SUBCELLULAR LOCALIZATION AND FUNCTION OF THE *ARABIDOPSIS THALIANA* SMALL GTPASE RABE, A HOST INTERACTING PROTEIN OF THE *PSEUDOMONAS SYRINGAE* VIRULENCE EFFECTOR AVRPTO

By

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ABSTRACT

SUBCELLULAR LOCALIZATION AND FUNCTION OF THE *ARABIDOPSIS THALIANA* SMALL GTPASE RABE, A HOST INTERACTING PROTEIN OF THE *PSEUDOMONAS SYRINGAE* VIRULENCE EFFECTOR AVRPTO

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Pseudomonas syringae pathovar *tomato* strain DC3000 (*Pst* DC3000) is a bacterial pathogen of tomato and of the model plant *Arabidopsis thaliana*. Like many Gram-negative bacterial pathogens of animals and plants, *Pst* DC3000 uses the conserved type III secretion system (TTSS) to deliver multiple virulence effector proteins directly into the host cell. Type III effectors collectively participate in causing disease, by mechanisms that are not well understood. Elucidating the virulence function of individual effectors is fundamental for understanding bacterial infection of plants.

Transgenic overexpression of AvrPto, one of *Pst* DC3000 virulence effector proteins, in Arabidopsis was previously shown to lead to suppression of basal defenses, thus enabling growth of non-pathogenic TTSS-defective bacteria in the transgenic plants. AvrPto interacts in the yeast two-hybrid system with the Arabidopsis RabE family of small GTPases, putative regulators of post-Golgi vesicle traffic to the plasma membrane. Although the function of RabE homologues in other eukaryotic organisms is well understood, the biological role of the Arabidopsis RabE proteins is obscure.

In this study, a live cell imaging approach was applied to investigate the subcellular localization of one of the five Arabidopsis RabE proteins, RabE1d, and of its mutant derivatives RabE1d-Q74L (predicted to be constitutively active) and RabE1d-

S29N (predicted to be constitutively inactive), fused to the green fluorescent protein (GFP). Microscopic analysis and cell fractionation studies revealed that transgenically expressed GFP-RabE1d and endogenous RabE proteins are associated with the Golgi apparatus and the plasma membrane in Arabidopsis leaves. Strikingly, upon transgenic expression of AvrPto *in planta*, the Golgi-localized pool of GFP-RabE1d was greatly reduced and often undetectable. Furthermore, RabE1d overexpression could partially counteract the AvrPto-induced susceptibility to TTSS-defective bacteria. This work uncovered a novel association between AvrPto virulence function and subcellular distribution of the RabE protein.

To explore a possible role of RabE in plant growth, development and defense against *Pst* DC3000, transgenic Arabidopsis plants overexpressing RabE1d or its S29N and Q74L mutant variants were used. Overexpression of wild-type RabE1d or of RabE1d-S29N resulted in plants that were morphologically and developmentally indistinguishable from wild-type Arabidopsis and were not altered in disease resistance. Interestingly, Arabidopsis plants expressing the mutant RabE1d-Q74L gained a significant degree of resistance to *Pst* DC3000, while their growth and development were similar to those of wild-type plants. In contrast, *RabE* silencing drastically affected Arabidopsis leaf morphology and rosette size (suggesting a role for RabE in plant growth and development) and had a complex effect on host defense.

This study identified an original case of a virulence effector of a plant-pathogenic bacterium that alters subcellular localization of a putative regulator of intracellular trafficking. Additionally, functional study of RabE1d laid the basis for further characterization of the role of the entire RabE family of small GTPases in Arabidopsis.

To Sofia and Giovanni, who always made me want to be a better person

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CHAPTER 1

Literature Review

INTRODUCTION

Multicellular organisms live in constant contact with microorganisms. While most microbes do not pose a threat for plant and animal health, many bacteria, fungi, protozoans and viruses have the potential of causing disease. Survival of plant and animal species is dependent on their ability to recognize potential pathogens and to mount effective defenses. Although higher eukaryotes have evolved elaborate self-protective mechanisms, many microorganisms can still cause disease, having developed equally sophisticated strategies to elude or suppress host defenses.

Microbial pathogens of plants are responsible for significant crop losses worldwide (Strange and Scott, 2005). Between 1988 and 1990, 13.3% of the world agricultural production, equivalent to $76.9 billion, was estimated to be lost due to pathogens (Baker et al., 1997). In light of the fast-growing human population and relative limitation of agricultural land, maximizing crop yield and quality and preventing losses due to pathogens are among the main concerns of modern society and scientific community. Understanding the molecular bases of plant-pathogen interactions is of fundamental importance for an effective reduction of plant diseases (Strange and Scott, 2005).

Recent research advancements are uncovering the depth and complexity of interactions between microbial pathogens and their eukaryotic hosts. Fascinating analogies are emerging in the way animals and plants ward off infectious diseases, and in the molecular mechanisms by which different microbes achieve pathogenicity on their hosts (Cao et al., 2001; Buttner and Bonas, 2003).

Gram-negative bacterial pathogens of plants (including *Pseudomonas*, *Ralstonia*, *Erwinia* and *Xanthomonas spp.*) and of animals (including *Yersinia*, *Salmonella*, and *Shigella spp.*) share an ancient virulence mechanism, the type III secretion system, that allows them to deliver disease-promoting proteins directly into the host cells (He et al., 2004; Galan and Wolf-Watz, 2006). Investigating the biochemical and molecular function of these secreted bacterial proteins has allowed identification of numerous eukaryotic cellular pathways and processes targeted by pathogens (Mota and Cornelis, 2005; Mudgett, 2005). One of the common themes in bacteria-caused diseases is that pathogens use these type III-secreted proteins to subvert the host cell metabolism and to create conditions favorable to their own growth; among the most common targets are the host cell secretory and endocytic pathways. A wealth of studies has explored in depth how bacterial pathogens of animals subvert the host cell trafficking pathways to their own benefit (Mota and Cornelis, 2005; Schlumberger and Hardt, 2006). It is not yet understood how phytopathogenic bacteria specifically interfere with trafficking, although many studies have indicated that type III effectors target components of the secretory pathway, or suppress its normal function (Bogdanove and Martin, 2000; Hauck et al., 2003; Nomura et al., 2005; Soylu et al., 2005).

HOST DEFENSES AGAINST MICROBIAL PATHOGENS

Innate immune responses in eukaryotes

The front line of defense against microbial pathogens in all eukaryotes is innate (or basal) immunity, an ensemble of non-specific preformed and inducible defenses that come into play in the early stage of interaction with microorganisms (Kimbrell and Beutler, 2001; Nurnberger et al., 2004; Ausubel, 2005). All pathogens need to overcome basal defenses to successfully colonize their hosts and cause disease.

Preformed defenses

Pre-existing defenses in animals include physical, physiological and chemical barriers. Physical barriers, such as the skin, mucosae and intestinal epithelium represent the first tier of defense. Temperature and pH of the animal body cavities can be considered as physiological barriers because they often are non-permissive for non-adapted microorganisms. Constitutively secreted antimicrobial compounds and peptides are additional limiting factors for potential pathogens (Schroder, 1999).

Similarly, plants are equipped with preformed structural, anatomical and chemical defenses. Surface wax, cuticle layers, trichomes, constitutively produced antimicrobial peptides (Broekaert et al., 1997) and toxic metabolites, along with the plant cell wall itself are remarkable obstacles against herbivores and microbial pathogens (Thordal-Christensen, 2003; Field et al., 2006).

Induced innate immune responses

Preformed barriers offer a very effective primary line of protection against pathogens. Microbes that can breach this layer of defense will come in contact with the host cells and trigger inducible immune responses. All higher eukaryotes express receptors that recognize evolutionarily conserved molecules found exclusively in microorganisms (Nurnberger et al., 2004). These molecules are commonly referred to as pathogen- or microbe-associated molecular patterns (PAMPs, or MAMPs) and include, among others, viral and fungal proteins, peptidoglycan of Gram-positive bacteria, lipopolysaccharide (LPS) of Gram-negative bacteria and bacterial flagellin (Akira et al., 2006). Receptors collectively called PRRs (pattern recognition receptors), located in the plasma membrane or cytoplasm of the animal cell, perceive PAMPs and initiate signal transduction cascades leading to innate immune responses (Akira et al., 2006). Certain PRRs, like Toll-like receptors, are inserted in the plasma membrane and detect microbial ligands extracellularly; conversely, the cytoplasmic NOD receptors, featuring a nucleotide binding site and leucine-rich repeats (NBS-LRR), are responsible for intracellular sensing (Athman and Philpott, 2004). The ultimate result of these receptors' stimulation is the host inflammatory response, important for controlling infection. Vertebrates feature an additional layer of surveillance, represented by specialized phagocytic cells, like macrophages (resident in many tissues throughout the body, such as the lungs, gut, liver and spleen) and neutrophils, which circulate in the blood. These types of cells participate in basal immune responses by actively finding, engulfing and killing microbes (Alberts et al., 2002).

Plants do not have mobile defender cells, but express inducible basal immunity triggered by PAMPs perception at the level of every single cell in contact with a potential pathogen. The model plant Arabidopsis, for example, can respond to a wide range of PAMPs including LPS, bacterial elongation factor EF-Tu (Kunze et al., 2004), bacterial flagellin and its conserved 22-aminoacid peptide flg22 (Gomez-Gomez et al., 1999). Leucine-rich repeat receptor-like protein kinases (LRR-RLKs), abundant on the plant cell surface, have been shown to serve as receptors for PAMPs (Nurnberger and Kemmerling, 2006). Some of these receptors have been recently identified and characterized in Arabidopsis. FLS2, a member of the LRR-RLK protein family, recognizes flagellin and the flg22 peptide (Gomez-Gomez and Boller, 2000). The EFR receptor kinase perceives Ef-Tu (Zipfel et al., 2006).

The events following PAMPs recognition include ion fluxes in and out of the cells, production of reactive oxygen species, activation of MAP kinase signaling pathways and transcriptional reprogramming. The final outputs of induced innate immunity include cell wall crosslinking, extracellular formation of heterogeneous appositions called "papillae", secretion of defense peptides and compounds, and expression of defense-related proteins (Chisholm et al., 2006). A complete signal transduction pathway has been described for the plant cell response to flagellin perception (Asai et al., 2002). The FLS2 receptor perceives flg22 in the extracellular milieu and initiates signal transduction. Downstream of FLS2, a MAP kinase cascade (MEKK1, MKK4/MKK5, and MPK3/MPK6) leads to expression and activation of the WRKY22 and WRKY29 transcription factors, which are key positive regulators of defense responses (Asai et al., 2002).

In any given plant species, innate immunity seems to be sufficient to confer broad-spectrum resistance against most microorganisms, including those capable of causing disease on other plant species. This phenomenon is usually referred to as "nonhost" resistance (Espinosa and Alfano, 2004; Nurnberger and Lipka, 2005).

Pathogen-specific and acquired immune responses

Vertebrates, in addition to non-specific basal immunity, express acquired (or adaptive) immune responses, mediated by specialized white blood cell types (T-cells and B-cells). Type B lymphocytes, or B cells, recognize non-self antigens and to specifically produce antibodies against them (Alberts et al., 2002).

Although plants lack an adaptive immune system similar to that developed by animals, they have achieved in a totally different way a significant level of specificity in their immune response. In the course of co-evolution with their microbial pathogens, plants have developed a remarkably effective defense mechanism, known as gene-for-gene resistance, which is pathogen- and cultivar-specific and can be envisioned as an additional layer of defense, superimposed on basal immunity (Abramovitch et al., 2006; Shan et al., 2007). The genetic basis of such resistance lies in the presence of a dominant allele of a resistance (*R*) gene in the host, and of the corresponding avirulence (*avr*) gene in the pathogen. R genes, with a few exceptions, encode intracellular receptor-like proteins with a nucleotide-binding site, leucine-rich repeats (NBS-LRR), and either a coiled-coil domain or a Toll-interleukin-1-like domain at the N terminus (Martin et al., 2003). Microbial *avr* genes typically encode a variety of proteins (effectors) secreted by the pathogen directly into the cytoplasm of the host cell. While the genetic basis of gene-

for-gene resistance is well understood, the biochemical mechanism is still obscure in the vast majority of cases. There have been only a few instances in which a direct interaction was demonstrated between a bacterial Avr and the corresponding host R protein (Scofield et al., 1996; Tang et al., 1996; Deslandes et al., 2003). Most Avr-R genetic interactions are currently interpreted with the "guard" hypothesis, whereby R proteins do not act as receptors for the Avr factors, but rather guard, or monitor, other host proteins (targets of effector virulence functions) for effector-induced modifications (Jones and Dangl, 2006).

The outcome of gene-for-gene resistance is rapid and localized cell death at the sites of contact with the pathogen (termed hypersensitive response, or HR), which limits further spread of the infection. The seminal discovery of gene-for-gene resistance in plants led to the basic distinction between compatible and incompatible interactions, where "compatible" refers to the interaction between a virulent pathogen and a susceptible host (the outcome of which is disease), and "incompatible" describes the interaction between an avirulent pathogen and a resistant host (no disease occurs in this case). The comparatively more recent understanding of nonhost resistance has brought on a more complex and multi-layered scenario, in which different forms of resistance partially overlap and complement each other to increase the immune coverage of plants (Thordal-Christensen, 2003; da Cunha et al., 2006; Jones and Dangl, 2006).

Systemic Acquired Resistance

Whereas the animal immune system can keep "memory" of encounters with individual pathogens and establish a specific long-lasting immune protection of the exposed individual, plants do not have a similar capability. They do, nonetheless, develop

a broad-range long-term systemic immunity, following localized exposure to pathogens, called Systemic Acquired Resistance (SAR) (Durrant and Dong, 2004). At the molecular level, SAR is characterized by systemic expression of defense markers, such as pathogenesis related (PR) proteins, a group of extracellular proteins with antimicrobial properties. SAR is strictly dependent on the signaling molecule salicylic acid (SA) (Gaffney et al., 1993), and can in fact be triggered by exogenous application of SA (Uknes et al., 1992). Furthermore, SAR is dependent on an intact SA signal transduction pathway, and on induction of the cellular secretory pathway (Wang et al., 2005). SAR expression in tissues far from an infection site implies the existence of long-distance signals whose identities are still elusive. While it has been conclusively demonstrated that SA is not the mobile signal that triggers SAR in distal tissues (Vernooij et al., 1994), several studies indicate that other signaling molecules, possibly lipid-derived, like jasmonates, may be playing this role (Grant and Lamb, 2006).

THE BACTERIAL TYPE III SECRETION SYSTEM

To successfully colonize their hosts and cause disease, microbial pathogens have evolved sophisticated strategies that enable them to evade or suppress basal defenses and to interfere with other cellular processes. Regardless of their lifestyle (intracellular for most animal pathogens, extracellular for plant pathogens), pathogenic bacteria need to subvert the host cell metabolism in order to gain access to nutrients, multiply and spread further. To achieve these goals, pathogenic bacteria produce and deliver to the host cells a variety of virulence-mediating proteins that interfere with host defense and housekeeping cellular pathways.

A powerful virulence mechanism that is conserved among several Gram-negative bacterial pathogens is the so-called type III secretion system (TTSS), used to deliver proteinaceous virulence factors directly into the host cells (He et al., 2004; Galan and Wolf-Watz, 2006). These proteins are referred to as type III effectors, because they are translocated into the host cell cytoplasm via the TTSS. The molecular machinery responsible for such translocation is very complex, consisting of more than 20 conserved proteins, including regulatory and structural components. Among the bacteria that employ the TTSS there are human pathogens, such as *Yersinia*, *Salmonella*, *Shigella*, *Chlamydia spp.*, enteropathogenic and enterohemorragic *Escherichia coli*, and plant pathogenic bacteria such as *Pseudomonas*, *Ralstonia*, *Erwinia* and *Xanthomonas spp.* (He et al., 2004). Regardless of the high conservation of core components, TTSS structure and substrates (translocated type III effectors) vary substantially among bacterial species. The most notable structural distinction is that between the extracellular portion of the

TTSS in animal and plant pathogenic bacteria (Buttner and Bonas, 2003; He et al., 2004). Commonly, each TTSS has a basal apparatus inserted in the inner and outer bacterial membranes. In animal pathogens, the extracellular component is a stiff needle-like structure. High resolution electron microscopy, combined with other techniques, allowed the development of detailed models of the *Salmonella typhimurium* (Marlovits et al., 2004) and of the *Shigella flexneri* (Sani et al., 2007) needle complex, highlighting the sophistication of this protein-translocation machinery.

In phytopathogenic bacteria, the TTSS basal apparatus is connected to a flexible filamentous appendage called a "pilus", which is sufficiently long to potentially cross the plant cell wall (Brown et al., 2001). The pilus elongates from the tip (Li et al., 2002) and functions as a conduit for the translocation of secreted proteins into the plant cell cytosol (Jin and He, 2001; Li et al., 2002). Structural and functional components of the type III secretion system in plant pathogenic bacteria are encoded by the *hrp* genes (for hypersensitive response and pathogenicity), so called because they are necessary for the bacterium to elicit HR in resistant plants and to cause disease in susceptible hosts (Lindgren, 1997).

Host cellular targets of bacterial virulence functions

Type III effectors of animal pathogens and their functions inside the host cell have been extensively studied. Many excellent reviews summarize the current knowledge on bacterial type III effectors and their host targets (Mota and Cornelis, 2005; Viboud and Bliska, 2005; Schlumberger and Hardt, 2006). Investigating the action mechanism of type III effectors in the eukaryotic cell has revealed how virulent pathogens subvert

certain host cellular functions, such as signal transduction, cytoskeleton dynamics, cell cycle progression and vesicular trafficking, to their benefit (Galan and Cossart, 2005). This interference is usually accomplished by physical interaction between the type III effectors and host cell regulatory proteins. An intriguing aspect of such interactions is that type III effectors often mimic the action of eukaryotic enzymes and regulators, acting, for example, as phosphatases, kinases, GTPase activating proteins and so on (Mota and Cornelis, 2005). In particular, several effectors seem to exert their virulence functions by modulating the activity of host small regulatory GTPases.

Comparatively less is known about virulence functions of type III effectors of phytopathogens (Grant et al., 2006). Interestingly, common themes can be identified across pathogens and host kingdoms, such as suppression of host basal defenses and modulation of host gene expression (Buttner and Bonas, 2003).

