for one of the *RabE* genes (primers are listed in Supplemental Table S2). Equal volumes of each PCR product were loaded on a 1% agarose gel.

flower mosaic virus 35S promoter. By convention, GFP was fused to the N terminus of RabE1d to preserve the C-terminal CAAX geranylgeranylation site that is critical for membrane association and function (Calero et al., 2003; Supplemental Fig. S1). Several independent transgenic lines were analyzed by confocal laser scanning microscopy (CLSM). GFP fluorescence was observed not only in intracellular punctate structures, consistent with the Golgi apparatus, as detected in heterologous tobacco cells (Zheng et al., 2005), but also prominently at the cell periphery (Fig. 3, A–E).

Leaf epidermal cells typically contain a very large vacuole that accounts for most of the cell volume. Fluorescence detected at the cell periphery may rep-

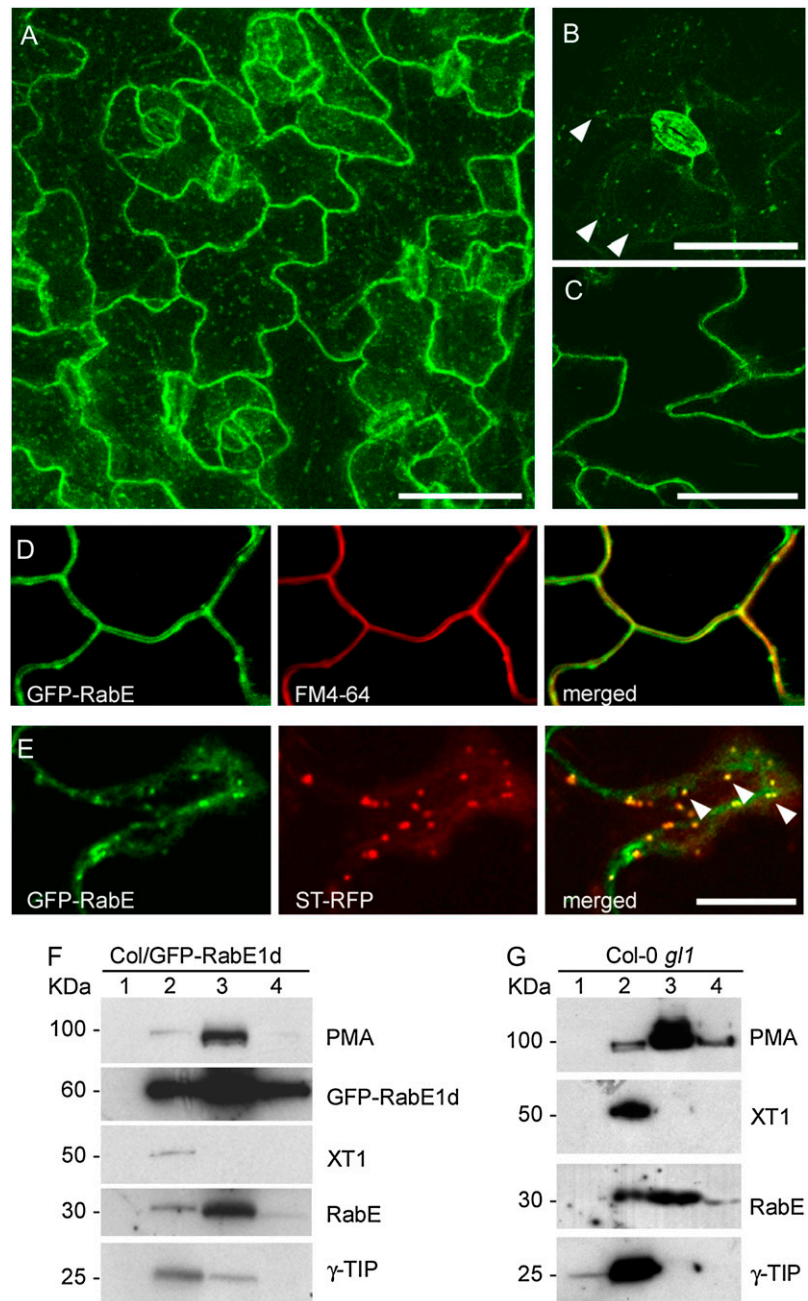
resent the PM, the vacuolar membrane (tonoplast), or the thin layer of cytoplasm that is between the PM and the tonoplast. To more precisely determine whether GFP-RabE1d was also localized at the PM, we stained live leaf tissue with the lipophilic dye FM4-64 (Fischer-Parton et al., 2000; Bolte et al., 2004). FM4-64 produces a bright red fluorescence in membranes but not in aqueous solutions and is rapidly incorporated into the PM. It is often used in microscopy as an endocytic tracker, because it is retained in the portions of PM that are internalized by endocytosis (Ueda et al., 2001). Within the first 15 to 30 min after incorporation (depending on the system used), FM4-64 selectively stains the PM. CLSM analysis revealed a precise overlap of GFP-RabE1d fluorescence with FM4-64 fluorescence immediately after staining, indicating that RabE1d is associated with the plasma membrane (Fig. 3D).

To investigate whether the punctate structures labeled by GFP-RabE1d correspond to the Golgi apparatus, we examined colocalization with rat sialyl transferase, a Golgi marker protein (Wee et al., 1998), fused to DsRed (ST-RFP). ST-RFP was transiently expressed in the GFP-RabE1d transgenic leaves via particle bombardment. Observation of cells coexpressing GFP-RabE1d and ST-RFP revealed overlapping fluorescence signals, confirming RabE1d's association with the Golgi apparatus (Fig. 3E). Taken together, our analysis of GFP-RabE1d localization revealed that, in the native Arabidopsis leaf cells, membrane-associated RabE1d is found not only in the Golgi apparatus, as reported previously in heterologous tobacco cells, but also in the PM.

#### Endogenous RabE Proteins Cofractionate with PM and Golgi Markers

GFP-RabE1d expression in the transgenic plants was driven by the cauliflower mosaic virus 35S constitutive promoter. To exclude the possibility that the observed RabE localization reflects patterns of only the transgenically expressed protein, we conducted additional analyses to determine the localization of endogenous RabE in transgenic as well as wild-type Arabidopsis plants. We performed subcellular membrane fractionation by centrifugation of clarified plant extracts on Suc step gradients (Zeng and Keegstra, 2008). This method allowed separation of the PM from fractions containing lighter membranes (Golgi, tonoplast). Western-blot analysis with an anti-RabE polyclonal antibody that reacts with all RabE family members revealed that the bulk of membrane-associated endogenous RabE proteins, as well as transgenically expressed GFP-RabE1d, were in the same fraction as the PM marker  $H^+$ -ATPase (Morsomme et al., 1998). A lower amount of endogenous RabE, as well as GFP-RabE1d, cofractionated with xylosyl transferase (XT1), a Golgi apparatus resident protein (Faik et al., 2002; Cavalier and Keegstra, 2006; Zeng and Keegstra, 2008; Fig. 3, F and G). A tonoplast marker,  $\gamma$ -tonoplast

