CHARACTERIZATION OF THE ARF GUANINE NUCLEOTIDE EXCHANGE FACTOR MIN7 AND THE RABE1 GTPASES OF ARABIDOPSIS

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

Cell and Molecular Biology

2011

ABSTRACT

CHARACTERIZATION OF THE ARF GUANINE NUCLEOTIDE EXCHANGE FACTOR MIN7 AND THE RABE1 GTPASES OF ARABIDOPSIS

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Pseudomonas syringae pv *tomato* DC3000 is a Gram-negative bacterium that utilizes a type three secretion system to translocate effector proteins into plant cells to promote pathogenesis. There are ~30 effectors secreted by *Pst* DC3000 and the effectors HopM1 and AvrPto have both been implicated in targeting host vesicle trafficking systems. HopM1 has been shown to promote disease by targeting HopM1 interactor 7 (MIN7). MIN7 contains the SEC7 domain, the catalytic domain of ADP-ribosylation factor (ARF) guanine nucleotide exchange factors (GEFs). ARF GEFs promote the exchange of GTP for GDP on ARF GTPases, but GEF activity has not been demonstrated for MIN7. In this research I show that the SEC7 domain of MIN7 is capable of promoting the exchange of GTP for GDP on an Arabidopsis ARF GTPase. In addition, I found that MIN7::DsRed was partially co-localized with at least five Arabidopsis ARFs.

min7 plants are compromised in salicylic acid (SA)-dependent defense and display hypersensitive cell death to benzothiadiazole (BTH), a functional analogue of SA. Two Arabidopsis lines with T-DNA insertions in the ARF-like B1 (*ARL-B1*) gene are found to be hypersensitive to BTH. Unlike *min7* plants, however, the ARL-B1 knockout plants are not compromised in BTH-induced defense. Thus, BTH hypersensitivity and deficient BTH-induced defense can be uncoupled.

AvrPto is an effector that compromises plant cell wall-based defense. AvrPto interacts in the yeast two-hybrid system with the RabE1 GTPases, a family of Rab GTPases predicted to be

involved in polarized secretion in Arabidopsis. I found that AvrPto interacts with wild type RAB-E1d and RAB-E1d-Q74L (predicted to be GTP bound and active) but not with RAB-E1d-S29N (predicted to be GDP-bound and inactive). To better understand the function of RabE1 in the cell, I used the yeast two-hybrid screen to identify two Arabidopsis interactors of RabE1, REI1 and REI2. REI1 is annotated as a receptor-like kinase, and REI2 contains a SEC14-like domain.

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Sheng Yang He for his patient mentoring. The members of my guidance committee, Drs. Richard Allison, Ken Keegstra, Susanne Thiem and Jonathan Walton, have been helpful and supportive.

I consider myself fortunate to have called the current and former members of the He lab colleagues. I continued projects initiated by Paula Hauck, Elena Bray Speth, and Kinya Nomura, and their commiseration was priceless. Only Kinya and I know the struggle of raising *min7* plants!

I have developed some very special friendships while at MSU and will always think fondly of a time when so many dear friends were so near. I enjoyed many wonderful evenings with Janet Paper, Heather Van Buskirk, and Colleen Doherty and was fortunate sit mere feet from my sweet friend Young Nam Lee for four years.

My parents, Richard and Carolyn Imboden, and my husband, Jake Davison, have given me unending support and encouragement. I would not have completed this without them.

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KEY TO ABBREVIATIONS

ARF-ADP-ribosylation factor

ARL-ARF-like protein

BTH-Benzothiadiazole

DN-Dominant negative

ETI-Effector-triggered immunity

ETS-Effector-triggered susceptibility

GA-Golgi apparatus

GAP-GTPase activating protein

GDI-Guanine nucleotide dissociation inhibitors

GEF-Guanine nucleotide exchange factor

MIN7-HopM1 interactor 7

NB-LRR-Nucleotide binding-leucine rich repeat protein

PAMP-Pathogen-associated molecular pattern

PM-Plasma membrane

PRR-Pattern recognition receptor

Pst DC3000-*Pseudomonas syringae* pv. *tomato* DC3000

PTI-PAMP-triggered immunity

SAR-Systemic acquired resistance

SA-Salicylic acid

T3SE-Type three secretion effector

T3SS-Type three secretion system

TGN-Trans-Golgi network

Chapter 1

Literature Review

INTRODUCTION

In the 1840s the late blight pathogen *Phytophthora infestans* brought the nation of Ireland to its knees with the destruction of its potato crop (Schumann and D'Arcy 2000). Loss of the staple crop lead to starvation and massive emigration from the country. *P. infestans* remains with us today (Fry and Goodwin 1997). Between 1990 and 1998, American farmers spent $287.8 million per year on fungicides to control potato light blight and experienced an estimated $210.7 million in lost revenue each year (Guenthner et al. 2001). Potatoes are not alone in pathogen vulnerability. It was estimated that 15% of worldwide agricultural losses for wheat, rice, maize, barley, potatoes, soybeans, sugar beet and cotton worldwide were due to pathogens (Oerke and Dehne 2004).

As the primary producers of biomass on the planet, plants are desirable sources of energy for both animals and microbes. We rely on domesticated plants as a source for food, fiber and fuel, and wild species as the basis of our ecological systems. In a world with a growing population and shifts in climate, it is more important than ever that we optimize the resources utilized for our food supply.

An essential step toward improving plant productivity is to better understand disease susceptibility and resistance and the inner workings of the plant cells. Pathogens have co-evolved with plants and have developed mechanisms to manipulate host plants. Elucidating how pathogens manipulate plants could assist us in understanding basic plant cellular processes (Bray Speth et al. 2007). Likewise, in defense against these threats, plants have evolved an elegant set of strategies that we are only beginning to elucidate in detail (Jones and Dangl 2006).

In my dissertation research I used a plant pathogen, the Gram-negative bacterium *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000), to probe the cellular processes of a

host plant, *Arabidopsis thaliana*. In particular, two bacterial effectors produced by this pathogen, AvrPto and HopM1, have attracted me to investigate components of the plant vesicle trafficking system: RabE1 GTPases and MIN7, an ADP-ribosylation factor (ARF) guanine nucleotide exchange factor (GEF).

A Model Pathosystem

To study the interaction between plants and pathogens researchers have developed a useful pathosystem with two model organisms, the Gram-negative bacterium *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) and the mustard plant *Arabidopsis thaliana* (Whalen et al. 1991; Katagiri et al. 2002). The genomes of both organisms are fully sequenced and genetically tractable, which enables mutational analysis of both pathogen and host (Arabidopsis Genome Initiative 2000; Buell et al. 2003).

Arabidopsis thaliana

A wild member of the *Brassicaceae* family, *Arabidopsis thaliana* has been developed into a model species due to a number of advantageous characteristics (Meyerowitz 1989). Arabidopsis are small plants that can produce relatively abundant seeds in a generation time of 5 to 6 weeks. Arabidopsis has a small genome, approximately 125 MB, and simple *Agrobacterium tumefaciens*-mediated floral dip can be used to rapidly transform Arabidopsis without tissue culture (Clough and Bent 1998). Arabidopsis research has been further facilitated by numerous tools developed by the Arabidopsis research community. Large collections of Arabidopsis mutants generated by transfer DNA (T-DNA) or transposon insertions are available to researchers, and natural variants (ecotypes) of the species have been collected all over the world that are adapted to different ecological conditions (Pigliucci 1998; Arabidopsis Biological Resource Center, www.abrc.osu.edu). Numerous public database have been developed for genomic, proteomic, and other 'omic' studies (Peng et al. 2009; Yilmaz et al. 2010; Swarbreck et al. 2007).

***Pseudomonas syringae* pv. *tomato* DC3000**

Pst DC3000 represents one of more than 50 pathovars of the species (Gardan et al. 1999). It is the causal agent of bacterial speck on tomato, but is also capable of infecting the model plant species *Arabidopsis thaliana* (Whalen et al. 1991). *Pst* DC3000 multiplies in many Arabidopsis ecotypes, including Col-0, leading to water soaking, tissue chlorosis, and necrosis.

Pst DC3000 enters the plant via wounds or natural openings such as stomata, but remains in the extracellular space. Like many other Gram-negative bacterial pathogens of humans and plants, *Pst* DC3000 carries a type three secretion system (T3SS) (Büttner and Bonas 2003). The T3SS is a needle-like structure responsible for translocation of effectors into the host cell to promote pathogenesis (Kubori et al. 1998; Galan and Wolf-Watz 2006). Additionally, *Pst* DC3000 produces the phytotoxin coronatine, a molecular mimic of the plant hormone jasmonic acid conjugated to isoleucine (JA-Ile), that facilitates entry via host plant stomata and suppresses host defense in the apoplast (Bender et al. 1989; Thines et al. 2007; Melotto et al. 2006).

Plant Immunity

As pathogens and plants have evolved so have the interactions between them. Plants have a number of preformed defenses such as leaf waxes, rigid cell walls, and constitutional expressed antimicrobials, but when these are overcome by pathogens, a plethora of induced defenses is activated (Heath 2000). The first line of induced defense in plants is pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Jones and Dangl 2006). This is the plant's response to conserved molecular patterns (such as flagellin) not easily lost by the pathogen during evolution. PTI can be overcome by pathogens through the virulence action of effector proteins, or effector-triggered susceptibility (ETS). However, the presence of effectors does not guarantee a successful infection because these effectors may be recognized by plant disease resistance proteins, leading to effector triggered immunity (ETI; also known as gene-for-gene resistance). Likewise, pathogen effectors that are not recognized by the host may target components of ETI to disable this type of plant immunity.

Both PTI and ETI are at least partially dependent upon salicylic acid (SA) signaling for immune responses (Tsuda and Katagiri 2010). SA is required both for the response of the plant at the site of infection as well as the ability to induce systemic acquired resistance (SAR) (Ryals et al. 1996). SA has antagonistic relationships with the hormones jasmonic acid (JA) and ethylene. *Pst* DC3000 uses the phytotoxin coronatine to suppress SA signaling by activating the JA signaling pathway (Brooks et al. 2005; Thines et al. 2007).

PAMP-Triggered Immunity (PTI)

PAMPs, also called microbe-associated molecular patterns (MAMPs) due to their presence in non–pathogenic species, are molecular patterns conserved by classes of microbes,

which includes bacterial flagellin, elongation factor Tu (EF-Tu), and lipopolysaccharides (LPS), and fungal chitin (Felix et al. 1999; Kunze et al. 2004; Zeidler et al. 2004; Kaku et al. 2006; Mackey and McFall 2006). They can be detected by plasma membrane (PM)-localized pattern recognition receptors (PRRs) (Nicaise et al. 2009). Most PRRs are receptor-like kinsases or receptor-like proteins (RLKs/RLPs). Flagellin and EF-Tu are recognized by RLKs FLS2 and EFR, respectively, which contain extracellular leucine-rich repeat (LRR) domains (Zipfel et al. 2004; Zipfel et al. 2006). Chitin is recognized by two receptors, CEBiP of rice and CERK1 of Arabidopsis, both of which contain extracellular LysM domains (Kaku et al. 2006; Miya et al. 2007). Whereas CERK1 contains an intracellular kinase domain, CEBiP lacks it. Receptors for other PAMPs, including LPS, have not been identified (Nicaise et al. 2009). Perception of PAMPs by PRRs leads to activation of mitogen-activated protein kinase (MAPK) signaling cascade, expression of defense genes including the *pathogenesis-related (PR*) genes, production of reactive oxygen species (ROS), callose deposition in the cell wall, and eventually restriction of microbial growth (Gomez-Gomez et al. 1999).

Effector-Triggered Susceptibility (ETS)

To overcome host PTI, pathogens utilize protein effectors secreted into the host cell. Approximately 30 effectors have been identified in the strain *Pst* DC3000 alone, and the *Pseudomonas* species is estimated to have about 200 effectors belonging to 60 different protein families (Lewis et al. 2009). It has been demonstrated that most of the *Pst* DC3000 effectors can suppress some forms of plant immunity (Hauck et al. 2003; Guo et al. 2009). Not surprisingly, several effectors have been found to directly target components of PTI and ETI (Figure 1-1). AvrPto and AvrPtoB are unrelated effectors with functional redundancy that have been reported

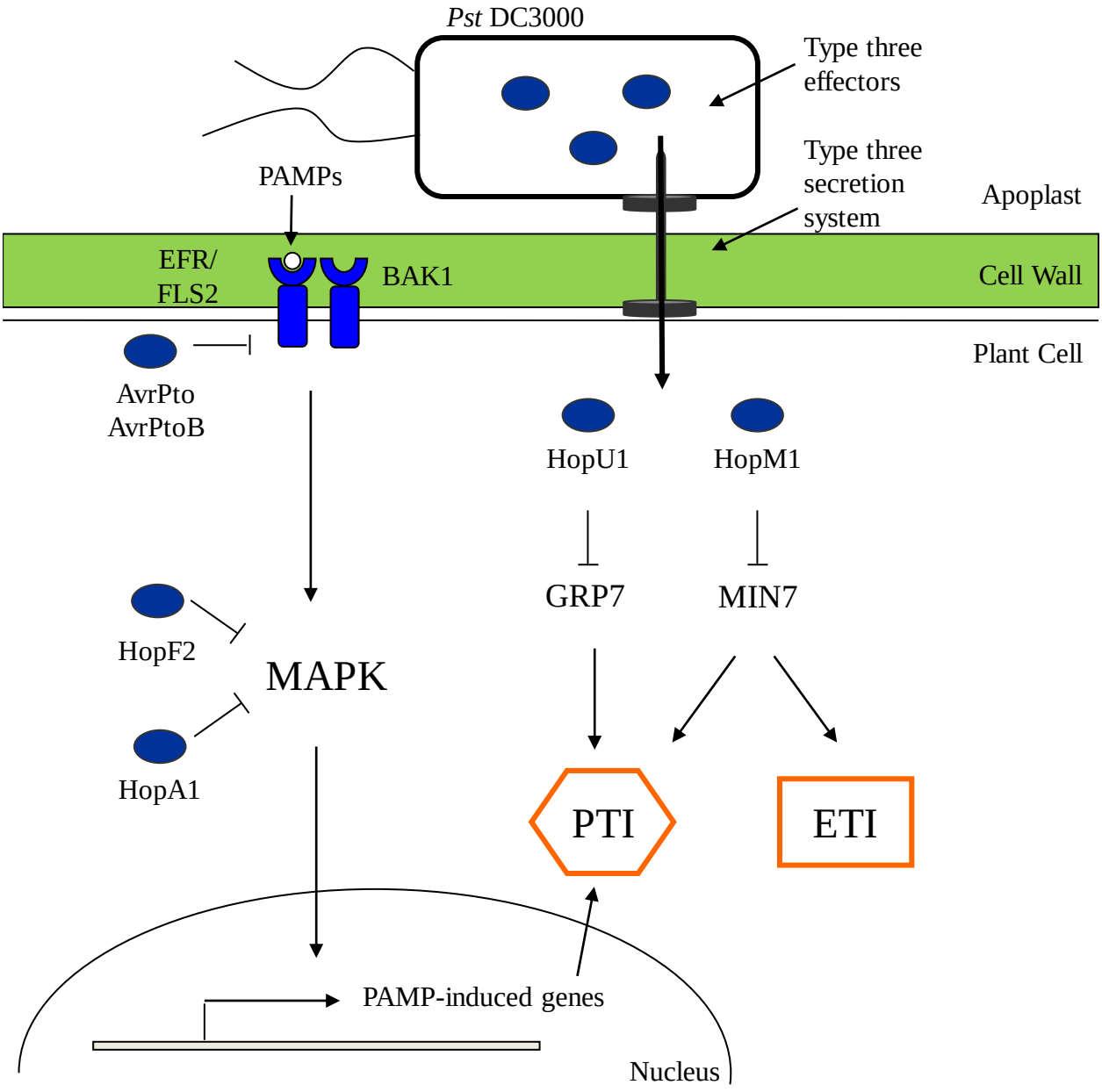

Figure 1-1. Host targets of *Pst* DC3000 effectors.

Pst DC3000 is an extracellular pathogen that uses a type three secretion system to translocate effectors into the plant host cell. AvrPto and AvrPtoB interfere with PAMP perception by targeting the PAMP receptors EFR and FLS2, and HopF2 and HopA1 interfere with MAPK signalling. Targeting of MIN7 and GRP7 by HopM1 and HopU1 suppresses immunity by less defined mechanisms. Adapted from Zhou and Chai, 2008.
For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis

to target the RLKs and interfere with the perception of PAMPs. AvrPtoB has E3 ubiquitin ligase activity, and it ubiquitinates FLS2 and CERK1 leading to their degradation via the proteasome and vacuole, respectively (Göhre et al. 2008; Gimenez-Ibanez et al. 2009). The binding of AvrPto to FLS2 and EFR prevents their autophosphorylation required for activation of downstream PTI signaling pathways (Xiang et al. 2008). There is also evidence that AvrPto and AvrPtoB both bind to BAK1, a PRR signaling partner, and interfere with the interaction between BAK1 and FLS2 (Shan et al. 2008). HopF2 and HopA1 impact PTI by interfering with the MAPK signaling downstream of PAMP perception. HopA1 does so by dephosphorylating components of the MAPK signaling pathway and HopF2 ADP-ribosylates MAP kinase kinases (Zhang et al. 2007; Y. Wang et al. 2010).

The host targets for several other DC3000 effectors have also been identified, but the target's function in plant immunity is less defined. HopU1 interferes with defense-related programmed cell death (also called hypersensitive response; HR), and does so by mono-ADP-ribosylating GRP7, an RNA-binding protein (Fu et al. 2007). Plants lacking *GRP7* support the growth of a *Pst* DC3000 T3SS mutant that is non-virulent in wild type Col-0 plants. The effector HopM1 mediates the degradation of Arabidopsis HopM1-interacting protein 7 (MIN7) in a proteasome-dependent manner (Nomura et al. 2006). MIN7 is an ADP-ribosylation factor (ARF) guanine nucleotide exchange factor (GEF), an activator of the ARF small GTPases that act as molecular switches in vesicle trafficking.

Effectors are not limited to affecting a single host process. In addition to targeting perception of PAMPs, AvrPtoB also has been shown to suppress plant cell death associated with ETI (Rosebrock et al. 2007), and the presence of AvrPto and AvrPtoB in the plant cell suppresses PAMP-inducible miRNAs (Navarro et al. 2008).

Effector-Triggered Immunity (ETI)

Although it shares some of the same signaling components and gene expression profiles as PTI, ETI is a faster, more robust response and includes programmed cell death (Tao et al. 2003). Effectors trigger an ETI only in specific resistant host genotypes in which they are recognized by host resistance (R) proteins, most of which are nucleotide binding (NB) leucine-rich repeat (LRR) proteins (Eitas and Dangl 2010). Effectors were originally called avirulence factors due to their association with pathogen avirulence and restriction of pathogen growth (Flor 1955).

Long before their molecular mechanisms were understood, R proteins were highly valued for breeding resistance into agricultural species to fight against pathogen infections (Flor 1971). R proteins recognize effectors through direct or indirect mechanisms. For example, the *Magnaporthe grisea* effector AvrPita interacts directly with the rice R protein PITA (Jia et al. 2000). More often, however, recognition is through an intermediate host protein. The unrelated *Pst* DC3000 effectors AvrPto and AvrPtoB activate the NB-LRR Prf by targeting the kinases Pto and Fen (Kim et al. 2002; Mucyn et al. 2006; Rosebrock et al. 2007). Similarly, NB-LRR-type R proteins detect bacterial effectors AvrRpt2, AvrB and AvrRpm1 by monitoring effector-induced phosphorylation or proteolysis of the intermediate host protein RIN4 (Mackey et al. 2002; Axtell and Staskawicz 2003).

Vesicle Trafficking

In eukaryotic cells, membrane-bound vesicles are used to transport proteins, lipids, and polysaccharides (Seabra and Wasmeier 2004; Jurgens 2004). For this to occur, vesicles must bud from the donor membrane, travel to their destination, dock with the target membrane, and release their cargo. This process is highly regulated and small GTPases are major components of the regulatory mechanism.

Vesicle Trafficking and Small GTPases

Several families of small GTPases exist in Arabidopsis: Rho of plant (Rop), Rab, Ran and ARF GTPases (Vernoud et al. 2003). Although functionally distinct they have structural and regulatory similarities. The activity of small GTPases is determined by guanine nucleotide-binding state. They are bound to GTP in the active state, and to GDP in the inactive state (Figure 1-2). GTPases are assisted in activation and deactivation by two types of enzymes, guanine-nucleotide exchange factors (GEFs) that promote GTP binding and GTPase activating proteins (GAPs) that promote GTP hydrolysis. An additional level of regulation is provided by guanine-disassociation inhibitors (GDIs), which recover small GTPases from the membrane and sequester them in the cytosol. GDIs have a binding pocket for the prenylation moiety that GTPases use as membrane anchors (Wu et al. 1996).