Uncovering the function of TTSS effectors of plant pathogens

It is perhaps not surprising that many type III effectors of plant pathogenic bacteria were initially identified based on their ability to trigger a hypersensitive response in specific plant species or cultivars. These type III effectors were therefore named "avirulence" (Avr) proteins, because they "betray" the pathogen's presence to the plant that has the genetic background to recognize it. In the context of dynamic co-evolution of plant pathogens and their hosts, the current hypothesis is that all type III effectors have a virulence function to begin with, but plants have evolved R proteins capable of recognizing the presence of some of these type III effectors, in a species- or cultivar-specific manner (Alfano and Collmer, 2004; Shan et al., 2007). Validating this

hypothesis, several studies have demonstrated how bacterial Avr proteins indeed contribute to pathogen virulence, in the absence of the cognate R proteins (Shan et al., 2000; Lim and Kunkel, 2005).

Ascribing virulence functions to the numerous type III effectors secreted by plant pathogenic bacteria is not an easy task (Chang et al., 2004). One of the critical issues that hinder studies on effector virulence functions is that of functional subtleness or redundancy. Deletion or mutation of individual effector genes in a bacterial pathogen, except in very few cases, does not result in a detectable change in virulence. Consequently, alternative methods have been adopted for characterizing effectors' functions. Stable or transient expression of single bacterial effectors *in planta* has proven to be one of the most powerful strategies for functional studies. This is also made possible by the development of model plant-pathogen systems, commonly used to simplify and hasten the study of complex interactions. Several agronomically important diseases, in fact, are difficult to study due to long generation time and poor laboratory adaptability of the host plants. Hence, the need to develop models that are amenable to genetic and molecular analyses.

PSEUDOMONAS SYRINGAE AND ARABIDOPSIS, A MODEL PATHOSYSTEM

Pseudomonas syringae is a Gram-negative phytopathogenic bacterium that grows epiphytically (on the leaf surface) on a wide variety of plants and is able to cause disease in susceptible hosts (Hirano and Upper, 2000). The *P. syringae* species includes about 40 different pathogenic variants (pathovars) that exhibit different host-specificities (Hirano and Upper, 2000).

P. syringae pathovar *tomato* DC3000 (*Pst* DC3000) is the causal agent of the bacterial speck disease in tomato. *Pst* DC3000 is also pathogenic on the model plant *Arabidopsis thaliana*, and thus is widely used in laboratories to investigate the mechanisms of plant response to bacterial infection (Preston, 2000; Katagiri et al., 2002). To initiate pathogenesis, *P. syringae* enters the intercellular space of leaves (apoplast) through stomata or wounds. In the apoplast of susceptible hosts, *Pst* DC3000 multiplies to high population levels (in the range of 10^8 cells/cm^2 of leaf). Disease symptoms develop gradually on infected leaves, starting with the appearance of water-soaking, followed by the formation of necrotic lesions surrounded by chlorotic halos.

The *Arabidopsis-Pst* DC3000 interaction represents one of the best studied model systems in plant pathology. Both the plant and pathogen genomes are fully sequenced (Buell et al., 2003; The Arabidopsis Genome Initiative, 2000) and they are the subjects of comprehensive genomic and functional genomics analyses, resulting in resources made readily accessible to the scientific community (www.arabidopsis.org; www.pseudomonas-syringae.org).

Virulence factors of *P. syringae* pv. *tomato* (*Pst*) DC3000

One major known virulence factor of *Pst* DC3000 is the phytotoxin coronatine (Bender et al., 1999). While it is not clear how coronatine reaches the host cell, its importance for virulence is highlighted by the observation that coronatine-lacking mutants of *Pst* DC3000 fail to multiply aggressively and cause disease when surface-inoculated onto Arabidopsis leaves (Melotto et al., 2006). Coronatine was believed for a long time to inhibit rapid induction of host defenses in the early stages of pathogen establishment and disease development (Mittal and Davis, 1995). Only recently, a study on bacterial entry through stomata has shed light on the key role played by coronatine. Melotto *et al.* (2006) showed that, while the plant closes its stomata upon recognizing the presence of bacteria or bacterial elicitors on the leaf surface, coronatine induces stomatal re-opening, allowing *Pst* DC3000 to enter more efficiently the apoplastic space, thus promoting colonization (Melotto et al., 2006).

The other component that is essential for virulence is the type III secretion system (TTSS). The *Pst* DC3000 TTSS delivers into the host cell an arsenal of at least thirty different type III effectors (Lindeberg et al., 2006; Schechter et al., 2006).Their collective key role in pathogenesis is inferred by the fact that *Pst* DC3000 *hrp*$^-$ mutants, impaired in regulation or secretion of type III effectors, lose the ability to multiply and cause disease in compatible hosts (Yuan and He, 1996; Roine et al., 1997).

The mechanisms by which individual type III effectors of *Pst* DC3000 affect host metabolism and promote symptom development are beginning to be elucidated. Targets being revealed include certain host pathways and processes such as basal defenses, gene expression, hormone responses and programmed cell death (Grant et al., 2006). At least

three effectors of *Pst* DC3000 (HopM1, AvrE, AvrPto) and two (AvrRpt2, AvrRpm1) of other *P. syringae* pathovars were found to be inhibitors of a specific cell wall-based immune response, which is callose deposition in papillae (Hauck et al., 2003; DebRoy et al., 2004; Kim et al., 2005).

The *Pst* DC3000 effector AvrPto

One of the most thoroughly investigated type III effectors of *Pst* DC3000 is AvrPto, a mostly hydrophilic 163 aa polypeptide of 18.3 KDa, expressed in bacteria during either compatible or incompatible interactions (Salmeron and Staskawicz, 1993).

AvrPto was initially identified as an avirulence protein in the *Pst*-tomato system. *Pseudomonas syringae* pv. *maculicola* transconjugants, expressing the *avrPto* gene cloned from an avirulent *Pst* strain (Race 0), became avirulent on tomato cultivars encoding the resistance gene *Pto* (Ronald et al., 1992). Soon after this discovery, a locus tightly linked to *Pto*, named *Prf*, was found to be also responsible, with *Pto*, for AvrPto-triggered resistance to *Pst* (Salmeron et al., 1994). Tomato *Pto* encodes a serine-threonine protein kinase (Martin et al., 1993), whereas *Prf* encodes a protein with leucine-zipper, nucleotide-binding, and leucine-rich repeat motifs (Salmeron et al., 1996). AvrPto interacts with the Pto kinase in the yeast two-hybrid system, and this interaction is the basis of the gene-for-gene specificity in this system (Tang et al., 1996). Interestingly, the AvrPto-Pto interaction has never been observed *in planta*.

While acting as an avirulence protein in the presence of the cognate R genes, AvrPto displays a virulence function in their absence. For instance, AvrPto was shown to slightly enhance the virulence of *Pst* strain T1 in tomato plants lacking Pto (Shan et al.,

2000). Deletion of AvrPto from the bacterial genome does not result in loss of avirulence, and this was explained when a second effector was identified, named AvrPtoB, which also interacts with the Pto kinase to trigger the hypersensitive response (Kim et al., 2002). Although AvrPto and AvrPtoB have redundant avirulence activities, they are very different proteins and have distinct and additive virulence functions: a double *avrPto/avrPtoB* bacterial mutant displays a larger decrease in virulence than either of the single mutants in susceptible tomato plants (Lin and Martin, 2005).

In Arabidopsis (which lacks Pto orthologs), AvrPto contributes to virulence by suppressing basal defenses. Inducible expression of this effector *in planta* promotes susceptibility to TTSS-deficient mutants of *Pst* DC3000 and suppresses callose deposition in papillae, a hallmark cell wall-based defense (Hauck et al., 2003). More recently, AvrPto was shown to be a potent suppressor of PAMP-induced gene expression and MPK3/MPK6 activation in Arabidopsis protoplasts, intercepting PAMP-dependent signaling upstream of MAPKKK (He et al., 2006).

The molecular and cellular mechanism by which AvrPto exerts its virulence functions is still unknown. Solution of its structure has not provided helpful clues in this regard (Wulf et al., 2004). A yeast two-hybrid screening for tomato proteins that interact with AvrPto yielded four interactors: a stress-related protein, a putative N-myristoyl transferase and two small GTPases, most similar to mammalian Rab8 (Bogdanove and Martin, 2000). AvrPto interaction with Rab8-like GTPases represents a particularly interesting finding, because Rab8 is a major regulator of polarized secretion in mammalian cells (Huber et al., 1993; Peranen et al., 1996; Gerges et al., 2004; Hattula et

al., 2006). This would indicate a potential interference of AvrPto with the plant cell secretory pathway.

VESICLE TRAFFICKING IN HOST-PATHOGEN INTERACTIONS

Protein and membrane traffic in the eukaryotic cell

Eukaryotic cells are functionally compartmentalized into membrane-bound organelles with specialized functions. Communication and transport between these compartments are vital to the cell and are accomplished through complex and tightly regulated pathways (Harter and Wieland, 1996; Sanderfoot and Raikhel, 2003; van Vliet et al., 2003; Jurgens, 2004). Some unique features differentiate the plant endomembrane system from that of animals or yeast. Plant cells, for instance, contain a large number of Golgi stacks disseminated throughout the cytoplasm, and large distinct vacuoles, functioning either as storage or lytic organelles (Jurgens, 2004).

Proteins, metabolites and membrane components to be transported between compartments are typically "packaged" in membrane-bound vesicles. Movement and target specificity of highly diverse vesicles are regulated at the molecular and biochemical level, through the involvement of sophisticated protein machineries and of the cytoskeleton. The main players of these pathways are proteins with conserved functions, shared between all eukaryotes. The secretory pathway and its components have been extensively characterized in yeast and mammalian cells, but are still comparatively less well understood in plants (Sanderfoot and Raikhel, 2003).

Small GTPases: key regulators of vesicle trafficking

Small monomeric GTPases represent a large superfamily of conserved regulatory proteins that control several processes in the eukaryotic cell. Based on their sequence,

they are subdivided into five families (Ras, Ran, Rho, Arf and Rab) with distinct functions (Takai et al., 2001). Ras GTPases regulate cell proliferation in yeast and animals; they represent the only group of small GTPases that has no known counterparts in plants (Vernoud et al., 2003). The Ran family regulates transport of RNAs and proteins across the nuclear envelope. The Rab, Arf and Rho families play distinct critical roles in intracellular vesicle trafficking.

Rab GTPases act as molecular switches to regulate budding of membrane-bound vesicles from a donor compartment, movement toward a target compartment, tethering and fusion of the vesicles with the target membrane (Zerial and McBride, 2001). Arf proteins modulate endocytic and secretory trafficking and organelle structure. Among their functions are recruitment of vesicle coat proteins and regulation of actin remodeling near the cell surface (D'Souza-Schorey and Chavrier, 2006). Arfs participate, for instance, in the biogenesis of clathrin-coated vesicles during endocytosis, and of COPI /COPII vesicles, which shuttle proteins between the ER and the Golgi apparatus. Rho GTPases play a critical role in the establishment and maintenance of cell polarization, mainly by controlling the actin cytoskeleton spatial organization (Park and Bi, 2007).

Most of the current knowledge on polarized vesicle trafficking in plant cells was gained studying growth in pollen tubes and root hairs, cells that show a highly polarized structure and tip growth. Several small GTPases of the Rab, Arf and Rho families were characterized as necessary regulators of tip growth, controlling vesicular trafficking, cytoskeleton organization and signaling (Cole and Fowler, 2006; Samaj et al., 2006).

The Rab family of small GTPases

Rab proteins represent the largest family of small GTPases, accounting for about 60 members in humans and 57 in Arabidopsis (Vernoud et al., 2003). Like all small monomeric GTPases, Rabs typically cycle between an "active" (GTP-bound) and an "inactive" (GDP-bound) state. Many accessory proteins are required for Rabs activation, inactivation and recycling back from target to donor compartment. Rab escort proteins (REPs) facilitate delivery of newly synthesized Rabs to the appropriate membrane and initial loading with GDP. Exchange of GDP for GTP, which activates Rab, is mediated by guanine nucleotide-exchange factors (GEFs). Hydrolysis of bound GTP is also mediated by accessory proteins, named GTPase-activating proteins (GAPs), which accelerate the otherwise very low intrinsic GTPase activity of Rabs. Finally, GDP dissociation inhibitors (GDIs) extract inactive GDP-Rab from the target membrane and escort it through the cytoplasm back to the donor membrane, to serve for another round of transport (Segev, 2001; Stenmark and Olkkonen, 2001).

The so-called "switch" regions of small GTPases adopt very different conformations in the GDP-bound and in the GTP-bound state; GTP-bound but not GDP-bound Rabs can interact with downstream components of the intracellular trafficking machinery, to achieve vesicle tethering and fusion with the target membrane.

Effectors of animal pathogens target the cell trafficking pathways

The cytoskeleton and membrane trafficking system have been known for a long time to be major targets of TTSS effectors produced by intracellular bacterial pathogens of animals. To reach their reproductive niche inside the host cell, these pathogens must

induce their own phagocytosis first, then they must influence the host endomembrane system in order to avoid fusion with the lysosome and degradation.

Small GTPases of the Rho subfamily are among the main known host targets of TTSS effectors of bacteria such as *Salmonella* and *Yersinia*, which manipulate host cytoskeleton dynamics during infection (Juris et al., 2002; Mota and Cornelis, 2005; Viboud and Bliska, 2005; Schlumberger and Hardt, 2006).

Recent studies revealed that human pathogens subvert cell trafficking also by targeting Rab GTPases. For example, the *Legionella pneumophila* effector protein DrrA/SidM acts as a potent GEF on Rab1, to control its intracellular distribution and to recruit it to the LCV (*Legionella*-containing vacuole) (Machner and Isberg, 2006; Murata et al., 2006). An analogous situation was observed in *Chlamydia trachomatis*, an obligate intracellular pathogen that multiplies in a non-lysosomal structure called an "inclusion". Several host Rab GTPases are recruited to the inclusion membrane, including Rab4A (Rzomp et al., 2003). A chlamydial inclusion membrane protein, CT229, interacts specifically with Rab4A and presumably mediates its recruitment to this highly specialized host-pathogen interface (Rzomp et al., 2006).

Similarly, during epithelial cells infection with *Salmonella enterica* serovar Typhimurium, a large number of Rabs were found to localize to the *Salmonella*-containing vacuole (SCV) membrane, including Rab7, which is normally responsible for late endosome fusion with the lysosome (Smith et al., 2007). Rab7 activity involves interaction with the microtubule motor complex, mediated by the bridging protein RILP. SifA, a secreted effector of *Salmonella*, was able to interact in vitro with Rab7 and was

shown to take part in uncoupling Rab7 from RILP (Harrison et al., 2004). This provides some clue on how the SCV escapes maturation into a lysosome.

The secretory and endocytic pathways in plant innate immunity

In the last few years, the evidence for a major role of the plant secretory pathway in defense against pathogens has been rapidly growing (Robatzek, 2007).

Among the most common plant defense responses that are associated with secretory processes is formation of callose-containing papillae. Papillae are heterogeneous appositions that form between the plasma membrane and the cell wall at the infection site and are believed to reinforce the cell wall, acting as a barrier against the invading microorganisms. Although callose, a β-1,3-glucan, is synthesized at the plasma membrane (Fink et al., 1987; Kauss, 1987), papillae deposition is coupled with polarized secretion (Assaad et al., 2004; Soylu et al., 2005). Interestingly, while PAMPs, as well as TTSS-deficient and nonhost bacteria, induce rapid papilla deposition, virulent bacteria suppress this response, via not yet understood mechanisms (Brown et al., 1993; Hauck et al., 2003; Keshavarzi et al., 2004; Soylu et al., 2005).

SNARE proteins (which include syntaxins) are essential components of the machinery that drives fusion of secretory vesicles with their appropriate target membrane. The Arabidopsis plasma membrane-localized syntaxin SYP121/PEN1 was found to be critical for nonhost resistance against *Blumeria graminis*, a fungal pathogen of barley (Collins et al., 2003). Upon fungal infection, PEN1 is rapidly recruited at the sites of papilla deposition, and seems to favor timely papilla formation; *pen1* mutant plants,

challenged with pathogens, produce papillae with a notable delay compared to wild-type plants (Assaad et al., 2004).

A key regulator of SAR and SA-dependent defense responses, NPR1, controls expression of genes encoding secretory pathway proteins in Arabidopsis. Null mutations in some of these genes cause increased susceptibility to *P. syringae* and a concomitant decrease in secretion of the PR1 marker protein (Wang et al., 2005).

One of *P. syringae* TTSS effectors, HopM1, targets several Arabidopsis proteins for degradation, including AtMIN7 (Nomura et al., 2006). AtMIN7 is a member of the GEF family of proteins that activate Arf GTPases, which are also involved in regulating vesicle trafficking.

Furthermore, an important role for the endocytic pathway in PAMP-dependent signaling has been recently identified. Upon perception of the flg22 peptide elicitor, the plasma membrane-localized FLS2 flagellin receptor fused to green fluorescent protein (GFP) rapidly disappears from the plasma membrane and is detected in vesicle-like structures, indicating internalization by endocytosis (Robatzek et al., 2006).

RATIONALE

Previous research conducted in our laboratory addressed the virulence function of the *Pst* DC3000 effector AvrPto in Arabidopsis. Transgenic expression of AvrPto in Arabidopsis plants caused suppression of host defense responses, resulting in susceptibility to TTSS-deficient mutants of *Pst* DC3000 (Hauck et al., 2003). AvrPto interacted in the yeast two-hybrid system with the Arabidopsis RabE proteins, small GTPases predicted to regulate post-Golgi traffic to the plasma membrane (P. Hauck).

This finding is particularly interesting because it suggests that AvrPto may exert at least one of its virulence functions by interfering with the host cell secretory pathway.

In this study, I applied a live cell imaging approach to visualize the Arabidopsis RabE proteins in the plant cell, and to investigate whether and how AvrPto affects RabE cell biology. The results of this study are described in Chapter 2 of this dissertation.

The RabE family of GTPases has not been thus far functionally characterized in Arabidopsis. I, therefore, pursued functional analysis of RabE by site-directed mutagenesis and *in planta* transgenic expression, with the dual purpose of investigating RabE role in the plant cell and in defense against pathogens. Chapter 3 of this dissertation illustrates the progress toward these goals.

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CHAPTER 2

Virulence function of the *Pseudomonas syringae* effector protein AvrPto is associated with altered intracellular localization of the small GTPase RabE in Arabidopsis

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I want to acknowledge the following contributions:
- Paula Hauck performed the yeast two-hybrid analysis and contributed Figure 2 - 3;
- Lori Imboden performed the yeast two-hybrid interaction assay between AvrPto and mutant variants of RabE1d and contributed Figure 2 - 5;
- Kinya Nomura characterized Arabidopsis plants expressing 6xHis-AvrPto and contributed Figure 2 - 1.