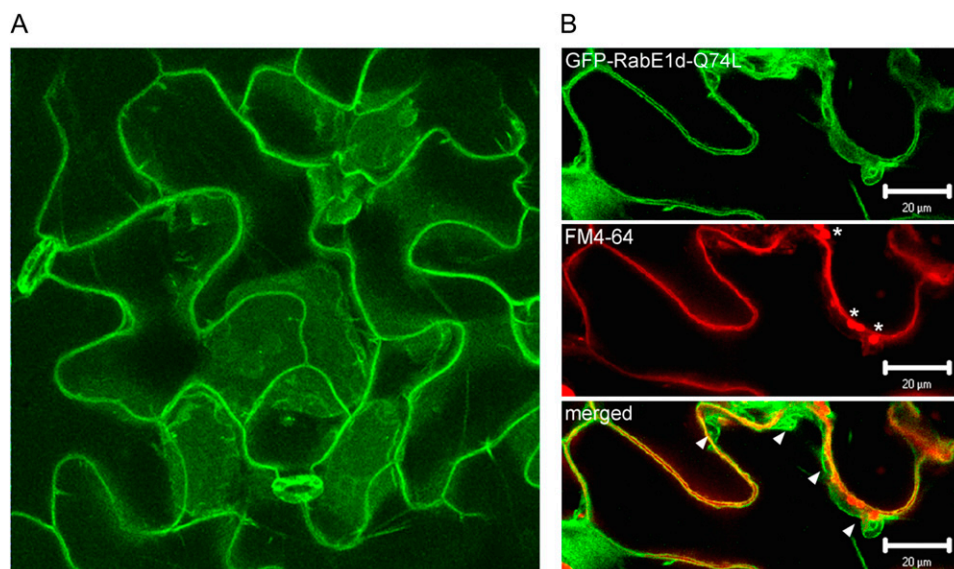
**Figure 3.** GFP-RabE1d localization in Arabidopsis leaf cells. Confocal microscopy images of the leaf epidermis of transgenic Arabidopsis plants expressing GFP-RabE1d are shown. A, Projection along the z axis of several optical planes intersecting the leaf epidermal cell layer. GFP-RabE1d is visible in intracellular punctate structures and at the cell periphery. B, Single focal plane intersecting a stomate and the cortical layer of epidermal cells. Arrowheads point at intracellular punctate structures. C, Single focal plane intersecting epidermal cells. The same pattern of distribution of GFP-RabE1d was observed in at least five independent transgenic lines. Bars = 50  $\mu$ m. D and E, Confocal microscopy images illustrating GFP-RabE1d colocalization with the FM4-64 dye at the plasma membrane (D) and with sialyl transferase (ST-RFP) at the Golgi apparatus (E). D, Single focal plane crossing adjacent epidermal cells (40 $\times$  oil-immersion objective, 4 $\times$  zoom). Left, GFP-RabE1d fluorescence, green; center, FM4-64 fluorescence, red; right, merged image, in which the yellow color results from the overlap of red and green. E, Single focal plane crossing the cytoplasm of a cell (40 $\times$  oil-immersion objective, 4 $\times$  zoom). Left, GFP-RabE1d fluorescence, green; center, ST-RFP fluorescence, red; right, merged image, in which the yellow color results from the overlap of red and green. Arrowheads point at some of the colabeled Golgi stacks in the merged image. Bars = 10  $\mu$ m. F and G, Western blots of cellular membrane fractions separated by ultracentrifugation on a Suc step gradient. Two different samples are shown: transgenic Arabidopsis overexpressing GFP-RabE1d (F) and nontransgenic Arabidopsis Col-0 *gl1* (G). Lanes 1 through 4 represent the four membrane fractions collected at the interfaces between layers of different Suc concentrations: 18% to 25% (lane 1), 25% to 34% (lane 2), 34% to 40% (lane 3), and 40% to 50% (lane 4). Equal volumes of each fraction were loaded on SDS-PAGE gels. PM-ATPase (PMA) is a plasma membrane marker, XT1 is a trans-Golgi resident protein, and  $\gamma$ -TIP is a marker for the tonoplast. GFP-RabE1d and endogenous RabE proteins were detected with a polyclonal anti-RabE antibody (courtesy of Dr. K. Nomura).



integral protein ( $\gamma$ -TIP), was found predominantly in the same fraction as the Golgi marker. Independent membrane fractionation experiments yielded similar results, although minor differences in protein levels and distribution among fractions were occasionally observed across western blots as a result of interexperiment variability. Overall, the membrane fractionation experiments complemented the live cell imaging results; together, they indicate that endogenous and ectopically expressed RabE proteins are not only localized at the Golgi apparatus but that the majority of membrane-associated RabE is in the PM in Arabidopsis leaf cells.

### GTP-Restricted RabE1d-Q74L Displays Altered Subcellular Localization

We also produced transgenic plants expressing RabE1d-Q74L, which is predicted to be constitutively active, as a GFP fusion. Unlike wild-type GFP-RabE1d, the GFP-RabE1d-Q74L fusion was not detected in intracellular punctate structures (i.e. Golgi) but was primarily found at the cell periphery (Fig. 4A). PM staining with FM4-64 revealed that the bulk of GFP-RabE1d-Q74L fluorescence did not overlap with the PM but was most likely localized in the tonoplast (Fig. 4B). We obtained from the Arabidopsis Biological



**Figure 4.** Intracellular localization of the mutant GFP-RabE1d-Q74L protein. A, Confocal microscopy image of a representative Arabidopsis leaf expressing GFP-RabE1d-Q74L. Projection along the z axis of several focal planes crossing the epidermal cell layer. Several independent transgenic lines were analyzed with similar results. B, GFP-RabE1d-Q74L is mostly localized in the tonoplast. Leaves were stained with FM4-64, to visualize the plasma membrane, and immediately observed. The image represents a single focal plane (40 $\times$  oil-immersion objective). Top, GFP fluorescence; middle, FM4-64 fluorescence (asterisks indicate autofluorescent chloroplasts in the mesophyll layer, below the epidermis); bottom, merged image (arrowheads indicate where the tonoplast is most clearly distinct from the plasma membrane). Invaginations and the formation of membranous structures are typical of the highly dynamic vacuolar membrane. Even in the areas where the plasma membrane and tonoplast are closest, green and red fluorescence are visibly distinct. Bars = 20  $\mu$ m.

Resource Center Arabidopsis transgenic lines expressing various endomembrane markers fused to GFP (Cutler et al., 2000). Comparison of GFP-RabE1d-Q74L fluorescence with that of either a PM-localized channel protein (line Q8) or a tonoplast marker (line Q5) revealed a localization pattern similar to that of the tonoplast, both in intact and in plasmolyzed leaf tissues (Supplemental Fig. S3). Plasmolysis in line Q8 resulted in the characteristic Hechtian strands (Oparka, 1994), typical of plasma membrane retracting from the cell wall; in mesophyll cells, the chloroplasts were clearly located on the inside of the GFP-labeled PM (Fig. 5). In contrast, plasmolyzed cells expressing GFP-RabE1d-Q74L did not display Hechtian strands, and chloroplasts in the mesophyll cells were distinctly on the outside of the GFP-labeled membrane (Fig. 5), further supporting RabE1d-Q74L localization in the tonoplast.