Despite their diverse functions, small GTPases share certain physical similarities. They all have a nucleotide-binding core that is composed of six β-sheets and five α-helices, and the nucleotide binding state alters the conformation of the Switch I and Switch II domains (Barnekow et al. 2009). Mutations have been identified that reduce the ability of small GTPases to alternate between the active and inactive forms (Der et al. 1986; Feig and Cooper 1988). One

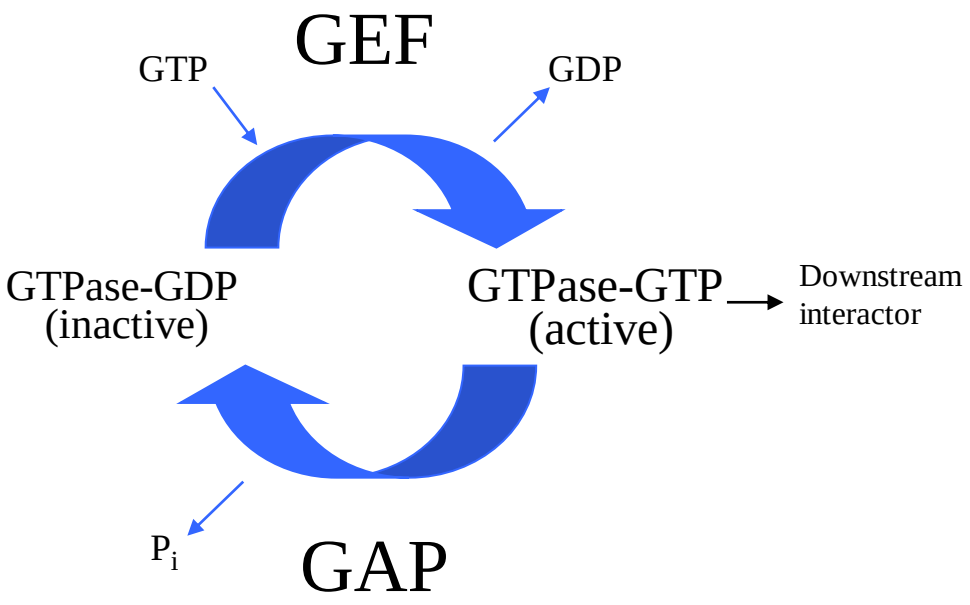

Figure 1-2. Schematic representation of the regulatory cycle of small GTPases

Small GTPases alternate between a GDP-bound inactive state and GTP-bound active state. In the GTP-bound state, small GTPases interact with downstream effectors. Guanine nucleotide exchange factors (GEFs) promote the exchange of GTP for GDP, and GTPase activating proteins (GAPs) facilitate GTP hydrolysis.

such mutation reduced the GTP binding affinity in favor of GDP, ensuring primarily GDP-bound or inactive GTPases in the cell (Feig and Cooper, 1988). It can act as a dominant-negative inhibitor by titrating out the downstream interactors of small GTPases. Alternatively, they can be fixed in an active state by a mutation that prevents GTP hydrolysis (Der et al. 1986). These types of mutations have been critical to the study of small GTPases because small GTPases often have high sequence similarity within families and have functional redundancy (Vernoud et al. 2003). Therefore, gene knock-out mutants may lack observable phenotypes.

ARF GTPases

The first ADP-ribosylation factor (ARF) GTPase was identified as a cofactor for the ADP-ribosylation of adenylate cyclase by the cholera toxin (Kahn and Gilman 1984). However, it was eventually recognized as a key player in the cellular traffic in eukaryotic cells by recruiting coat proteins to budding vesicles (Serafini et al. 1991; Donaldson et al. 1992). The ARF family of proteins includes the ARF GTPases, ARF-like (ARL) GTPase, and Sar1, and they have been recognized as regulators of vesicle budding, coatamer recruitment, and cytoskeletal re-arrangement (Kahn 2009).

ARF GTPases contain canonical small GTPase domains but are distinguished by an N-terminal amphipathic helix modified by myristoylation that is required for membrane localization (Franco et al. 1995; Antonny et al. 1997). Indeed, truncation of the helix results in mislocalization of the protein in the cell (Matheson et al. 2008). Myristoylation and membrane localization are required for activation (Antonny et al. 1997). Mammalian Sar1 and ARL3 lack myristoylation sites and contain instead a N-terminal acetylation site that is required for membrane localization (Huang et al. 2001; Setty et al. 2004).

ARF GTPases are activated by GEFs, some of which carry a highly conserved SEC7 domain (Peyroche et al. 1996; Casanova 2007). The SEC7 domain is catalytic and has been demonstrated to be sufficient for substrate specificity (Macia et al. 2001). There are five families of SEC7 GEFs identified in mammals and yeast, but only two families, the BIG and GBF, are represented in Arabidopsis (Memon 2004; Anders and Jürgens 2008). The first Arabidopsis SEC7 protein shown to promote GTP/GDP exchange on an ARF protein was GNOM, a member of the GBF family (Steinmann et al. 1999). GNOM is localized to endosomal compartments and is required for the endosomal recycling of the polar auxin transport protein PIN1 (Geldner et al. 2001; Geldner et al. 2003). Subsequent work reveals some overlapping function with another ARF GEF from the GBF family, GNOM-like (GNL), which is localized to the Golgi apparatus (GA) and PM (Richter et al. 2007; Teh and Moore 2007). Incorrect localization of PIN1 has also been observed in the *ben1/min7* mutant, implicating a role of MIN7 in PIN1 recycling (Tanaka et al. 2009).

There are 15 predicted ARF GAPs in Arabidopsis based on the presence of the ARF GAP domain (AGD) (Vernoud et al. 2003). Two ARF GAPs, AGD5/Nevershed1 (NEV1) and VAN3/SFC, have a demonstrated ability to promote the hydrolysis of GTP on Arabidopsis ARFs (Stefano et al. 2010; Liljegren et al. 2009). AGD5 is trans-Golgi network (TGN)-localized with ARF-A1c/ARF1 and has promiscuous GAP activity *in vitro* (Stefano et al. 2010). VAN3/SFC was identified in a screen for mutants with altered vein patterning and is required for the normal transport of PIN1 (Deyholos et al. 2000; Sieburth et al. 2006). VAN3/SFC partially co-localizes with the ARF GEF GNOM and both are required for endosomal recycling (Naramoto et al. 2010).

There are 19 annotated ARF and ARF-like (ARL) GTPases in Arabidopsis (Vernoud et al. 2003). The 12 ARF GTPases are subdivided into four families, ARFA1, ARFB1, ARFC1 and

ARFD1, and seven ARL GTPases in four families, ARLA1, ARLB1, ARLC1 and ARL1. The ARFs characterized from Arabidopsis have thus far displayed similar intracellular localization and molecular function to predicted othologues from mammals and yeast. In other systems, ARF1 has been extensively characterized in the recruitment of coat proteins to budding vesicles at the GA (Vasudevan et al. 1998). Arabidopsis ARF1/ARF-A1c has been localized to the GA and function in trafficking between the endoplasmic reticulum (ER) and the GA (Pimpl et al. 2000; Stefano et al. 2006a). However, ARF1/ARF-A1c is also localized to the TGN (Xu and Scheres 2005; Stefano et al. 2006a; Matheson et al. 2007). ARF-B1a of Arabidopsis is localized to the PM as is its yeast and mammalian orthologue ARF6 (Matheson et al. 2008). However, in Arabidopsis this ARF is also localized to the GA.

Progress has also been made with the characterization of ARL GTPases in Arabidopsis. ARL1 has been localized to the TGN and early endosomes and binds to GRIP domain proteins (Latijnhouwers et al. 2005; Stefano et al. 2006b). Little is known about the molecular function of ARL-C1/Titan5, but loss of expression is embryo-lethal in Arabidopsis (McElver et al. 2000).

Rab GTPases

Rab GTPases are small GTPases that function in several steps of vesicle transport, from initiation of the vesicle to tethering at the target membrane (Stenmark 2009). In addition to the common small GTPase domains, Rab GTPases contain a C-terminal hypervariable domain and geranylgeranylation site (Pfeffer 2005). The hypervariable domain has been implicated in targeting of Rabs, which is critical to their function (Chavrier et al. 1991). The geranylgeranyl moiety is anchored in the membrane when the RAB GTPase is active and masked by a guanine-

disassociation inhibitor (GDI) when cytosolically localized and inactive (Magee and Newman 1992; Soldati et al. 1993).

Numerous Arabidopsis Rab GTPases have been investigated in recent years, aided by confocal microscopy of fluorescently-tagged proteins and the use of mutants with fixed guanine nucleotide states, particularly the dominant-negative (DN), GDP-fixed state. Subcellular localization reveals that many Rabs share organelle specificity with yeast and mammalian orthologues (Nielsen et al. 2006). However, Rab GTPases within the same family that display similar intracellular localization may have distinct functions (Pinheiro et al. 2009). Additionally, plants have unique cellular structures such as the phragmoplast and the chloroplast that necessitate plant-specific Rab functions.

Arabidopsis contains 57 Rab GTPases, which are divided into eight families based on sequence and predicted functional similarities to yeast and mammalian Rab proteins (Vernoud et al. 2003). The largest Rab family in Arabidopsis is RabA which is composed of 26 GTPases. It is similar to the mammalian Rabll family that contains only three proteins (Nielsen et al. 2006). In pea and Arabidopsis, RabA proteins are found in largely distinct compartments with some overlap (Inaba et al. 2002; Chow et al. 2008). Expression of the DN form of RabA family members interferes with pollen tube tip growth (de Graaf et al. 2005; Silva et al. 2010). This interference may be due to the disruption of cytokinesis observed in plants expressing the DN form of RabA (Chow et al. 2008).

The DN form of RAB-G3e interferes with the development of tracheary elements, which are plants cells that transport water and minerals (Kwon et al. 2008). Interestingly, overexpression of wild type RAB-G3e increases the rate of endocytosis in plant cells and confers increased osmotic stress tolerance to plants.

The RabE family is most similar to Rab8 family of mammals and yeast that is involved in polarized secretion (Vernoud et al. 2003). Expression of the DN form of RAB-E1d increases the accumulation of secGFP (a modified GFP designed for secretion) in the cell (Zheng et al. 2005). RABE1-d has been localized to both the GA and the PM (Zheng et al. 2005; Bray Speth et al. 2009).

Rabs belonging to the RabD1/D2 and RabF1/F2 families have both distinct and overlapping functions and cellular localization. RabF1 and RabF2 localize to distinct but overlapping compartments while utilizing the same exchange factor, VSP9a (Ueda et al. 2004; Goh et al. 2007). RabD1 and RabD2 are found in both the GA and the TGN and regulate trafficking between the ER and GA (Pinheiro et al. 2009). However, the DN forms of RabD1 and RabD2 inhibit ER-GA traffic by precipitation of different Rab interactors.

Vesicle Trafficking and Pathogenesis

During pathogen attack, a large redistribution of resources occurs in the plant cells. Pathogens are perceived by plants through detection of PAMPs or effectors, and this triggers downstream signaling and changes in transcription (Felix et al. 1999). Papillae, cell wall appositions composed of callose, phenolics and reactive oxygen species, are established at the site of pathogen in a trafficking-dependent manner (Assaad et al. 2004). Peroxisomes travel to the site of infection (Lipka et al. 2005). Pathogenesis-related (PR) proteins and phytoalexins are secreted into the apoplast (van Loon et al. 2006; Pedras and Yaya 2010). These processes are thought to be dependent upon the vesicle trafficking systems in the cell. Interestingly, manipulation of host vesicle traffic is an important virulence strategy in bacterial pathogenesis in

mammals, and over the last decade, the importance of vesicle trafficking in plant pathogenesis has also begun to emerge.

PEN1 was discovered in a screen for nonhost Arabidopsis mutants with increased penetration by powdery mildew fungus (*Blumeria graminis f. sp. hordei* (*Bgh*)), and the PEN1 orthologue ROR2 performs the same function in barley (Collins et al. 2003). PEN1 and ROR2 are syntaxins, proteins that form part the SNARE complex required for vesicle fusion, and PEN1 is required for the establishment of the papillae (Assaad et al. 2004). PEN1 complexes with synaptosomal-associated protein of 33 kD (SNAP33) and vesicle-associated membrane protein (VAMP) 721 or 722 to mediate exocytosis and cargo release (Kwon et al. 2008). Another syntaxin, *Nicotiana benthemiana* SYP132, is necessary for secretion of the protein pathogenesis related 1a (PR1a) into the apoplast and contributes to ETI and PTI (Chinchilla et al. 2006).

Penetration resistance mediated by ROR2 is dependent upon other canonical components of vesicle trafficking, barley ADP-ribosylation factor (ARF) GTPases ARF-A1b and ARF–A1c (Böhlenius et al. 2010). *ARF-A1b/c* silenced barley plants have reduced penetration resistance to *Bgh*. Barley ARF-A1b and ARF-A1c are required for ROR2 localization to the papillae and the deposition of callose, but not the formation of the other papillae components.

Manipulation of vesicle traffic components is also found in plant-bacterial interactions. The PAMP receptor FLS2 is a PM-localized protein that directly binds the flagellin peptide flg22, triggering PTI (Chinchilla et al. 2006; Robatzek 2006). Upon ligand binding, FLS2 is endocytotically recycled and inhibition of endocytosis is correlated with loss of PTI-associated ROS production (Robatzek 2006; Serrano et al. 2007). This indicates that the endocytosis of the receptor is linked to its function in PAMP-triggered signaling. The actin cytoskeleton is involved in vesicle trafficking in plants (Boevink et al. 1998), and recently, it was demonstrated

that Arabidopsis actin depolymerizing factor 4 (ADF4) contributes to AvrPphB-mediated ETI (Tian et al. 2009). Finally, the Arabidopsis ARF GEF MIN7 is degraded in the presence of the *Pst* DC3000 effector HopM1 (Nomura et al. 2006). In the absence of MIN7 (i.e., in *min7* mutant plants), HopM1 is not essential for bacterial multiplication.

RATIONALE

When this work was initiated, little was known about the vesicle trafficking pathways regulated by MIN7 or RabE1. MIN7 was predicted to be a GEF but activity had not been demonstrated. It was not known which Arabidopsis ARFs or ARLs are co-localized with MIN7 in the cell. Similarly, downstream interactors of RabE1 had not been identified. I attempted to address these questions in my research. In chapter 2, I will describe my work with MIN7 and the Arabidopsis ARF GTPases. In chapter 3, I will summarize my work with RabE1. In chapter 4, I will give my perspective on the work completed and describe the future direction of these projects.

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Chapter 2

Characterization of MIN7: An Arabidopsis ADP-ribosylation Factor Guanine Nucleotide Exchange Factor

I would like to thank James Kremer for contributing to Figures 2-2 and 2-5.

ABSTRACT

HopM1-interacting protein 7 (MIN7) is one of eight SEC7-domain-containing proteins in Arabidopsis, and based on the presence of the SEC7 domain, MIN7 is predicted to be an ADP-ribosylation factor (ARF) guanine nucleotide exchange factor (GEF). ARF GEFs promote the exchange of GTP for GDP on ARF GTPases and activate ARF GTPases, which are critical regulators of vesicle trafficking in eukaryotes. MIN7 is a host target of HopM1, an effector secreted into the host cell by the phytopathogenic bacterium *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000. HopM1 mediates degradation of MIN7 via the 26S proteasome. However, MIN7 GEF activity has not been demonstrated, and it is not known which Arabidopsis ARFs or ARLs are MIN7 substrates.

Using the SEC7 domain of MIN7, I was able to demonstrate GEF activity *in vitro*. In the presence of MIN7-SEC7, there was an approximate threefold increase in GTPγS binding by the ARF-A1c. *In planta*, MIN7::DsRed partially co-localizes with two members of the ARF-A1 family, ARF-A1c::GFP and ARF-A1d::GFP, as well as representatives of the three additional families of ARFs in Arabidopsis, ARF-D1a::GFP, ARF-B1a::GFP and ARF-C1::GFP.

min7 mutant plants are compromised in benzothiadiazole (BTH)-induced defense and display enhanced cell death to high concentrations (>300 μM) of BTH, an analogue of salicylic acid. I identified two T-DNA insertion lines (*arlB1-1* and *arlB1-2*) in the gene *ARLB1*. Both of these lines showed enhanced cell death in response to treatment with 300 μM BTH. However, both lines maintained normal BTH-induced defense. These results suggest that BTH hypersensitivity can be uncoupled from defects in BTH-induced defense.

INTRODUCTION

The yeast protein SEC7 was first identified as a protein necessary for secretion and integrity of the Golgi apparatus (GA) (Bussey et al. 1983). The connection to ADP-ribosylation factor (ARF) GTPases became apparent with the discovery that overexpression of the human ARF4 could suppress the *sec7* mutation in yeast (Deitz et al. 1996). Later that year two separate groups demonstrated that two human proteins, Gea1 and ARNO, which share a domain with yeast SEC7, could promote the exchange of GTP for GDP on human ARF1 (Cherfils et al. 1998; Béraud-Dufour et al. 1998). All subsequently discovered guanine nucleotide exchange factors (GEFs) for ARF GTPases in animals, yeast, and plants carry the SEC7 domain (Cox et al. 2004).

The SEC7 domain is composed of 10 helices and a hydrophobic groove for ARF binding and has an invariant glutamate finger that wedges between the GDP and the substrate ARF (Cherfils et al. 1998; Béraud-Dufour et al. 1998). Dislodging the GDP allows for GTP binding and activation of the ARF GTPase. In the active, GTP-bound state ARF GTPases are membrane localized (Antonny et al. 1997). ARF GTPases regulate vesicle trafficking through the recruitment of coat proteins to budding vesicles. There are 12 ARF and 7 ARF-like (ARL) GTPases in Arabidopsis (Vernoud et al. 2003).

ARF GEFs are divided into small (<100 kDa) and large (>100 kDa) ARF GEFs and are further subdivided into 6 subfamilies based on the presence of domains beyond the SEC7 domain (Gillingham and Munro 2007b). Only two of these subfamilies, GBF/GEA and BIG/SEC7, are found in Arabidopsis and both are comprised of large ARF GEFs (Cox et al. 2004). A total of eight ARF GEFs are predicted in Arabidopsis (Swarbreck et al. 2007). Since there are fewer GEFs than ARFs, at least some of the GEFs must have multiple ARF substrates.

GNOM was the first identified ARF GEF in Arabidopsis and is the best characterized (Steinmann et al. 1999). GNOM regulates polar auxin transport by regulating the endosomal recycling of the PIN1 protein to the plasma membrane (PM) (Geldner et al. 2003). It is one of two ARF GEFs in Arabidopsis, the other being BIG2, that have been shown to promote GTP/GDP exchange on ARF GTPases *in vitro* (Steinmann et al. 1999; Nielsen et al. 2006; Anders et al. 2008). BIG2 (At1g01950) is required for development of the embryo sac (Pagnussat et al. 2005).

Arabidopsis MIN7/BIG5/BEN1 is the first plant SEC7-domain ARF GEF that is implicated in bacterial pathogenesis. The association of MIN7 with pathogenesis was discovered in the study of *Pseudomonas syringae* pv *tomato* DC3000 infection in Arabidopsis (Nomura et al. 2006). *Pst* DC3000 uses a type three secretion system to translocate effectors into the host cell to promote pathogenesis (Büttner and Bonas 2003). *Pst* DC3000 has approximately 30 effectors, including HopM1 (Nomura et al. 2006). HopM1 interacts with MIN7 and promotes MIN7 degradation through the 26S proteasome (Nomura et al. 2006). The *min7* mutant plants are more susceptible to the ΔCEL mutant, in which *hopM1* and several other effector genes are deleted, indicating that, without the host target MIN7 in the plant, HopM1 is not necessary for infection. The *min7* plants are also compromised in BTH-induced dependent defense (Nomura et al. submitted). It is well established that pre-treatment of plants with the SA analog benzothiadiazole (BTH) leads to induction of systemic acquired resistance (SAR) and restriction of *Pst* DC3000 growth (Friedrich et al. 1996). However, *min7* plants are compromised in BTH-induced defense and allow more growth of *Pst* DC3000 than wild type Col-0 plants when pre-treated with BTH. Furthermore, *min7* plants display hypersensitivity to high concentrations of BTH (K. Nomura and S.Y. He, unpublished). BTH hypersensitivity has also been observed in

Arabidopsis *bip2* and *syp132* mutants (Wang et al. 2005; Kalde et al. 2007). BiP2, an endoplasmic reticulum (ER) resident protein, and SYP132, a syntaxin are both required for secretion of pathogenesis-related protein 1 (PR1), a marker protein of BTH-induced defense (Wang et al. 2005; Kalde et al. 2007).

How MIN7 regulates plant defense is not understood. Being an ARF GEF, MIN7 likely participates in plant defense-associated vesicle traffic in specific subcellular compartments. Recently, MIN7 has been localized to the trans-Golgi network (TGN)/early endosome (EE) in Arabidopsis root cells and in leaf tissue (Tanaka et al. 2009; Nomura et al. submitted). In *min7* mutant plants, the recycling of PIN1 to the PM (Tanaka et al. 2009) and secretion of several putative defense-associated proteins (Nomura et al. submitted) are affected. This result suggests that MIN7 potentially controls the traffic of several different cargoes (e.g., PM-localized PIN1 and secreted defense proteins) in the TGN/EE. Despite these insights, several critical questions remain unknown concerning the function of MIN7. The most basic question is whether MIN7 is capable of promoting GTP/GDP exchange on an ARF GTPase. If yes, which ARFs and ARLs are substrates of MIN7 in Arabidopsis? In this chapter, I describe my research aimed at addressing these questions.