ABSTRACT

Many Gram-negative pathogenic bacteria cause disease in their animal or plant hosts by means of multiple virulence effector proteins, delivered into the host cell via the type III secretion system (TTSS). Elucidating individual effectors' functions is fundamental for understanding bacterial infection of plants. We have previously shown that transgenic overexpression of the *Pseudomonas syringae* pv. *tomato* strain DC3000 effector AvrPto in the host plant Arabidopsis leads to suppression of basal defenses and enables multiplication of non-pathogenic TTSS-defective bacteria. AvrPto localization at the host plasma membrane is required for its avirulence and virulence functions in tomato and in Arabidopsis protoplasts. In this study, we confirmed that membrane localization is required for AvrPto to exert its virulence function in transgenic Arabidopsis plants as well. Furthermore, we found that AvrPto interacted in yeast two-hybrid assay with the Arabidopsis RabE small GTPase proteins, which are homologous to mammalian Rab8 and yeast Sec4p, known regulators of polarized secretion. Microscopic analysis and cell fractionation studies revealed that transgenically expressed GFP-RabE1d and endogenous RabE proteins were associated with the Golgi apparatus and the plasma membrane in Arabidopsis leaves. Strikingly, transgenic expression of membrane-localized, but not soluble, AvrPto in Arabidopsis impaired the normal localization of RabE1d at the Golgi apparatus. Such effect on RabE1d localization depended on its nucleotide-binding state. Overexpression of RabE1d could partially counteract the AvrPto-induced susceptibility to TTSS-defective bacteria. Our experiments uncover a novel association between the AvrPto virulence function and RabE subcellular distribution.

INTRODUCTION

A common virulence mechanism, shared by many Gram-negative bacterial pathogens of plants and animals, is the delivery of bacterial proteins directly into the host cell via the type III secretion system (TTSS) (Buttner and Bonas, 2003; He et al., 2004; Mota and Cornelis, 2005). These proteins, collectively called TTSS effectors, alter the host cellular processes to favor pathogen growth. To carry out virulence functions, effectors interact with (and often biochemically modify) critical regulatory components of basic host cellular functions. Bacterial pathogens of animals, such as *Salmonella* and *Yersinia*, employ various type III effectors to interfere with host cytoskeleton dynamics, vesicle trafficking, signal transduction, apoptosis, and potentially other pathways (Mota and Cornelis, 2005). Only recently, effectors from phytopathogenic bacteria and their host targets have started to be elucidated. A major virulence activity of TTSS effectors of *Pseudomonas syringae* seems to be suppression of host defenses (Alfano and Collmer, 2004; Mudgett, 2005; Nomura et al., 2005; Abramovitch et al., 2006; Desveaux et al., 2006; Nomura et al., 2006).

Studying the function of effectors delivered by bacterial pathogens of plants is greatly facilitated by the use of model host-pathogen systems such as the *Arabidopsis thaliana-P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) interaction. *Pst* DC3000 is the causal agent of bacterial speck disease on Arabidopsis and tomato; its pathogenicity is dependent on a functional TTSS. *Pst* DC3000 mutant strains that are defective in the TTSS, such as *hrcC*$^-$ (formerly *hrpH*$^-$) and *hrpA*$^-$, are unable to multiply and to cause disease on host plants, behaving as non-pathogens (Yuan and He, 1996; Roine et al.,

1997). *Pst* DC3000, like several other plant pathogenic bacteria, produces an array of 30 or more type III effectors (Lindeberg et al., 2006; Schechter et al., 2006). The function of most of these proteins in the host cell has yet to be determined. A common feature of type III effectors is that bioinformatic analysis of protein sequences, in most cases, does not provide clues to their function. Moreover, mutation or deletion of single effectors often does not result in reduced virulence, indicating functional subtlety and/or redundancy, when delivered by bacteria during infection.

Our group previously showed that AvrPto, one of *Pst* DC3000 TTSS effectors, when transgenically expressed in Arabidopsis, greatly compromises plant basal defenses and promotes susceptibility to non-pathogenic bacteria, such as TTSS-defective mutants (Hauck et al., 2003). Other studies have shown that AvrPto expression promotes susceptibility to nonhost *P. syringae* strains (He et al., 2006) and suppresses pathogen-associated molecular pattern (PAMP)-induced signal transduction, early defense gene expression and other associated responses (Kang et al., 2004; Li et al., 2005; Oh and Collmer, 2005; He et al., 2006). However, the molecular mechanism by which AvrPto exerts its virulence function(s) in the plant cell is still elusive. The best understood function of AvrPto is that of triggering gene-for-gene resistance in tomato plants carrying the *Prf* resistance gene (Mucyn et al., 2006). Such avirulence function depends on physical interaction, demonstrated in yeast two-hybrid assay (Y2H), between AvrPto and the Pto kinase (Scofield et al., 1996; Tang et al., 1996). However, AvrPto contributes to *Pst* DC3000 virulence in tomato lacking Prf, and this virulence function does not require Pto (Shan et al., 2000a). Arabidopsis has no known ortholog(s) of Pto or Prf.

To gain insight into the virulence effects of AvrPto in Arabidopsis, we conducted a Y2H screen for Arabidopsis proteins that interact with AvrPto. Among the interactors, we identified members of the RabE family of small GTPases, closely related to mammalian Rab8 (Huber et al., 1993), *Saccharomyces pombe* Ypt2 (Craighead et al., 1993), and *Saccharomyces cerevisiae* Sec4p (Goud et al., 1988), which are well-known regulators of polarized secretion. Interestingly, a previous Y2H screening of a tomato cDNA library for AvrPto-interacting proteins also yielded two small GTPase proteins (named Api2 and Api3) that are similar to Rab8 (Bogdanove and Martin, 2000). Rab proteins are key regulators of vesicle formation and transport between membrane-bound cellular compartments (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). The identification of Rab GTPases as AvrPto interactors in two different host species (Arabidopsis and tomato) raises the intriguing possibility that one of the virulence functions of this effector may be to perturb intracellular vesicle trafficking in the plant. Interestingly, small GTPases regulating cytoskeleton dynamics and vesicle trafficking are among the most common host targets of TTSS effectors produced by animal bacterial pathogens (Harrison et al., 2004; Machner and Isberg, 2006; Murata et al., 2006; Rzomp et al., 2006; Smith et al., 2007). Importance of vesicle traffic and the secretory pathway for plant defense and bacterial virulence was recently highlighted by several studies (Collins et al., 2003; Wang et al., 2005; Nomura et al., 2006) and reviews (Field et al., 2006; Robatzek, 2007).

Very little is known about the function of RabE GTPases in the Arabidopsis cell. Rab proteins, in general, are considered as specific markers of endomembrane compartments. This specificity prompted us to apply a live cell imaging approach to

visualize the Arabidopsis RabE proteins in the plant cell, and to investigate whether and how AvrPto affects RabE cell biology. We found that RabE1d is associated with both the plasma membrane (PM) and the Golgi apparatus in Arabidopsis cells. *In planta* expression of AvrPto impaired RabE1d localization at the Golgi, without affecting its localization at the PM. Such effect on RabE1d subcellular distribution was dependent on AvrPto localization at the host membrane and on the RabE1d nucleotide binding state. Furthermore, RabE1d overexpression in Arabidopsis transgenic plants reduced the virulence effect of AvrPto. Our data suggest that one of the mechanisms by which AvrPto carries out its virulence function in the Arabidopsis cell is by interfering with subcellular localization of RabE proteins, which likely leads to perturbation of intracellular vesicle trafficking.

MATERIALS AND METHODS

Yeast two-hybrid screen

Arabidopsis proteins that interacted with AvrPto of *Pst* DC3000 were identified by following the Matchmaker LexA-based Y2H system manual (Clontech Laboratories Inc., Palo Alto, CA). Two Arabidopsis cDNA libraries, made using RNA from pathogen-infected and uninfected *Landsberg erecta* plants (kindly provided by Dr. J. Jones, Sainsbury Laboratory, UK), were screened. The *avrPto* coding sequence was amplified from *Pst* DC3000 genomic DNA by PCR (sense primer: 5'-GCGAATTCCGAACCATGGGAAATATATGTGTC-3'; antisense primer: 5'-GCCTCGAGATTGCCAGTTACGGTA-3'; the *Eco*RI and *Xho*I restriction sites, used for cloning, are underlined) and cloned into pNLexA, to serve as bait in the Y2H screening.

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 was on average 100 μE, and the temperature was kept constant at 20°C. Bacteria were cultured in low-salt LB medium (10g/l Tryptone, 5g/l Yeast Extract, 5g/l NaCl), supplemented with the appropriate antibiotics. 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, Friendship, WV). Titer of the bacterial inoculum was $1x10^6$ colony forming units (CFUs)/ml, unless

otherwise indicated. Plants were inoculated by vacuum-infiltration, and bacteria enumeration in leaves was conducted as described (Katagiri et al., 2002).

AvrPto and 6xHis-AvrPto transgenic plants

Generation of transgenic Arabidopsis plants that express AvrPto under the control of the dexamethasone (DEX)-inducible promoter was previously described (Hauck et al., 2003). To generate 6xHis-AvrPto-expressing plants, a 6xHistidine tag was added to the N-terminus of AvrPto by PCR, and the PCR product was cloned into the pTA7002 binary vector (Aoyama and Chua, 1997) for DEX-inducible expression in Arabidopsis.

***RabE* cloning and mutagenesis**

The *RabE1d* (At5g03520) coding sequence was amplified from Arabidopsis Col-0 cDNA using the rabE-5' and rabE-3' primers (Table 2.1), containing the *Eco*RI and *Bam*HI restriction sites, respectively. The PCR product was ligated into a TOPO vector (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 (Table 2 - 1). 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 for introducing 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 (Table 2 - 1). RabE1d-S29N and RabE1d-Q74L amplification products were introduced in a TOPO vector by TA-cloning and sequenced.

Primer	Sequence
rabE-5'	5'-gaattc**atg**gcggttgcgccggcaag-3'
rabE-3'	5'-ggatccgagcaatcatactc**cta**aac-3'
Q74L-for	5'-cactgctggtc**t**agaacgtttc-3'
Q74L-rev	5'-gaaacgttct**a**gaccagcagtg-3'
S29N-for	5'-gtggggaaga**a**ttgtttgttac-3'
S29N-rev	5'- gtaacaaacaa**t**tcttccccac-3'

Table 2 - 1: Primers for *RabE* cloning and mutagenesis.

Restriction sites are underlined (*Eco*RI for rabE-5', *Bam*HI for rabE-3').
Start and stop codons are in bold; single nucleotide changes are in bold and underlined.

GFP-RabE1d transgenic plants

RabE1d and *RabE1d-S29N*, cloned in the TOPO vector as described above, were subcloned in the *Eco*RI and *Bam*HI sites of the binary expression vector pEGAD (Cutler et al., 2000), downstream of the enhanced green fluorescent protein (hereafter 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 (gl1)*, as well as AvrPto-expressing 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 correct size GFP-RabE fusion proteins was verified by western blot.

Protein extraction and immunoblotting

Total proteins were extracted as follows: approximately 20 mg (fresh weight) of fresh or frozen leaf tissue were ground with a pestle in a microfuge tube in the presence of 100 µl of 1× SDS-PAGE loading buffer [90 mM Tris-HCl pH 8.0, 100 mM DTT, 3% SDS, 22.5% sucrose, 10 µl/ml Protease Inhibitor Cocktail for Plant Cell Extracts (Sigma), bromophenol blue (to saturation)]. Extracts were immediately heated at 80°C for 10 minutes and then frozen at -20°C. Before loading on gel, extracts were thawed at room temperature and centrifuged at 20,000×*g* for 2 minutes, to pellet debris. An equal volume of each sample was used for SDS-PAGE electrophoresis. Total proteins were

separated on precast gradient gels (4-20%, ISC BioExpress), then transferred onto Immobilon-P membrane (Millipore, MA) using a semi-dry transfer apparatus (SEMI PHOR, Hoefer Scientific Instruments, San Francisco, CA). Protein detection was carried with the following primary antibodies: anti-AvrPto (raised in rabbit against recombinant AvrPto protein expressed in *E. coli*, Cocalico Biological, Inc.), anti-RabE (raised in chicken against recombinant RabE protein expressed in *E. coli*, Cocalico Biological, Inc.), anti-XT1 (Faik et al., 2002; Cavalier and Keegstra, 2006), anti-PM ATPase (Morsomme et al., 1998), and anti-γTIP (gift of Dr. N. Raikhel, unpublished).

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 5 ml of ice-cold extraction buffer [50 mM HEPES pH 7.5, 100 mM KCl, 10 mM EDTA, 1 mM DTT, and 10 µl/ml Protease Inhibitor Cocktail for Plant Cell Extracts (Sigma)] containing 34% sucrose. The extract was homogenized with a Polytron immersion blender (3 pulses of 10 seconds each), filtered through a single layer of Miracloth and centrifuged for 10 minutes at 10,000×*g*, to remove most unbroken chloroplasts and nuclei. The supernatant was adjusted to 40% sucrose in about 10 ml final volume (concentration was determined with a refractometer), and layered on top of a 5 ml cushion of 50% sucrose, in clear ultracentrifugation tubes. The homogenate was subsequently layered with 10 ml of 34% sucrose, 8 ml of 25% sucrose and 8 mL of 18% sucrose. All sucrose solutions were prepared in the same buffer used for extraction. Gradients were centrifuged at 100,000×*g* for 3 hours, at 4°C, in a SW28 rotor (Beckman). After centrifugation, the membrane-

containing interphases were collected, diluted with sucrose-free extraction buffer, and membranes were collected by ultracentrifugation (1 hour at 100,000×*g*). Membrane pellets were resuspended in equal volumes of SDS-PAGE loading buffer and heated at 80°C for 10 minutes. Protein electrophoresis and western blot were performed as described above.

Confocal microscope 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× or a 40× 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 Inc.). For FM4-64 staining, detached Arabidopsis leaves were submerged in 8.2 μM FM4-64 (Molecular Probes, Leiden, The Netherlands) in water for 15 minutes. 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

Transient expression in Arabidopsis leaves was achieved by biolistic transformation. The binary vector bearing rat sialyl transferase fused to DsRed (ST-RFP)

was a kind gift of Dr. F. Brandizzi (Saint-Jore et al., 2002). Gold particles (1.0 μm, Bio-Rad) were coated with the pEGAD::*RabE1d* or ST-RFP plasmid DNA as described by Zhang *et al.* (Zhang et al., 2001). Arabidopsis leaves were harvested and arranged on MS agar plates (4.3 g/l MS salts, 0.8% agar, pH 5.7). The DNA-coated particles were delivered into the lower leaf epidermis with a particle gun (Dupont), using 1100 psi rupture discs, under a vacuum of 25 in Hg. After bombardment, leaves were incubated in the sealed plates at room temperature, and fluorescence was observed 24 hours post transformation.

For co-imaging 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.

RESULTS

AvrPto membrane localization is required for virulence function in Arabidopsis plants

Hauck *et al.* (2003) showed that AvrPto protein expression in transgenic Arabidopsis plants compromised basal defense and allowed increased multiplication of TTSS-defective mutant bacteria. To characterize this phenomenon further, we wanted to determine if membrane localization of AvrPto is necessary for these effects. First, we needed to confirm membrane-association of AvrPto in these transgenic Arabidopsis plants. Consistent with studies showing that AvrPto is targeted to the host plasma membrane in transgenic tobacco and tomato plants (Shan et al., 2000b), and in Arabidopsis protoplasts (He et al., 2006), AvrPto was exclusively detected in the membrane fraction, but not in the soluble fraction, of the stable transgenic Arabidopsis plants (Fig. 2 - 1, A).

We also generated Arabidopsis plants that express AvrPto carrying an N-terminal 6xHis tag, under the control of the DEX-inducible promoter (Aoyama and Chua, 1997). In contrast to wild-type AvrPto, 6xHis-AvrPto did not localize to the membrane fraction, but remained in the soluble fraction (Fig 2 - 1, A). This result is consistent with studies showing that host-mediated myristoylation of a conserved glycine at the N-terminus of AvrPto is responsible for AvrPto association with the host membrane. Site-directed mutagenesis of the myristoylation site disrupts AvrPto plasma membrane localization (Shan et al., 2000b; He et al., 2006). Our result suggests that addition of the short 6xHis tag to the N-terminus of wild-type AvrPto was sufficient to disrupt AvrPto membrane

localization, most likely by preventing its myristoylation. Significantly, while expression of membrane-localized AvrPto in Arabidopsis promoted susceptibility to TTSS-deficient mutants, expression of soluble 6xHis-AvrPto did not (Fig 2 - 1, B). This result demonstrates that the virulence function of transgenically expressed AvrPto depends not only on protein production, but on association with the host membrane.

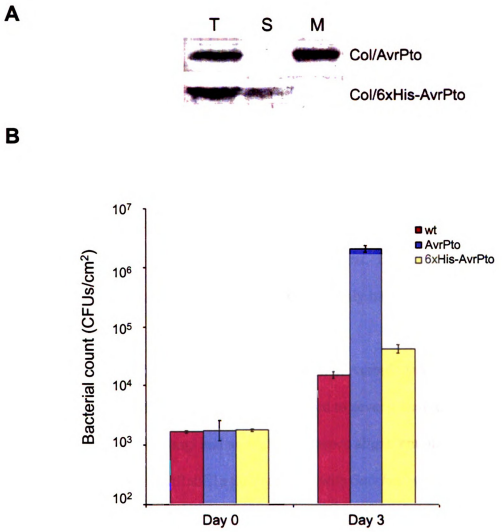

Figure 2 - 1: Membrane localization is critical for AvrPto virulence function.

(A) Western blot analysis: anti-AvrPto antibody was used to detect AvrPto and 6xHis-AvrPto in transgenic plants extracts. Transgene expression was induced by spraying the plants with 30μM DEX, 24h prior to protein extraction. Total extracts were centrifuged for 1h at 100,000 × *g*, at 4°C, to separate the membrane fraction (pellet) from the soluble fraction (supernatant). T = total extract; S = soluble fraction; M = membrane fraction.

(B) AvrPto and 6xHis-AvrPto expression was induced by spraying the plants with 30μM DEX 24 h prior to bacterial inoculation, and then at 24 h intervals during the course of the experiment. *hrcC*⁻ TTSS-deficient mutant bacteria were syringe-infiltrated in the plant leaves at a concentration of 10^6 CFUs/ml.

Figure contributed by Kinya Nomura.