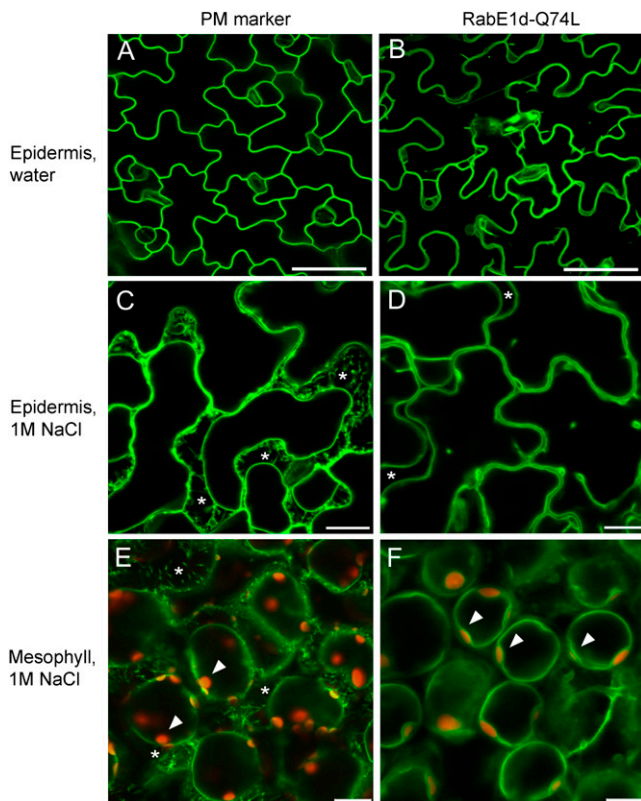
#### Challenge with *P. syringae* Bacteria Promotes Focal Accumulation of GFP-RabE1d

To investigate whether intracellular distribution of the RabE1d protein was perturbed in the presence of bacteria, we performed microscopic observation of transgenic GFP-RabE1d plants challenged with various strains of *Pst* DC3000. Leaves were syringe inoculated with bacteria at  $1 \times 10^8$  colony-forming units (cfu) mL<sup>-1</sup> and analyzed by CLSM at 5 to 6 h after

inoculation. We observed that bacterial inoculation caused polarized GFP-RabE1d accumulation in mesophyll cells (Fig. 6A). Interestingly, whereas such a phenomenon was restricted to a few sparse cells in leaves challenged with wild-type virulent *Pst* DC3000 (Fig. 6D) or with the nonpathogenic TTSS-defective mutant *hrpA*<sup>-</sup> (Fig. 6C), focal accumulation of GFP-RabE1d was widespread in leaves inoculated with the avirulent strain *Pst* DC3000 (*avrRpt2*; Fig. 6E). These results suggest that the GFP-RabE1d subcellular localization is dynamic and can respond to bacterial infection, especially in a gene-for-gene interaction.

#### *RabE1d* Cosuppression in Transgenic Plants Resulted in Altered Plant Growth and Morphology

A large percentage of GFP-RabE1d transgenic Arabidopsis plants generated in this study showed cosuppression of the transgene and of endogenous *RabE*, as demonstrated by western-blot analysis (Fig. 7A). Severe reduction of the endogenous RabE protein level in transgenic plants invariably correlated with a distinct morphological phenotype (Fig. 7, B and C). Rosette leaves developed normally for the first 3 weeks, when Arabidopsis development is usually slower, and plants were indistinguishable from Col-0 plants. In the following 2 weeks, when Arabidopsis size increases more rapidly, the leaves of *RabE*-cosuppressed plants did not fully elongate (Fig. 7D); midribs remained

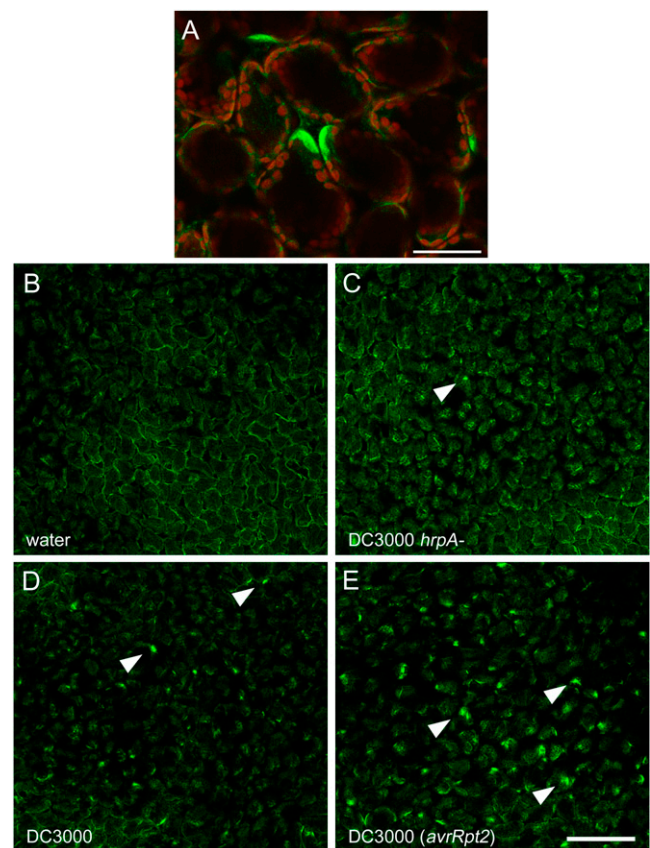


**Figure 5.** Localization pattern of GFP-RabE1d-Q74L compared with the localization of a PM marker protein. A, C, and E, Arabidopsis expressing a GFP fusion to the PM integral protein PIP2A (line Q8). B, D, and F, Arabidopsis expressing GFP-RabE1d-Q74L. In C to F, samples were plasmolyzed in 1 M NaCl. Asterisks indicate areas between plasmolyzed cells; Hechtian strands are noticeable in C and E (PM) but not in D and F (RabE-Q74L). Arrowheads indicate chloroplast autofluorescence (red); chloroplasts are on the inside of the PM (E), while GFP-RabE1d-Q74L fluorescence is on the cytoplasmic side of the chloroplasts. All images are single focal planes. Bars = 50  $\mu\text{m}$  (A and B), 20  $\mu\text{m}$  (C), and 10  $\mu\text{m}$  (D–F).