METHODS AND MATERIALS

Cloning

Nineteen *ARF* and *ARL* sequences were identified in Arabidopsis and 18 of those are expressed in leaf tissue, the site of infection of *Pst* DC3000 (Vernoud et al. 2003; Swarbreck et al. 2007). For protein expression in *E. coli*, primers were designed to delete the first 17 amino acids that form an amphipathic helix: *Δ17ARF-A1c*-F 5'-ATCGGATCCATGCGTATTCTGATGGTTG-3' and *Δ17ARF-A1c*-R 5'-AAAACTCGAGCTATGCCTTGCTTGCGAT-3' (Nielsen et al. 2006). *BamHI* and *XhoI* restriction sites are underlined The amphipathic helix prevents GEF binding to ARFs in the absence of lipid membranes and is routinely deleted for *in vitro* assays (Antonny et al. 1997). When truncated an ARF is used, the number of amino acids deleted from the N-terminus will be indicated (e.g., the first 17 amino acids are deleted from Δ17ARF-A1c). The SEC7 domain of MIN7 was identified based on the SEC7 domain of BIG2 that has been shown to activate ARF-A1c/ARF1 and sequence alignment with Arabidopsis, yeast and human ARF GEFs. Primers used to amplify the MIN7 SEC7 domain are: *MIN7-SEC7*-F 5'-TGGATCC**ATGCATCATCATCATCATCAC**TCTACTGGA GACCAATTGAAACC-3' and *MIN7-SEC7*-R 5'-AATCGGCCGTTAGAGCTTCTTCATGGTGTCATCGTC-3' (Nielsen et al. 2006). *BamHI* and *EagI* restriction sites are underlined and the sequence for the 6xhistidine tag is in bold. The SEC7 domain of MIN7 will be referred to as GST::MIN7-SEC7$_{556\text{-}772}$ hereinafter. *ARF* and *ARL* sequences were amplified from cDNA generated from leaf total RNA with the Elongase polymerase mix (Invitrogen, Carlsbad, CA) and cloned in pCR2.1-TOPO (Invitrogen, Carlsbad, CA; Table 2-1) or pENTR/d-TOPO (Invitrogen, Carlsbad, CA; Table 2.1). Constructs in pCR2.1-TOPO were subcloned into pET42a (EMD, Darmstadt, Germany) for protein expression in *E. coli*. Constructs in pENTR/d were recombined into the destination

vector pMDC83, in which a C-terminal GFP fusion will be generated using LR Clonase II (Invitrogen, Carlsbad, CA).

Sequence alignment of Arabidopsis *ARF* and *ARL* GTPases

Arabidopsis *ARF* and *ARL* coding sequences (CDS) and proteins sequences from TAIR database were used for ClustalW2 alignment (Vernoud et al. 2003; Swarbreck et al. 2007).

Purification of MIN7-SEC7$_{556-772}$ and Δ17ARF-A1c

MIN7- SEC7$_{556-772}$ and Δ17ARF-A1c were expressed from the pBR322-derived vector pET42a (EMD Biosciences/Novagen, Darmstadt, Germany) with an N-terminal tag that consists of glutathione-S-transferase (GST), a 6x histidine tag, and the S epitope tag from ribonuclease A. The N-terminal tag can be completely removed by Factor Xa. Proteins were expressed in *E. coli* BL21 (DE3) cells grown in low-salt Luria-Bertani broth (10g/l tryptone, 5g/l yeast extract, 5g/l NaCl) overnight at 20°C following induction by isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were collected and resuspended in 5 ml lysis buffer (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM MgCl2, 1 mM DTT, 5 mM EDTA, 1 mg/ml lysozyme, Benzonase, Complete Mini EDTA-free Protease inhibitor cocktail (Roche, Mannheim, Germany)). Cells were sonicated and centrifuged to collect cell debris. The supernatant was applied to GST-bind resin (EMD, Darmstadt, Germany) and incubated overnight at 4°C. GST::MIN7-SEC7$_{556-772}$ was eluted from the resin with glutathione. MIN7- SEC7$_{556-772}$ was concentrated in a Microcon YM-10 spin column and resuspended in storage buffer (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 5 mM EDTA). Glycerol was added to a final concentration of 10 %, and protein solutions were stored at -80°C. GST::Δ17ARF-A1c on resin was incubated with Factor Xa (NEB, Ipswich, MA) overnight at 4°C to release Δ17ARF-A1c. A Microcon

YM-10 spin column was used to replace the buffer with Mg^{2+}-free buffer containing EDTA (50 mM HEPES pH7.5, 100 mM KCl, 5 mM EDTA, 1 mM DTT). EDTA chelates Mg^{2+}, destabilizing guanine nucleotides from GTPases. GDP was added to the reaction at a final concentration of 100 μM to ensure that, upon addition of Mg^{++}, GTPases would be bound predominantly by GDP. A Microcon YM-10 spin column was used to replace the buffer with storage buffer. Glycerol was added (final concentration of 50%) and protein solutions were stored at -80°C. Proteins were quantified using the DC Protein Assay kit (Bio-Rad, Hercules, CA) and visualized on Denville Blue (Denville Scientific, Metuchen, NJ)-stained SDS-PAGE gels. Purification protocols were adapted from Gillingham and Munro (2007).

GTP/GDP exchange assay

In 100 μl assay buffer (50 mM HEPES-KOH pH7.5, 1 mM DTT, 100 mM KCl, 1 mM $MgCl_2$) at room temperature, 2.5 μM GTPase, 50 nM GEF or 500 nM GEF, GST or buffer, and 50 μM [^{35}S] GTPγS (~ 800 CPM/pmol) were combined. Samples (2.5 μl) were taken at 2, 5 and 10 minutes after the addition of GTPγS and added to 300 μl ice-cold stop buffer (50 mM HEPES-KOH, pH7.5, 1 mM DTT, 100 mM KCl, 10 mM $MgCl_2$). Samples were spotted on BA85 protran filter using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA) and washed three times with ice cold stop buffer. Filters were dried and immersed in scintillation fluid, and radioactivity was enumerated by a scintillation counter.

Transient Expression in *Nicotiana benthemiana*

Agrobacterium tumefaciens strain GV3101 containing plant expression plasmids (35S::MIN7-DsRed or DEX::HopM1=GFP (Nomura et al. submitted) and 35S::ARF-GFP) was grown to mid-log phase at 30°C. Cells were pelleted and resuspended in infiltration media (10 mM

$MgS0_4$, 10 mM MES, pH 5, 300 μM acetosyringone) to OD_{600} =0.4 (for 35S::MIN7::DsRed), 0.1 (for 35S::ARF:GFP), or 0.1 (DEX::HopM1::GFP). Bacteria were infiltrated into leaves of *N. benthemiana* at the 5 to 7 leaf stage for confocal microscopy. Flowering plants were used for HopM1 toxicity assays.

Laser Scanning Confocal Microscopy

For transient expression of MIN7::DsRed and ARF::GFP, two days after infiltration with Agrobacteria leaf samples were excised and viewed by sequential scanning using an Olympus FluoView 1000 Laser Scanning Confocal Microscope (Center Valley, PA) with a 60X objective lens. GFP-tagged proteins were excited by the 488 nm argon laser diode and emissions were collected through a 500-545 nm band pass filter. DsRed-tagged proteins were excited with the 559 laser diode and emissions were collected through a 570-600 nm band pass filter. Microscopy images are composites of multiple scans taken through multiple planes of the Z axis (or depth of the sample), composites of multiple scans taken in one plane on the Z axis over time or single scans of a single plane on the Z axis. Images were processed using the Olympus Fluoview Viewer Version 2.0b.

Immunoblot analysis

To determine expression of ARF::GFPs in tobacco, a cork borer was used to sample tissue two days following Agrobacterium infiltration. Tissue was ground in 5X SDS loading buffer (100 mM Tris-HCl, pH6.8, 200 mM DTT, 4% SDS, 20% glycerol) and heated at 95°C for 10 minutes. Proteins were separated on 12% SDS-PAGE gels and blotted to PVDF membrane by semi-dry transfer. Protein detection was carried out with an anti-GFP antibody (Abcam, Cambridge, MA).

Confirmation of T-DNA insertion lines

All available T-DNA insertion lines were acquired from the Arabidopsis Biological Resource Center (ABRC). For confirmation of the T-DNA insertion, DNA was extracted and amplified using the Extract N' AMP system (Sigma, St. Louis, MO) in a PCR reaction that include two primers corresponding to the genomic sequences flanking the insertion and the left border sequence of the T-DNA insertion (Table 2.2).

RT-PCR

Total RNA was extracted from Arabidopsis leaf tissue of Col-0 and the At5g52210 T-DNA insertion lines Salk_062390 (*arlB1-1*) and Salk_120386 (*arlB1-2*) using a RNA extraction kit (RNeasy Plant Mini Kit, Qiagen, Valencia, CA). First-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was then used as a template for PCR with gene-specific primers: *arlB1*-1 5'-AATCATATGATGTTTTCTCTTATGTCT-3', *arlB1*-2 5'-CTATGAATTTGGCACAGGAGTGTAC-3', *arlB1*-3 5'-AGAATGGCTGGTTGGAGTAATG-3' and *arlB1*-4 5'TGATGCAAAGATTGTGGTCTG-3'.

BTH Hypersensitivity

Commercial Actiguard (Syngenta, Greensboro, NC) containing 50% benzothiadiazole (BTH) was suspended in water to a concentration of 300 μM. BTH solution or water was sprayed on 3 to 5 week old plants. Plants were covered in plastic wrap and monitored over 4-10 days for development of chlorosis and necrosis.

Bacterial inoculation and enumeration

Arabidopsis plants were grown in potting soil in growth chambers maintained at 20°C with a 12-h day length at 100 $\mu Em^{-2}s^{-1}$. Four to five week old plants were infiltrated with 10^6 CFU/ml *Pst* DC3000 following a published procedure (Katagiri et al. 2002). Leaf samples were collected

using a cork borer, ground, serially diluted, and spotted on low-salt Luria Bertani agar plate (10 g/L tryptone, 5 g/L yeast extract, 5g/L NaCl) containing 100 mg/L rifampicin. Colony forming units were counted and calculated per square centimeter of leaf tissue. To induce the defense response, plants were treated with 50 μM BTH or water 24 hours prior to inoculation with *Pst* DC3000.

RESULTS

GST::MIN7-SEC7$_{556-772}$ promotes the exchange of GTPγS for GDP *in vitro*

To date, only two SEC7 domain-containing proteins from Arabidopsis have been shown to have GEF activity. GNOM weakly promotes GTP/GDP exchange on the human ARF1 and BIG2 has been shown to promote exchange activity on Arabidopsis Δ17ARF-A1c (Steinmann et al. 1999; Nielsen et al. 2006; Anders et al. 2008). I sought to test whether MIN7 also has ARF GEF activity, as indicated by the presence of the SEC7 domain.

An established method for determination of GEF activity is to measure the ability to exchange GTP for GDP on ARF GTPases *in vitro* (Peyroche et al. 1996; Chardin et al. 1996; Steinmann et al. 1999; Macia et al. 2001; Gillingham and Munro 2007a). MIN7 is a large protein (~195 KDa) and the full length protein has been difficult to express and purify in the quantity needed for *in vitro* assays (K. Nomura and S.Y. He, unpublished). It has been demonstrated that the SEC7 domain is sufficient for both exchange activity and ARF specificity *in vitro* (Pacheco-Rodriguez et al. 1998; Macia et al. 2001; Nielsen et al. 2006; Zeeh et al. 2006; Gillingham and Munro 2007a). I identified the boundaries of the MIN7-SEC7 domain by sequence alignment to other SEC7 domains used previously for exchange assays (Figure 2-1). The SEC7 domain was cloned in the pET42 vector with an N-terminal tag that includes GST. GST::MIN7- SEC7$_{556-772}$ was successfully expressed in *E. coli* and purified from bacteria lysate using a GST binding resin (Figure 2-2). ARF-A1c was used for the exchange assay because it was previously co-localized with MIN7 in Arabidopsis root cells (Tanaka et al. 2009). The N-terminal amphipathic helix was deleted to prevent interference with GEF binding *in vitro* (Antonny et al. 1997). Δ17ARF-A1c was also cloned in the pET42a vector with a N-terminal tag that included GST. Δ17ARF-A1c was successfully expressed in *E. coli* and purified from

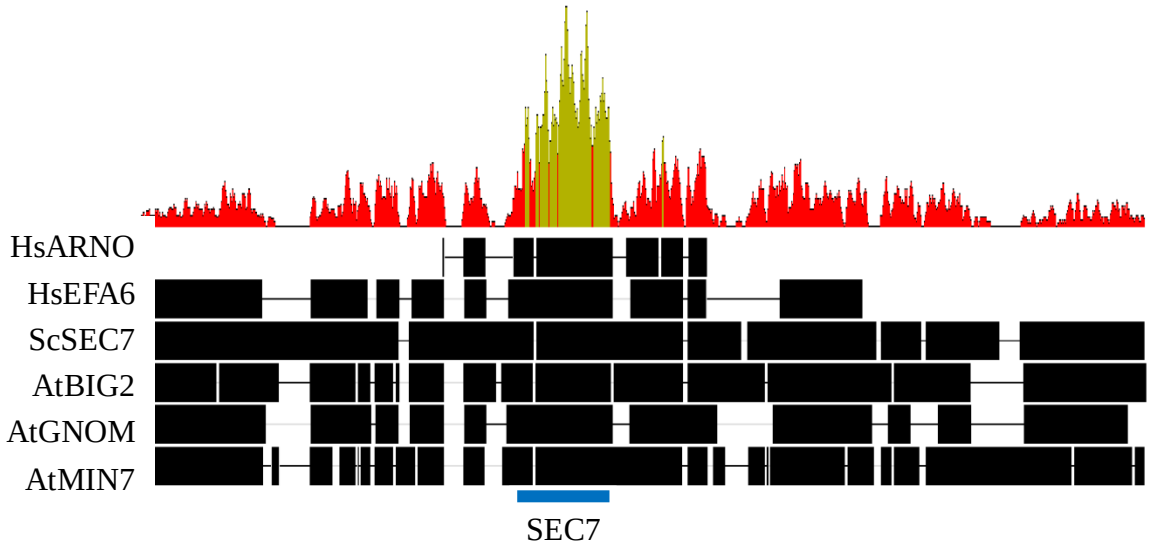


Figure 2-1. Multiple sequence alignment of SEC7 domain-containing ARF GEFs

An alignment of the SEC7 domain-containing proteins was used to determine the boundaries of the SEC7 domain of MIN7. GEF amino acid sequences from Arabidopsis (GNOM, BIG2, MIN7), yeast (SEC7), and human (ARNO, EFA6) are represented by horizontal black bars. The lines connecting the horizontal bars represent gaps in the sequence alignment. Top: Amino acid identity is represented by red and yellow peaks and degree of conservation is proportional to the height of the peak. Yellow peaks highlight the region of highest conservation. The blue line indicates the SEC7 domain. The alignment was performed by James Kremer using ClustalW with a PAM250 substitution matrix.

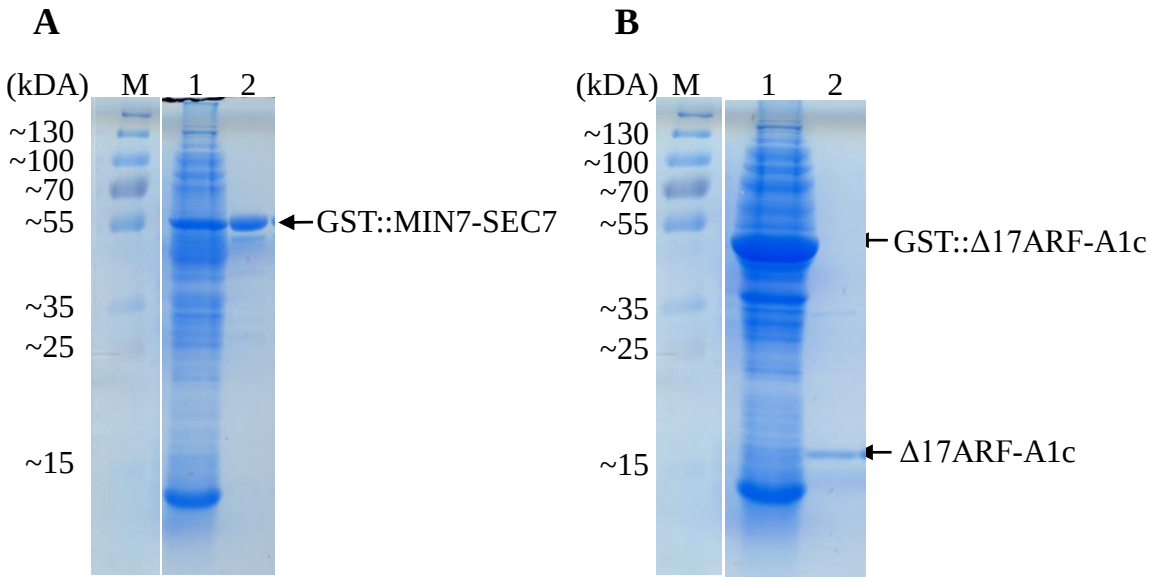


Figure 2-2. Expression in *E. coli* and Purification of MIN7-SEC7$_{556-772}$ and Δ17ARF-A1c

A. Expression in *E. coli* and purification of MIN7-SEC7$_{556-772.}$ Protein marker (lane M).

E. coli containing a pET42 derivative expressing GST:: MIN7-SEC7$_{556-772}$ (lane 1). Protein purified from bacterial homogenate by GST binding resin and eluted with glutathione (lane 2). B. Expression in *E. coli* and purification of Δ17ARF-A1c. Protein marker (lane M). *E. coli* containing a pET42 derivative expressing GST::Δ17ARF-A1c (lane 2). Δ17ARF-A1c purified from bacterial homogenate by GST binding resin and GST tag removed by Factor Xa (lane 2). Proteins were separated on 12% SDS-PAGE and stained with Denville Blue.

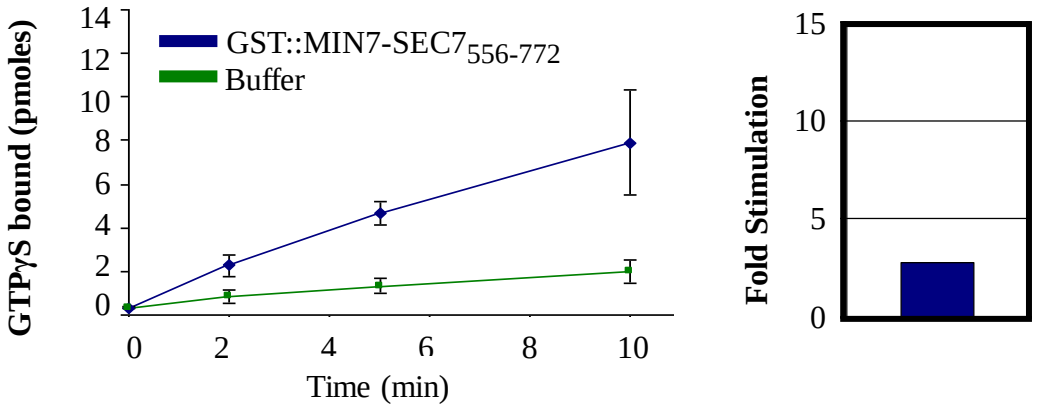
bacterial lysate using a GST binding resin (Figure 2-2). The N-terminal tag was removed with Factor Xa.

In the absence of GST::MIN7-SEC7$_{556-772}$, less than 1 picomole of GTPγS was bound by 250 nmol Δ17ARF-A1c. However, in the presence of 5 pmol GST::MIN7-SEC7$_{556-772}$, the same quantity of Δ17ARF-A1c bound approximately threefold more GTPγS Δ17ARF-A1c than in reactions with the buffer alone (Figure 2.3A). This experiment was repeated three times, with similar results, indicating that ARF GEF activity is present. I also conducted an experiment using a higher amount of GST::MIN7-SEC7$_{556-772}$ and with an additional GST control. Less than 1 picomole of GTPγS was bound by 250 nmol Δ17ARF-A1c in the presence of 50 pmol GST or buffer but GTPγS binding was increased ~13-fold upon addition of 50 pmol GST::MIN7-SEC7$_{556-772}$ (Figure 2-3B). This experiment was only performed once due to time constraints. Therefore, it is not known if the marked increase in GTPγS binding is reproducible, but it is consistent with earlier experiments showing an increase in the presence of GST::MIN7-SEC7$_{556-772}$ over buffer alone.