AvrPto interacts with the Arabidopsis RabE small GTPases in Y2H assay

To gain insights into the molecular basis of AvrPto-mediated promotion of Arabidopsis susceptibility to bacterial infection, we conducted Y2H screening of two Arabidopsis cDNA libraries, using AvrPto as bait. AvrPto was found to interact with several Arabidopsis proteins, 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, At5g16840) and several putatively chloroplast- or mitochondria-targeted proteins. Because AvrPto is localized at the host membrane, we chose to focus on RabE, the only interactor that is also predicted to be membrane-localized.

There are five highly similar RabE proteins in Arabidopsis (Rutherford and Moore, 2002; Vernoud et al., 2003), closely related to several characterized regulators of the secretory pathway in fungi and animals. Sequence alignment of the five *Arabidopsis thaliana* RabE proteins (AtRabE1a through E1e) with *Saccharomyces pombe* Ypt2 (SpYpt2), human Rab8a (HsRab8a), *Drosophila melanogaster* Rab8 (DmRab8) and *Saccharomyces cerevisiae* Sec4p (ScSec4p) illustrates this high similarity (Figure 2 - 2).

Arabidopsis contains a total of 57 Rabs, classified into eight families (RabA through H), based on conserved motifs and similarity to the equivalent Rab classes in yeast and animals (Rutherford and Moore, 2002; Vernoud et al., 2003). We examined the ability of AvrPto to interact with representative members of the other Rab protein families. We cloned and expressed *RabA1a*, *B1b*, *C1*, *D2a*, *F2a*, and *G3a* in yeast; none interacted with AvrPto in the Y2H system (Fig. 2 - 3, A). In addition, we investigated whether AvrPto interacts with other members of the RabE family. 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 (Figure 2 - 3, B). Thus, it appears that AvrPto interacts specifically with the Arabidopsis RabE family of GTPases.

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                                          PM1                          PM2
AtRabE1b/1-216  -----MAAPPARARADYDYLIKLLLIGDSGVGKSCLLLRFSDGSFTTSFITTIGIDFKIR
AtRabE1c/1-216  -----MAAPPARARADYDYLIKLLLIGDSGVGKSCLLLRFSDGSFTTSFITTIGIDFKIR
AtRabE1a/1-216  -----MAAPPARARADYDYLIKLLLIGDSGVGKSCLLLRFSDGSFTTSFITTIGIDFKIR
AtRabE1d/1-216  -----MAVAPARARSDYDYLIKLLLIGDSGVGKSCLLLRFSDDTFTTSFITTIGIDFKIR
AtRabE1e/1-218  -----MAVAPARARSDYDYLIKLLLIGDSGVGKSCLLLRFSDDTFTTSFITTIGIDFKIR
SpYpt2/1-200    ----------MST-KSYDYLIKLLLIGDSGVGKSCLLLRFSEDSFTPSFITTIGIDFKIR
HsRab8a/1-207   ------------MAKTYDYLFKLLLIGDSGVGKTCVLFRFSEDAFNSTFISTIGIDFKIR
DmRab8/1-207    ------------MAKTYDYLFKLLLIGDSGVGKTCILFRFSEDAFNTTFISTIGIDFKIR
ScSec4p/1-215   MSGLRTVSASSGNGKSYDSIMKILLIGDSGVGKSCLLVRFVEDKFNPSFITTIGIDFKIK

                          PM3
AtRabE1b/1-216  TIELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGILLVYDVTDESSFNNIRNWIRNIEQ
AtRabE1c/1-216  TIELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGILLVYDVTDESSFNNIRNWIRNIEQ
AtRabE1a/1-216  TIELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGILLVYDVTDESSFNNIRNWIRNIEQ
AtRabE1d/1-216  TVELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGILLVYDVTDESSFNNIRNWMKNIEQ
AtRabE1e/1-218  TVELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGILLVYDVTDESSFNNIRNWMKNIEQ
SpYpt2/1-200    TIELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGILLLYDVTDKKSFDNVRTWFSNVEQ
HsRab8a/1-207   TIELDGKRIKLQIWDTAGQERFRTITTAYYRGAMGIMLVYDITNEKSFDNIRNWIRNIEE
DmRab8/1-207    TIELDNKKIKLQIWDTAGQERFRTITTAYYRGAMGIMLVYDITQEKSFENIKNWIRNIEE
ScSec4p/1-215   TVDINGKKVKLQLWDTAGQERFRTITTAYYRGAMGIILVYDVTDERTFTNIKQWFKTVNE

                      G2                                G3
AtRabE1b/1-216  HASDNVNKILVGNKADMDESKRAVPKSKGQALADEYGIKFFETSAKTNLNVEEVFFSIAK
AtRabE1c/1-216  HASDNVNKILVGNKADMDESKRAVPTAKGQALADEYGIKFFETSAKTNLNVEEVFFSIGR
AtRabE1a/1-216  HASDSVNKILVGNKADMDESKRAVPKSKGQALADEYGMKFFETSAKTNLNVEEVFFSIAK
AtRabE1d/1-216  HASDNVNKILVGNKADMDESKRAVPTAKGQALADEYGIKFFETSAKTNLNVENVFMSIAK
AtRabE1e/1-218  HASDSVNKILVGNKADMDESKRAVPTSKGQALADEYGIKFFETSAKTNQNVEQVFLSIAK
SpYpt2/1-200    HASENVYKILIGNKCDCED-QRQVSFEQGQALADELGVKFLEASAKTNVNVDEAFFTLAR
HsRab8a/1-207   HASADVEKMILGNKCDVND-KRQVSKERGEKLALDYGIKFMETSAKANINVENAFFTLAR
DmRab8/1-207    NASADVEKMLLGNKCELTD-KRQVSKERGEQLAIEYGIKFMETSAKASINVEEAFLTLAS
ScSec4p/1-215   HANDEAQLLLVGNKSDMET--RVVTADQGEALAKELGIPFIESSAKNDDNVNEIFFTLAK


AtRabE1b/1-216  DIKQRLADTDSRAEPATIKISQTDQA-AGAGQATQKSACCGS-
AtRabE1c/1-216  DIKQRLSDTDSRAEPATIKISQTDQA-AGAGQATQKSACCGT-
AtRabE1a/1-216  DIKQRLADTDARAEPQTIKINQSDQG-AGTSQATQKSACCGT-
AtRabE1d/1-216  DIKQRLTETDTKAEPQGIKITKQDT--AASSSTAEKSACCSYV
AtRabE1e/1-218  DIKQRLTESDTKAEPQGIKITKQDANKASSSSTNEKSACCSYV
SpYpt2/1-200    EIKKQKIDAENEFSNQANNVDLG-NDRTVKR-------CC---
HsRab8a/1-207   DIKAKMDKKLEGNSPQGSNQGVKITPDQQKRSSFFR--CVLL-
DmRab8/1-207    DIKAKTEKRMEANNPPKGGHQLKPMDSRTKDSWLSR--CSLL-
ScSec4p/1-215   LIQEKIDSNKLVGVGNGKEGNISINSGSGNSS---KSNCC---
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Figure 2 - 2: ClustalW alignment of the five Arabidopsis RabE proteins and their closest homologues in other organisms.

Yellow boxes highlight the highly conserved nucleotide-binding domain residues (PM = phosphate/magnesium-binding domain; G = guanine base-binding domain). Blue boxes highlight Rab-specific residues (Stenmark and Olkkonen, 2001). Amino acids in red are commonly mutated in functional studies, to create Rab variants that have a higher affinity for GDP than for GTP (S or T in PM1), or that cannot hydrolyze GTP (Q in PM3). Mutating the N in G2 results in Rabs that cannot bind any nucleotide. The two C-terminal C residues represent geranylgeranylation sites.

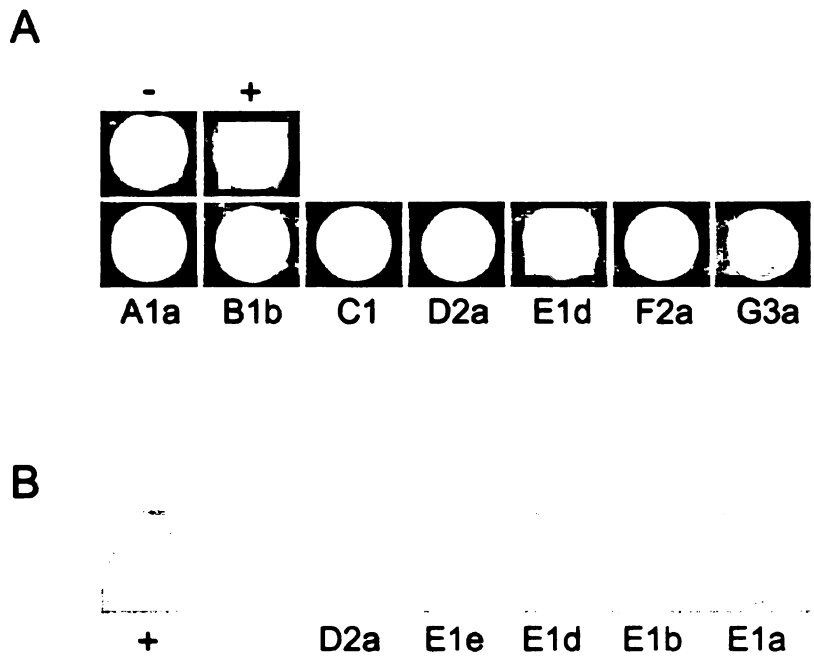

Figure 2 - 3: AvrPto interacts with Arabidopsis RabE in the yeast two-hybrid system.

(A) Y2H assay demonstrating that AvrPto (in the bait vector pNLexA) interacts with Arabidopsis RabE1d, but not with other members of the Rab superfamily (in the prey vector pB42AD). Interaction is visualized by development of the blue color on media containing X-Gal.
(-) empty vectors, negative control; (+) pLexA-A53 + pB42AD-T, positive control.

(B) Y2H assay demonstrating AvrPto (in pNLexA) interaction with four of the five Arabidopsis RabE proteins (in pB42AD). Interaction is visualized by development of the blue color on media containing X-Gal. Yeast expressing AvrPto in pNLexA and RabD2a in pB42AD is shown as negative control.
(+) pLexA-A53 + pB42AD-T, positive control.

Figure contributed by Paula Hauck.

Gene expression analysis of the Arabidopsis *RabE* gene family

Some Arabidopsis Rab families are uncommonly large. Careful analysis of genome duplications showed that 44 out of 57 *Rab* genes reside in duplicated regions. Of the five *RabE* genes, for instance, *RabE1d* and *E1e* appear to be deriving from a major duplication event between chromosomes III and V, and the same holds true for *RabE1b* and *E1c* (Rutherford and Moore, 2002).

The high degree of sequence identity among RabE proteins (equal or higher than 86%) suggests functional redundancy. Members of gene families are, in some cases, preferentially expressed in different tissues, or at specific developmental stages, or in response to stresses. We, therefore, investigated whether this is the case with the five *RabE* genes, by gathering information on their expression through the TAIR website (www.arabidopsis.org). *In silico* analysis revealed that all five genes are expressed in all Arabidopsis tissues and developmental stages. *RabE1d* and *E1e* (encoding 94% identical proteins) were the only two family members whose expression was much lower in pollen than in all other tissues. According to the TAIR database, *RabE1d* was the most highly expressed in rosette leaves, immediately followed by *RabE1c*. The *RabE1a*, *E1e* and *E1b* genes had the lowest expression levels. RT-PCR analysis on rosette leaves confirmed these data (Figure 2 - 4).

To gain insight on potential up- or down-regulation of members of the RabE family in responses to pathogens or other stresses, the expression pattern of the *RabE* genes was analyzed with the AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp) (Schmid et al., 2005), across several publicly available microarray datasets. 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 on whether the five RabE proteins have redundant or distinct functions, we chose to utilize RabE1d (At5g03520), which has the highest mRNA expression level in rosette leaves.

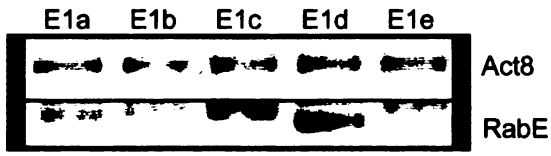

Figure 2 - 4: RT-PCR showing *RabE* gene expression in rosette leaves.

RT-PCR reaction products representing the five *RabE* genes, reverse-transcribed and amplified from Arabidopsis Col-0 rosette leaf total RNA. A single reverse transcription reaction was performed, and equal amounts of the resulting cDNA were used as template in PCR reactions. Each PCR reaction contained primers for the *Actin8* gene, in addition to primers for one of the *RabE* genes (procedure described in detail in Chapter 3; primers are listed in Table 3 - 1). Five out of 25µl of PCR product were loaded on 1% agarose gel; 21 cycles of amplification were used for *Act8*, 25 for the *RabE* genes.

AvrPto preferentially interacts with GTP-bound RabE

In the cell, Rab GTPases act as molecular switches that cycle between an "active" GTP-bound and an "inactive" GDP-bound state. Interconversion between the two forms is stimulated by accessory proteins. A guanosine nucleotide exchange factor (GEF) converts a GDP-bound Rab into the GTP-bound form, whereas a GTPase-activating protein (GAP) stimulates the Rab low intrinsic GTPase activity, causing hydrolysis of GTP into GDP (Novick and Brennwald, 1993). Mutation of conserved residues is widely used to alter the nucleotide-exchange and the GTP-hydrolysis activities of Rab proteins for functional analysis (Stenmark et al., 1994). Substitution of a conserved serine or threonine with asparagine in the PM1 nucleotide-binding domain results in Rabs that have a stronger preference for GDP than for GTP, whereas substitution of a conserved glutamine with leucine in the PM3 catalytic domain inhibits both intrinsic and GAP-stimulated GTP hydrolysis (Figure 2 - 2) (Stenmark et al., 1994). We engineered the mutant forms RabE1d-S29N and RabE1d-Q74L by site-directed mutagenesis.

In the Y2H system, AvrPto interacted with only wild-type RabE1d or RabE1d-Q74L (predicted to be mostly in the GTP-bound form), but not with RabE1d-S29N (predicted to be mostly GDP-bound) (Fig 2 - 5). Nucleotide-binding state is known to affect the conformation of Rab proteins (Stenmark and Olkkonen, 2001). Our results indicate that the active GTP-bound form of RabE, but not the inactive GDP-bound, is in the appropriate conformation for interacting with AvrPto.

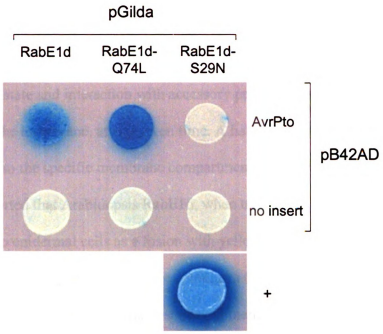

Figure 2 - 5: Wild-type RabE and RabE-Q74L, but not RabE-S29N, interact with AvrPto in Y2H.

Yeast two-hybrid assay demonstrating interaction between AvrPto (in the prey vector pB42AD) and RabE1d or RabE1d-Q74L, but not RabE1d-S29N (all in the bait vector pGILDA). For this experiment only, the mutant RabE1d proteins, as well as wild type RabE1d as a control, were modified by substituting the two C-terminal conserved cysteine residues, which are sites of geranylgeranylation (Figure 2 - 2), with glycine and serine, to prevent prenylation and membrane association.
As a negative control, yeast expressing the three RabE1d versions in pGILDA and empty pB42AD vector is shown. Interaction in visualized by development of blue color on X-Gal-containing media.
(+) pLexA-A53 + pB42AD-T, positive control.

Figure contributed by Lori Imboden.

RabE1d is associated with Golgi apparatus and plasma membrane

Each Rab protein is normally present in cells in two pools, one of which is cytoplasmic, the other is 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 (YFP), was detected in the Golgi apparatus and in the cytoplasm (Zheng et al., 2005). However, AvrPto was reported to be localized at the host plasma membrane (Shan et al., 2000; He et al., 2006). To determine RabE localization in Arabidopsis cells, we created stable transgenic plants that express RabE1d fused with enhanced green fluorescent protein (GFP), under the control of the CaMV 35S promoter. GFP was fused to the N-terminus of RabE1d, to preserve the C-terminal CAAX geranylgeranylation site, critical for membrane association and function (Calero et al., 2003). Several independent transgenic lines were analyzed by confocal laser scanning microscopy. GFP fluorescence was observed in intracellular punctate structures consistent with the Golgi apparatus, as detected in tobacco cells (Zheng et al., 2006), but also at the cell periphery (Fig 2 - 6).

Leaf epidermal cells typically contain a very large vacuole that accounts for most of the cell volume. Fluorescence detected at the cell periphery may represent the PM, or the vacuolar membrane (tonoplast), or even the thin layer of cytoplasm that is between the PM and the tonoplast. To more precisely determine whether GFP-RabE was also localized at the PM, we stained live leaf tissue with the lipophylic dye FM4-64 (Fischer-

Parton et al., 2000; Bolte et al., 2004). FM4-64, which produces a bright red fluorescence when it is in membranes but not in aqueous solutions, is rapidly incorporated in 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 15-30 minutes after incorporation (depending on the system used), FM4-64 will selectively stain the PM. Confocal microscope analysis revealed overlap of GFP-RabE1d fluorescence with FM4-64 fluorescence, immediately after staining, suggesting RabE association with the plasma membrane (Fig 2 - 7, A).

To investigate whether the punctate structures labeled by GFP-RabE1d corresponded to the Golgi apparatus, we examined co-localization 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-RabE transgenic leaves, via particle bombardment. Observation of cells co-expressing GFP-RabE1d and ST-RFP revealed overlapping fluorescence signals, confirming RabE association with the Golgi apparatus (Fig 2 - 7, B).

Taken together, microscopic analysis of GFP-RabE1d localization suggested that, in native Arabidopsis leaf cells, membrane-associated RabE1d is found not only in the Golgi apparatus, as previously reported, but also in the PM.

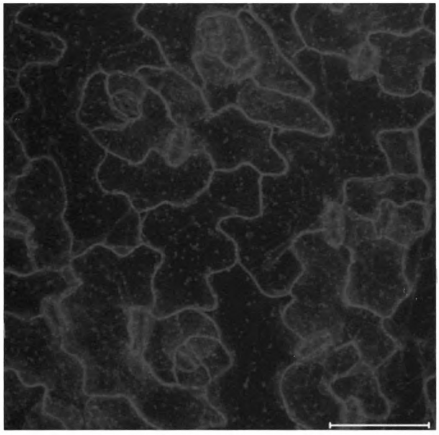

Figure 2 - 6: GFP-RabE1d localization in Arabidopsis leaf cells.