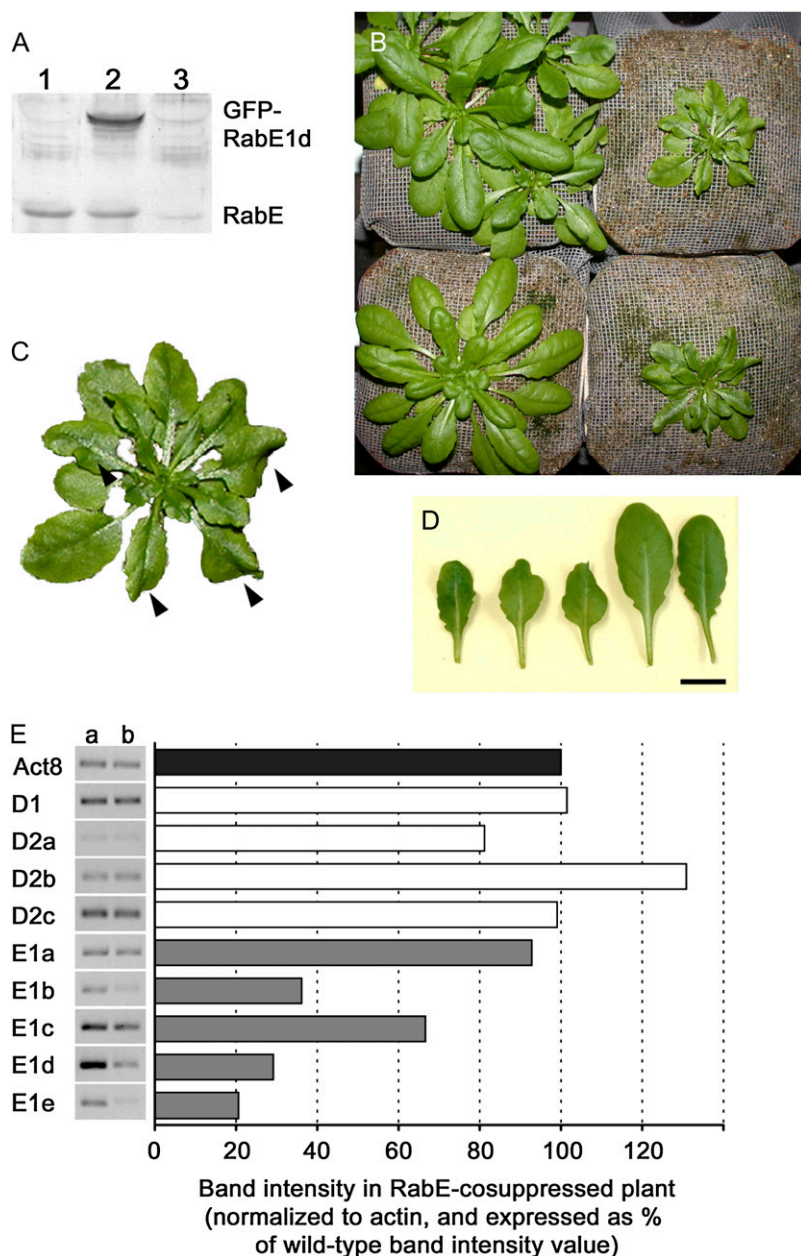
short, while the leaf lamina continued to expand, producing a characteristic wavy phenotype. Mature (6- to 7-week-old) *RabE*-cosuppressed plants were significantly smaller than wild-type plants and had short midribs and petioles. *RabE*-cosuppressed plants flowered at the same time as wild-type Arabidopsis, although their stems were much shorter than wild-type stems and the plants produced fertile seeds. The progeny of selected silenced lines (B8, B11, and B13) maintained silencing and had the same phenotype as the parental plants.

Expression of the *RabE* gene family was analyzed by RT-PCR in the cosuppressed lines and compared with expression in wild-type plants. RT-PCR demonstrated that not all *RabE* gene family members are equally affected by cosuppression. *RabE1d* and *RabE1e* were knocked down most severely, followed by *RabE1b* and *RabE1c*. *RabE1a* showed only slight down-regulation (Fig. 7E). Given the high degree of sequence similarity

among small GTPases of the Arabidopsis Rab superfamily, we tested whether other closely related Rabs were affected by silencing. The closest relatives of the RabE clade in Arabidopsis are the four RabD proteins (RabD1, -D2a, -D2b, and -D2c). RabD was previously characterized as a regulator of the early secretory pathway, being involved in transport from the endoplasmic reticulum to the Golgi (Batoko et al., 2000; Zheng et al., 2005). RT-PCR revealed that transcripts of all four *RabD* genes were present at similar levels in *RabE1d*-silenced plants and in wild-type Arabidopsis (Fig. 7E). Silencing in the transgenic plants, therefore, is specific and limited to the *RabE* genes, primarily *RabE1d* and *-E1e*.



**Figure 6.** Focal accumulation of GFP-RabE1d in response to bacteria. Leaves of transgenic GFP-RabE1d Arabidopsis were syringe inoculated with various strains of *Pst* DC3000 at a density of  $1 \times 10^8$  cfu mL<sup>-1</sup>. A, Confocal microscopy observation at 6 h after inoculation revealed focal accumulation of the fluorescent GFP-RabE1d in mesophyll cells. Such accumulation is limited to a few cells in response to virulent and nonpathogenic bacteria, whereas it is widespread in response to avirulent bacteria, which trigger gene-for-gene resistance. Bar = 50  $\mu\text{m}$ . B, No bacteria. C, *Pst* DC3000 *hrpA*<sup>-</sup> mutant bacteria, nonpathogenic. D, Wild-type *Pst* DC3000, virulent. E, *Pst* DC3000 (*avrRpt2*), avirulent. Three independent transgenic lines (A1, A5, and A14) yielded similar results. For B to E, all panels represent Z stacks of the same number of optical planes intersecting the leaf mesophyll (arrowheads indicate some of the points of accumulation). A second, independent set of images is shown in Supplemental Figure S4. Bar = 200  $\mu\text{m}$ .



**Figure 7.** *RabE* cosuppression adversely affects Arabidopsis plant size and leaf morphology. A, Western blot (with anti-RabE antibody) illustrating how *RabE*-cosuppressed plants (lane 3) have a considerably lower amount of endogenous RabE protein compared with both the wild type (lane 1) and GFP-RabE1d overexpressors (lane 2). B, *RabE*-cosuppressed plants (top and bottom right) have smaller size and altered morphology compared with wild-type Arabidopsis (top left) and *RabE1d*-overexpressing plants (bottom left). C, Enlarged image of the *RabE*-cosuppressed plant in B (top right); arrowheads point to the wavy leaves. D, Size comparison of rosette leaves from 5-week-old *RabE*-cosuppressed (three left) and wild-type (two right) Arabidopsis plants. Bar = 10 mm. E, RT-PCR analysis of expression of the five *RabE* and four *RabD* genes in wild-type (lane a) and in *RabE*-cosuppressed (lane b) Arabidopsis plants. Equal volumes of the PCR samples were loaded on a 1% agarose gel. The gel was photographed with a Bio-Rad imager, and Quantity One software was used to quantify the bands. Intensity values, normalized to those of *Actin8*, are represented in the chart as percentages of the wild-type value.

**RabE-Cosuppressed Plants Are Not Compromised in Resistance to *Pst* DC3000**

We performed pathogenesis assays with *Pst* DC3000 to establish whether the partially *RabE*-cosuppressed plants are impaired in their defenses against *P. syringae*. *Pst* DC3000 consistently caused disease symptoms and multiplied on *RabE*-cosuppressed plants, reaching similar population levels as on wild-type Arabidopsis (Supplemental Fig. S2). In several instances, however, we observed that older (6- to 7-week-old) or environmentally stressed *RabE*-cosuppressed plants displayed some degree of basal resistance, possibly due to stress caused by *RabE* down-regulation (Supplemental Fig. S2). We did not observe a consistent and reproducible difference between wild-type Col-0 plants and *RabE*-

cosuppressed plants in the multiplication of *Pst* DC3000 (*avrRpt2*) or a TTSS-defective mutant of *Pst* DC3000, the *hrpA*<sup>-</sup> mutant (Supplemental Fig. S2).