Co-Expression of MIN7 and ARFs in *N. benthemiana*

MIN7 has been localized to the TGN/EE compartment in Arabidopsis and tobacco based on confocal microscopy studies using a MIN7 antibody and MIN7::DsRed (Tanaka et al. 2009; Nomura et al. submitted). To identify potential ARF/ARL substrates of MIN7 *in vivo*, I sought to identify the ARFs or ARLs that co-localize with MIN7. MIN7::DsRed and individual ARF GTPases with a C-terminal GFP tag were expressed separately or together in *N. benthemiana* leaf cells. Two days after infiltration, MIN7::DsRed was observed in mobile small (~1 μM)

A



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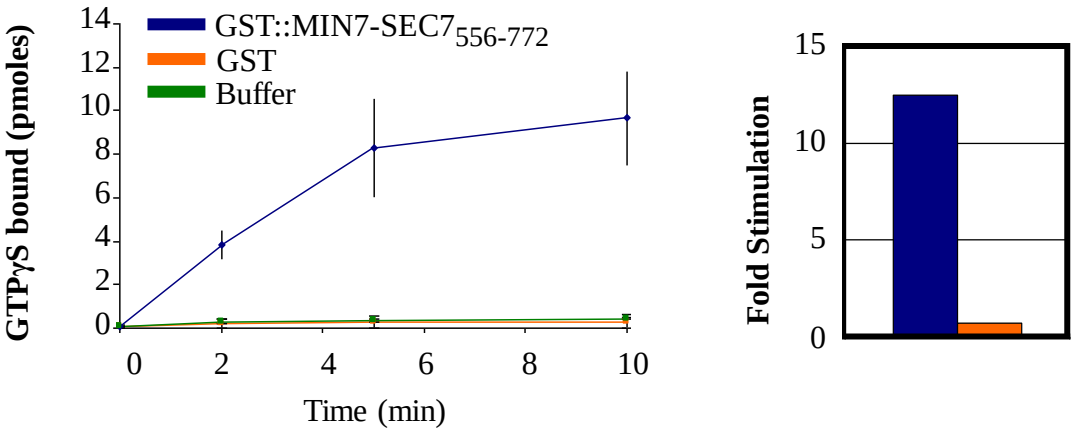


Figure 2-3. MIN7-SEC7$_{556-772}$ stimulates exchange of GTP for GDP on Δ17ARF-A1c

A. GTP/GDP exchange activity was measured by binding of [^{35}S] GTPγS by 250 nmol Δ17ARF-A1c in the presence of 5 pmol GST::MIN7-SEC7$_{556-772}$ or buffer. A representative of three replicates is shown. B. A single experiment comparing the binding of [^{35}S] GTPγS by 250 nmol Δ17ARF-A1c in the presence of 50 pmol GST::MIN7-SEC7$_{556-772}$, 50 pmol GST or buffer. Left graphs: Means and standard errors (n=3) of [^{35}S] GTPγS binding over time. Right graphs: Fold increase in [35S] GTPγS binding in the presence of GST::MIN7-SEC7$_{556-772}$ or GST over that in the presence of buffer only 2 minutes after addition of [35S] GTPγS

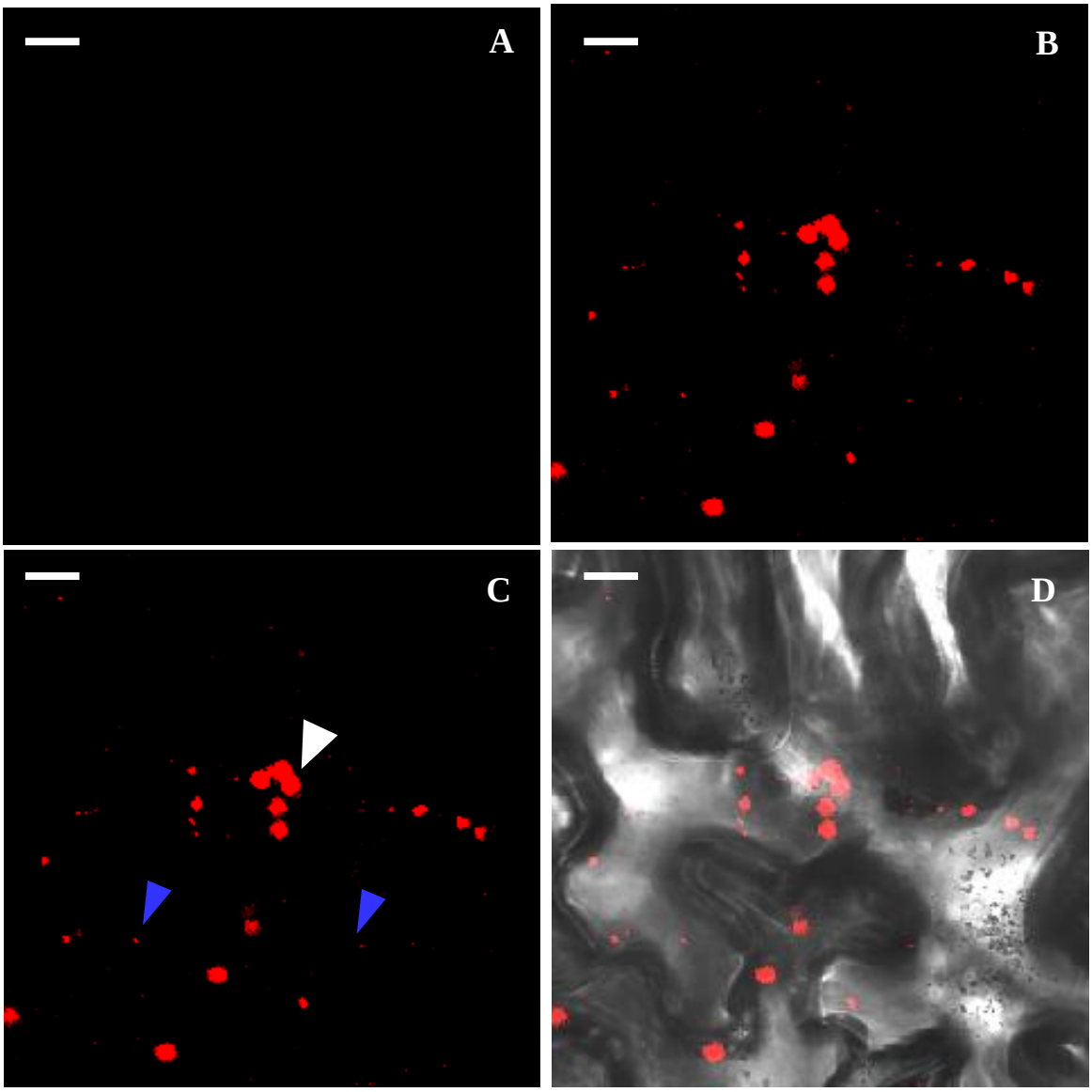


Figure 2-4. MIN7::DsRed localizion in *N. benthemiana* leaf cells

Confocal microscopy images of *N. Benthemiana* leaf cells transiently expressing MIN7::DsRed two days after *A. tumefaciens*-mediated transformation. MIN7 localizes to mobile small (~1 μM) punctate structures (blue arrows) and large (up to ~10 μM), mobile structures (white arrows). A. GFP signal shown as a negative control. B. DsRed signal. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are a composite of 10 scans taken in the same plane every 12 seconds. Scale bar=10 μM.

punctate structures, consistent with its TGN/EE localization (Figure 2-4). All of the ARFs that could be amplified from leaf RNA (ARF-A1e::GFP, ARF-A1c::GFP, ARF-A1a::GFP, ARF-A1d::GFP, ARF-A1f::GFP, ARF-D1a::GFP, ARF-B1a::GFP, ARF-C1, ARF-B1c::GFP, and ARF-B1b::GFP) were transiently expressed in tobacco with MIN7::DsRed at least once. All of the ARF::GFPs examined had some overlapping signal with MIN7-DsRED (Figures 2-11 to 2-15 and data not shown), and five ARFs (ARF-A1d::GFP, ARF-A1c::GFP, ARF-D1a::GFP, ARF-B1a::GFP and ARF-C1::GFP) from the four ARF families (ARF-A1, B1, C1 and D1) were chosen for further analysis (Figure 2-5). Data is shown only for the five ARFs analyzed multiple times.

ARF-A1d::GFP, ARF-A1c::GFP, ARF-D1a::GFP, and ARF-C1::GFP were also observed in mobile small (~1 μM) punctate structures (Figures 2-6 to 2-9). No large mobile structures were found. Of these, ARF-A1c has previously been localized to the Golgi apparatus (GA) and TGN, which appear as small punctate structures. ARF-B1a::GFP appeared primarily at the cell periphery, which is consistent with its published PM localization, and some in intracellular punctate structures (Figure 2-10).

In the co-expression experiments, MIN7::DsRED partially overlaps with all ARF::GFPs tested in small (~1-5 μM) and large, mobile punctate structures (~5-10 μM) (Figures 2-11 to 2-15). In all cases, independent ARF::GFP signal was found without MIN7::DsRed signal, but very little MIN7::DsRed signal occurred independent of sites of co-localization (Figures 2-11 to 2-13). However, when co-expressed with ARF-B1a and ARF-C1, independent MIN7::DsRed signal was observed in addition to the mobile structures. MIN7::DsRed signal co-localized with ARF-B1a only in intracellular mobile structures, but not at the PM (Figure 2-14 and Figure 2-15).

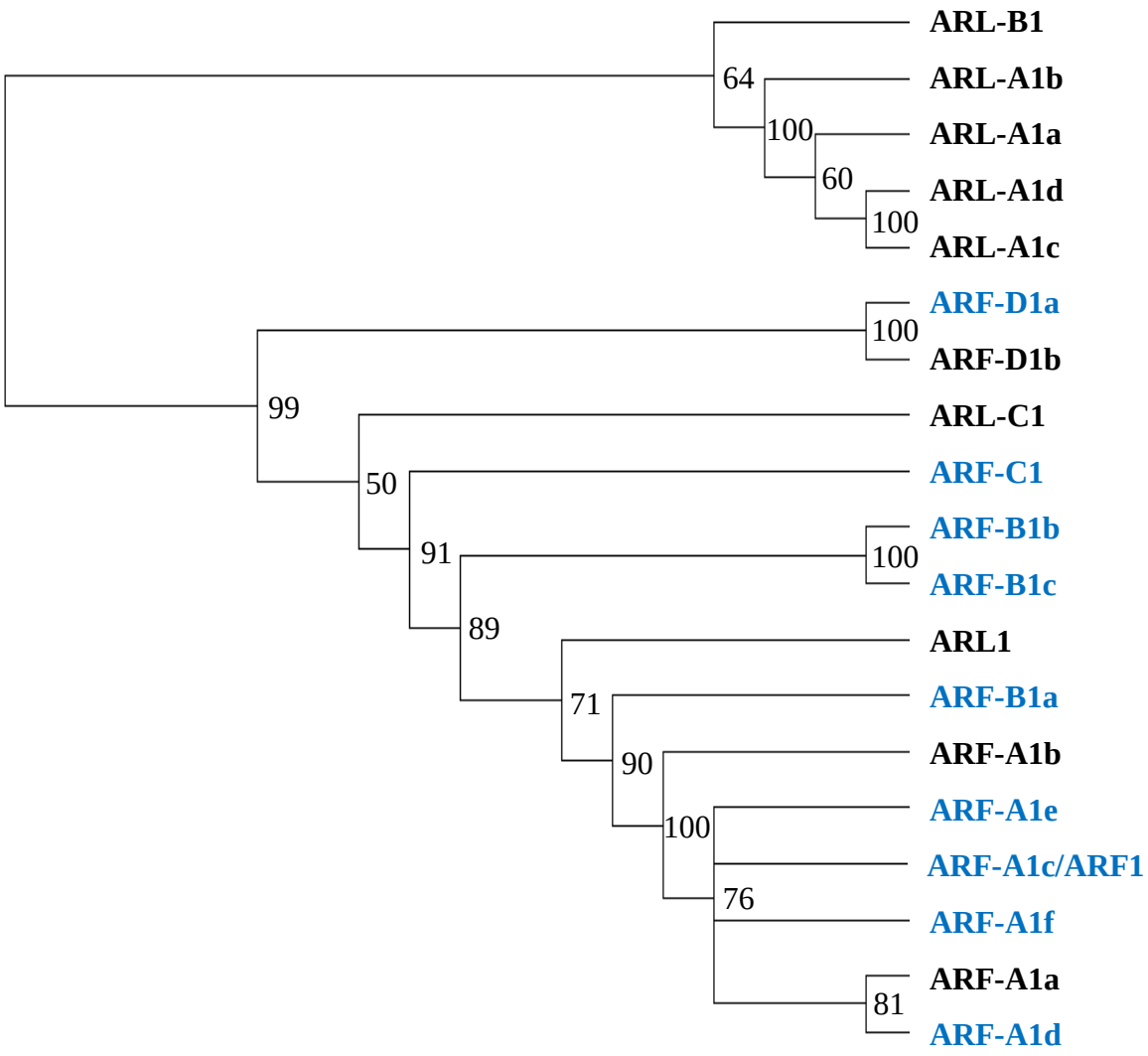


Figure 2-5. The phylogenetic relationship of Arabidopsis ARF and ARL GTPases

Unweighted pair group method with arithmetic (UPGMA) mean tree of full length MUSCLE-aligned Arabidopsis ARF and ARL amino acid sequences. Bootstrap values are indicated on each branch. Alignment and tree were contributed by James Kremer. ARFs fused to GFP and analyzed by confocal microscopy are in blue.

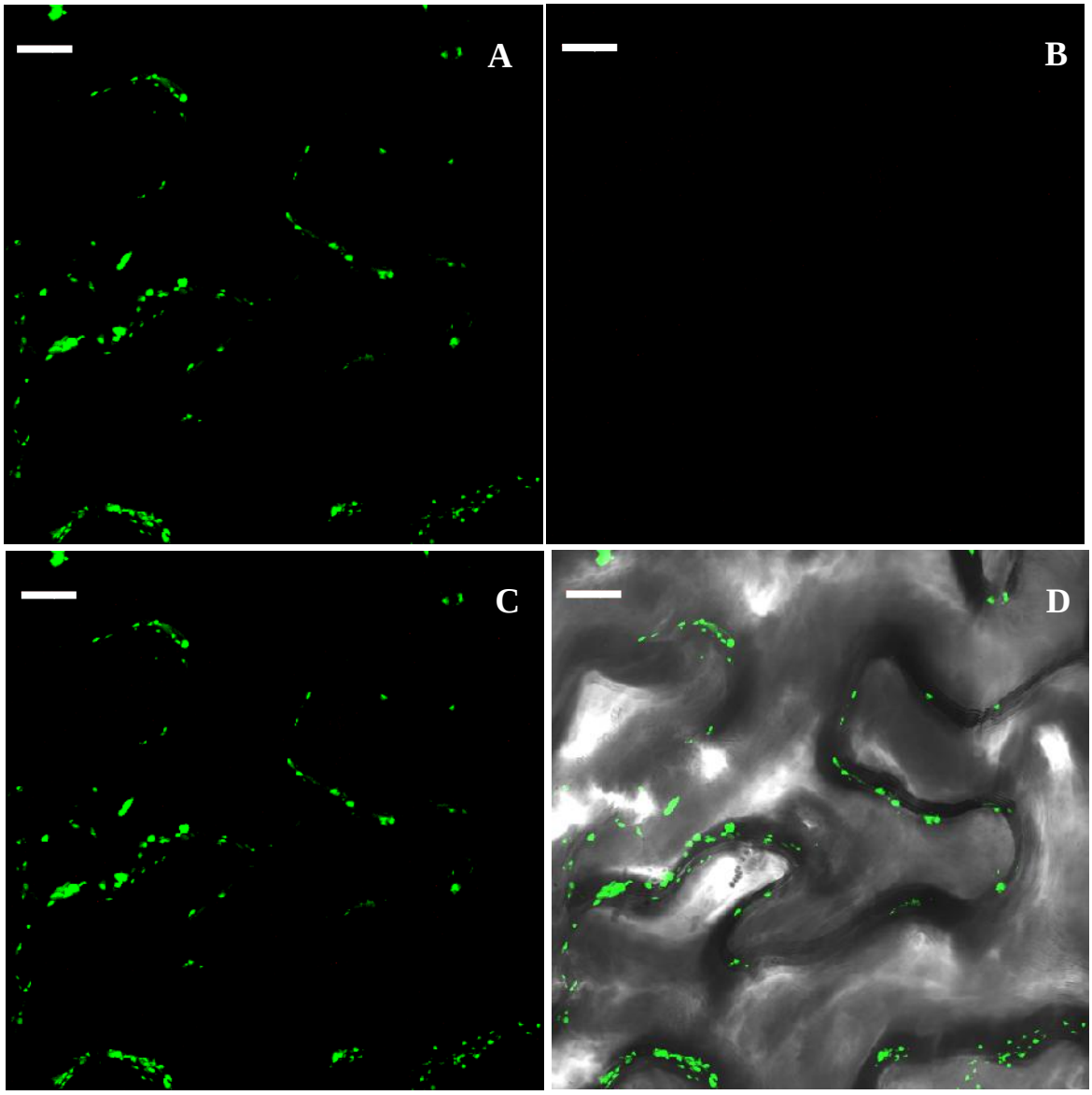


Figure 2-6. ARF-A1c::GFP localization in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-A1c::GFP two days after *A. tumefaciens*-mediated transformation. ARF::A1c localizes to mobile small (~1 μM) punctate structures. A. GFP signal. B. DsRed signal shown as a negative control. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are a composite of 10 scans taken in the same plane every 12 seconds. Scale bar=10 μM.

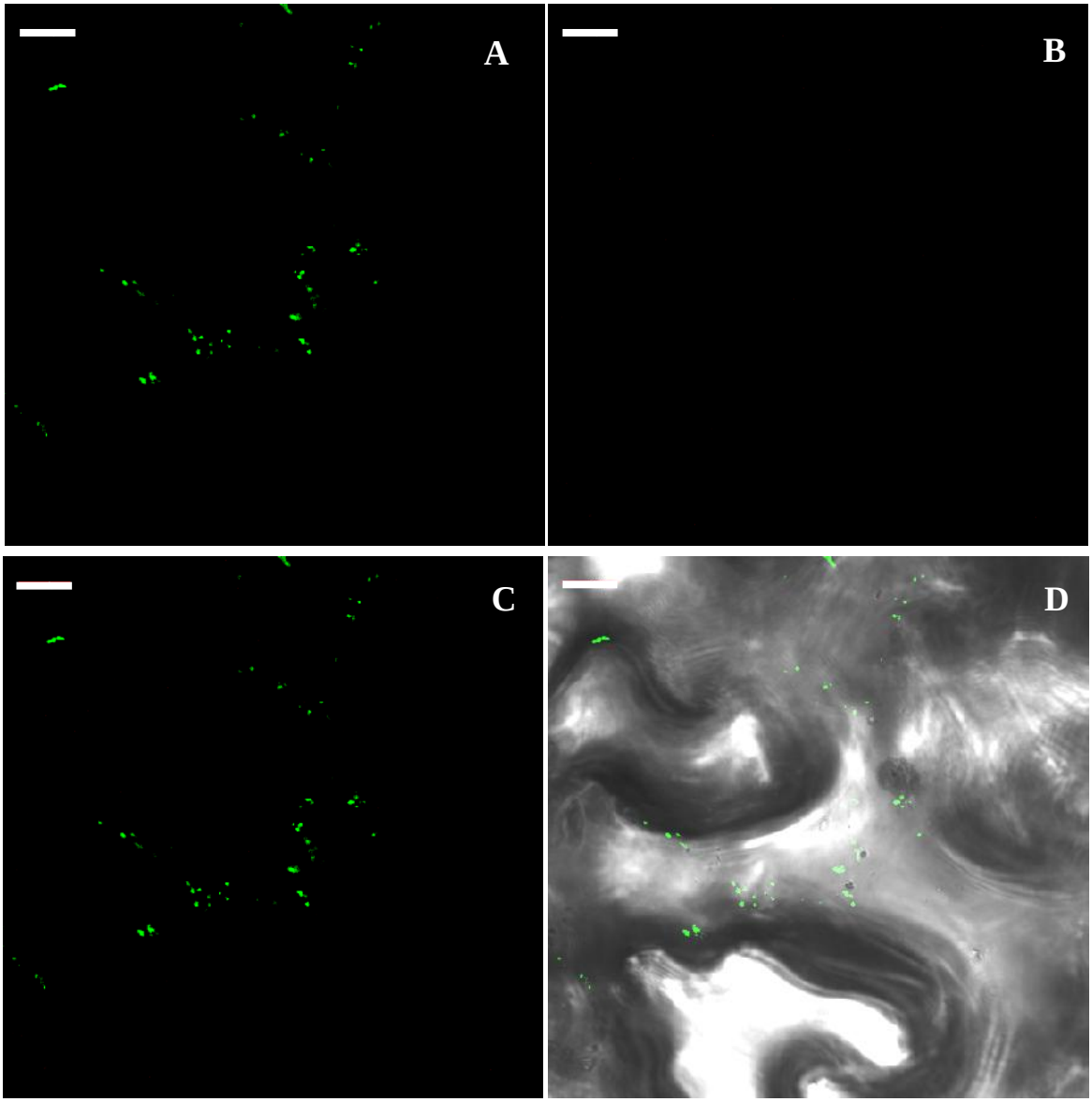


Figure 2-7. ARF-A1d::GFP localization in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-A1d::GFP two days after *A. tumefaciens*-mediated transformation. ARF::A1d localizes to mobile small (~1 μM) punctate structures. A. GFP signal. B. DsRed signal shown as a negative control. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are a composite of 10 scans taken in the same plane every 12 seconds. Scale bar=10 μM

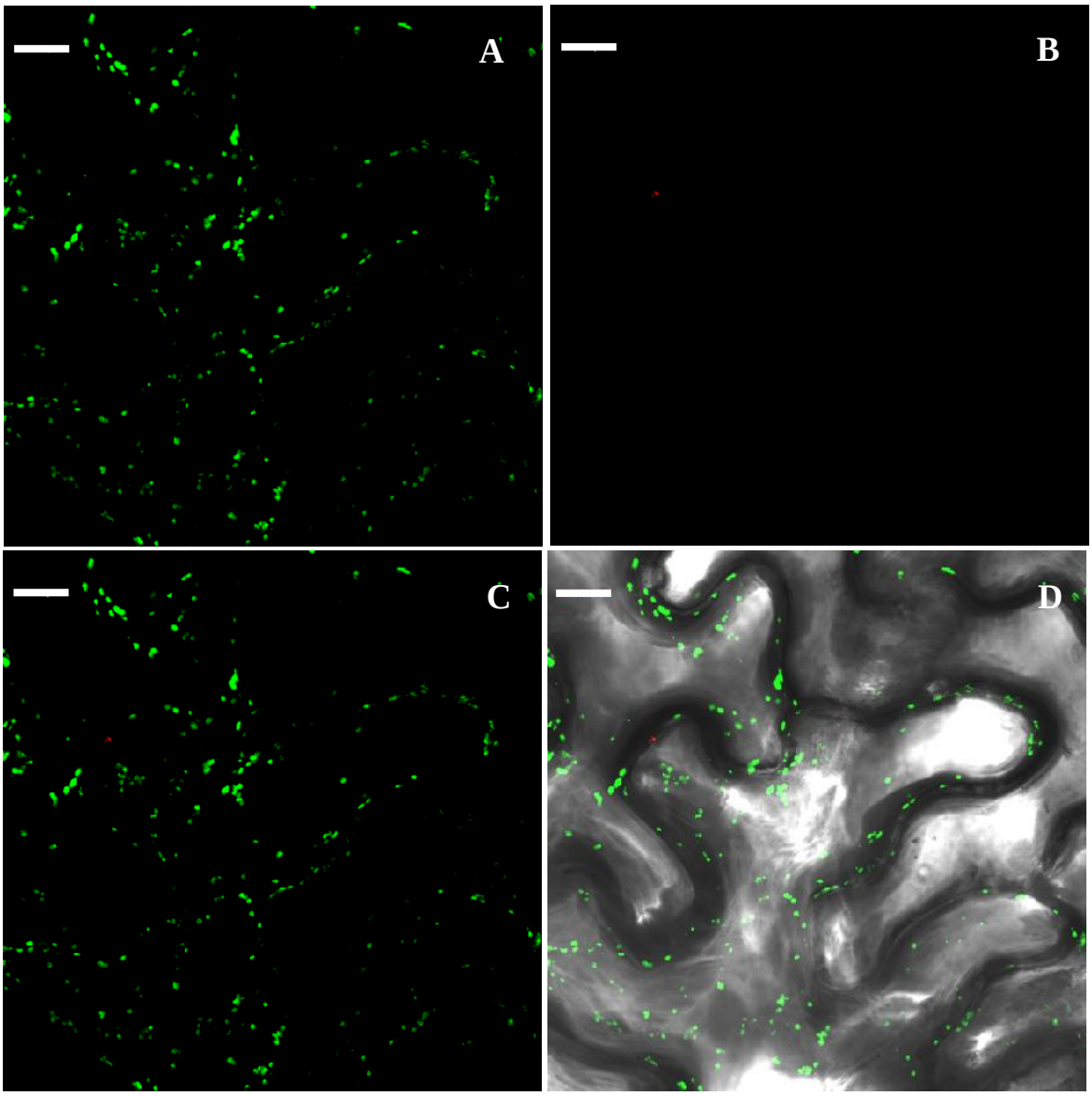


Figure 2-8. ARF-D1a::GFP localization in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-D1a::GFP two days after *A. tumefaciens*-mediated transformation. ARF-D1a::GFP localizes to mobile small (~1 μM) punctate structures. A. GFP signal. B. DsRed signal shown as a negative control. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. All images are composites of scans taken along multiple planes of the Z axis. Scale bar=10 μM.