Confocal microscope image of the leaf epidermis of a transgenic Arabidopsis plant expressing GFP-RabE1d. This image represents 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.
Scale bar = 50μm.

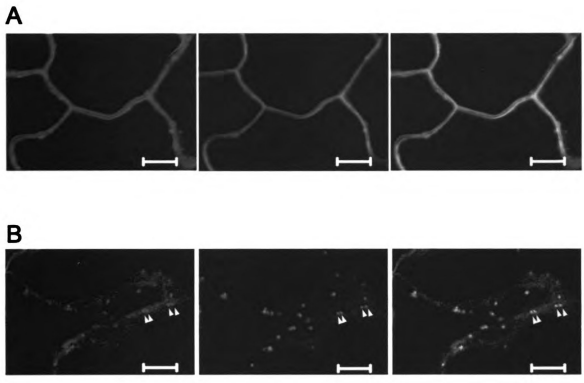

Figure 2 - 7: GFP-RabE1d is localized at the plasma membrane and at the Golgi apparatus in Arabidopsis cells.

(A) Confocal microscope image showing overlapping fluorescence of GFP-RabE1d and FM4-64, at the plasma membrane. The image is a single focal plane crossing adjacent cells (40x oil immersion objective, 4x zoom). Left panel: GFP-RabE1d fluorescence, green; center panel: FM4-64 fluorescence, red; right panel: merged image, in which the yellow color results from the overlap of red and green.
Scale bar = 10 μm.

(B) Confocal microscope image showing overlapping fluorescence of GFP-RabE and RFP fused to sialyl transferase (ST), a marker of the Golgi apparatus. The image is a single focal plane crossing the cytoplasm of a cell (40x oil immersion objective, 4x zoom). Left panel: GFP-RabE1d fluorescence, green; center panel: ST-RFP fluorescence, red; right panel: merged image, in which the yellow color results from the overlap of red and green. The arrowheads point at some of the co-labeled Golgi stacks.
Scale bar = 10 μm.

Endogenous RabE co-fractionates with PM and Golgi markers

GFP-RabE1d expression in the transgenic plants was driven by the strong 35S constitutive promoter. To exclude the possibility that the observed localization of RabE reflects patterns of only the overexpressed protein, we analyzed the localization of endogenous RabE in transgenic as well as wild-type Arabidopsis plants. We performed subcellular fractionation by centrifugating clarified plant extracts on sucrose step gradients. Our flotation method allowed separation of the PM from a fraction containing lighter membranes (Golgi, tonoplast). The endogenous RabE proteins, as well as the transgenically expressed GFP-RabE1d, were detected in both fractions, with the bulk of membrane-associated RabE 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 co-fractionated with XT1, a Golgi apparatus resident protein (Faik et al., 2002; Cavalier and Keegstra, 2006) (Fig. 2 - 8) . The tonoplast marker, γTIP, was found predominantly in the same fraction as the Golgi marker. Overall, the membrane fractionation experiments complemented the microscope analysis; together, they indicate that endogenous and ectopically expressed RabE proteins are not only localized at the Golgi apparatus, but a significant pool is associated with the PM in Arabidopsis leaf cells.

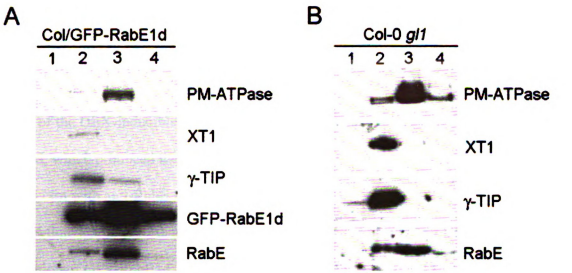

Figure 2 - 8: Detection of subcellular localization of endogenous RabE and transgenically expressed GFP-RabE1d by membrane fractionation technique.

Western blot of membrane fractions from **(A)** transgenic plants overexpressing GFP-RabE1d and **(B)** wild-type Arabidopsis plant extract.
Total membranes were separated by flotation on a sucrose step gradient. Lanes 1 through 4 represent the 4 membrane fractions collected at the interfaces between layers of different sucrose concentration, after ultracentrifugation: 18-25% (1), 25-34% (2), 34-40% (3) and 40-50% (4). Equal volumes of each fraction were loaded on SDS-PAGE gel. PM-ATPase is a PM marker, XT1 is a trans-Golgi resident protein, and γ-TIP is a marker for the tonoplast. GFP-RabE1d and endogenous RabE proteins were detected with a polyclonal chicken anti-RabE antibody developed for this study (K. Nomura).

AvrPto expression in Arabidopsis alters RabE localization at the Golgi

To investigate a possible effect of AvrPto on RabE *in vivo*, we produced double transgenic plants by transforming pEGAD::*RabE1d* into AvrPto-expressing plants (Hauck et al., 2003). In these double transgenic plants, expression of GFP-RabE1d is under the control of CaMV 35S promoter, whereas AvrPto expression is under the control of the DEX-inducible promoter. Several independent transgenic lines were obtained, which produced the fusion protein, as confirmed by microscope analysis and by western blot with anti-RabE antibodies (Figure 2 - 9).

Microscopic examination revealed a striking effect of AvrPto on the subcellular distribution of GFP-RabE1d. After AvrPto induction, the level of GFP-RabE1d in the Golgi apparatus was greatly reduced and, in some experiments, was undetectable (Figure 2 - 10). This effect seems specific to the Golgi-associated pool of GFP-RabE1d, as localization of GFP-RabE1d at the plasma membrane appeared unperturbed.

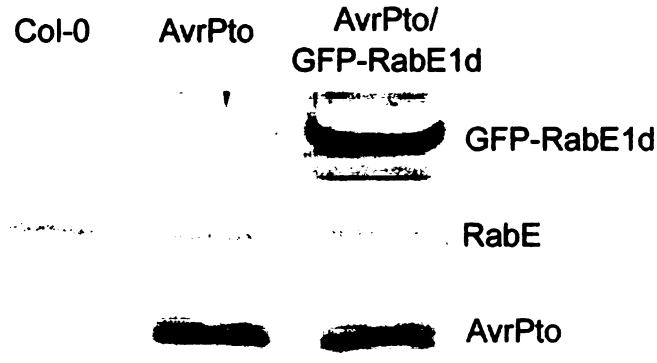

Figure 2 - 9: Western blot indicating expression of GFP-RabE1d and of AvrPto in leaves of double-transgenic plants.

GFP-RabE1d was constitutively expressed (under the CaMV 35S promoter); AvrPto expression was induced by spraying the leaves with 30μM DEX, 24 hours prior to protein extraction. Equal volumes of protein extracts, containing roughly equal amounts of proteins, were loaded in each lane. At least three independent transgenic lines were analyzed (not shown), showing similar protein expression levels.

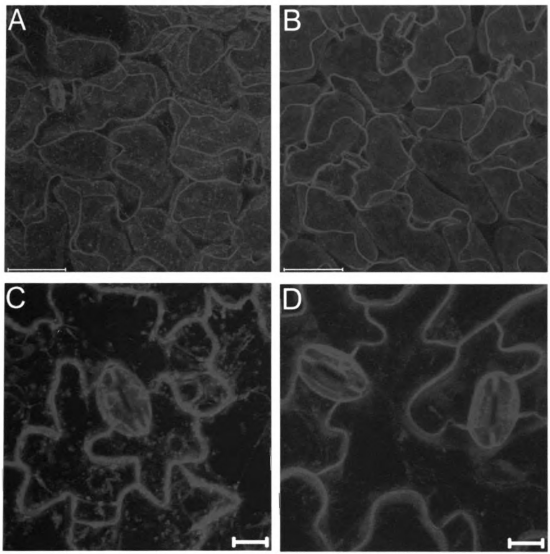

Figure 2 - 10: AvrPto expression alters intracellular distribution of GFP-RabE1d.

Confocal microscopic images of Arabidopsis plants co-expressing GFP-RabE1d and AvrPto. AvrPto expression was induced by spraying plants with 30μM DEX; water was sprayed on a different set of plants, as a control.

(A) and **(B)** represent water- and DEX- treated samples, respectively. Each image is a projection along the Z-axis of several focal planes intersecting the upper epidermis and the palisade mesophyll cell layers. Scale bar = 50 μm.

(C) and **(D)** represent water- and DEX- treated samples, respectively, at higher magnification. Scale bar = 10 μm.

To further characterize this phenomenon, we generated Arabidopsis Col-0 plants expressing GFP-RabE1d-S29N (under the control of CaMV 35S promoter) and AvrPto (under the control of DEX-inducible promoter). RabE1d-S29N failed to interact with AvrPto in the Y2H system, as shown in Fig. 2 - 5. In the absence of inducer, the subcellular distribution of GFP-RabE1d-S29N closely mirrored that of wild-type RabE1d. The GFP-RabE-S29N protein was partly cytosolic, as demonstrated by diffuse fluorescence and abundant cytoplasmic strands. A pool of GFP-RabE1d-S29N was observed in association with membranes; the distribution was reminiscent of that of wild-type RabE, at the cell periphery and in intracellular punctate structures (Figure 2 - 11, A). A closer look at the peripheral localization, using FM4-64 as a PM marker, revealed that the RabE-S29N mutant was also localized at the PM (Figure 2 - 11, B).

Interestingly, DEX-induction of AvrPto expression in the transgenic plant did not affect GFP-RabE1d-S29N localization at the Golgi (Figure 2 - 12).

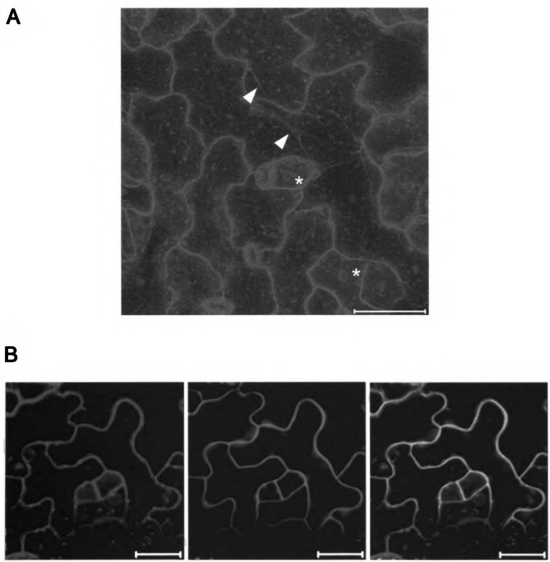

Figure 2 - 11: Subcellular distribution of the GFP-RabE1d-S29N protein.

(A) Confocal microscope image of a representative Arabidopsis leaf expressing GFP-RabE1d-S29N. Projection along the Z-axis of several focal planes crossing the epidermal cell layer. Arrowheads point at cytoplasmic strands, and asterisks mark fluorescence in the perinuclear region. Scale bar = 50μm.

(B) Overlapping fluorescence of GFP-RabE-S29N and FM4-64 at the plasma membrane. From left to right: GFP-RabE1d-S29N, green (left); FM4-64, red (center); merged image, in which yellow results from the overlap of green and red (right). The image represents a single focal plane. Scale bar = 20μm.

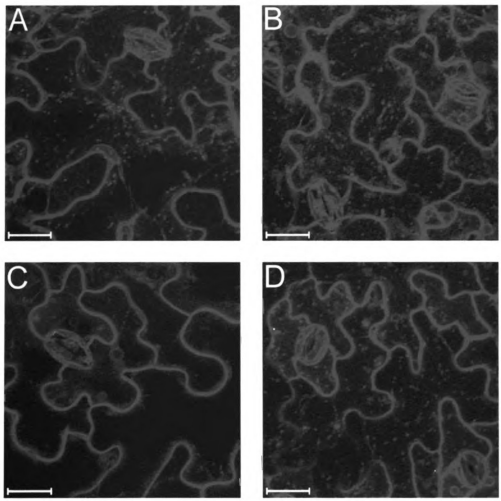

Figure 2 - 12: Intracellular localization of RabE-S29N is unaffected by AvrPto expression.

Confocal microscope images of double transgenic plants expressing either GFP-RabE1d and AvrPto (left panels), or GFP-RabE1d-S29N and AvrPto (right panels). Expression of AvrPto was induced with DEX. A 30μM DEX solution in water, or water alone (as a control), were infiltrated in leaves with a needle-less syringe. Control and treated leaves were detached and observed 24 hours after infiltration.

(A) and **(B)** represent, respectively, RabE1d and RabE1d-S29N localization upon water infiltration.

(C) and **(D)** represent, respectively, RabE1d and RabE1d-S29N localization upon AvrPto induction.

All images are projections along the Z-axis of several focal planes. Scale bar = 20 μm.

We extended our analysis of AvrPto-induced RabE relocalization to the transgenic Arabidopsis plants expressing 6xHis-AvrPto. As shown in Figure 2 - 1, 6xHis-AvrPto was not membrane-associated and did not exert its virulence function in Arabidopsis. We investigated whether soluble 6xHis-AvrPto can affect RabE subcellular localization. GFP-RabE1d was transiently expressed in Arabidopsis leaves that also expressed 6xHis-AvrPto or AvrPto (as a control). Remarkably, GFP-RabE1d fluorescence at the Golgi apparatus was unaltered in the presence of 6xHis-AvrPto, whereas the Golgi-associated fluorescence was greatly reduced in the presence of untagged AvrPto (Fig. 2 - 13). This result indicates that reduction of the Golgi-localized RabE1d pool requires AvrPto localization at the host membrane.

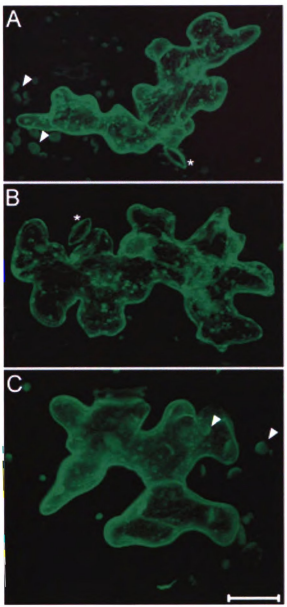

Figure 2 - 13: 6xHis-AvrPto does not affect RabE localization at the Golgi.

Confocal microscope images of Arabidopsis epidermal leaf cells transiently expressing GFP-RabE1d. A 30 μM DEX solution was infiltrated with a needle-less syringe in leaves of Col-0 *gl1* **(A)** and of transgenic Arabidopsis expressing 6xHis-AvrPto **(B)** or AvrPto **(C)**, respectively. Two to three hours after DEX infiltration, leaves were detached and the pEGAD::*RabE1d* plasmid was delivered by particle bombardment. Transformed leaves were observed 24 hours after bombardment.
The arrowheads indicate autofluorescent chloroplasts in the mesophyll layer (beneath the epidermal layer); the asterisks indicate autofluorescence from adjacent stomatal guard cells. Scale bar = 10μm.

RabE overexpression reduces AvrPto virulence function in transgenic plants

In Arabidopsis protoplasts, expression of the tomato Pto protein partially relieved the AvrPto-mediated suppression of flg22 marker gene induction, likely due to AvrPto sequestration by Pto (He et al., 2006). We investigated whether RabE1d overexpression could similarly reduce the virulence effect of AvrPto in transgenic plants. To address this question, we examined the ability of the TTSS-defective *hrpA*$^-$ mutant to multiply in AvrPto/GFP-RabE1d double transgenic plants. We found that GFP-RabE1d overexpression partially restricted the multiplication of *hrpA*$^-$ mutant bacteria to a level that was intermediate between those achieved on wild-type Arabidopsis and on AvrPto single transgenic plants (Fig. 2 - 14, A).

Importantly, the increased resistance from overexpression of GFP-RabE1d was not caused by a nonspecific, broad-spectrum resistance mechanism because, when challenged with *Pst* DC3000, Arabidopsis expressing GFP-RabE1d remained as susceptible as wild-type Arabidopsis (Fig. 2 - 14, B). These results therefore suggest that RabE1d overexpression specifically counteracts the virulence function of AvrPto. *Pst* DC3000 secretes many effectors; therefore the unaltered susceptibility of EGFP-RabE1d transgenic plants to this bacterium reinforces the notion that one or more other effectors produced by *Pst* DC3000 may be functionally redundant to AvrPto.

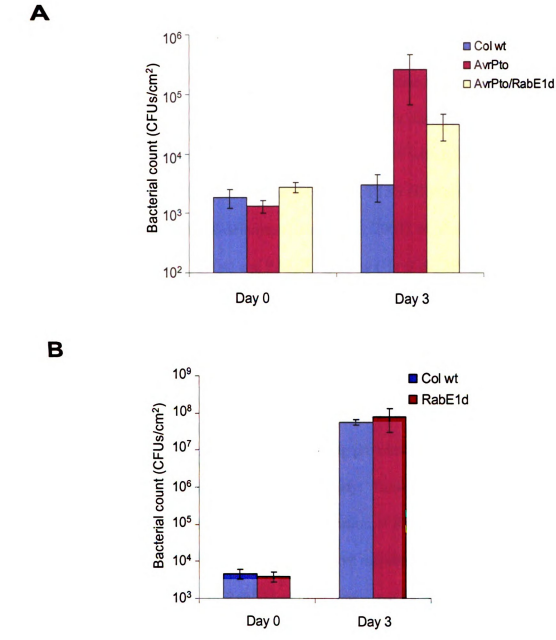

Figure 2 - 14: RabE overexpression limits AvrPto-induced bacterial multiplication.

(A) Multiplication of the TTSS-deficient hrpA- mutant on wild-type Arabidopsis, AvrPto-expressing plants and AvrPto/RabE1d-expressing plants. A 0.3µM DEX solution was sprayed on the plants 24 hours before bacterial inoculation, and then every 24 h throughout the entire course of the experiment. Bacteria were vacuum-infiltrated in the leaves at a titer of 10^6 CFUs/ml.

(B) Multiplication of *Pst* DC3000 on wild-type Arabidopsis and on RabE1d-expressing plants. Bacteria were vacuum-infiltrated in the leaves at a titer of 10^6 CFUs/ml.

DISCUSSION

To successfully colonize their hosts and cause disease, bacterial pathogens of plants and animals have evolved virulence mechanisms, such as the TTSS, that enable them to evade or suppress host defenses and to interfere with host cellular functions. AvrPto of *Pst* DC3000 is one of several bacterial TTSS effectors shown to act inside the plant cell to suppress basal defenses (Hauck et al., 2003), nonhost-resistance-associated cell death (Kang et al., 2004) and PAMP signaling (He et al., 2006; Hann and Rathjen, 2007). However, the exact mechanism by which AvrPto exerts its virulence function(s) is not yet understood.