**Transgenic Expression of RabE1d-Q74L Confers Resistance against *Pst* DC3000**

GFP-RabE-Q74L plants did not exhibit significant alterations in growth and development, other than the appearance of minute sparse indentations in mature rosette leaves about 2 weeks prior to bolting. The origin and significance of these indentations are unclear; however, we have been unable to show, using trypan blue staining, that they develop as a consequence of cell death.



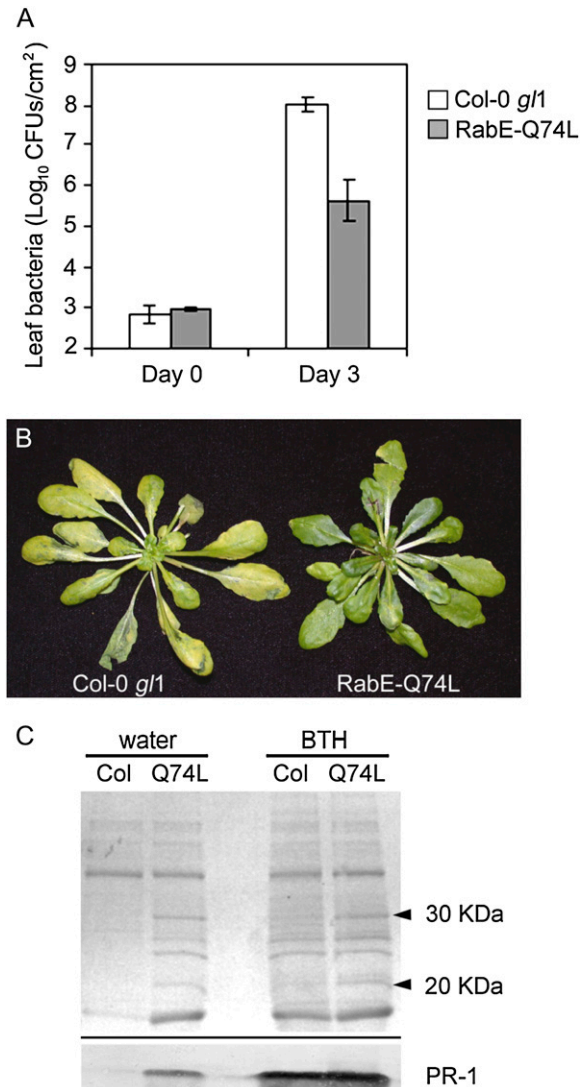
Although transgenic expression of constitutively active GFP-RabE1d-Q74L did not globally affect plant growth or development (as did *RabE* cosuppression), it had a remarkable effect on plant responses to *P. syringae* infection. Upon challenge with *Pst* DC3000, the GFP-RabE1d-Q74L-expressing plants displayed a considerable degree of resistance, reflected by bacterial multiplication being consistently restricted (10- to 100-fold) compared with multiplication on wild-type *Arabidopsis* (Fig. 8A). This observation was consistent across several experiments on different transgenic lines. Visible disease symptoms, namely chlorosis and necrosis, were also markedly reduced (Fig. 8B). This enhanced resistance phenotype apparently requires the constitutively active form of RabE1d, as transgenic expression of GFP-RabE1d did not result in enhanced resistance (Supplemental Fig. S2).

#### Constitutive Secretion of Extracellular Proteins in GFP-RabE1d-Q74L-Expressing Plants

Up-regulation of the secretory pathway was recently demonstrated in systemic acquired resistance (SAR; Wang et al., 2005), and plants undergoing SAR accumulate secreted proteins in the apoplast, including antimicrobial polypeptides (Uknes et al., 1992). Because RabE proteins are predicted to be involved in regulating secretory vesicle trafficking, the enhanced resistance to *Pst* DC3000 in GFP-RabE1d-Q74L-expressing plants could be caused by constitutive stimulation of defense-associated secretion. To test this possibility, *Arabidopsis* wild-type and GFP-RabE1d-Q74L-expressing plants were sprayed with benzothiadiazole (BTH), a synthetic activator known to trigger SAR in plants (Lawton et al., 1996), or with water as a control. Three days later, total protein secretion in the apoplast and secretion of the extracellular defense protein PR1 (for pathogenesis-related protein 1) were monitored. Intercellular wash fluid (IWF) collected from water-treated GFP-RabE1d-Q74L-expressing plants contained PR1 and several unknown proteins that were absent from the water-treated *Arabidopsis* wild-type IWF, indicating a constitutive activation of secretory and defense pathways. BTH application resulted in similar levels of secreted PR1 and several other proteins in the apoplast of both wild-type and transgenic plants (Fig. 8C). Interestingly, some protein bands were exclusively detected in the IWF of water- and BTH-treated RabE1d-Q74L-expressing plants but not in the BTH-treated wild-type plant IWF (Fig. 8C, arrowheads). These unique extracellular proteins associated with expression of RabE1d-Q74L suggest that other secretory pathways, in addition to the SAR pathway, are activated in these plants.

#### DISCUSSION

Plant RabE GTPases are predicted to be involved in mediating secretory vesicle traffic from the Golgi to the



**Figure 8.** Constitutive expression of RabE-Q74L confers resistance to *Pst* DC3000. A, Bacterial multiplication in GFP-RabE1d-Q74L-expressing plants (gray bars) compared with that in wild-type *Arabidopsis* (white bars). *Pst* DC3000 was syringe infiltrated at a density of  $1 \times 10^5$  cfu mL<sup>-1</sup>. B, Disease symptoms 3 d after vacuum inoculation with *Pst* DC3000 at a density of  $1 \times 10^6$  cfu mL<sup>-1</sup>. Left, *Arabidopsis* Col-0 *gl1* (wild type); right, *Arabidopsis* expressing GFP-RabE-Q74L. C, Accumulation of extracellular proteins in plants expressing GFP-RabE1d-Q74L. Proteins in the IWF from wild-type (Col) and RabE1d-Q74L-expressing (Q74L) plants were separated by SDS-PAGE. Top, Coomassie Brilliant Blue-stained gel representing total proteins; the arrowheads indicate bands that seem to be exclusive to the Q74L plants. Bottom, Western blot with the anti-PR-1 antibody (a gift of Dr. X. Dong).

PM. Therefore, they could play fundamental roles in the secretion of extracellular matrix molecules, thereby influencing plant growth and development, as well as in the secretion of extracellular defense molecules in response to pathogen infections. RabE proteins from both tomato (Bogdanove and Martin, 2000) and *Arabidopsis* (this study) interact with a pathogen effector

protein, AvrPto, in Y2H assays. Because a number of studies have implicated vesicle trafficking in pathogen defense (Collins et al., 2003; Assaad et al., 2004; Wang et al., 2005; Nomura et al., 2006; Kalde et al., 2007), physical interaction between AvrPto and RabE GTPases could indicate a potential role of RabE GTPases in plant defense. However, to date, only one report has documented subcellular localization of Arabidopsis GFP-RabE1d transiently expressed in heterologous tobacco epidermal cells (Zheng et al., 2005). Subcellular localization of RabE GTPases in native Arabidopsis cells and the biological role of RabE GTPases in regulating plant growth or defense at the whole-plant level remained unknown.