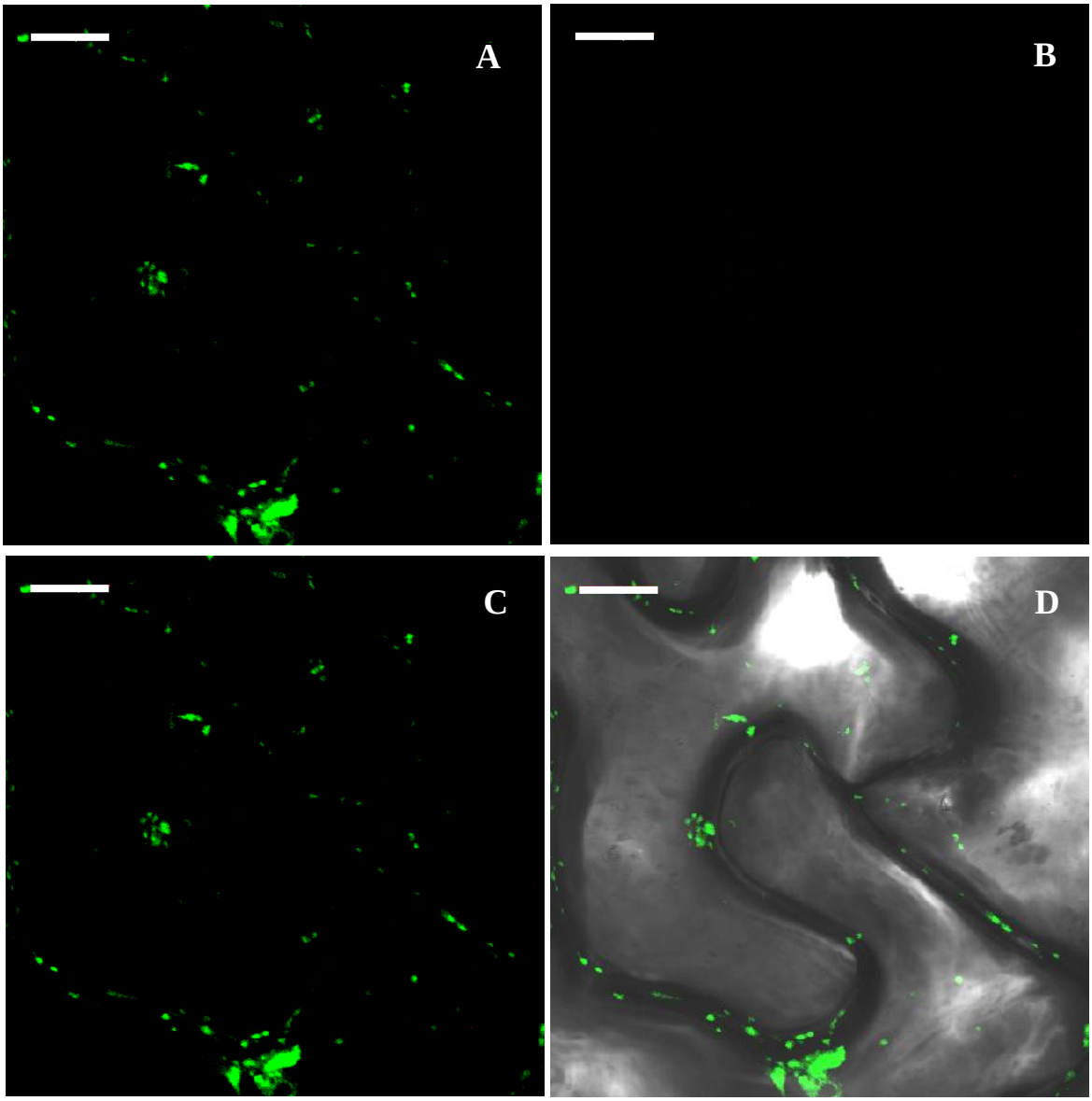


Figure 2-9. ARF-C1::GFP localization in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-C1::GFP two days after *A. tumefaciens*-mediated transformation. ARF-C1::GFP localizes to mobile small (~1 μM) punctate structures. A. GFP signal. B. DsRed signal shown as a negative control. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. All images are composites of scans taken along multiple planes of the Z axis. Scale bar=10 μM.

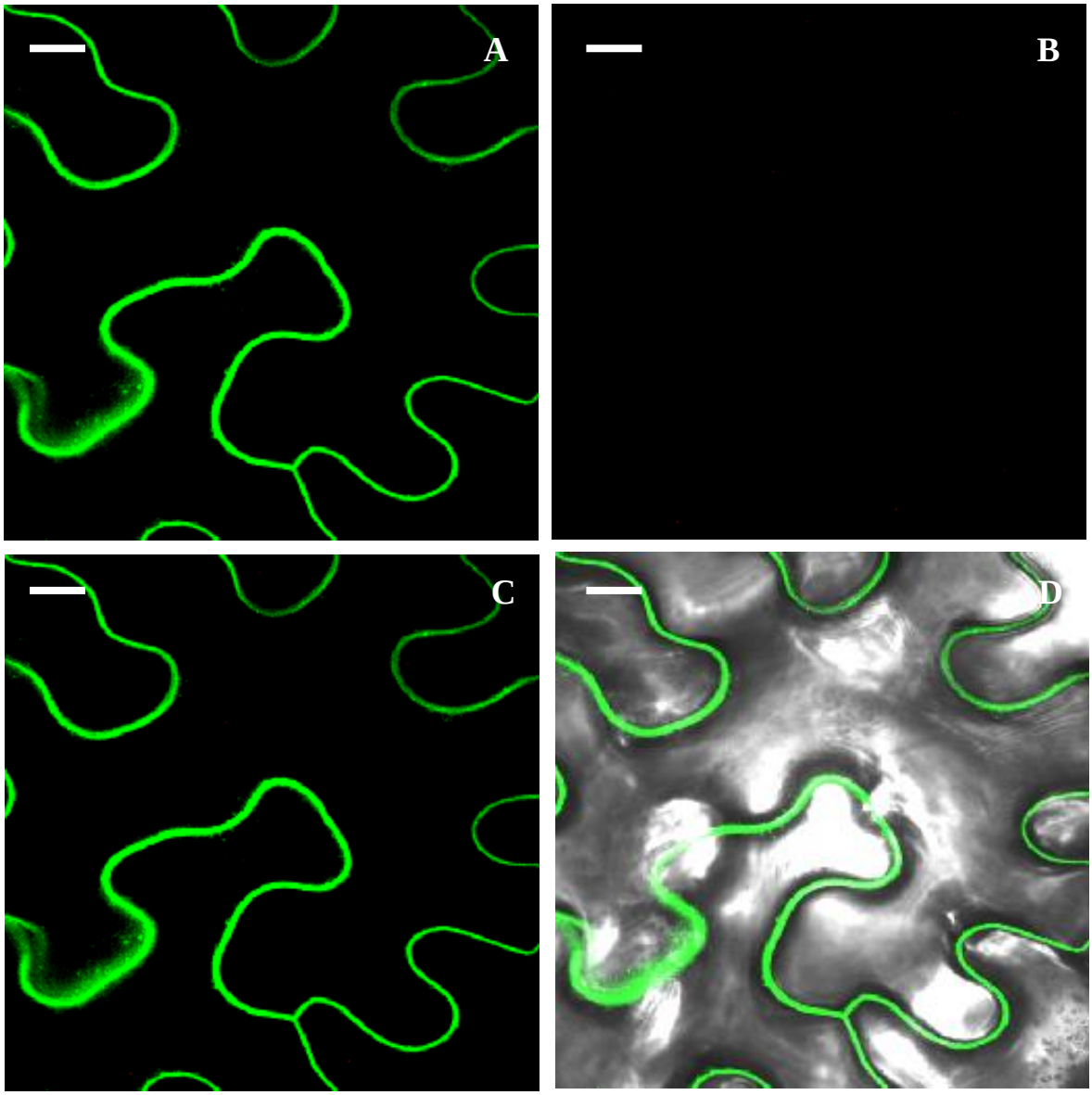


Figure 2-10. ARF-B1a ::GFP localization in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-B1a::GFP two days after *A. tumefaciens*-mediated transformation. ARF-B1a localizes along the cell periphery. A. GFP signal. B. DsRed signal shown as a negative control. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are composites of 10 scans taken in the same plane every 12 seconds. Scale bar=10 μM.

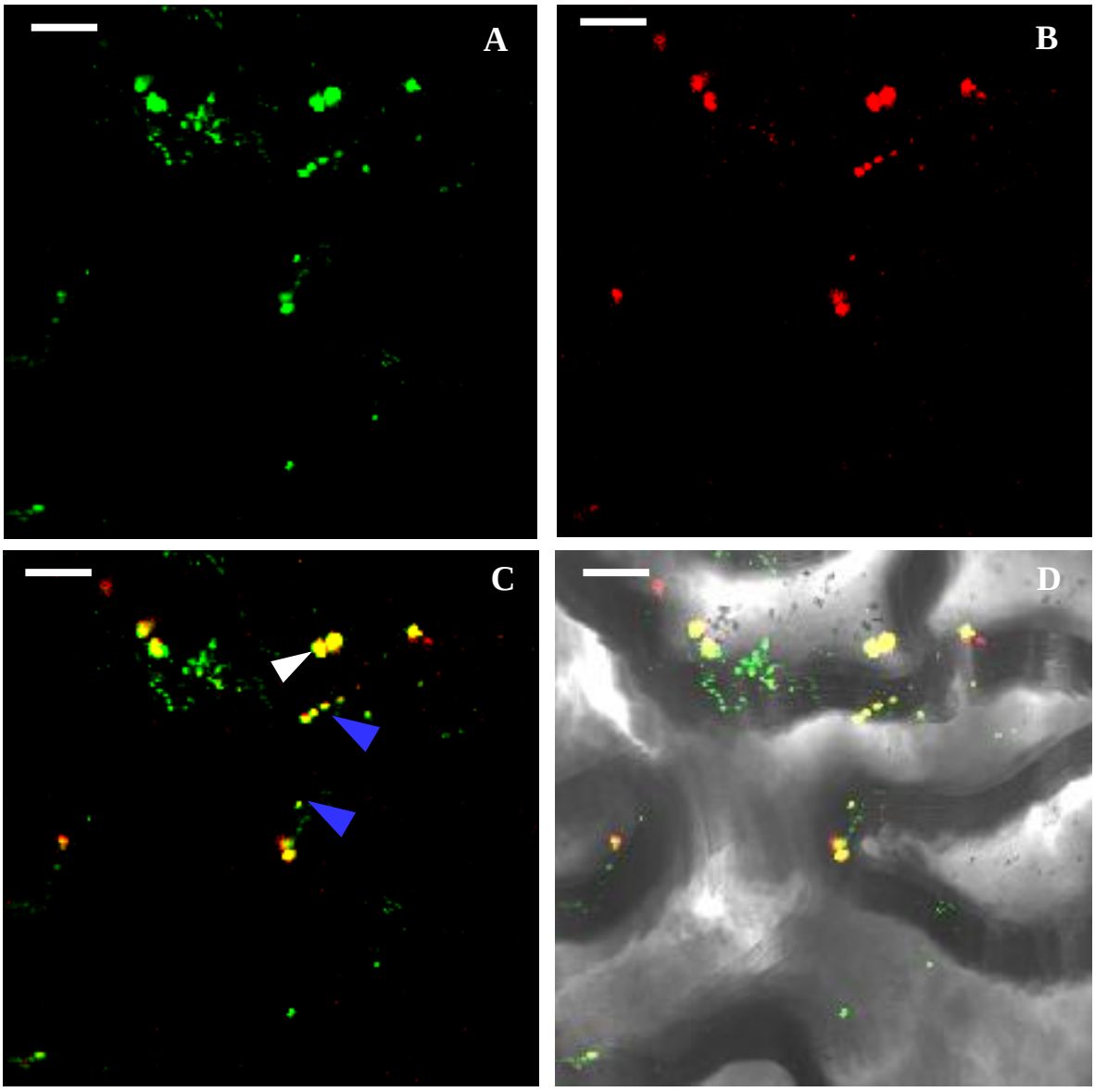


Figure 2-.11. Overlapping localization of ARF-A1c::GFP and MIN7::DsRed in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-A1c::GFP and MIN7::DsRed two days after *A. tumefaciens*-mediated transformation. MIN7::DsRed and ARF-A1c localize to mobile small (~1-5 μM) punctate structures (blue arrows) and large (~5-10 μM), mobile structures (white arrow). A. GFP signal. B. DsRed signal. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are composites of 10 scans taken in the same plane every 12 seconds. Scale bar=10 μM.

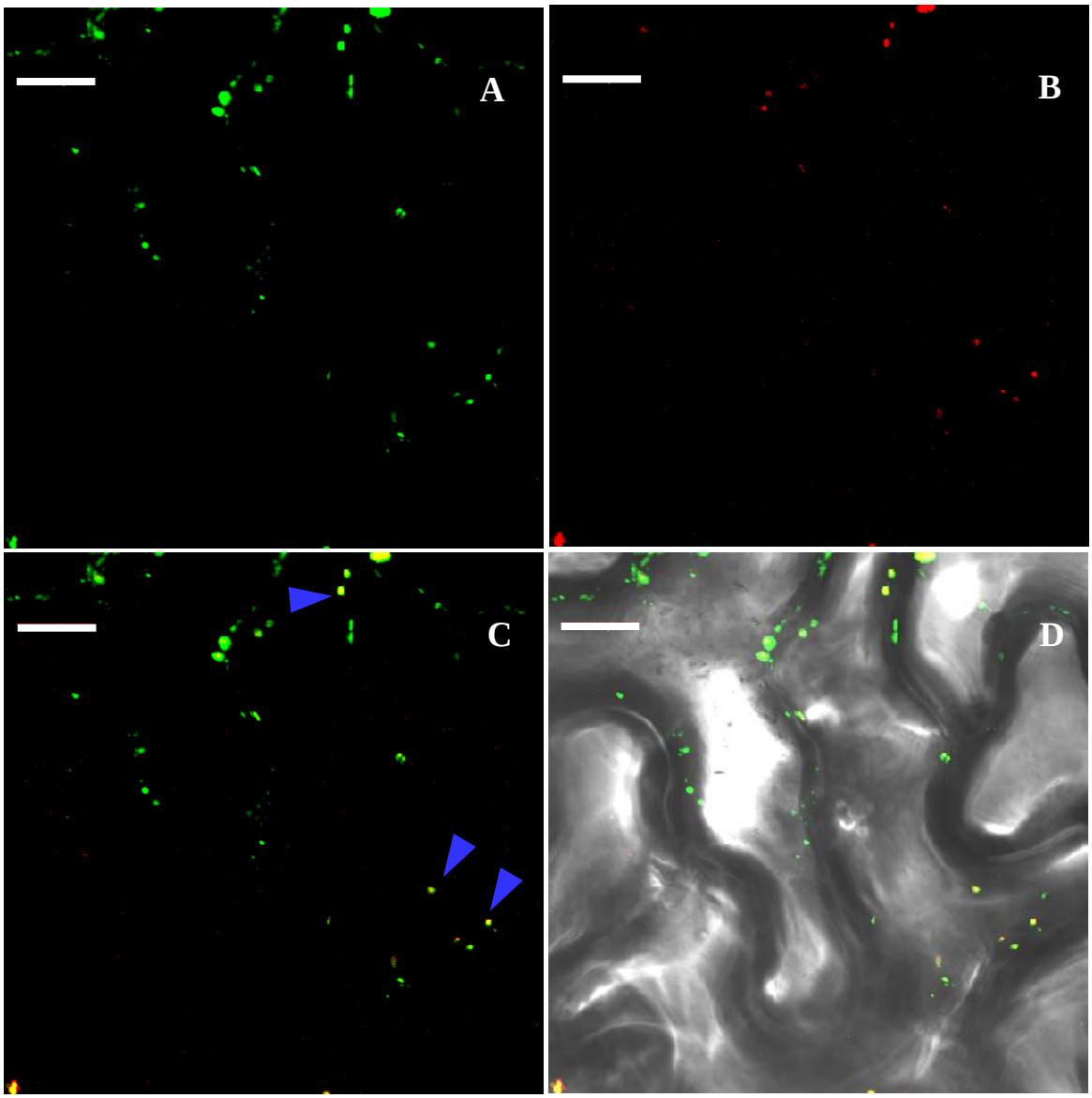


Figure 2-12. Overlapping localization of ARF-A1d::GFP and MIN7::DsRed in *N. benthemiana* leaf cells

Confocal microscopy images of *N. benthemiana* leaf cells transiently expressing ARF-A1d::GFP and MIN7::DsRed two days after *A. tumefaciens*-mediated transformation. MIN7::DsRed and ARF-A1d::GFP localize to mobile small (~1-5 μM) punctate structures (blue arrows). A. GFP signal. B. DsRed signal. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are composites of 10 scans taken in the same plane every 12 seconds. Scale bar=10 μM

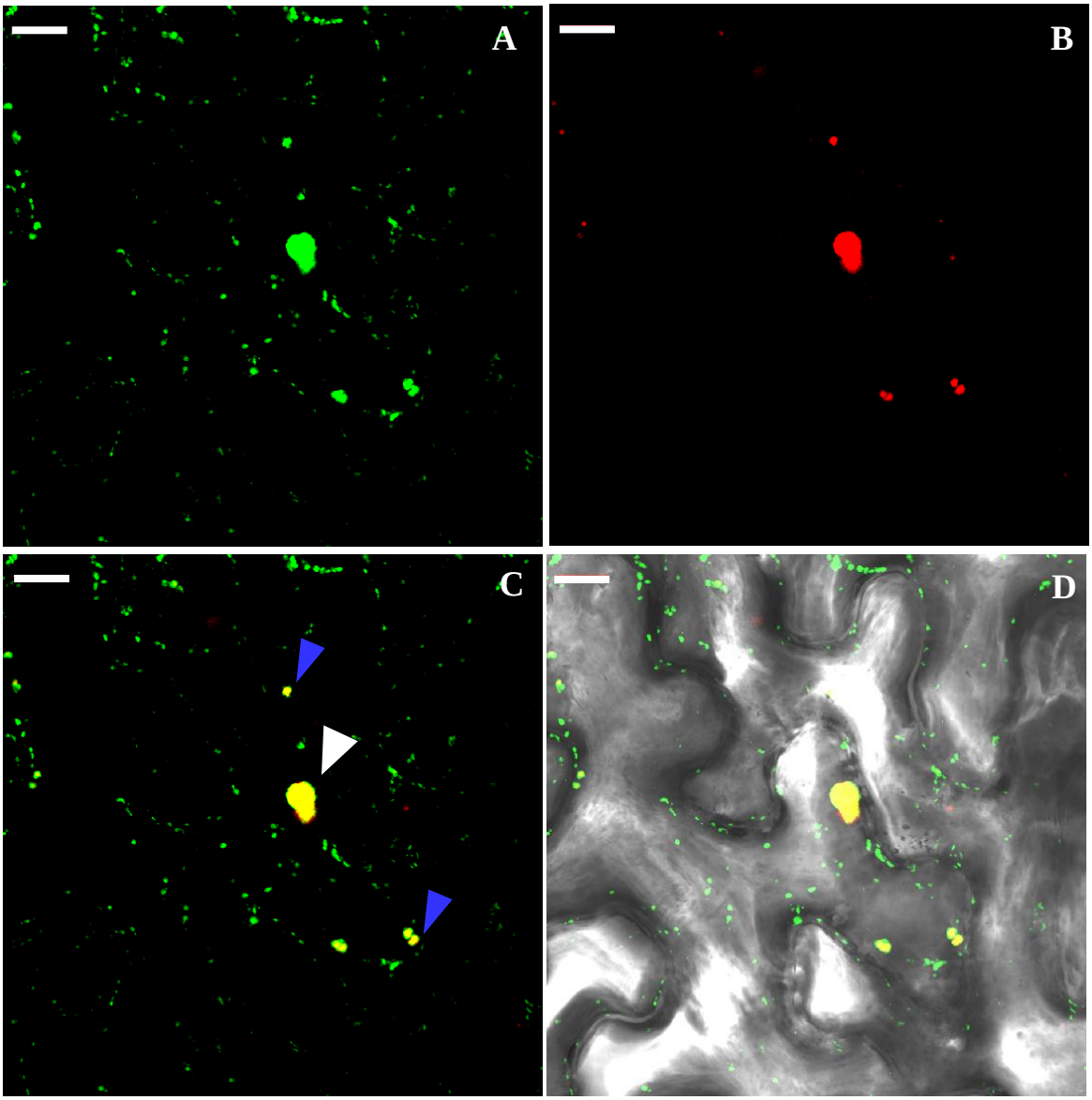


Figure 2-.13. Overlapping localization of ARF-D1a and MIN7::DsRed in *N. benthemiana* leaf cells

Confocal microscopy images of *N. Benthemiana* leaf cells transiently expressing ARF-D1a::GFP and MIN7::DsRed two days after *A. tumefaciens*-mediated transformation. MIN7::DsRed and ARF-D1a localize to mobile small (~1-5 μM) punctate structures (blue arrows) and large (~5-10 μM), mobile structures (white arrow). A. GFP signal. B. DsRed signal. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. All images are composites of scans taken along multiple planes of the Z axis. Scale bar=10 μM.