Bogdanove and Martin (2000) first identified small GTPases as AvrPto interactors in tomato, which, together with our identification of the Arabidopsis RabE family of small GTPases as AvrPto-interacting proteins, prompted us to conduct in-depth cell biology experiments, described in this study. These experiments revealed a striking AvrPto-dependent effect on the Golgi-localization of RabE1d, and showed that this effect requires both the AvrPto localization to the host membrane and the appropriate nucleotide-binding state of RabE1d. Furthermore, we found that overexpression of RabE1d alone was sufficient to partially counteract the AvrPto-induced susceptibility to nonpathogenic *hrpA*$^-$ mutant bacteria, without activating a nonspecific resistance to *Pst* DC3000 (Figure 2 - 8). This result implicates the involvement of RabE1d in AvrPto-induced susceptibility in Arabidopsis and shows a first example of a plant pathogen effector altering subcellular localization of a host small GTPase implicated in vesicle traffic.

The inability of AvrPto expression to affect Golgi-localization of the RabE1d-S29N mutant, which does not interact with AvrPto in Y2H assay (Figure 2 - 2), suggests that AvrPto-dependent alteration of RabE1d localization requires physical interaction between the two proteins. AvrPto was shown to be located exclusively at the host PM in tomato and Arabidopsis (Shan et al., 2000; He et al., 2006). In this study, we found that a substantial pool of RabE1d is also localized at the PM. Despite the apparent co-localization of AvrPto and RabE1d to the same host membrane, we were unable to detect stable *in planta* interaction using co-immunoprecipitation methods. It was previously reported that *in vivo* co-immunoprecipitation could not be accomplished in interaction studies involving some Rab proteins (Monier and Goud, 2005). Co-immunoprecipitation of yeast Sec4p and its effector Sec15p was only achieved by using chemical crosslinkers to stabilize the interaction (Guo et al., 1999). In addition, AvrPto may represent a particularly difficult bait for co-immunoprecipitation experiments: even the interaction between AvrPto and its best-known host target, the tomato Pto kinase, was demonstrated in Y2H (Scofield et al., 1996; Tang et al., 1996) but has not yet been documented *in planta*. It is possible that AvrPto interaction with its host target proteins may be inherently transient and unstable or require special conditions. The lack of demonstrable stable interaction *in planta*, however, does not affect our conclusion that AvrPto specifically alters Golgi-localization of wild-type RabE1d, but not of the RabE1d-S29N mutant, which does not interact with AvrPto in yeast.

Previous studies showed that localization to the host PM is crucial for the virulence and avirulence activities of AvrPto in transgenic tobacco and tomato plants (Shan et al., 2000b), and in Arabidopsis protoplasts (He et al., 2006). It is therefore very

interesting to find that N-terminally tagged 6xHis-AvrPto, which was not targeted to the host membrane and had no virulence function in Arabidopsis, failed to affect RabE subcellular localization (Fig. 2 - 8). Rab proteins undergo cyclical interconversion between the GDP- and the GTP-bound form, accompanied by shuttling between donor and target membranes. Given AvrPto localization and its preference for GTP-RabE, our results can be best explained by a model in which PM-localized AvrPto encounters GTP-bound RabE1d and traps it, directly or indirectly, so that RabE1d can no longer be recycled into the cytoplasm and back to the Golgi/ trans-Golgi network (TGN). Alternatively, PM-localized AvrPto mediates a RabE1d modification so that, either the modified RabE1d cannot be extracted by host proteins to be recycled back to the Golgi/TGN, or the modified RabE1d disrupts Golgi integrity when it reaches the Golgi apparatus.

Rab proteins are often considered as molecular markers of specific cellular compartments in which they function. Arabidopsis RabE1d localization at the Golgi apparatus had been previously described (Zheng et al., 2005). In this study, we further characterized RabE subcellular distribution and discovered that a substantial amount of protein is associated with the PM, in addition to the Golgi, in Arabidopsis cells. Based on its localization in the plant cell, and on the role of its yeast and mammalian homologs, RabE is likely to function in mediating traffic of secretory vesicles between the Golgi or TGN and the PM. Although the identity of RabE-dependent trafficking cargo remains unknown, an attractive possibility is that the RabE family of small GTPases controls polarized secretion of some type of antimicrobial peptides/compounds or papilla components. Interestingly, callose deposition in papillae, a hallmark response to non-

pathogenic bacteria, is suppressed in plants expressing AvrPto (Hauck et al., 2003). Although callose is synthesized at the plasma membrane (Scheible and Pauly, 2004), papillae contain enzymes and numerous other structural compounds, and their deposition is associated with polarized vesicle trafficking (Huckelhoven et al., 1999; Assaad et al., 2004). RabE proteins may also participate in a vesicle trafficking pathway that targets PAMP receptors to the PM to establish plant basal defense. Future research is needed to test these hypotheses and to determine whether AvrPto interferes with the RabE-mediated vesicle trafficking route to impair delivery of antimicrobials and/or PAMP receptors during infection. It also remains to be determined whether AvrPto affects the subcellular localization of the other four RabE family members (i.e., RabE1a, b, c, and e). As shown in Fig. 2 - 2, AvrPto interacts with four RabE GTPases, suggesting potential redundancy among RabE family members.

The availability of sophisticated techniques for imaging live cells, such as confocal laser scanning microscopy with fluorescent tags, has opened up a whole new realm of possibilities for investigating plant-pathogen interactions (Koh and Somerville, 2006). One of the obvious advantages is the ability to examine such interactions at the single cell level, rather than at the global tissue scale. Cellular responses such as movement of the nucleus, focal accumulation of secretory vesicles and other organelles at the site of pathogen attack, papillae deposition and reorganization of actin microfilaments, have been extensively documented (Koh and Somerville, 2006). While the overall reorganization of plant cells upon microbe infection has been known for a long time, only recently individual plant proteins were identified whose subcellular localization is altered in response to pathogens or pathogen-derived elicitors (Lipka and

Panstruga, 2005). For instance, the Arabidopsis PM syntaxin PEN1 focally accumulates at the sites of attempted penetration by *Blumeria graminis*, defining microdomains reminiscent of lipid rafts in animal cells (Assaad et al., 2004; Bhat et al., 2005). The PM-localized FLS2 flagellin receptor, upon perception of the flg22 peptide elicitor, rapidly disappears from the PM and is detected in vesicle-like structures, indicating endocytosis (Robatzek et al., 2006).

Our study provides an example in which a virulence-promoting bacterial TTSS effector alters the subcellular localization of a host vesicle trafficking regulatory protein. Further analysis of RabE function in vesicle traffic may shed light not only on *P. syringae* pathogenesis and host immunity, but importantly also on the plant cellular vesicle traffic system.

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CHAPTER 3

Investigating RabE function in Arabidopsis

ABSTRACT

The virulence effector protein AvrPto, produced by the plant pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pst* DC3000), interacts in the yeast two-hybrid system with the *Arabidopsis thaliana* RabE family of small GTPases, putative regulators of post-Golgi vesicle traffic to the plasma membrane. When expressed in the plant cell, AvrPto altered the subcellular distribution of RabE, while RabE overexpression was sufficient to partially counteract the virulence function of AvrPto. These findings suggest that *Pst* DC3000 may use AvrPto (and possibly other effectors) to interfere with the host vesicle trafficking system. Although the function of RabE homologs in other eukaryotic organisms is well understood, a specific role of the RabE proteins in Arabidopsis growth, development, or defense has not yet been established. To gain insights into the biological function of RabE proteins in Arabidopsis, we produced and studied transgenic Arabidopsis plants that overexpressed either the wild-type RabE1d protein or its mutant derivatives RabE1d-S29N and RabE1d-Q74L, which are expected to be in the GTP-bound (active) or GDP-bound (inactive) state, respectively. We found that Arabidopsis plants expressing the GTP-bound mutant RabE1d-Q74L gained a significant degree of resistance to *Pst* DC3000, while their growth and development are similar to those of wild-type plants. In contrast, partial silencing of *RabE* genes drastically affected Arabidopsis leaf morphology and rosette size (suggesting a role of RabE proteins in plant growth and development) and had a complex effect on host defense.

INTRODUCTION

Rab proteins are conserved regulators of vesicle trafficking between membrane-bound compartments in eukaryotic cells. They participate in vesicle formation, transport along the cytoskeleton, tethering and fusion to the target membranes. Their functional specificity is determined, in part, by their unique subcellular distribution (Stenmark and Olkkonen, 2001; Zerial and McBride, 2001). The *Arabidopsis thaliana* genome encodes 57 Rab proteins, divided in eight subfamilies (RabA to RabH) based on sequence similarity (Rutherford and Moore, 2002; Vernoud et al., 2003). The RabE clade includes five highly similar proteins, whose biological function in Arabidopsis is not well-understood. RabE GTPases are highly similar to a class of eukaryotic Rabs, including yeast Sec4p and animal Rab8, extensively characterized regulators of vesicle transport from the trans-Golgi network (TGN) to specific regions of the plasma membrane (PM).

S. cerevisiae Sec4p is associated with the cytoplasmic side of the PM and of secretory vesicles directed to specific regions of the PM, such as the budding sites, and is essential for exocytosis in yeast (Goud et al., 1988). During bud formation, Sec4p is localized at the tip of the daughter cell (Novick and Brennwald, 1993; Walch-Solimena et al., 1997). In addition to Sec4p, several other fungal Sec4-like proteins have been identified and studied. CLPT1 of the plant-pathogenic fungus *Colletotrichum lindemuthianum* is a Sec4-like GTPase that is required for protein secretion and pathogenesis. CLPT1 can complement the *S. cerevisiae sec4-8* mutant (Dumas et al., 2001). Expression of the nucleotide-binding-deficient CLPT1-N123I form in *C. lindemuthianum* results in a dominant-negative phenotype, blocking secretion and leading

to accumulation of intracellular vesicular aggregates (Siriputthaiwan et al., 2005). CLPT1 is thus believed to contribute to fungal pathogenesis by regulating the transport of secretory vesicles that may deliver extracellular enzymes potentially involved in pathogenesis (Siriputthaiwan et al., 2005). Rab8 directs vesicle trafficking to the basolateral membrane in polarized Madin-Darby Canine Kidney (MDCK) epithelial cells (Huber et al., 1993). In addition, Rab8 promotes the polarized transport of newly synthesized membrane proteins in fibroblasts (Peranen et al., 1996) and is involved in cell morphogenesis (Hattula et al., 2006). In rat brain, Rab8 is a critical component of the cellular machinery that controls both constitutive cycling and regulated delivery of a specific type of receptors (AMPA-type glutamatergic receptors, AMPARs) into synapses (Gerges et al., 2004).

Unlike its fungal and animal counterparts, plant RabE proteins have only recently begun to be characterized. The RabE1d subcellular localization results presented in Chapter 2 of this dissertation, together with previously published data (Zheng et al., 2005), support the hypothesis that the Arabidopsis RabE proteins function in trafficking between the Golgi apparatus and the PM.

Fungal and bacterial infections, in plants, are often associated with activation (or suppression) of extracellular defense responses, including secretion of antimicrobial phytoalexins and formation of cell wall appositions known as papillae (Snyder and Nicholson, 1990; Snyder et al., 1991; Brown et al., 1995; Soylu et al., 2005; Field et al., 2006). An important role for an intact secretory pathway in plant defense against pathogens has been demonstrated in several studies (Snyder and Nicholson, 1990; Snyder et al., 1991; Collins et al., 2003; Assaad et al., 2004; Soylu et al., 2005; Wang et al.,

2005; Field et al., 2006). However, the molecular mechanisms underlying vesicle trafficking leading to defense, and the specific cargos transported by these vesicles have yet to be elucidated.

The physical interaction between RabE proteins and AvrPto detected in the yeast two-hybrid system, the AvrPto-induced alteration of RabE1d subcellular localization, and the ability of transgenically overexpressed RabE1d to partially counteract the virulence effect of AvrPto altogether suggest that RabE may mediate intracellular transport events important for establishing defenses against bacterial pathogens. Virulent *Pst* DC3000 may use AvrPto, and potentially other effectors, to interfere with RabE-dependent trafficking, therefore weakening plant defenses. In addition, RabE proteins may have a function in basic cellular traffic necessary for plant growth and development. As a first step toward understanding a possible role of the RabE family of proteins in plant defense, growth, and/or development at the whole plant level, we generated transgenic plants overexpressing wild-type RabE1d , RabE1d-Q74L (predicted to be active and preferentially GTP-bound) or RabE1d-S29N (predicted to be inactive and preferentially GDP-bound form), and examined these plants for morphological and developmental phenotypes and response to pathogen infection.

MATERIALS AND METHODS

Transgenic plants

Generation of Arabidopsis plants expressing RabE1d and RabE1d-S29N was described in the Materials and Methods section of Chapter 2 of this dissertation. Plants expressing GFP-RabE-Q74L were produced by subcloning the *RabE1d-Q74L* sequence into the *Eco*RI and *Bam*HI sites of the binary expression vector pEGAD (Cutler et al., 2000), in frame with the *GFP* sequence. The binary vector was introduced in *A. tumefaciens* strain GV3850 via triparental mating, for plant transformation. Arabidopsis Col-0 *glabrous (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 correct size GFP-RabE fusion proteins was verified by western blot.

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 was on average 100 $\mu E\ m^{-2}\ sec^{-1}$, and the temperature was kept constant at 20°C. *Pst* DC3000 bacteria were cultured in low-salt LB medium (10g/l Tryptone, 5g/l Yeast Extract, 5g/l NaCl), supplemented with 100μg/ml Rifampicin. For plant surface inoculation, 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 0.05% Silwet L-77 (OSI Specialties, Friendship, WV). Titer of the bacterial inoculum was $5x10^7$ colony forming units (CFUs)/ml. Arabidopsis plants growing on mesh-covered pots were submerged for a few seconds in the bacterial suspension, to completely coat the leaves, and incubated for three days under a tight-fitting dome, to maintain high humidity. Bacteria enumeration in leaves was conducted on day 3 post-inoculation, as previously described (Katagiri et al., 2002).

Protein extraction and immunoblotting

Total proteins were extracted as follows: approximately 20 mg (fresh weight) of fresh or frozen leaf tissue were ground with a pestle in a microfuge tube in the presence of 100 µl of 1× SDS-PAGE loading buffer [90 mM Tris-HCl pH 8.0, 100 mM DTT, 3% SDS, 22.5% sucrose, 10 µl/ml Protease Inhibitor Cocktail for Plant Cell Extracts (Sigma), bromophenol blue (to saturation)]. Extracts were immediately heated at 80°C for 10 minutes and then frozen at -20°C. Before loading on gel, extracts were thawed at room temperature and centrifuged at 20,000×*g* for 2 minutes, to pellet debris. An equal volume of each sample was used for SDS-PAGE electrophoresis. Total proteins were separated on precast gradient gels (4-20%, ISC BioExpress), then transferred onto Immobilon-P membrane (Millipore, MA) using a semi-dry transfer apparatus (SEMI PHOR, Hoefer Scientific Instruments, San Francisco, CA). Protein detection was carried with the following anti-AvrPto and anti-RabE antibodies (developed by Dr. K. Nomura).

Confocal microscope 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× or a 40× 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 Inc.). For FM4-64 staining, detached Arabidopsis leaves were submerged in 8.2 μM FM4-64 (Molecular Probes, Leiden, The Netherlands) in water for 15 minutes. 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.

RNA extraction

Total RNA was extracted from 100 mg of Arabidopsis leaf tissue with the RNeasy Plant Mini Kit (QUIAGEN), according to the manufacturer's specifications. RNA concentration in samples was determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

RT-PCR analysis

RNA reverse transcription and target gene amplification (RT-PCR) were performed using the RNA LA PCR Kit (AMV), Ver. 1.1 (TaKaRa, Japan). Reverse

transcription reaction mixture was prepared according to the manufacturer's protocol (5mM $MgCl_2$, 1 × RNA PCR Buffer, 1 mM dNTP mixture, 1 unit/μl RNase Inhibitor, 0.25 units/μl AMV Reverse Transcriptase, 0.125 μM Oligo-dT Adaptor Primer, RNase-free water and 1 μg total RNA). For amplification of the RabE and RabD transcripts, a single RT reaction was carried in a total volume of 50 μl, and incubated in a thermal cycler for 30 minutes at 45°C, followed by 5 minutes at 99°C and 5 minutes at 5°C. Five microliters of reverse transcribed cDNA were used as template in each of ten PCR reactions 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 Table 1. Each PCR reaction contained 2.5 mM $MgCl_2$, 1 × LA PCR Buffer II, 0.2 μM Forward Primer and 0.2 μM Reverse Primer, sterilized distilled water and 5 μl of the RT reaction described above, in a final volume of 25 μl. The reactions were placed in a thermal cycler and amplification was performed under the following conditions: 94°C, 2 minutes (1 cycle), 94°C, 30 seconds, 54°C, 30 seconds, 72°C, 1 minute (22 or 25 cycles), 72°C, 1 minute (1 cycle), 4°C. Ten microliters of the PCR reactions were 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).

Gene	Locus	Forward and Reverse Primers
Actin8 (ACT8)	At1g49240	F: 5'-GCTTCATCGGCCGTTGCATTTC-3' R: 5'-GATCCCGTCATGGAAACGATGTCTC-3'
AtRabD1	At3g11730	F: 5'-CTCGGAAACGCAGTCTTCAGC-3' R: 5'-GCTTATTCAAGACACAGCGACATGG-3'
AtRabD2a	At1g02130	F: 5'-GATCTCTGGCTCTGTATCGCTCG-3' R: 5'-GGATATTGCTAGGCTGGTCACGTC-3'
AtRabD2b	At5g47200	F: 5'-CTGAATTGACTGCCGGAGATTCC-3' R: 5'-GATGATCGAAAGAGGAGTGGTGAC-3'
AtRabD2c	At4g17530	F: 5'-CATCACCGACGAAGATCACGG-3' R: 5'-GCGAATTAAGAGGAGCAGCAGC-3'
AtRabE1a	At3g53610	F: 5'-CCGACGATCTATCTTCCCCGAGTAG-3' R: 5'-GACAGGCGTCGTGGACCC-3'
AtRabE1b	At5g59840	F: 5'-CCAACAAGGTCTCTTCTCTTCTC-3' R: 5'-CAACTTTGGAGCCTTTTGGGAC-3'
AtRabE1c	At3g46060	F: 5'-GTCGTCCGCCATAACCTTC-3' R: 5'-CACTTCACCCCCAAACTTTTTTCG-3'
AtRabE1d	At5g03520	F: 5'-GTTTCTGACGATGGCGGTTGC-3' R: 5'-CAGCAAGCTGACTTCTCGGCTG-3'
AtRabE1e	At3g09900	F: 5'-GGCTGTCTCCGGCGAGAAG-3' R: 5'-CATAGGACGATCCCTTGAATGATGC-3'

Table 3 - 1: Gene-specific primers for RT-PCR.