Using a combination of confocal microscopy and cell membrane fractionation on transgenic plants expressing GFP-RabE1d and on wild-type plants, we show not only that RabE proteins are localized in the Golgi, as reported by Zheng and colleagues (2005), but also that a prominent fraction is associated with the PM of Arabidopsis leaf cells. Because a hallmark feature of Rab proteins is that they localize to the specific membrane compartments in which they function, association of RabE with both the Golgi and the PM provides strong support for a role in mediating the traffic of secretory vesicles from the Golgi to the PM (i.e. instead of other post-Golgi target membranes), as suggested previously by Zheng et al. (2005).

Interestingly, the RabE-Q74L protein expressed in Arabidopsis as a GFP fusion displayed an unanticipated localization pattern. The bulk of fluorescent protein was localized at the vacuolar membrane, and we could not detect, by CLSM fluorescence in the Golgi or in the PM, the predicted destination of the active form of RabE. After delivery of vesicles to the target membrane, Rab proteins are usually recycled back to the cytoplasm and then incorporated again into the donor membrane for multiple rounds of vesicle transport. Such recycling is dependent on hydrolysis of GTP into GDP, because inactivated GDP-bound Rabs are extracted from the target membrane by accessory proteins that mediate their recycling (Segev, 2001). The Q74L mutation is predicted to inactivate the GTP hydrolysis activity of RabE. Presumably, RabE-Q74L cannot be dephosphorylated and, thus, cannot be recycled to the cytoplasm. It is possible that a default nonspecific endocytosis pathway, such as that observed for the FM4-64 dye (Ueda et al., 2001), could carry the GTP-bound RabE-Q74L protein from its target location, the PM, to the tonoplast, where it accumulates.

Analysis of *RabE*-cosuppressed plants clearly indicates that RabE GTPases play a role in plant growth, as evidenced by dwarf plant sizes and altered leaf morphology in *RabE*-cosuppressed plants. Rosette leaves of *RabE*-cosuppressed lines developed relatively normally for the first 3 weeks, when Arabidopsis development is usually slower. However, when Arabidopsis size increases more rapidly, in the 4th and 5th weeks, the leaves of *RabE*-cosuppressed plants were

not able to fully elongate; leaf midribs and petioles remained short, yielding characteristically wavy leaves. Inflorescences emerged at the same time as in the wild type, but the overall stature of the *RabE*-cosuppressed plants remained much shorter. These observations lead us to suggest that RabE-mediated vesicle traffic from the Golgi to the PM is required for the rapid elongation of certain tissues (e.g. leaf midribs and stems) associated with rapid plant growth. The dwarf phenotype of *RabE*-cosuppressed plants was likely caused by simultaneous down-regulation of more than one *RabE* gene, as suggested by reduced expression of *RabE1d* and *-E1e* and, to a lesser extent, *RabE1a*, *-E1b*, and *-E1c* (Fig. 6E). Consistent with this speculation, knockout mutants of *RabE1d*, caused by T-DNA insertions, did not exhibit any defect in growth and development (data not shown). Notably, cosuppression did not affect the expression of *RabD* genes, the closest homologues of *RabE* genes; therefore, the observed phenotypes can be ascribed to partial silencing of multiple *RabE* gene family members.

Besides our interest in determining the subcellular localization of RabE GTPases in native Arabidopsis cells and a possible role of RabE GTPases in growth and development at the whole plant level, another major motivation for this work was to investigate a potential role of RabE GTPases in plant defense. Bogdanove and Martin (2000) first identified RabE GTPases as AvrPto interactors in tomato and discussed the possibility that these GTPases could be virulence targets of AvrPto. In an independent Y2H screen for AvrPto-interacting Arabidopsis proteins, we also isolated a RabE GTPase. We found that AvrPto interacted with all tested members of the RabE family but not with members of other Rab families of Arabidopsis (Fig. 1). Moreover, AvrPto interacted with a RabE mutant (*RabE1d*-Q74L) predicted to be GTP bound but not with a RabE mutant (*RabE1d*-S29N) predicted to be GDP bound. Finally, we found that the majority of membrane-associated *RabE1d* is localized at the PM, where AvrPto is known to be located in tomato and Arabidopsis cells (Shan et al., 2000; He et al., 2006). Despite the intriguing specificity in Y2H assays and the apparent colocalization of AvrPto and *RabE1d* to the same host membrane, we have been unable to detect AvrPto-*RabE1d* interaction using in vivo coimmunoprecipitation or in vitro pull-down methods. Physical interaction of AvrPto with confirmed virulence targets FLS2 and BAK1 was demonstrated using both in vitro and in vivo methods (Shan et al., 2008; Xiang et al., 2008). Thus, our results suggest that RabE proteins may not be host targets of AvrPto. Alternatively, RabE GTPases may interact with AvrPto in the plant cell only transiently and/or may require special conditions.

Interestingly, GFP-*RabE1d* plants challenged with the avirulent strain *Pst* DC3000 (*avrRpt2*) manifested dramatic focal accumulation of GFP-*RabE1d* in mesophyll cells (Fig. 5). GFP-*RabE1d* focal accumulation was limited to a few sporadic cells when plants were

inoculated with wild-type virulent *Pst* DC3000 (i.e. in a susceptible interaction) or with the *hrpA*<sup>-</sup> mutant (which activates plant basal defenses). Polarized accumulation of RabE GTPase during a gene-for-gene interaction could be part of the host cell response to direct defense-associated vesicle trafficking toward invading pathogens. If so, the GFP-RabE1d fusion may be a useful cellular marker for future study of vesicle trafficking in the gene-for-gene response in Arabidopsis. Accumulation of other vesicle traffic-associated regulators (such as t-SNAREs and v-SNAREs) at the site of pathogen penetration has also been observed in fungal infections (Assaad et al., 2004; Huckelhoven, 2007; Kwon et al., 2008).

*RabE* cosuppression in Arabidopsis, under the conditions reported in this study, did not result in increased susceptibility to *P. syringae* bacterial strains. RabE proteins, therefore, may not be required for establishing defenses against this pathogen. Alternatively, partial down-regulation of RabE proteins may be insufficient to confer a discernible defense phenotype. A complete knockout of all five *RabE* genes would be necessary to resolve this question. However, the dwarf phenotype of partially *RabE*-cosuppressed plants suggests that it is unlikely that completely *RabE*-deficient plants would be viable and/or suitable for bacterial infection assays.