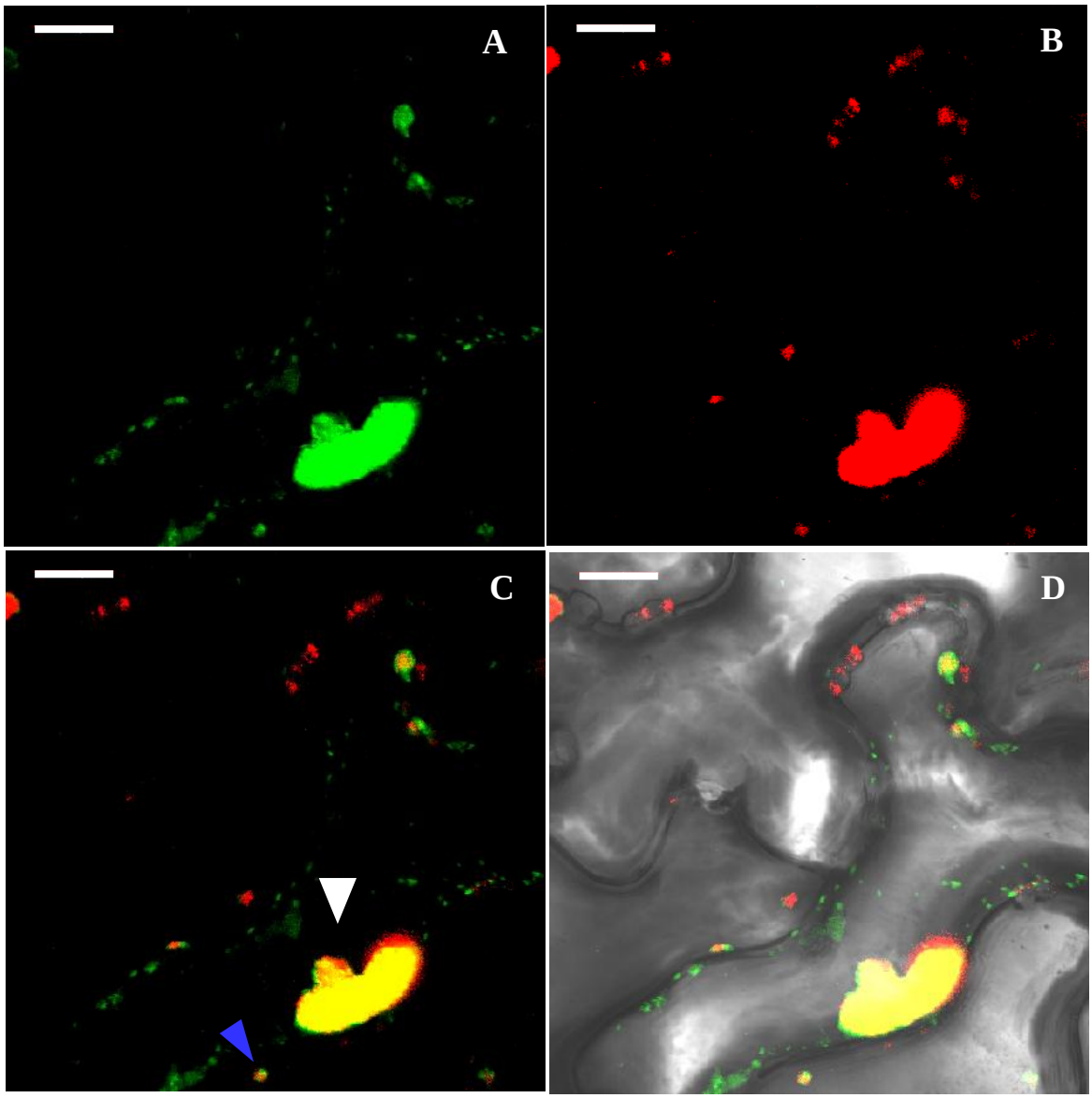


Figure 2-14. Overlapping localization of ARF-C1 and MIN7::DsRed in *N. benthemiana* leaf cells

Confocal microscopy images of *N. Benthemiana* leaf cells transiently expressing ARF-C1::GFP and MIN7::DsRed two days after *A. tumefaciens*-mediated transformation. MIN7::DsRed and ARF-C1 localize to mobile small (~1-5 μM) punctate structures (blue arrow) and large (~5-10 μM), mobile structures (white arrow). A. GFP signal. B. DsRed signal. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. All images are composites of scans taken along multiple planes of the Z axis. Scale bar=10 μM.

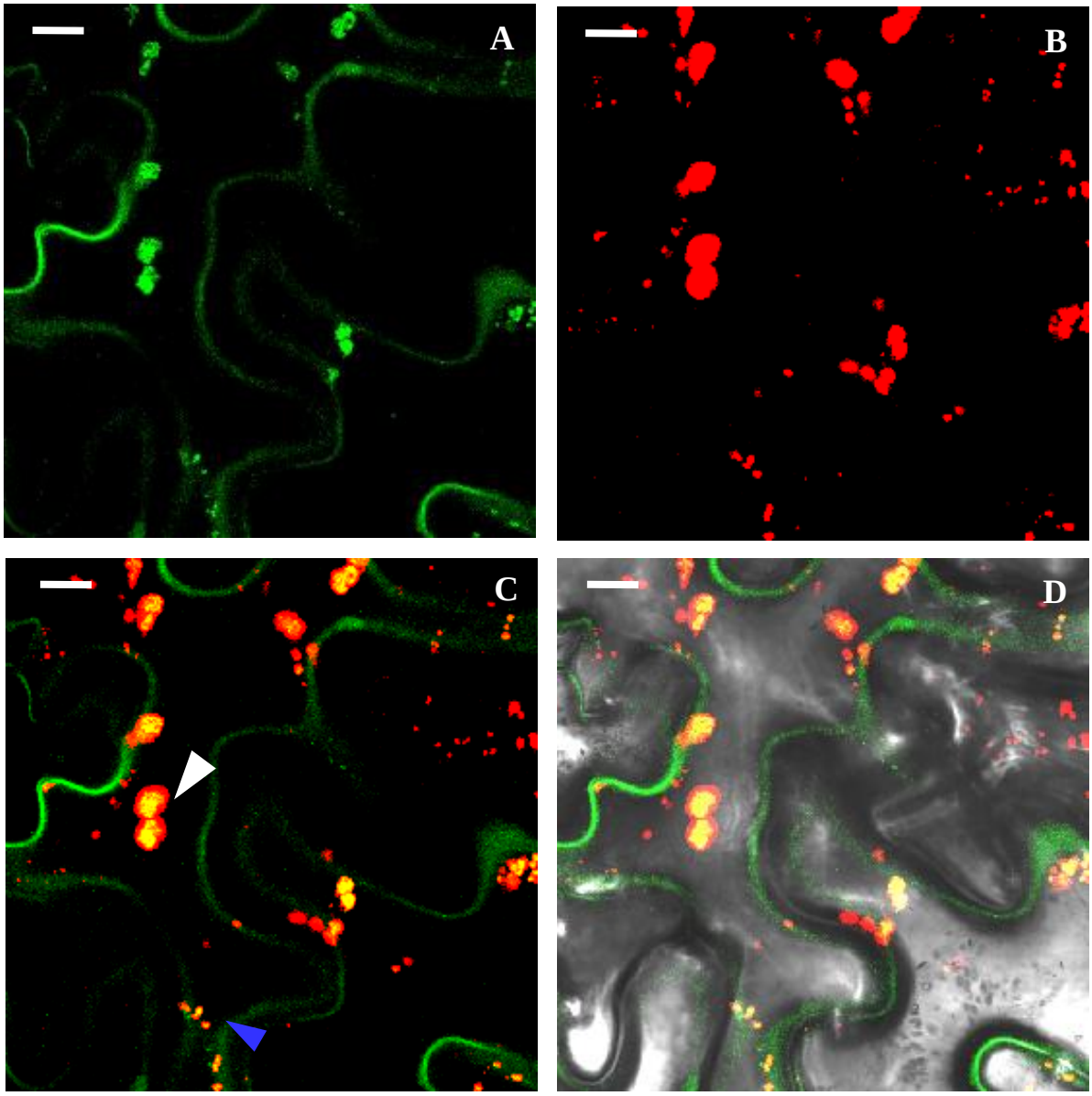


Figure 2-15. Overlapping localization of ARF-B1a and MIN7::DsRed in *N. benthemiana* leaf cells

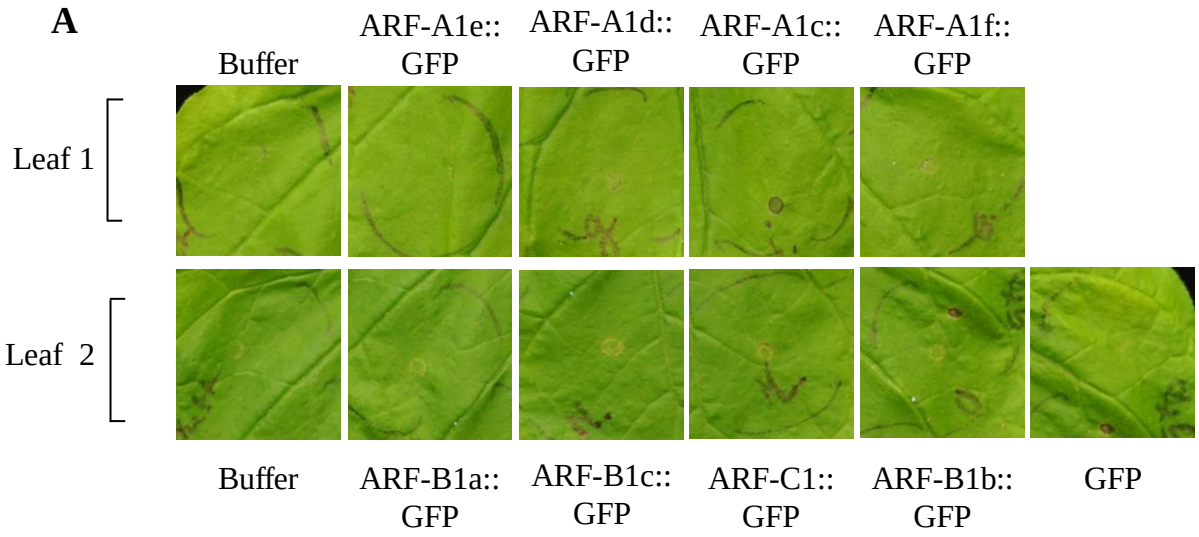
Confocal microscopy images of *N. Benthemiana* leaf cells transiently expressing ARF-B1a::GFP and MIN7::DsRed two days after *A. tumefaciens*-mediated transformation. MIN7::DsRed and ARF-B1a localize to mobile small (~1-5 µM) punctate structures (blue arrow) and large (~5-10 µM), mobile structures (white arrow) and ARF-B1a localizes to the cell periphery. Please note that co-localization was observed only in some of the intracellular punctuate structures (indicated by blue or white arrows), but not at the cell periphery. A. GFP signal. B. DsRed signal. C. Merged images of A and B. D. Merged images of A and B with bright field image in the background. Images are composites of 10 scans taken in the same plane every 12 seconds. Scale bar=10 µM.

HopM1-toxicity in tobacco expressing ARF::GFP

Previous studies have shown that HopM1 promotes cell death in Arabidopsis and tobacco leaves (DebRoy et al. 2004; Nomura et al. submitted). Because HopM1 degrades MIN7, which is expected to activate its ARF GTPase(s), I wanted to know whether overexpression of any of the ARF GTPases could reduce HopM1-induced cell death in tobacco leaves. ARF-A1e::GFP, ARF-A1d::GFP, ARF-A1c::GFP, ARF-A1f::GFP, ARF-D1a::GFP, ARF-B1a::GFP, ARF-B1c::GFP, ARF-C1::GFP, ARF-B1b::GFP or GFP were expressed individually and co-expressed in *N. benthemiana* with HopM1::GFP under a dexamethasone (DEX)-inducible promoter. Without DEX induction, cell death was observed in leaves transformed with the HopM1::GFP construct, indicating that basal-level expression of HopM1::GFP was sufficient to cause cell death in *N. benthemiana*. In contrast, expression of ARF::GFPs alone did not cause any visible symptom (Figure 2-16A). ARF::GFP expression did not reproducibly delay the onset of or reduce the severity of HopM1::GFP-induced cell death, compared to GFP or buffer controls (Figure 2-17A). Expression of ARF::GFPs was confirmed by immunoblot assay (Figure 2-16B and Figure 2-17B).

BTH hypersensitivity can be uncoupled from BTH-induced defense

BTH is an analog of the defense signaling hormone SA and is a potent inducer of BTH-induced defense (Friedrich et al. 1996). Some defense-associated vesicle traffic mutants (e.g., *bip2* and *pen1*) display hypersensitivity to high levels of BTH relative to Col-0 plants (Wang et al. 2005; Kalde et al. 2007). Interestingly, *min7* plants are also compromised in BTH-induced defense (Nomura et al. submitted). I reasoned that if BTH hypersensitivity is linked to



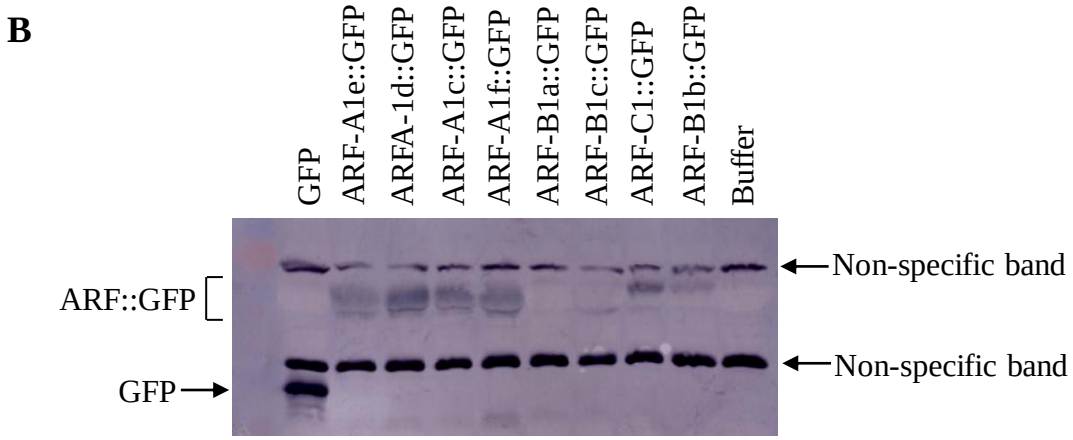
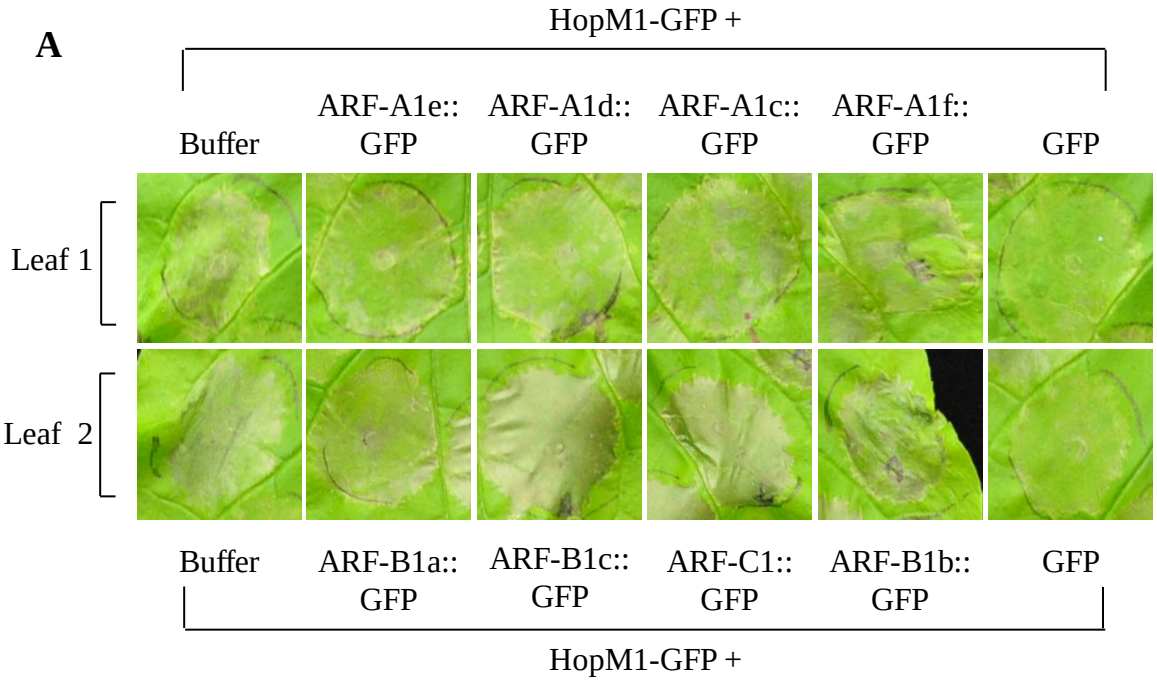


Figure 2-16. Expression of Arabidopsis ARFs does not cause tissue necrosis in *N. benthemiana* leaves

A. *N. benthemiana* leaves two days after infiltration of *Agrobacterium tumefaciens* GV3101 containing pMDC83 derivative that expresses a ARF-GFP fusion or GFP alone. Upper row and lower row represent two different leaves. B. Western analysis of total protein extracts from *N. benthemiana* leaves two days after infiltration with *A. tumefaciens* as descried for A. GFP or ARF-GFP fusions were detected with an anti-GFP antibody.



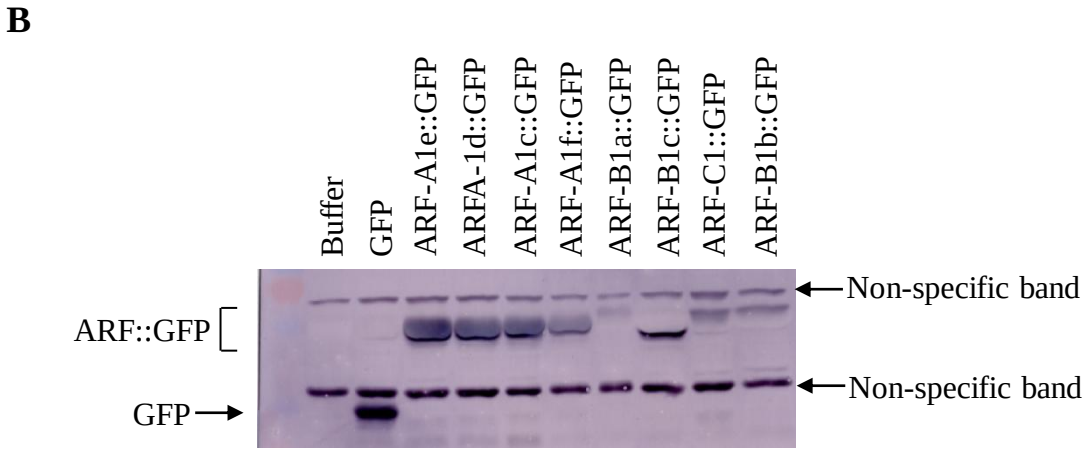


Figure 2-17. Expression of ARF::GFP does not reduce HopM1-mediated cell death in *N. benthamiana*

A. Cell death in *N. benthemiana* leaves two days after infiltration of *Agrobacterium tumefaciens* GV3101 containing pMDC83 (ARFs) or pBAR (HopM1) derivative that expresses proteins indicated. Upper row and lower row represent two different leaves. B. Western analysis of total protein extracts from *N. benthemiana* two days after infiltration with *A. tumefaciens* as described for A. GFP or ARF-GFP fusions were detected with an anti-GFP antibody.

defense-associated trafficking, screening T-DNA insertion lines for BTH hypersensitivity may indicate specific ARFs or ARLs with a role in pathogenesis.