BTH treatment

Benzothiadiazole (BTH; Actigard) was prepared at a final concentration of 300 μM in water and sprayed onto potted GFP-RabE1d-Q74L and Col-0 *gl1* Arabidopsis plants. A separate set of plants was sprayed with water, as a control. Plants were covered with a tight-fitting dome and assayed for responses 3 days after BTH application.

Intercellular Wash Fluid (IWF) collection and analysis

BTH-treated and control plants were harvested three days after treatment. Whole plants were vacuum-infiltrated for 2 minutes with distilled water containing 0.002% Silwet L-77 (OSI Specialties, Friendship, WV). 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 400 × *g* for 20 minutes, at 4°C. The IWF volume was measured with a micropipette, and the appropriate volume of 5 × SDS-PAGE loading buffer was immediately added. Samples were heated at 85°C for 5 minutes, then frozen or loaded on acrylamide gel.

Callose staining

Pst DC3000 from a fresh culture plate was inoculated into liquid LB and grown to mid-logarithmic phase. Bacteria were pelleted by centrifugation (10 minutes at 2,000 × *g*) and resuspended in distilled water. A bacterial suspension of $O.D._{600}$= 0.4 (equivalent to 2×10^8 CFUs/ml), or water, were infiltrated with a needle-less syringe in leaves of Arabidopsis Col-0 *gl1* and of EGFP-RabE1d-Q74L plants. Six hours after infiltration, leaves were collected and vacuum-infiltrated with 95% ethanol, followed by incubation at

50°C for 30 minutes. The 95% ethanol was replaced with 75% ethanol and the leaves were incubated overnight at room temperature. Cleared leaves were rinsed in 50% ethanol, then in water, and placed in staining solution [150 mM K_2HPO_4, pH 9.5, and 0.01% aniline blue (Sigma)] for about 15 minutes at room temperature. Stained leaves were mounted in 50% glycerol on microscope slides and observed with a Zeiss Axiophot D-7082 fluorescence microscope with an excitation filter of 365 nm, a 400 nm dichroic mirror and a 450 nm long-pass emission filter.

RESULTS

GFP-RabE1d-Q74L displays a unique subcellular localization pattern

Rab proteins engineered by substitution of a conserved glutamine with leucine in the PM3 catalytic domain (Figure 2 - 2) are unable of catalyzing both intrinsic and GAP-stimulated GTP hydrolysis, but are not affected in their nucleotide-binding properties (Stenmark et al., 1994). Rabs carrying this mutation were demonstrated to be mostly in the GTP-bound (active) state, and often have a constitutive-active phenotype.

Transgenic expression of GFP-RabE-Q74L had no significant effects on Arabidopsis growth and development, other than appearance of minute sparse indentations in mature rosette leaves, one or two weeks prior to bolting. Interestingly, unlike wild-type GFP-RabE1d (see Chapter 2), GFP-RabE1d-Q74L was not observed in association with any intracellular punctate structures, but was exclusively found at the cell periphery (Figure 3 - 1, A). Initial observation of the fusion protein peripheral distribution was suggestive of a tonoplast, rather than PM localization. To confirm this suspicion, we obtained seeds of Arabidopsis transgenic lines expressing GFP fusions to a PM-localized channel protein (line Q8) and to a tonoplast marker (line Q5) (Cutler et al., 2000) from ABRC (Arabidopsis Biological Resource Center, Ohio State University). Comparison of our GFP-RabE1d-Q74L localization with that of the PM and tonoplast markers further suggested RabE1d-Q74L was not located at the PM (Figure 3 - 1, B). Staining with FM4-64 confirmed that the bulk of GFP-RabE1d-Q74L fluorescence was indeed not overlapping with the PM membrane, but labelled the tonoplast (Figure 3 - 2).

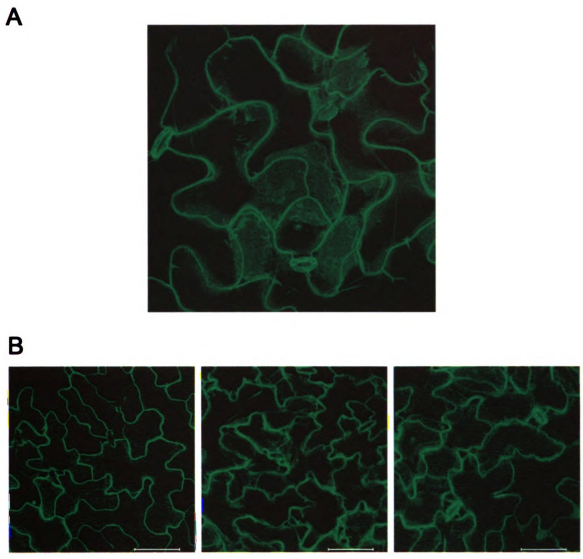


Figure 3 - 1: Localization of GFP-RabE1d-Q74L in transgenic Arabidopsis.

(A) Confocal microscope image of a representative Arabidopsis leaf expressing GFP-RabE1d-Q74L. Projection along the Z-axis of several focal planes crossing the epidermal cell layer.

(B) Localization pattern of GFP-RabE1d-Q74L compared to a PM marker and a tonoplast marker. From left to right: line Q8, expressing a GFP fusion to the Plasma membrane Integral Protein PIP2A (left); line Q5, expressing a fusion to delta-TIP (Tonoplast Integral Protein), a vacuolar membrane channel protein (center); Arabidopsis expressing GFP-RabE1d-Q74L (right). Scale bar = 50μm.

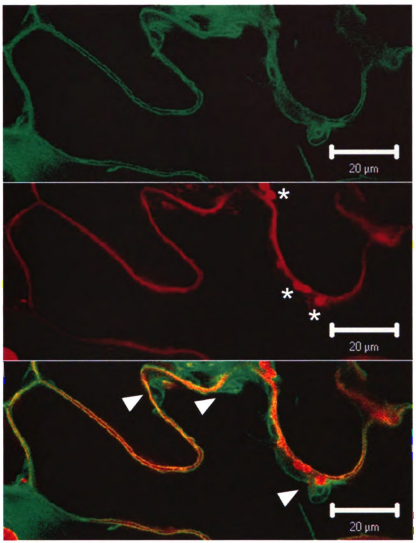


Figure 3 - 2: GFP-RabE-Q74L is primarily localized in the tonoplast.

Confocal microscope image of epidermal leaf cells of Arabidopsis expressing GFP-RabE-Q74L, indicating that GFP-RabE1d-Q74L accumulates mostly in the tonoplast. Leaves were stained with FM4-64, to visualize the PM, and immediately observed. The image represents a single focal plane (40x oil-immersion objective).

(A) GFP fluorescence;

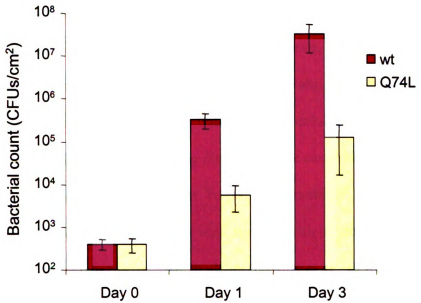
(B) FM4-64 fluorescence (the asterisks indicate autofluorescence of chloroplasts in the mesophyll layer, below the epidermis);

(C) Merged image: arrowheads point at places where the tonoplast is most clearly distinct from the PM. Invaginations and formation of membranous structures are typical of the highly dynamic vacuolar membrane. Even in the areas where the PM and tonoplast are closest, still green and red fluorescence are visibly distinct.

RabE1d-Q74L confers resistance against *Pst* DC3000

Although overexpression of constitutively active GFP-RabE1d-Q74L did not affect plant growth or development, 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 restricted 10- to 100-fold, compared to multiplication on wild-type Arabidopsis (Figure 3 - 3, A). Visible disease symptoms, namely chlorosis and necrosis, were also markedly reduced (Figure 3 - 3, B).

A



B

Figure 3 - 3: RabE-Q74L overexpression confers resistance to *Pst* DC3000.

(A) Bacterial multiplication in GFP-RabE1d-Q74L-expressing plants (Q74L), compared to that in wild-type Arabidopsis (Col). *Pst* DC3000 was vacuum-infiltrated at a density of 10^5 CFUs/ml.

(B) Disease symptoms 3 days after inoculation with *Pst* DC3000 at a density of 10^5 CFUs/ml. On the left, Arabidopsis Col-0 *gl1* (wild-type); on the right, Arabidopsis expressing GFP-RabE-Q74L.

Up-regulation of the secretory pathway was recently demonstrated in Systemic Acquired Resistance (SAR) (Wang et al., 2005), and it was known for a long time that SAR-expressing plants accumulate in the apoplast secreted proteins, which include 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, protein secretion in the apoplast and secretion of the extracellular marker of plant defenses PR1 (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 other proteins in the apoplast, in both wild-type and transgenic plants (Figure 3 - 4). 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 plants IWF. These unique extracellular proteins, associated with expression of RabE1d-Q74L suggest that additional secretory pathways are activated in these plants, in addition to the SAR pathway.

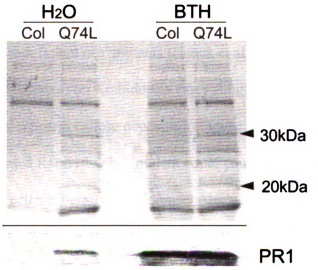


Figure 3 - 4: Accumulation of extracellular proteins in plants expressing GFP-RabE1d-Q74L

Proteins in the intercellular wash fluid from wild type (Col) and RabE1d-Q74L-expressing plants (Q74L) were separated by SDS-PAGE. In the top panel, Coomassie Blue-stained gel, representing total proteins; the arrowheads indicate bands which seem to be exclusive to the Q74L plants. In the bottom panel, western blot with the anti-PR1 antibody (gift of Dr. X. Dong, Duke Univ.).

Another cellular event that is associated with defense against microbes and involves the secretory pathway is deposition of callose-containing papillae. Virulent bacteria are able to suppress papilla formation in a TTSS-dependent manner (Brown et al., 1995; Hauck et al., 2003). Interestingly, AvrPto expression in Arabidopsis is sufficient to suppress bacteria-induced callose deposition, and this correlates with elevated susceptibility to non-pathogenic TTSS-deficient *hrp*$^-$ mutants (Hauck et al., 2003). When inoculated at high density ($2x10^8$ CFUs/ml) on Arabidopsis expressing GFP-RabE1d-Q74L, virulent *Pst* DC3000 failed to suppress callose deposition (Figure 3 - 5) suggesting that the transgenically expressed RabE1d-Q74L mutant could counteract the ability of *Pst* DC3000 to suppress cell wall-associated defense. Absence of callose deposits in the water-infiltrated leaves demonstrates that GFP-RabE1d-Q74L expression is not promoting constitutive callose deposition. This experiment, rather, indicates that the transgene expression is counteracting specifically *Pst* DC3000-mediated suppression of callose production.

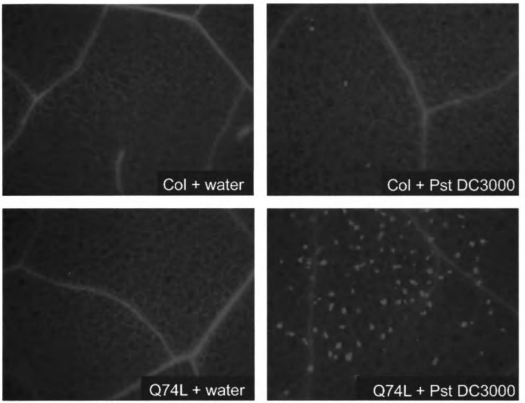


Figure 3 - 5: Pst DC3000 fails to suppress callose deposition in resistant RabE1d-Q74L-expressing plants.

Callose staining results on Col and Q74L leaves infiltrated with either water or *Pst* DC3000. Callose deposits are visible as bright spots against the dark background. Six to eight leaves per treatment were analyzed; the pictures represent the average callose distribution observed.
This experiment was done twice, with comparable results.

RabE1d-S29N expression does not alter plant growth, development or disease susceptibility

The serine/threonine to asparagine mutation in the PM1 domain of Rabs (Fig. 2 - 2), which greatly increases Rab affinity for GDP over that for GTP, was found to confer, in most cases, a dominant-negative phenotype. The dominant-negative effect is often associated with an impaired ability of the mutant Rab to be delivered to the apropriate membrane compartment. This is often interpreted as the result of highly increased affinity of the mutant Rabs for guanine exchange factor (GEF) (Burstein et al., 1992), and consequent sequestration of this important activating protein (Peranen et al., 1996).

As described in Chapter 2, however, the intracellular distribution of the GFP-RabE-S29N protein was similar to that of wild-type RabE, at both the cell periphery (plasma membrane) and in intracellular punctate structures. GFP-RabE1d-S29N-overexpressing plants were phenotypically and developmentally indistinguishable from wild-type Arabidopsis. When surface-inoculated with *Pst* DC3000, GFP-RabE1d-S29N-overexpressing plants exhibited a similar degree of susceptibility as wild-type plants (Figure 3 - 6). Taken together, these results suggest that transgenic expression of RabE1d-S29N does not affect growth, development or disease resistance.

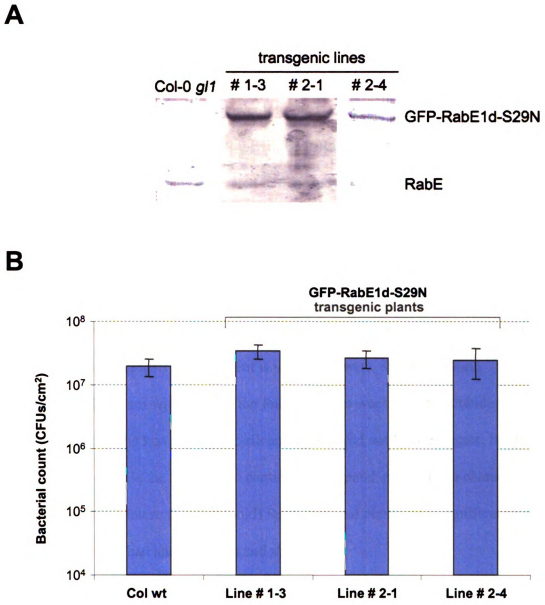


Figure 3 - 6: *Pst* DC3000 growth on plants overexpressing GFP-RabE1d-S29N

(A) Western blot analysis, with anti-RabE primary antibody, indicating expression of the GFP-RabE1d-S29N fusion protein in different transgenic lines. Endogenous RabE proteins are also detected (lower band).

(B) Bacterial population in Arabidopsis leaves 3 days after surface-inoculation with *Pst* DC3000 at a density of $5x10^7$ CFUs/ml.

Occurrence of *RabE*-silencing in transgenic plants

When creating transgenic plants, it is common to generate, in some individuals, transgene silencing; if gene silencing spreads from the transgene to the endogenous copy, it is generally a post-transcriptional event, termed co-suppression (Vaucheret et al., 1998). A large percentage of GFP-RabE1d, GFP-RabE1d-Q74L, and GFP-RabE1d-S29N transgenic Arabidopsis plants generated in this study showed co-suppression of the transgene and of endogenous RabE, as demonstrated by western blot analysis using a polyclonal antibody that reacts with all RabE proteins (Figure 3 - 7). Severe reduction of the overall endogenous RabE protein level in transgenic plants invariably correlated with a distinct morphological phenotype. Rosette leaves developed normally for the first 3-4 weeks (when Arabidopsis development is usually slower), the plants being indistinguishable from wild-type. In the following two weeks, when Arabidopsis size increases rapidly, the leaves of RabE-silenced plants did not fully elongate; midribs remained short, while the leaf lamina continued to expand, producing a characteristic wavy phenotype. Mature (5-6 week-old) RabE-silenced plants were significantly smaller than wild-type and had short midribs and stems.

RabE-silenced plants flowered at the same time as wild-type Arabidopsis, and produced fertile seeds. The progeny of a selected silenced line (B11) also manifested silencing and had the same phenotype as the parental plant. Interestingly, RabE-silenced plants spontaneously arose also among the progeny of established GFP-RabE1d overexpressors.

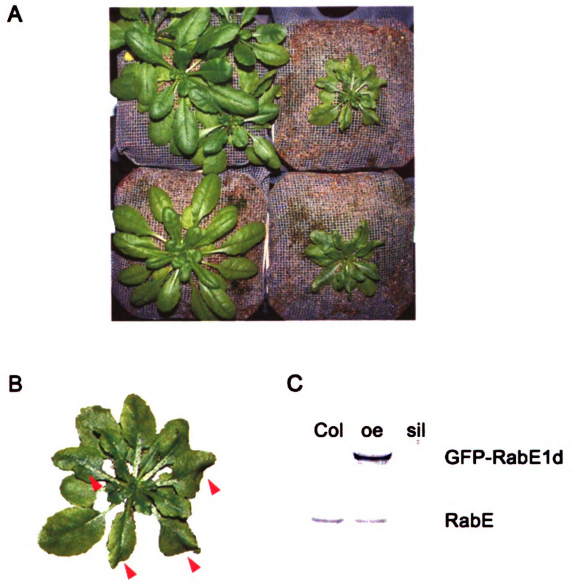


Figure 3 - 7: RabE silencing severely affects plant morphology.

(A) Size and morphology of RabE1d-overexpressing plants (bottom left) and RabE-silenced plants (top and bottom right), compared to Arabidopsis wild type (top left).

(B) Enlarged picture of the RabE-silenced plant in (A) top right; the arrowheads point at the wavy leafs.

(C) Western blot (with anti-RabE antibody) illustrating how RabE-silenced plants (sil) have a considerably lower amount of endogenous RabE, compared to both wild-type (Col) and GFP-RabE1d-overexpressors (oe).

Effect of *RabE* co-suppression on the expression of individual *RabE* genes

The *RabE* gene family expression profile was analyzed by RT-PCR in the co-suppressed lines, in comparison to wild type Arabidopsis. The RT-PCR demonstrated that not all *RabE* gene family members are equally affected by co-suppression. *RabE1d* and *RabE1e* were the most severely knocked-down, followed, to a lesser extent, by *RabE1b*. *RabE1a* and *RabE1c* showed only mild down-regulation (Figure 3 - 8). 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 the transcripts of all four *RabD* genes were present at similar levels in *RabE*-silenced plants and in wild-type Arabidopsis (Figure 3 - 8). Silencing, in the transgenic plants, is therefore specifically limited to the *RabE* genes, primarily *RabE1d* and *E1e*.