Transgenic expression of the RabE1d-Q74L variant, on the other hand, conferred in Arabidopsis a significant degree of resistance to *Pst* DC3000. It remains to be determined whether this resistance is caused by a direct effect of RabE1d-Q74L, due to enhancement of defense-related vesicle traffic, or rather is triggered by an indirect effect, due to overall perturbation of cellular vesicle traffic. We found that the IWF collected from water-treated GFP-RabE1d-Q74L plants contained PR1 and several unknown proteins that were absent from the IWF of water-treated wild-type Arabidopsis, indicating constitutive activation of secretory and defense pathways in these plants. BTH application resulted in similar levels of secreted PR1 and other proteins in the apoplast in both wild-type and RabE1d-Q74L transgenic plants (Fig. 8C). However, some of these extracellular proteins are detected only in the IWF of water- and BTH-treated RabE1d-Q74L-expressing plants but not in the IWF of BTH-treated wild-type plants. These unique extracellular proteins associated with the expression of RabE1d-Q74L suggest the activation of other secretory pathways in these plants in addition to the SAR pathway. Interestingly, activation of SAR and resistance to *Pst* DC3000 in the GFP-RabE1d-Q74L transgenic plants did not correlate with a dwarf phenotype, a common phenotype of Arabidopsis mutants that are constitutively resistant to pathogens (Lorrain et al., 2003). Further analysis of the RabE-Q74L transgenic plants may provide novel insights into plant defense and indicate possible avenues for engineering resistance in crops.

## MATERIALS AND METHODS

### Y2H Assay

We identified Arabidopsis (*Arabidopsis thaliana*) proteins that interacted with AvrPto of *Pseudomonas syringae* pv *tomato* strain DC3000 using the Matchmaker LexA-based Y2H system (Clontech Laboratories). Two Arabidopsis cDNA libraries, constructed from infected and uninfected Landsberg *erecta* plants (kindly provided by J. Jones), were screened. The AvrPto coding sequence was amplified from *Pst* DC3000 genomic DNA by PCR (sense primer, 5'-GCGAATTCCGAACCATGGGAAATATATGTGTC-3'; antisense primer, 5'-GCCCTCGAGATTGCCAGTTACGGTA-3') and cloned into pNLexA to serve as bait in the Y2H screen.

### RabE Cloning and Mutagenesis

We amplified the *RabE1d* (At5g03520) coding sequence from Arabidopsis Col-0 cDNA using the rabE-5' and rabE-3' primers (Supplemental Table S1), containing the *EcoRI* and *BamHI* restriction sites, respectively. The PCR product was ligated into a TOPO vector (pCR2.1; Invitrogen) and sequenced. Single nucleotide changes were introduced in the *RabE1d* sequence by two-step overlapping PCR, to generate the RabE1d-S29N and RabE1d-Q74L mutant derivatives. RabE1d-S29N was obtained through a G → A substitution; in the first PCR step, two overlapping *RabE1d* fragments were amplified using the primer combinations rabE-5'/S29N-rev and rabE-3'/S29N-for (Supplemental Table S1). The products were purified from agarose gel, mixed, and used as template for a second PCR amplification step, with the rabE-5' and rabE-3' primers. The presence of an overlapping region allowed annealing of the two gene fragments and amplification of the full-length coding sequence. A similar procedure was used to introduce the Q74L mutation through an A → T substitution. In this case, the following primer combinations were used in the first PCR step: rabE-5'/Q74L-rev and rabE-3'/Q74L-for (Supplemental Table S1). *RabE1d*-S29N and *RabE1d*-Q74L amplification products were introduced into pCR2.1-TOPO and sequenced.

### GFP-RabE1d Transgenic Plants

*RabE1d* and the mutant variant *RabE1d*-Q74L were subcloned in the *EcoRI* and *BamHI* sites of the binary expression vector pEGAD (Cutler et al., 2000), downstream of the enhanced GFP sequence, to create translational fusions. The binary vector was introduced in *Agrobacterium tumefaciens* strain GV3850 via triparental mating for plant transformation. Arabidopsis Col-0 *glabrous* (Col-0 *gl1*) plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected based on resistance to the herbicide Basta (glufosinate). A solution containing 0.012% glufosinate (Finale concentrate; AgrEvo Environmental Health) and 0.025% Silwet L-77 was sprayed on 2-week-old seedlings growing in soil. Surviving T1 plants were screened for GFP fluorescence with a Zeiss Axiophot microscope, and expression of the correctly sized GFP-RabE fusion proteins was verified by western blotting with an anti-RabE polyclonal antibody (described below).

### Protein Extraction and Immunoblotting

Total proteins were extracted as follows: approximately 20 mg (fresh weight) of fresh or frozen leaf tissue was ground with a pestle in a microfuge tube in the presence of 100  $\mu$ L of 1 $\times$  SDS-PAGE loading buffer (90 mM Tris-HCl, pH 8.0, 100 mM dithiothreitol, 3% SDS, 22.5% Suc, 10  $\mu$ L mL<sup>-1</sup> Protease Inhibitor Cocktail for plant cell extracts [Sigma], and bromophenol blue [to saturation]). Extracts were immediately heated at 80°C for 10 min and then frozen at -20°C. Before loading, extracts were thawed at room temperature and centrifuged at 20,000g for 2 min to pellet debris. An equal volume of each sample was used for SDS-PAGE. Total proteins were separated on precast gradient gels (4%–20%; ISC BioExpress), then transferred onto Immobilon-P membranes (Millipore) using a semidry transfer apparatus (SEMI PHOR; Hoefer Scientific Instruments). Protein detection was carried out with the following primary antibodies: anti-RabE (raised in chicken against recombinant RabE protein expressed in *Escherichia coli*; Cocalico Biological), anti-XT1 (Faik et al., 2002; Cavalier and Keegstra, 2006), anti-PM ATPase (Morsomme et al., 1998), and anti- $\gamma$ -TIP (a gift of Dr. N. Raikhel).

### Cell Membrane Fractionation

Leaves were harvested and weighed immediately prior to extraction. Leaf tissue (2.5 g) was ground with a cold mortar and pestle in the presence of 5 mL

of ice-cold extraction buffer (50 mM HEPES, pH 7.5, 100 mM KCl, 10 mM EDTA, 1 mM dithiothreitol, and 10  $\mu\text{L mL}^{-1}$  Protease Inhibitor Cocktail for plant cell extracts [Sigma]) containing 34% Suc (*w/v*). The extract was homogenized with a Polytron immersion blender (three pulses of 10 s each), filtered through a single layer of Miracloth, and centrifuged for 10 min at 10,000g to remove most unbroken chloroplasts and nuclei. The supernatant was adjusted to 40% Suc in about a 10-mL final volume (concentration was determined with a refractometer) and layered on a 5-mL cushion of 50% Suc in clear ultracentrifugation tubes. The homogenate was subsequently layered with 10 mL of 34% Suc, 8 mL of 25% Suc, and 8 mL of 18% Suc (*w/v*). All Suc solutions were prepared in the same buffer used for extraction. Gradients were centrifuged at 100,000g for 3 h, at 4°C, in an SW28 rotor (Beckman). After centrifugation, the membrane-containing interphases were collected and diluted with Suc-free extraction buffer, and membranes were collected by ultracentrifugation (1 h at 100,000g). Membrane pellets were resuspended in equal volumes of SDS-PAGE loading buffer and heated at 80°C for 10 min. Equal volumes were loaded on SDS-PAGE gels. Protein electrophoresis and western blotting were performed as described above.