Eighteen ARF and ARL lines with T-DNA insertions in 14 genes were found to be homozygous for insertions in *ARF* or *ARL* genes (Table 2-3). The homozygous T-DNA insertion lines were tested for hypersensitivity to BTH. Two lines with insertions in *ARL-B1* developed chlorosis and necrosis in response to treatment with 300 μM BTH (Figure 2-18). These two lines, Salk_062390 (*arlB1-1*) and Salk_120386 (*arlB1-2*), were tested for transcript accumulation of *ARL-B1* by RT-PCR. The *arlB1-2* line had no transcript (Figure 2-19A), whereas *arlB1-1* had reduced transcript accumulation, compared with that in Col-0 plants (Figure 2-19B). Next, the two lines were tested for the ability to restrict the growth of *Pst* DC3000 after induction of BTH-induced defense by treatment with 50 μM BTH (Figure 2-20). Previous work indicated that compromised BTH-induced defense and hypersensitivity to BTH were linked (Wang et al. 2005; Kalde et al. 2007). However, no difference in *Pst* DC3000 growth was seen between Col-0, *arB1-1*, and *arlB1-2* plants, suggesting that BTH hypersensitivity can be uncoupled from compromised BTH-induced defense in *arlB1* plants.

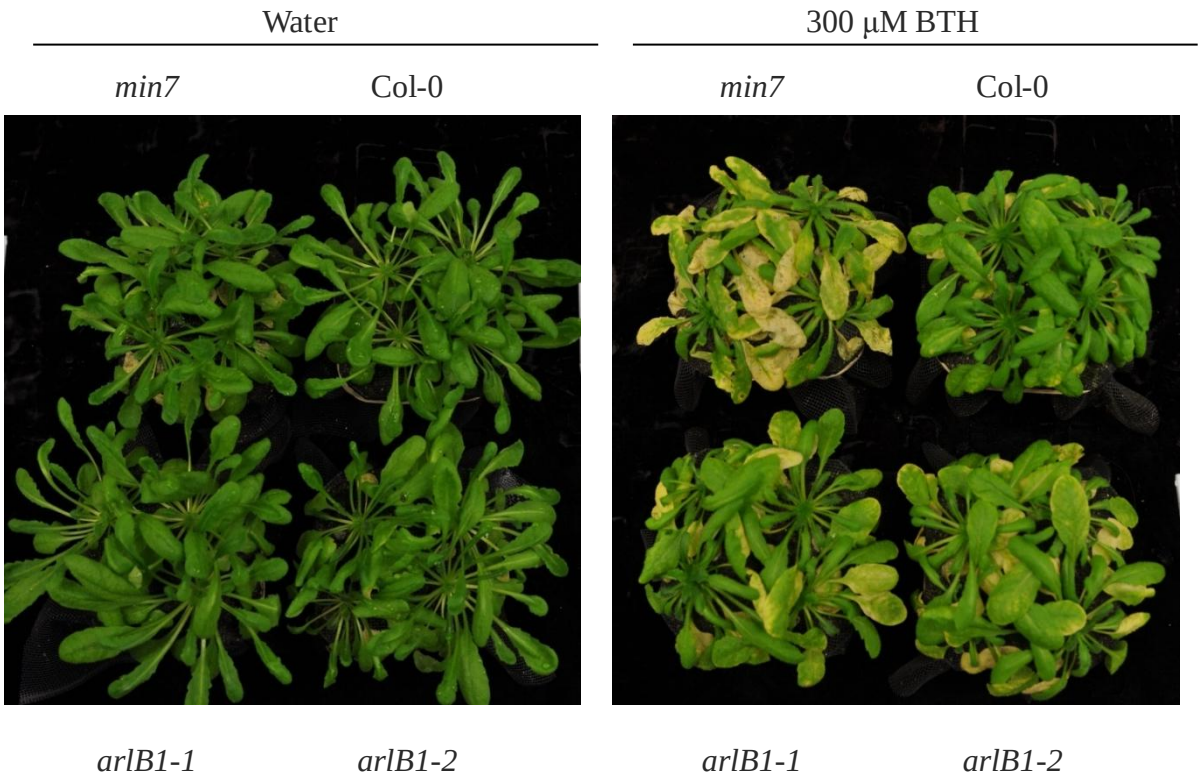


Figure 2-18. BTH hypersensitivity of *min7* and *arlb1* plants

Plants were treated with water (left) or 300 μM BTH (right) and monitored for the development of chlorosis and necrosis. The images were taken 9 days after treatment.

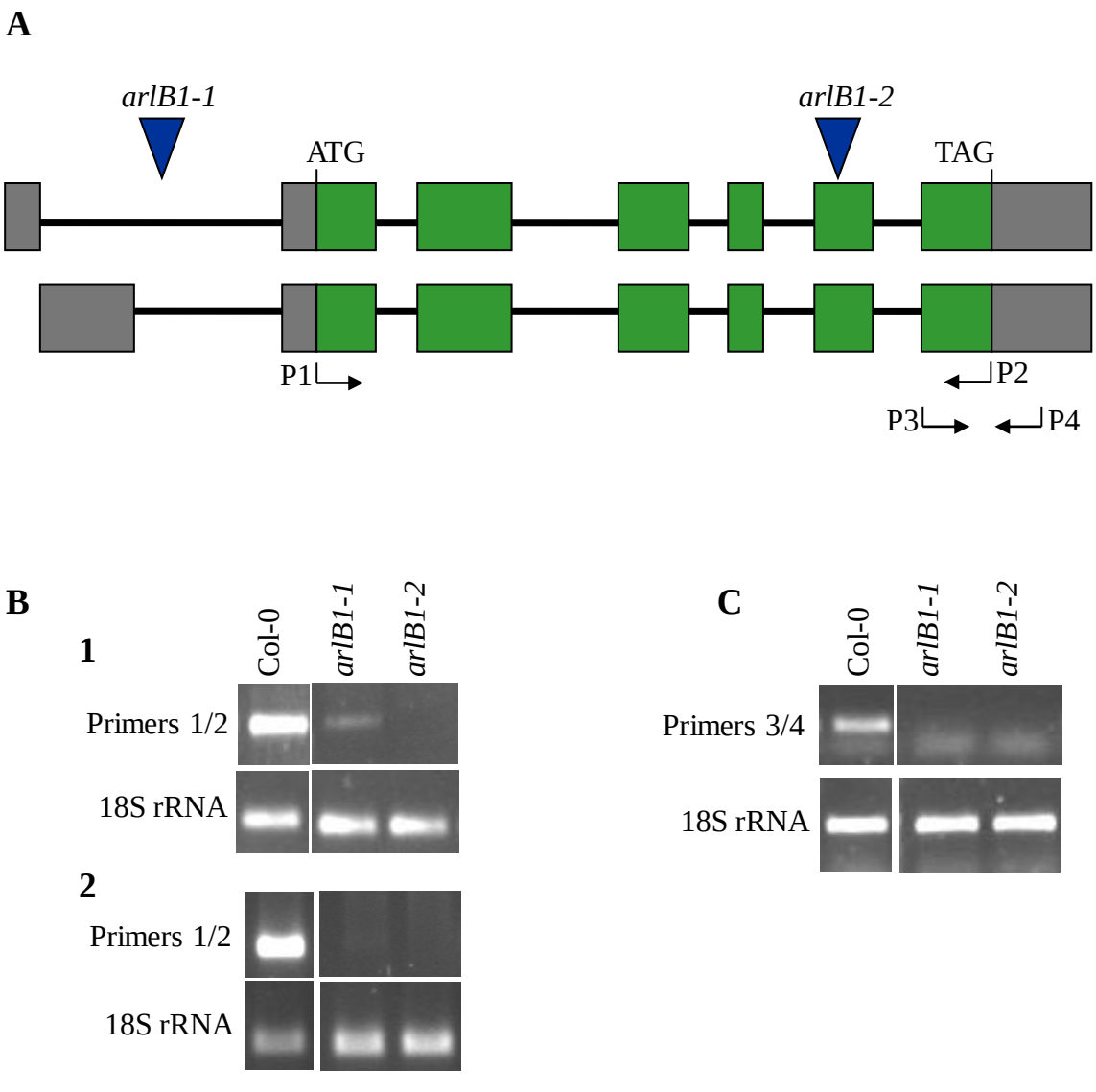


Figure 2-19. Characterization of T-DNA insertions in *arlb1* lines

A. Map of T-DNA insertions (indicated by triangles) in *ARLB1* (adapted from the Arabidopsis Information Resource). Untranslated regions (UTRs) are in gray, introns in thin lines, and exons in green. Blue arrows indicate locations of primers used in this study. Differences in the length of the upper and lower cDNA clones reflect alternative splicing in the 5'UTR. B. RT-PCR analysis of *ARLB1* expression in Col-0, *arlb1-2*, and *arlb1-1* with primers 1/2. Tissue samples from B-1 and B-2 were taken from different *arlB1-1* plants. *arlB1-1* has a lower level of transcript than Col-0, but the quantity varied. *arlB1-2* has no detectable transcript in any experiment. Primers for 18S rRNA were included as a control for quantity of RNA. C. RT-PCR analysis of *ARLB1* expression in Col-0, *arlb1-2*, and *arlb1-1* with primers 3/4. No transcript was detected in *arlB1-1* or *arlB1-2*.

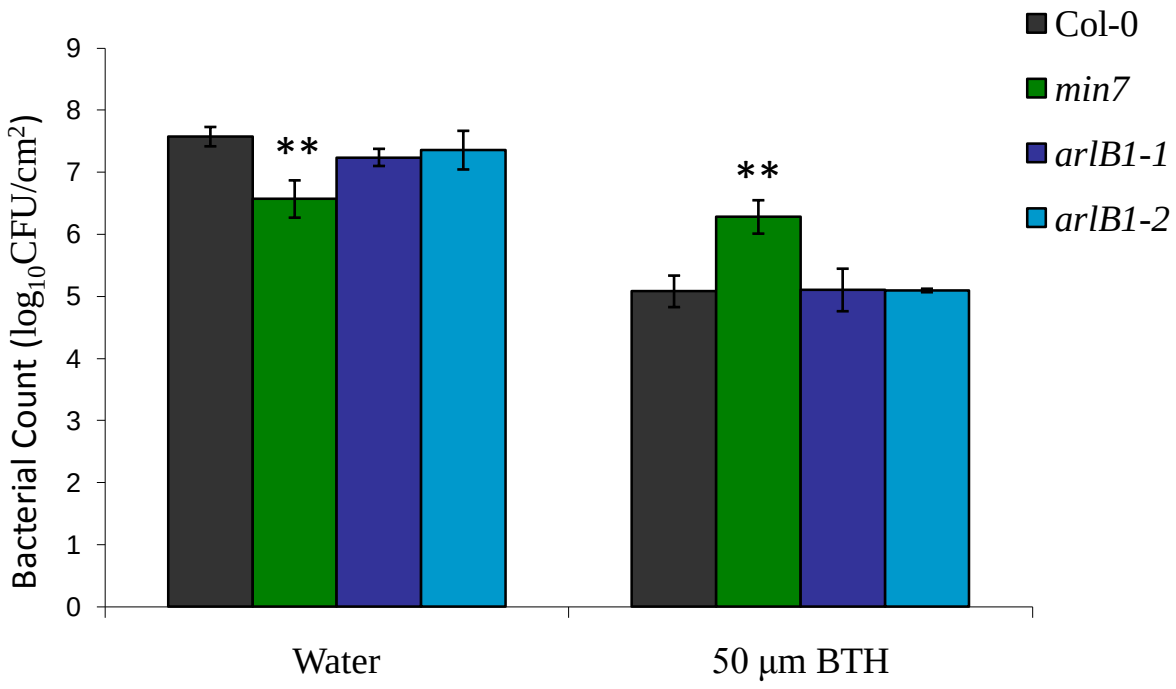


Figure 2-20 *min7* plants are compromised in BTH-induced defense and *arlB1-1* and *arlB1-2* plants are not

Plants were treated with 50 μM BTH or water 24 hours prior to inoculation with 10^6 CFU/ml *Pst* DC3000. The number of bacteria in infected leaves was determined three days after inoculation. Bars indicates standard error (n=4). ** indicate significant difference at a P value of <0.01 between Col-0 and *min7* as determined by a two-tailed t-test. There was no significant difference between *arlB1-1* or *arlB1-2* and Col-0.

DISCUSSION

The Arabidopsis protein MIN7 was predicted to be an ARF GEF based on a conserved SEC7 domain. ARF GEFs regulate the ARF family of small GTPases critical for the regulation of vesicular budding (Gillingham and Munro 2007b). ARF GEFs and ARF GTPases are found in all eukaryotes and only a small fraction of ARFs and their GEFs are well characterized. MIN7 has attracted attention recently as a regulator of PIN1 localization and as a target for the *Pst* DC3000 effector HopM1 (Tanaka et al. 2009; Nomura et al. 2006). Here I have shown that MIN7 has GEF activity on an Arabidopsis ARF GTPase, as evidenced by an increase in [^{35}S]GTPγS binding relative to controls. This is the third time an ARF GEF from Arabidopsis has been demonstrated to have this activity, and the second time this activity has been shown on an Arabidopsis ARF GTPase (Steinmann et al. 1999; Nielsen et al. 2006).

My research did not determine if MIN7 is a GEF specifically for ARF-A1c. In many cases, GEF activity has been established initially using an ARF that is not necessarily a specific substrate of the GEF in question. For example, the GEF activity of GNOM was established using human ARF1 as a substrate with a threefold increase in GTP binding observed (Steinmann et al. 1999). Typically, if a GEF is able to promote binding of GTP to an ARF GTPase by more than 30 fold over binding in the absence of the GEF, it can be considered specific for that ARF GTPase (Gillingham and Munro 2007a). The small stimulation of MIN7-SEC7 on ARF-A1c suggests that MIN7 may act on an alternative ARF GTPase in Arabidopsis. However, it is also possible that the conditions used in my GEF assay were not optimal for MIN7. For example, the MIN7-SEC7 domain I used was based on similarity to other SEC7 domains used successfully for exchange assays, but it may not be ideal for MIN7 (Macia et al. 2001; Nielsen et al. 2006; Gillingham and Munro 2007a). Greater activities may be detected with larger MIN7 fragments

or the full-length protein. However, soluble full-length MIN7 could not be expressed in *E. coli* (K. Nomura and S.Y. He, unpublished). Alternatively, MIN7 may vary from human and yeast GEFs in the reaction conditions (i.e., buffer composition, pH, etc.) for full activity.

When transiently expressed in tobacco, MIN7::DsRed is localized to small punctate structures consistent with TGN/EE localization. It also appears in larger structures that may be aggregates formed as a result of MIN7 overexpression. All of the ARF::GFPs examined localize in part to the large structure in the presence of MIN7, but the total amount of overlapping signal between MIN7 and the ARFs is low. This may be due to the fact that MIN7::DsRed expression is low and inconsistent. However, none of the ARFs were localized to large, mobile structures in the absence of MIN7. There are at least two explanations for this (F. Brandizzi, personal communication). One is that the MIN7-associated aggregates non-specifically precipitate proteins from the cytosol. Because ARFs are expected to be localized to the cytosol in the inactive state, they may be co-precipitated with MIN7 (Antonny et al. 1997). Alternatively, all of the ARF::GFPs tested may localize, at least in part, to the TGN. The two ARFs previously localized in the plant cell, ARF-A1c and ARF-B1a, are both partially localized to the TGN (Xu and Scheres 2005; Stefano et al. 2006; Matheson et al. 2007; Matheson et al. 2008)). Therefore, both the large and small punctate structures to which MIN7 and the ARFs co-localized may be TGN or derived from TGN. It is possible that the ARFs may appear at many membranes, but are only activated and deactivated at membranes where specific GEFs and GAPs are localized. It is not known how membrane specificity is determined for ARF and GEF localization nor is it known the how the relationship between ARFs and GEFs affects that localization.

HopM1 transiently expressed in *N. benthemiana* leaves triggers cell death. Because HopM1-mediated *Pst* DC3000 virulence in Arabidopsis is associated with cell death promotion

(DebRoy et al. 2004) and because HopM1 mediates the degradation of MIN7, I considered that overexpressing the ARF GTPases may counter the action of HopM1 and could reduce HopM1::GFP-triggered cell death in *N. benthamiana*. Indeed, overexpression of an ARF has been used to a rescue mutant GEF phenotype (Deitz et al. 1996). However, ARF::GFP overexpression was not sufficient to reduce HopM1::GFP toxicity in *N. benthemiana*. It is possible that the Arabidopsis ARF GTPases expressed in tobacco may not be activated by the tobacco ARF GEFs to counteract the effect of HopM1::GFP or that HopM1::GFP toxicity in plant cells could be operating through a GEF-independent pathway.

My research establishes that MIN7 is an active ARF GEF in Arabidopsis, providing further supporting evidence for the notion that MIN7 is a component of the Arabidopsis vesicle trafficking system involved in defense (Nomura et al. 2006; submitted). Previous studies have shown that Arabidopsis mutants defective in the regulators of defense-associated vesicle traffic (such as PEN1 and BIP2) display hypersensitivity to BTH. Recently, *min7* mutant plants were found to be hypersensitive to BTH and MIN7 is required for BTH-induced defense against *Pst* DC3000 (Nomura et al. submitted). Interestingly, I found that the *arlB1* mutants are also hypersensitive to BTH treatment. However, the ability to restrict *Pst* DC3000 growth after treatment with BTH remains intact in *arlB1* mutant plants. Therefore, BTH hypersensitivity may be linked to perturbations of plant trafficking systems, but is not necessarily an indicator of compromised BTH-induced defense.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kinya Nomura, who discovered that MIN7 is a target of HopM1, elucidated the *min7* phenotypes and who built the 35S::MIN7-DsRed construct. Dr. Young Nam

Lee supplied the 35S::HopM1::GFP construct. Drs. Christy Mecey and Melinda Frame provided technical assistance with confocal microscopy, and Dr. Federica Brandizzi assisted with interpretation of the microscopy images. Weining Huang cloned full-length MIN7 from Arabidopsis. I would like to thank Jackson Gehan and Weining Huang for selecting the *ARF* and *ARL* T-DNA insertion lines, and Dr. Elena Bray Speth for assistance confirming T-DNA insertions in those lines. Megha Gulati assisted with the amplification of full length ARFs and ARLs from total RNA, and Matt Oney and Robert Parker contributed the ARF-A1f::GFP clone. Dr. Allison Gillingham provided protocols for the GTP/GDP exchange assay.

Table 2-1. Primers for cloning full-length *ARF* GTPases in pENTR/d-TOPO. The CACC sequence was added to the 5' end of the inserts for directional cloning in pENTR/d-TOPO.

At Locus		Primer Sequence
At3g62290	*ARF-A1e*	5' 5'-**CACC**ATGGGTCTATCCTTCGGAAAGT-3'
		3' 5'-AGCCTTGTTTGCGATGTTG-3'
At1g70490	*ARF-A1d*	5' 5'-**CACC**ATGGGGTTGAGTTTCGCCAA-3'
		3' 5'-TGCCTTGCCAGCGATGTT-3'
At2g47170	*ARF-A1c*	5' 5'-**CACC**ATGGGGTTGTCATTCGGA-3'
		3' 5'-TGCCTTGCTTGCGATGTT-3'
At1g10630	*ARF-A1f*	5' 5'-**CACC**ATGGGGCTTTCATTTGCA-3'
		3' 5'-AGCTTTGCTAGCAATGTTGTTG-3'
At1g02440	*ARF-D1a*	5' 5'-**CACC**ATGGGGACGACTCTGGGA-3'
		3' 5'-CATTCTTTCAGCATTTTTCAACAG-3'
At3g03120	*ARF-B1c*	5' 5'-**CACC**ATGGGTCAAACTTTTCGCAA-3'
		3' 5'-AAACGAGGGACCAACTGATG-3'
At3g22950	*ARF-C1*	5' 5'-**CACC**ATGGGAGCATTCATGTCGA-3'
		3' 5'-ACTCGTGGCTTTACCGGTAA-3'
At5g17060	*ARF-B1b*	5' 5'-**CACC**ATGGGTCAAGCTTTTCGTAAGC-3'
		3' 5'-AAACGAGTGGCCAACCGAT-3'

Table 2-2. Primers used to confirm the insertion of T-DNA in Salk lines LP primers are upstream of the insertion and RP are downstream of the insertion. LBa1 primer corresponds to the sequence of the T-DNA insertion.