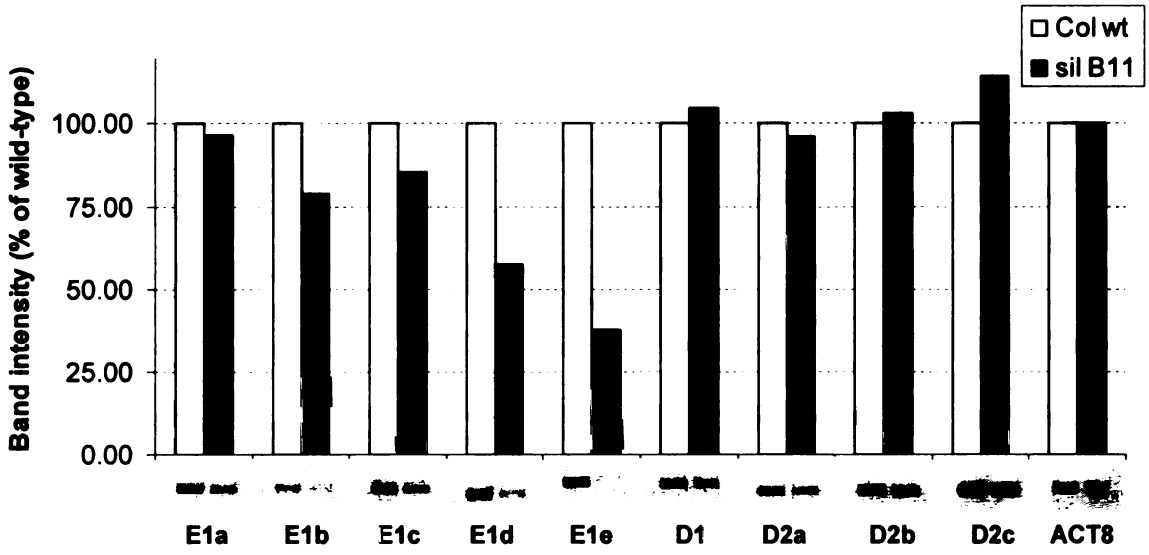


Figure 3 - 8: Expression of the *RabE* and *RabD* genes in *RabE*-silenced plants.

RT-PCR analysis of expression of the five *RabE* and four *RabD* genes in the *RabE*-silenced Arabidopsis plants. Equal volumes of the PCR reactions were loaded on 1% agarose gel. The gel was photographed with a Bio-Rad imager and the Quantity One software was used to quantify the bands. Intensity values, normalized to those of *Actin8*, are represented in the chart as percent of wild-type value.

***RabE*-silenced plants exhibit complex responses to *Pst* DC3000 infection and PAMP-induced resistance**

The *RabE*-silenced plants, although morphologically abnormal, represent an opportunity for exploring the effect of partially down-regulating the production of more than one RabE member on plant defense against pathogens. An interesting observation revealed that RabE may play a positive role in establishment of PAMP-induced basal resistance. It was previously shown that pre-treatment of Arabidopsis leaves with the conserved flagellin peptide flg22 could induce resistance against *P. syringae*, restricting bacterial multiplication up to 100-fold (Zipfel et al., 2004). In my experiments, I confirmed that flg22-induced basal resistance was associated with 100-fold reduction in *Pst* DC3000 population in wild-type plants (Figure 3 - 9). However, flg22 pretreatment of *RabE*-silenced plants caused a significantly lower degree of resistance to *Pst* DC3000, only about 10-fold reduction in *Pst* DC3000 multiplication (Figure 3 - 9). However, *Pst* DC3000 multiplied on *RabE*-silenced plants to levels that were consistently 0- to 10-fold lower than on wild-type Arabidopsis (across several experiments). This result suggests a low level of basal resistance in *RabE*-silenced plants, possibly due to general stress. Therefore, RabE-silenced plants seem to have two opposing phenotypes with regard to pathogen responses: they have a reduced ability to exhibit flg22-induced resistance, but, at the same time, they seem to have a slightly elevated constitutive basal resistance against *Pst* DC3000, perhaps through a distinct mechanism.

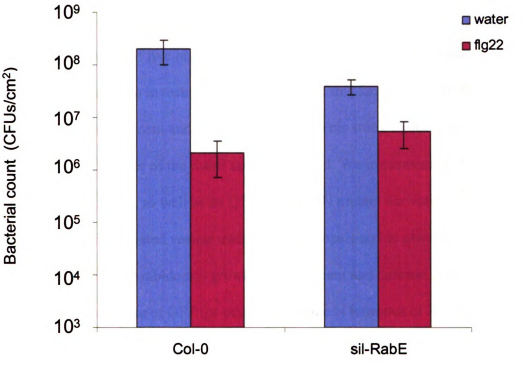


Figure 3 - 9: RabE-silenced plants exhibited complex responses to bacterial infection and PAMP-induced resistance.

The flg22 peptide was infiltrated in the leaves with a needle-less syringe, at a concentration of 2μM. Water was infiltrated in a separate set of leaves, as a control. Plants were inoculated with *Pst* DC3000 24 hours after flg22 treatment. Bacteria (10^5 CFUs/ml) were inoculated by vacuum-infiltration. Bacteria in leaves were enumerated on Day 3 post-inoculation.

DISCUSSION

The presence of five highly similar *RabE* genes in Arabidopsis presents a significant challenge to investigating the function of this family of small GTPases in plant growth, development and pathogen defense. This study focused on functional analysis of one member of the RabE family, RabE1d. We implemented stable transgenic expression of RabE1d, as well as its Q74L and S29N mutant derivatives, hoping to alter the normal RabE-mediated vesicle traffic in Arabidopsis and to observe any effects from such perturbation on Arabidopsis growth, development and defense. Many studies have shown that the presence of GFP (or other tags) at the N terminus of Rab proteins does not affect their subcellular localization or function, in both plant (Ueda et al., 2001; Ueda et al., 2004) and animal systems (Bucci et al., 2000; Mesa et al., 2001; Galperin and Sorkin, 2003), but allows simultaneous analysis of protein function and subcellular localization. We therefore used GFP-fused RabE1d, as well as its Q74L and S29N mutant derivatives, for functional study.

We found that transgenic expression of the RabE1d-Q74L variant (expected to be in active conformation) conferred on Arabidopsis a significant degree of resistance to *Pst* DC3000. Based on the data gathered in this study, it is not yet possible to discern whether this resistance is a direct effect of the mutated protein, due to enhancement of defense-related vesicle traffic, or rather an indirect effect, due to overall perturbation of cellular vesicle traffic, not necessarily associated with defense. There are numerous examples of constitutively resistant Arabidopsis mutants, such as *dnd* (disease, no death), *cim* (constitutive immunity) and *cpr* (constitutive PR-expression), which display upregulated

defenses against *P. syringae* and other pathogens. Common characteristics of these mutants include accumulating high levels of salicylic acid and PR proteins, and being significantly dwarfed, or otherwise morphologically altered, compared to wild type plants (Bowling et al., 1994; Clarke et al., 1998; Yu et al., 1998; Li et al., 2001; Maleck et al., 2002). In this respect, the RabE1d-Q74L-expressing plants may be particularly interesting and unique, because they exhibit a high level of resistance to *P. syringae* without being adversely affected in overall plant size or leaf shape. However, more experiments will be needed to further characterize growth, development and pathogen resistance in these transgenic plants.

In addition to enhanced resistance, the GFP-RabE1d-Q74L transgenic plants displayed unexpected localization of the fusion protein at the vacuolar membrane (tonoplast). The peculiar GFP-RabE1d-Q74L localization pattern could be interpreted as passive flow of the protein from the PM to the tonoplast via endocytosis. According to the general Rab recycling model, after its synthesis, RabE is supposed to be delivered to the donor compartment (e.g., the Golgi apparatus) in which it functions, and loaded with GDP. GDP exchange with GTP activates the protein, promoting interaction with the downstream machinery needed for vesicle targeting and fusion with the target membrane (e.g., the PM). Once vesicle delivery is completed, Rabs are inactivated by GTP hydrolysis. A GDP dissociation inhibitor (GDI) extracts GDP-bound (but not GTP-bound) Rab from the membrane and escorts it through the cytosol back to the donor compartment, for a new transport cycle. It is possible that RabE-Q74L, upon reaching the PM, cannot be extracted by GDI because it remains in its GTP-bound state. A default non-specific endocytosis pathway such as that observed for the FM4-64 dye (Ueda et al.,

2001) could carry the RabE-Q74L protein to the tonoplast, where it accumulates. If this is the case, a more accurate microscopic analysis may reveal a low transient pool of GFP-RabE1d-Q74L at the PM and Golgi apparatus, which was not detectable during my routine observation. Alternatively, co-expression with AvrPto may be useful: as described in Chapter 2, AvrPto interacts with wild-type RabE or RabE-Q74L in Y2H. Transgenic expression of AvrPto *in planta* results in GFP-RabE mislocalization, which we interpreted as AvrPto "trapping" active RabE proteins at at the PM. Based on this model, AvrPto may similarly "trap" the GFP-RabE1d-Q74L protein at the PM, resulting in increased PM and reduced tonoplast level of the fluorescent protein. Further experiments are needed to examine this possibility.

In contrast to the GFP-RabE1d-Q74L transgenic plants, Arabidopsis plants expressing RabE1d-S29N (expected to be in inactive conformation) were similar to wild-type plants in their morphology and pathogen response. Furthermore, the subcellular localization pattern of GFP-RabE1d-S29N was similar to that of GFP-RabE1d (as shown in Chapter 2). These results could be explained in at least two ways. The first possibility is that GFP-RabE1d-S29N, in Arabidopsis, has a dominant-negative effect on endogenous RabE1d protein (and possibly other RabE members) but this RabE protein is not necessary for plant development or defense. The second possibility is that GFP-RabE1d-S29N is not exerting a dominant-negative effect. We could not distinguish between these two possibilities, although the remarkable effect of RabE downregulation on plant size and morphology (seen in RabE-silenced plants) seems to argue against the first hypothesis.

Transgene-mediated *RabE* co-suppression correlated with significant changes in plant size and leaf morphology, and with complex defense phenotypes. Co-suppression affected not only *RabE1d,* but also other *RabE* genes (Figure 3 - 8). Therefore, the multiple phenotypes of *RabE*-silenced plants are likely caused by simultaneous silencing of more than one *RabE* gene. Consistent with this speculation, individual knock-out mutants of *RabE1d* and *RabE1b*, two of the most severely silenced genes, did not exhibit any defect in growth and development, nor in disease susceptibility to *Pst* DC3000 (data not shown). Unfortunately, a T-DNA insertion upstream of the *RabE1e* gene did not affect gene expression, leaving thus open the possibility that *RabE1e* downregulation may alone be responsible for the *RabE1d*-silenced plants phenotypes. As loss-of-function mutants provide a powerful tool in the study of gene functions, additional analysis of *RabE1e*, *RabE1a*, and *RabE1c* single mutants and various combinations of multiple *RabE* gene mutations is needed to further assess the biological function(s) of the RabE family of GTPases in Arabidopsis.

Pathogenesis assays with and without flg22 pre-treatment unveiled a complex overlap of two apparently opposing defense responses in the *RabE*-silenced plants. The origin of the low but reproducible constitutive resistance exhibited by *RabE*-silenced plants is unclear; resistance could be caused by an indirect effect of long-term perturbation of the *RabE1d*-mediated traffic, resulting in nonspecific general stress on plants. On the other hand, flg22-induced resistance was reduced in the *RabE*-silenced plants, suggesting that RabE may be involved in regulating trafficking events that mediate PAMP-triggered cellular responses. In the future, inducible RNAi-mediated *RabE* gene silencing may circumvent the low constitutive resistance observed in stable

RabE-silenced transgenic plants, enabling a more definitive evaluation of the apparently positive role of RabE proteins in PAMP-triggered plant resistance.

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CHAPTER 4

Conclusions and future perspectives

A very significant amount of crops are lost every year to pathogens worldwide (Baker et al., 1997; Strange and Scott, 2005). In order to devise effective strategies for preventing such losses, it is critical that we deepen our understanding of the molecular and cellular bases of plant-pathogen interactions. Gram-negative plant pathogenic bacteria use the highly conserved type III secretion system (TTSS) to deliver virulence-mediating proteins inside the plant cell. Bacterial virulence effectors are apparently not generic bullets aimlessly shot in the host cell cytoplasm through the TTSS. Rather, they are precisely targeted at those host cellular processes the pathogen needs to manipulate to its own advantage (Grant et al., 2006). One of the major current endeavors in the field of plant pathology is that of understanding individual effector functions. Importantly, identifying the host targets of pathogen effectors and studying their role in the host cell is often a way to gain insight on previously uncharacterized plant cell functions.

In the absence of a circulatory system and of specialized cell types, such as those found in animals, plants fight their battle against microbial invaders at the level of each single cell in contact with a potential pathogen. Every plant cell can be envisioned as a battleground, where pathogens deploy their virulence factors to interfere with specific host targets. It was known for a long time that the plant cell responds to pathogen attack with cytoskeleton and organelle rearrangements, secretion of antimicrobial compounds and peptides, papilla deposition and more (Lipka and Panstruga, 2005; Field et al., 2006). Most of the knowledge in this field was obtained by studying plant cell defense responses against fungal pathogens. The recent application of sophisticated microscopy techniques to the study of plant pathology allows us to take a closer look at the fascinating interaction between plants and pathogens at the cellular and subcellular level (Koh and

Somerville, 2006). The use of live cell imaging techniques for investigating the effect of disease-promoting virulence factors of bacterial pathogens on the plant cell is a relatively new and unexplored field.

My dissertation work focused on investigating intracellular localization and function of the poorly characterized Arabidopsis RabE small GTPases, which were previously found to interact in yeast two-hybrid with the *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 effector AvrPto. I specifically pursued analysis of RabE1d, one of the five highly similar Arabidopsis RabE GTPases, and therefore, the following conclusions apply to RabE1d, and more investigation will be necessary to find out whether they can be extended to other members of the family.

The first part of this study demonstrated that transgenically expressed GFP-RabE1d and endogenous RabE proteins are localized at the Golgi apparatus and at the plasma membrane (PM) in Arabidopsis cells. Analysis of GFP-RabE1d subcellular localization in the presence of AvrPto revealed a novel and interesting phenomenon. AvrPto expression *in planta* induced a remarkable change in GFP-RabE1d intracellular distribution, which was dependent on AvrPto membrane-localization and on RabE1d nucleotide-binding state. The Golgi-localized pool of RabE1d, but not of GDP-bound RabE1d-S29N, was greatly reduced in the presence of membrane-associated, but not soluble, AvrPto. Furthermore, overexpression of RabE1d proved to be sufficient to specifically counteract AvrPto virulence function. These results, altogether, revealed a novel connection between the virulence function of the bacterial effector AvrPto and the subcellular distribution of RabE, a putative regulator of plant intracellular vesicle

trafficking. In the future, it will be interesting to perform more in-depth microscopic analysis, to understand whether AvrPto is specifically impairing RabE localization at the Golgi, without affecting overall Golgi integrity, or whether the Golgi itself (or part of it) is disassembled as a consequence of AvrPto virulence function.

The second part of my dissertation focused on investigating the biological function of RabE in Arabidopsis growth, development and response to bacterial pathogens. A major obstacle toward this functional analysis was represented by the presence of five highly similar *RabE* genes in Arabidopsis. I chose to begin the analysis by exploring the effect of constitutively overexpressing RabE1d and its mutant derivatives Q74L and S29N in stable transgenic plants. Overexpression of RabE1d-Q74L resulted in particular interesting phenotypes. The mutant protein targeted GFP to the tonoplast, a novel and unexpected subcellular localization and, most importantly, the transgenic plants manifested a notable resistance to *P. syringae* infection, which correlated with constitutive activation of defense and secretion pathways and with callose deposition that was not suppressed by the pathogen. Remarkably, these transgenic plants were not negatively affected in their growth and development, whereas the vast majority of all known constitutively resistant Arabidopsis mutants are dwarfed or otherwise morphologically altered.

Future work will be necessary to further characterize the actual mechanism underlying RabE1d-Q74L-mediated defense responses in these plants. It is known that activation of SAR is accompanied by accelerated secretion of certain PR proteins (Wang et al., 2005). However, I detected the presence of several unique extracellular protein bands in the intercellular wash fluid (IWF) of BTH-treated RabE1d-Q74L-expressing

plants, but not in the IWF of BTH-treated wild-type plants (Figure 3 - 4), suggesting that additional secretory pathways (perhaps specific to RabE) are activated in these plants, in addition to the SAR-associated pathway. To unequivocally assign RabE a role in trafficking and in defense, identification of a marker (protein or compound) that is transported or secreted in a RabE-dependent fashion is absolutely critical. One possible approach toward this goal would be that of determining the identity of those proteins, recovered in the IWF of plants expressing RabE1d-Q74L, which appeared to be unique to the transgenic plants (Figure 3 - 4). Also, my current results were obtained from constitutive expression of RabE1d-Q74L. As long-term overexpression may be more likely to activate SAR than short-term gene expression, it will be of interest in the future to produce transgenic plants that conditionally express the RabE1d-Q74L transgene (e. g., dexamethasone-inducible) to separate SAR-dependent protein secretion from a possible RabE-specific secretory process.

In the process of selecting transgenic plants overexpressing RabE1d or its mutant derivatives, I noticed a significant number of primary transformants with short, curly leaves, smaller than their sibling plants. Molecular characterization of these individuals revealed that they were *RabE*-cosuppressed plants. The overall level of RabE proteins in these plants is considerably lower than in wild-type Arabidopsis, due to partial silencing of the *RabE1d*, *E1e* and *E1b* genes, primarily. The pathogenesis assays performed on these plants revealed a complex overlay of distinct defense responses, including a low basal level of constitutive resistance, and an apparent impairment in PAMP-induced defenses. More work is needed to dissect these responses. Specifically, in-depth analysis of individual *RabE* gene knock-out mutants, and of different combinations of individual

mutations (obtained by crosses), may shed light on the contribution of different *RabE* genes to the plant's response to pathogens. Moreover, the low basal level of constitutive resistance could be caused by long-term silencing of multiple *RabE* genes. If so, chemically inducible RNAi-mediated silencing of *RabE* genes could be used to attempt uncoupling the basal resistance observed in silenced-RabE plants from the possible defect in PAMP-triggered defense responses.

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