### Confocal Microscopy Analysis and Imaging

Pieces of leaves were sampled randomly and mounted in water. Imaging was performed using an LSM510 META inverted confocal laser scanning microscope (Zeiss) and either a 20 $\times$  or a 40 $\times$  oil-immersion objective. For GFP-RabE fluorescence analysis, the 488-nm excitation line of an argon ion laser was used, with a 505- to 530-nm band-pass filter, in the single-track facility of the microscope. Images were processed with the LSM Image Browser version 3.1 (Zeiss) and with the Adobe Photoshop Elements version 5.0 software (Adobe Systems). For FM4-64 staining, detached Arabidopsis leaves were submerged in 8.2  $\mu\text{M}$  FM4-64 (Molecular Probes) in water for 15 min. Leaves were rinsed in distilled water and observed immediately. For imaging GFP-RabE1d and FM4-64 fluorescence, the 488-nm excitation line was used; GFP fluorescence was collected with a 505- to 530-nm band-pass filter, and FM4-64 fluorescence was collected with a 615-nm long-pass filter.

### Biolistic Transformation

ST-RFP transient expression in Arabidopsis leaves was achieved by biolistic transformation. The binary vector was a gift of Dr. F. Brandizzi (Saint-Jore et al., 2002). Gold particles (1.0  $\mu\text{m}$ ; Bio-Rad) were coated with the ST-RFP plasmid DNA as described by Zhang et al. (2001). Arabidopsis leaves were harvested and arranged on Murashige and Skoog agar plates (4.3 g L<sup>-1</sup> Murashige and Skoog salts, 0.8% agar, pH 5.7). The DNA-coated particles were delivered into the lower leaf epidermis with a particle gun (DuPont), using 1,100-psi rupture discs under a vacuum of 25 in mercury. After bombardment, leaves were incubated on the sealed plates at room temperature, and fluorescence was observed at 24 h after transformation. For coimaging GFP-RabE1d and ST-RFP, the argon ion laser excitation lines of 488 nm (for GFP) and 543 nm (for DsRed) were used. GFP fluorescence was collected with a 505- to 530-nm band-pass filter, and DsRed fluorescence was collected with a 615-nm long-pass filter.

### Plant Growth and Bacterial Multiplication Assay

Arabidopsis plants were grown in soil, in growth chambers, under a 12-h-dark/12-h-light cycle. The light intensity averaged 100  $\mu\text{E m}^{-2} \text{s}^{-1}$ , and the temperature was kept constant at 20°C. *Pst* DC3000 bacteria were cultured in low-salt Luria-Bertani medium (10 g L<sup>-1</sup> tryptone, 5 g L<sup>-1</sup> yeast extract, and 5 g L<sup>-1</sup> NaCl) supplemented with 100  $\mu\text{g mL}^{-1}$  rifampicin. For multiplication assays in plants, bacterial liquid cultures were incubated at 30°C to the mid to late logarithmic phase. Bacteria were collected by centrifugation and resuspended in sterile water with the addition of 0.004% Silwet L-77 (OSI Specialties). Titer of the bacterial inoculum was  $1 \times 10^5$  cfu mL<sup>-1</sup>, unless otherwise indicated. Arabidopsis leaves were inoculated by syringe infiltration, and bacteria enumeration in leaves was conducted as described previously (Katagiri et al., 2002).

### RNA Extraction and RT-PCR Analysis

Total RNA was extracted from 100 mg of Arabidopsis leaf tissue with the RNeasy Plant Mini Kit (Qiagen), according to the manufacturer's specifica-

tions. RNA concentration in samples was determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop). RNA RT and target gene amplification (RT-PCR) were performed using the RNA LA PCR Kit (AMV), version 1.1 (TaKaRa). RT reaction mixture was prepared according to the manufacturer's protocol (5 mM MgCl<sub>2</sub>, 1 $\times$  RNA PCR buffer, 1 mM deoxynucleoside triphosphate mixture, 1 unit  $\mu\text{L}^{-1}$  RNase inhibitor, 0.25 units  $\mu\text{L}^{-1}$  AMV reverse transcriptase, 0.125  $\mu\text{M}$  oligo[dT] Adaptor Primer, RNase-free water, and 1  $\mu\text{g}$  of total RNA). For amplification of the *RabE* and *RabD* transcripts, a single RT reaction was carried out in a total volume of 50  $\mu\text{L}$  and incubated in a thermal cycler for 30 min at 45°C, followed by 5 min at 99°C and 5 min at 5°C. Five microliters of reverse-transcribed cDNA was used as template in each of 10 PCRs with gene-specific primer pairs designed to amplify the five *RabE* gene family members, the four *RabD* genes, and the *Actin8* gene as a control. Primer sequences are listed in Supplemental Table S2. Each PCR sample contained 2.5 mM MgCl<sub>2</sub>, 1 $\times$  LA PCR buffer II, 0.2  $\mu\text{M}$  forward primer and 0.2  $\mu\text{M}$  reverse primer, sterilized distilled water, and 5  $\mu\text{L}$  of the RT reaction described above in a final volume of 25  $\mu\text{L}$ . The reactions were placed in a thermal cycler, and amplification was performed under the following conditions: 94°C for 2 min (one cycle); 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min (22 or 25 cycles); 72°C for 1 min (one cycle); and then 4°C. Ten microliters of the PCR samples was loaded on a 1% agarose gel. Gels were photographed with a Bio-Rad Gel Documentation System, and band intensity was analyzed with the Quantity One software (Bio-Rad).

### BTH Treatment

BTH (Actigard) was prepared at a final concentration of 300  $\mu\text{M}$  in water and sprayed onto potted GFP-RabE1d-Q74L and Col-0 *g11* Arabidopsis plants. A separate set of plants was sprayed with water as a control. Plants were covered with a tight-fitting clear plastic dome and assayed for responses at 3 d after BTH application.

### IWF Collection and Analysis

BTH-treated and control plants were harvested 3 d after treatment. Whole plants were vacuum infiltrated for 2 min with distilled water containing 0.002% Silwet L-77 (OSI Specialties). The plants were placed in conical centrifuge tubes (Nalgene) containing a mesh septum placed about 2 cm above the bottom. IWF was collected by centrifuging the infiltrated plants at 400g for 20 min at 4°C. The IWF volume was measured with a micropipette, and the appropriate volume of 5 $\times$  SDS-PAGE loading buffer was immediately added. Samples were heated at 85°C for 5 min, then frozen or loaded onto an acrylamide gel.

### Supplemental Data

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

**Supplemental Figure S1.** ClustalW alignment of the five Arabidopsis RabE proteins and their closest homologues in other organisms.

**Supplemental Figure S2.** Bacterial multiplication assays.

**Supplemental Figure S3.** Localization pattern of GFP-RabE1d-Q74L.

**Supplemental Figure S4.** Focal accumulation of GFP-RabE1d in response to bacteria.

**Supplemental Table S1.** Primers for *RabE* cloning and mutagenesis.

**Supplemental Table S2.** Gene-specific primers for RT-PCR.

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