Salk insertion line		Primer Sequence
130670	LP	5'-ATGCCGCTAAGATTTTGAGTG-3'
	RP	5'-GAAGCAGTTGGCATCATTTTG-3'
128880	LP	5'-TCAATAAGTACTATTGCAAGTCGC-3'
	RP	5'-AAACGCATACTCAATTGCAGC -3'
136703	LP	5'-GGAAGAAACGTATAGGAATTTTTGG-3'
	RP	5'-CAAGACCTTTGTGGACCTACG -3'
013103	LP	5'-ATCACTCCTCCGACTCCTTTC-3'
	RP	5'-GATTCCTCTTCTGCTTGTTTGG-3'
107687	LP	5'-CTGGAGAGAACTCGTTGTTGG-3'
	RP	5'-CGAGAAAAAGGTGAAATCGAAG-3'
039612	LP	5'-TCCAGGCACAATACACGAAAG-3'
	RP	5'-CAAGCTCGGAGAGATTGTCAC-3'
027659	LP	5'-GCGAGATAAAACCGGTAGGAG-3'
	RP	5'-CAGTCTCCACGTTGAATCCTG-3'
090913	LP	5'-AAGAAAATAACTTTACCAATGGCG -3'
	RP	5'-GTTTGCTTTGGATGTAGGTGC-3'
027975	LP	5'-CAAATCTGATCTGGGCTTCTCTG-3'
	RP	5'-GTTCTGGTTTCCCCTTAACTCGTG-3'
137117	LP	5'-GAGGAAGCTGCTGCCCAAATG-3'
	RP	5'-CGATTGTGGGAACAGTAGACAGAAC-3'
112741	LP	5'-TTAGATCGAGAGAGGATCGGG -3'
	RP	5'-TGCAACATACTGTTTTCAACTGG-3'

Table 2-2. Continued

Salk insertion line		Primer Sequence
145860	LP	5'-TTCTGATTCATCATGCCACAG-3'
	RP	5'-GGATATACATTCGGATACTGGTTC-3'
045932	LP	5'-5'-TGACTCTGATTCTGCTTTCAGG -3'
	RP	5'-CGGAGATTGTTCAGGATTTTG -3'
08193C	LP	5'-GTTTAATTCGATGATTTGGAGTGC-3'
	RP	5'-CGCTCTGTCAGACACAGCTTC-3'
096522	LP	5'-GATTAGTTCTTCGACTTTGAATGC-3'
	RP	5'-ATCACTCCTCCGACTCCTTTC-3'
059077C	LP	5'-CAGCTCTGGTTCCTAAAATATGTTC -3'
	RP	5'-GAATTCTCCTCCACGGATCTC-3'
062390	LP	5'-CACTAGGCTAATTTTGATCTTCCTG-3'
	RP	5'-GTCTATGAATTTGGGACAGGAGTG-3'
120386	LP	5'-CCACCAAAGAGAATATGCTTG-3'
	RP	5'-GCTAGCCAAAATGATGCAAAG-3'
120433	LP	5'-TCAACTTAGAAAGAAGAACGCAG-3'
	RP	5'-AATTGCGATCAAGGAAACAAG -3'
079031C	LP	5'-TCACTTGTTCTTTCCGTCCAG-3'
	RP	5'-TCAAACCATTTTCTGATGGATTC-3'
057736	LP	5'-CGCACCTTTCAATTCATCTTC -3'
	RP	5'-GGAAGAAACGTATAGGAATTTTTGG-3'
LBa1		5'-TGGTTCACGTAGTGGGCCATCG-3

Table 2-3. T-DNA insertion lines screened for BTH hypersensitivity. Arabidopsis lines confirmed to carry a T-DNA insertion in the *ARF* or *ARL* genes indicated. Homozygous lines have the insetion in both copies of the gene, and heterozygous lines have insertions in only one copy.

At Locus	Gene name	Salk insertion line	Insertion Homozygous	Insertion Heterozygous
At3g62290	*ARF-A1e*	130670	x	
		128880	x	
At2g47170	*ARF-A1c*	136703	x	
		013103		x
At1g23490	*ARF-A1a*	107687	x	
At1g70490	*ARF-A1d*	039612		x
At5g14670	*ARF-A1b*	027659	x	
		090913	x	
At1g02440	*ARF-D1a*	019966	x	
At3g22950	*ARF-C1*	027975	x	
At3g03120	*ARF-B1c*	137117	x	
At5g17060	*ARF-B1b*	112741	x	
		145860		x
At5g67560	*ARL-A1d*	045932	x	
		08193C		x
At3g49870	*ARL-A1c*	096522	x	
		059077C	x	
At5g52210	*ARL-B1*	062390	x	
		120386	x	
At2g24765	*ARL1*	120433	x	
		079031C	x	
		057736	x	

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Chapter 3

Characterization of RabE1: An Arabidopsis Rab GTPase

Figures 3-1 and 3-2 were previously published in Plant Physiology.

Bray Speth, E., L. Imboden, P. Hauck, and S.Y. He. 2009. Subcellular Localization and Functional Analysis of the Arabidopsis GTPase RabE. *Plant Physiology*. 149:1824 -1837

ABSTRACT

The RabE1 family is one of eight Rab GTPase families in Arabidopsis and is predicted to function in polarized secretion from the Golgi apparatus to the plasma membrane. As critical regulators of vesicle trafficking, Rab GTPases are an important component of plant defense. Like other small GTPases, Rabs alternate between a GTP-bound active state and a GDP-bound inactive state. Several members of the Arabidopsis RabE1-family were previously identified as yeast two-hybrid interactors of a virulence effector, AvrPto, of the phytopathogenic bacterium *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000. I discovered that AvrPto interacts with wild type RAB-E1d and RAB-E1d-Q74L, which is predicted to be in GTP-bound active state, but not RAB-E1d-S29N, which is predicted to be in the GDP-bound inactive state. This result suggests that AvrPto selectively interacts with the active form of RabE1 GTPases. Additionally, I have used the yeast two-hybrid system to identify two Arabidopsis proteins that interact with the RabE1 GTPases. RabE1-interactor 1 (REI1) is annotated as a receptor-like kinase, and RAB-E1-interactor 2 (REI2) is predicted to be a phosphotidyl inositol transfer protein (PITP). However, *rei2* mutant plants maintained normal resistance to *Pst* DC3000 when pre-treated with flg22 (a pathogen-associated molecular pattern) prior to inoculation.

INTRODUCTION

Rab GTPases are one of several classes of Ras-like small GTPases that regulate vesicle trafficking in eukaryotic cells. There are 57 Rab GTPases in Arabidopsis and they fall into eight families, RabA through RabH (Vernoud et al. 2003). Rabs have been shown to be involved in vesicle budding, vesicle movement along the cytoskeleton to the target membrane, target-membrane determination, and vesicle tethering to the target membrane (Seabra and Coudrier, 2004; Stenmark, 2009).

Like other Ras-like GTPases, Rab GTPases have a nucleotide binding core and moving switch domains found in all classes of small GTPases. These switch domains change conformation when Rab GTPases alternate between a GTP-bound active state and a GDP-bound inactive state (Stroupe and Brunger 2000). The switch is assisted by two classes of enzymes. The guanine nucleotide exchange factors (GEFs) promote the exchange of GTP for GDP, thereby activating the Rab GTPase, and GTPase activating proteins (GAPs) promote GTP hydrolysis, inactivating the GTPase (Becker et al. 1991; Burton and De Camilli 1994; Barr and Lambright 2010). Rab GTPases also have a C-terminal hypervariable domain containing two cysteines at variable positions within the last five residues. These two cysteines are the sites of prenylation, which is important for membrane anchoring (Pfeffer 2005).

In the active state, Rab GTPases recruit functional interactors (Grosshans et al. 2006). Indeed, the identification of downstream interactors has helped elucidate the specific functions of some Rabs. For example, the role of the yeast Rab GTPase SEC4p in transport of vesicles was inferred from its interaction with a myosin motor (Wagner et al. 2002). Interestingly, despite the high similarity between Rab proteins, there is great diversity among downstream Rab interactors and no domains common to all Rab interactors can be identified via sequence analysis

(Barnekow et al. 2009). Thus, Rab GTPase interactors are generally sought with biochemical methods, such as protein pull-down and yeast two-hybrid (Y2H).

Cell trafficking components are frequent targets of human pathogen effectors. The human Rab GTPase Rab7, for example, is targeted by two different pathogens, *Helicobacter pylori* and *Mycobacterium tuberculosis* (Via et al. 1997; Terebiznik et al. 2006). Recently, trafficking components have also been implicated as targets of plant pathogen effectors (Nomura et al. 2006).

AvrPto is one of the approximately 30 effectors produced by the phytopathogen *Pseudomonas syringae* pv *tomato* (*Pst*) DC3000 (Ronald et al. 1992; Salmeron and Staskawicz 1993). *Pst* DC3000 uses a type three secretion system (T3SS) to secrete effectors into the host cell (Büttner and Bonas, 2003). It has been demonstrated that many effectors can promote susceptibility in the host, but can also be recognized by disease resistance (R) proteins in specific resistant plants (Eitas and Dangl, 2010). Bacteria secreting AvrPto trigger effector-triggered immunity (ETI) in resistant tomato plants expressing the serine-threonine kinase Pto and the NB-LRR protein Prf (Martin et al. 1993; Xiao et al. 2003). In the absence of Pto or Prf (such as in Arabidopsis), AvrPto suppresses basal defense responses associated with pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) (Hauck et al. 2003). Arabidopsis plants expressing AvrPto are able to support growth of a T3SS-deficient mutant, and genome-wide gene expression data in AvrPto-expressing plants show a bias towards suppression of genes coding for secreted proteins. Formation of the callose-rich papillae in the plant cell wall is a hallmark of plant defense, and it is suppressed by AvrPto (Hauck et al. 2003). Although callose biosynthesis occurs at the plant cell wall, the formation the papillae is dependent upon a functional trafficking system (Assaad et al. 2004). Taken together, these results suggest that

AvrPto promotes bacterial infection by down-regulating plant defenses, possibly by affecting the plant vesicle trafficking directly or indirectly.

In search for Arabidopsis targets of AvrPto using yeast two-hybrid (Y2H) screens our lab identified a small GTPase that belongs to the RabE1 family (Bray Speth et al. 2009). Previously, four tomato proteins (AP1, AP2, AP3 and AP4) were identified as interactors of AvrPto in Y2H screens and among these are two small GTPases that are similar to Arabidopsis RabE proteins and the mammalian RAB8 protein (Bogdanove and Martin 2000). The RabE1 family of GTPases in Arabidopsis includes five members, RAB-E1a through RAB-E1e. Four of the five members of the RabE1 family (RAB-E1a, -E1b, -E1d, and –E1e) interact with AvrPto (Bray Speth et al. 2009). However, no interactions were detected between AvrPto and representatives of five additional Arabidopsis Rab GTPase families, RAB-A1a, -B1b, -C1, -D2a, -F2a, and -G3a (Bray Speth et al. 2009).

Based on similarities to yeast and mammalian Rab GTPases, RabE1 GTPases are predicted to be involved in polarized trafficking between the Golgi apparatus (GA) and the plasma membrane (PM) (Vernoud et al. 2003). Expression of a dominant-negative, GDP-fixed version of RAB-E1d increases the amount of SecGFP (a modified GFP designed for secretion) that accumulates in the intracellular space (Zheng et al. 2005). Cellular localization data also support this prediction. YFP::RAB-E1d and YFP::RAB-E1c are localized to the GA in tobacco leaf cells and Arabidopsis root cells (Camacho et al. 2009) whereas GFP::RAB-E1d was localized to both the GA and the PM in leaf cells (Zheng et al. 2005; Bray Speth et al. 2009). Additionally, YFP::RAB-E1d was localized to the cell plate in dividing cells (Chow et al. 2008). To date, one interactor has been identified for the Arabidopsis RabE1 family. RabE1 proteins

interact with a phosphotidylinositol-4-phosphate 5-kinase and RAB-E1d stimulates its kinase activity *in vitro* (Camacho et al. 2009).

I further characterized the RabE1-AvrPto interaction and searched for RabE1-interacting Arabidopsis proteins with the goal of increasing our understanding of the RabE1-controlled vesicle traffic pathway in Arabidopsis.

METHODS AND MATERIALS

RNA Extraction and Reverse Transcriptase-PCR (RT-PCR)

Total RNA was extracted from Arabidopsis leaf tissue using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was then used as a template for PCR with gene specific primers (Table 3-1).

Construction of RAB-E1d mutants

Previously, amino acid mutations were generated in RAB-E1d at positions conserved among small GTPases that result in inhibition of GTP-hydrolysis (Q74L) or reduction GDP disassociation (S29N) (Bray Speth et al. 2009). GTPases with the Q74L mutation should be found predominantly in the GTP-bound form, whereas the S29N mutation should leave GTPases bound to GDP. The *RAB-E1d* coding regions carrying either of the two mutations were cloned into the Matchmaker LexA Y2H bait vector, pGilda (Clontech, Mountain View, CA). An additional set of RAB-E1d mutants was generated via site-directed mutagenesis to replace the two cysteines in the C-terminal region to prevent geranylgeranylation that is necessary for membrane localization. RAB-E1d, RAB-E1d-S29N, and RAB-E1d-Q74L inserts in pGilda were amplified with the following primer in which the two cysteines (-CCXXX) were changed to glycine and serine (-GSXXX) and cloned into pGilda: RAB-E1d-R 5'-GCCGCATCGTCTTCT ACAGCCGAGAAGTCAGCT**G**GCT**C**TAGTTACGTTTAGCTCGAG AA-3'. Codons for modified residues are in bold and the *Xho*I restriction site is underlined.

Yeast Two-Hybrid

The Matchmaker LexA Y2H system (Clontech, Mountain View, CA) was used to screen Arabidopsis cDNA libraries generated from pathogen-challenged and healthy Arabidopsis

(Courtesy of Dr. J. Jones, Sainsbury Laboratory, UK and Dr. J. Dangl, University of North Carolina, Chapel Hill, NC). Yeast competent cells expressing RAB-E1d-Q74L from the pGilda bait plasmid were prepared using the Zymo Frozen-EZ Yeast Transformation II Kit (Orange, CA) and transformed with the cDNA libraries. Transformants ($2x10^7$ colony forming units) were screened by selecting for colonies expressing both the *LEU2* (the leucine biosynthetic gene) and β-galactosidase reporters. These colonies grew in leucine-minus medium and appeared blue on plates containing the β-galactosidase substrate 5-bromo-4-chloro-3-hydroxyindole (X-gal). Positive colonies were restreaked to obtain individual blue colonies, and inserts in the pB42AD prey plasmid were amplified with the following primers: pB42AD 5' (5'–CCAGCCTCTTGCTG AGTGGAGATG–3') and pB42AD 3' (5'–CGTCAGCAGAGCTTCACCATTG–3'). Prey inserts were sequenced and identified via BLAST search of Arabidopsis gene sequences in the Genbank databases.

Confirmation of T-DNA insertion

T-DNA insertion lines (Salk_114805, Salk_047863) for At1g75370 (*REI2*) were acquired from the Arabidopsis Biological Resource Center (ABRC). For confirmation of T-DNA insertion, DNA was extracted and amplified using the Extract-N-Amp system (Sigma, St. Louis, MO). The PCR reaction included two primers from the genomic sequence of the gene flanking the insertion: 114805-3' (5'-AACACCATGATAACCATGAGGGTAG-3') or 047863-3' (5'-TCACCAGCAC ACCTTCTTTTTC -3') and 114805/047863-5' (5'-CTCGGATTCTTCTGAA GATTTCTC -3'), and one primer from the left border of the T-DNA (LBa1 primer: 5'-TGGTTCACGTAGTGGG CCATCG-3'). Homozygous T-DNA insertion plants were screened for the production of full-length *REI2* transcript in Salk_114805 and Salk_047863 lines by RT-PCR. Primers correspond to unique regions of *REI2* surrounding the insertion site: 5'-

(5’CTCGGATTCTTCTGAAGATTTC TC-3’) and 3’ (5’- CTCTCGAGTCACCAGCACACCT TCTTTTTC-3’).

Bacterial inoculation and enumeration

Arabidopsis plants were grown in potting soil in growth chambers maintained at 20°C and a 12-h day length at 100 $\mu Em^{-2}s^{-1}$. Four-to-five week old plants were infiltrated with 10^6 colony forming unites (CFU)/ml bacteria following a published procedure (Katagiri et al. 2002). Leaf samples were collected using a cork borer, ground, serially diluted, and spotted on low-salt Luria Bertani broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) containing 100 mg/L rifampicin. Bacterial CFUs were counted and calculated per square centimeter of leaf tissue. To elicit PTI, plants were infiltrated with 1 μM flg22 peptide 24 hours prior to bacterial inoculation.

RESULTS

All five members of the *RAB-E1* family are expressed in leaf tissue

RAB-E1b (At5g59840) was identified as a Y2H interactor of AvrPto, which is produced by the foliar pathogen *Pst* DC3000 (Bray Speth et al. 2009). Therefore, the expression of *RabE1* family members in leaf tissue was investigated. RT-PCR analysis confirmed the expression of *Rab-E1a*, *-E1b*, *-E1c*, *-E1d*, and *–E1e* in the leaf (Figure 3-1), and due to the high level of expression of *Rab-E1d*, it was chosen for further analysis.

AvrPto interacts with wild-type RAB-E1d and RAB-E1d-Q74L but not RAB-E1d-S29N

It is known that the GTP-bound active form, but not the GDP-bound inactive form, of Rab GTPases interacts with their downstream interactors to regulate vesicle traffic (Grosshans et al. 2006). I wanted to know whether the AvrPto interaction was specific to one particular form of RAB-E1d. Accordingly, RAB-E1d was modified by site-directed mutagenesis at specific residues that are known to generate GTP-fixed (Q74L) or GDP-fixed (S29N) GTPases (Der et al. 1986; Feig and Cooper, 1988). Also, the predicted prenylation site (-CCXXX) was changed to glycine and serine (-GSXXX). Prenylation is critical for membrane attachment of GTPases (Pfeffer, 2005) and to promote entry into the nucleus for Y2H analysis, these residues are often mutated or deleted (Brondyk and Macara 1995). Both RAB-E1d-GS (may be GTP- or GDP-bound) and RAB-E1d-Q74L-GS (predicted to be GTP-bound) interacted with the AvrPto (Figure 3-2). However, there was no interaction detected between AvrPto and RAB-E1d S29N-GS.

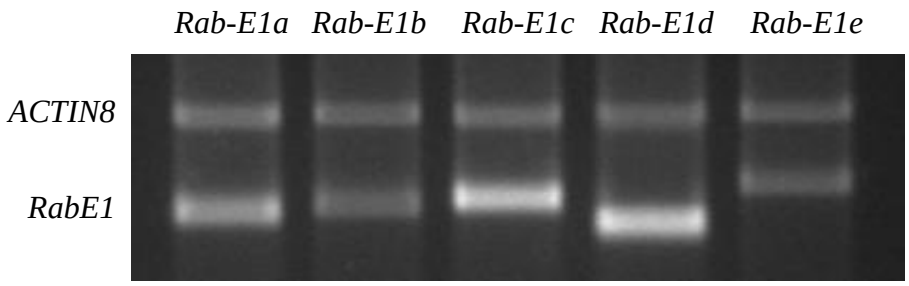

Figure 3-1. Accumulation of *RabE1* transcript in Arabidopsis leaf tissue. Each reaction included gene specific primers and primers for *ACTIN8* as an internal control.

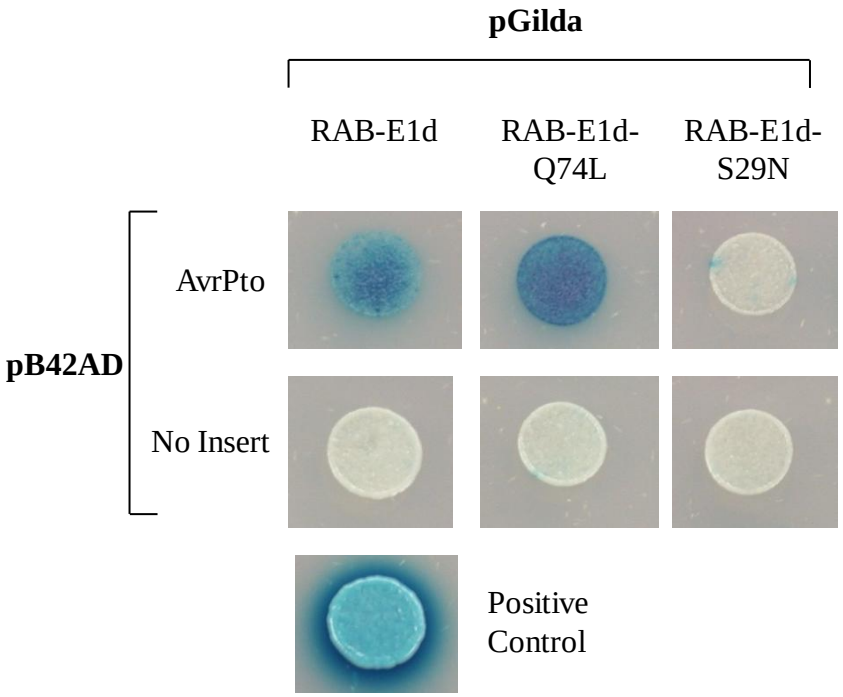

Figure 3-2. **AvrPto interacts with wild type RAB-E1d and RAB-E1d-Q74L but not RAB-E1d-S29N in the yeast two-hybrid system**.

RAB-E1d was expressed from the pGilda vector and AvrPto was expressed from pB42AD. As negative control, pGilda expressing RAB-E1d, RAB-E1-Q74L or RAB-E1d-S29N was expressed in yeast with the empty pB42AD vector. The postive control expresses pB42AD-T and pLexA53. Blue color indicates protein-protein interaction. The RABE1d proteins have C-terminal cysteine residues substituted to glycine and serine to prevent prenylation.

RAB-E1-family GTPases interact with two Arabidopsis proteins in the Y2H system

Although research on Arabidopsis Rab GTPases has expanded in recent years, many Rab GTPases are poorly characterized and only a few interactors have been identified. Interactors are desirable as they may indicate the function of a RAB GTPase (Grosshans et al. 2006). Therefore, I performed multiple screens of an Arabidopsis library with RAB-E1d, RAB-E1a and RAB-E1d Q74L-GS and generated a list of nine potential interactors (Table 3-2). The identified proteins were named RabE1 interactors (REIs).

The Arabidopsis protein REI1 (At5g38990) is predicted to be a membrane-localized receptor like kinase (RLK) (Swarbreck et al. 2007). It is one of at least 610 RLKs in Arabidopsis (Shiu and Bleecker 2001). A ~300 aa fragment from the C-terminal half of REI1 was recovered from the screen using both RAB-E1d-Q74L-GS and RAB-E1a. For further tests, a larger C-terminal cytoplasmic portion of the protein ($REI1_{464-880}$) was cloned and tested in Y2H with the wild-type, Q74L, and S29N versions of RAB-E1d and with additional RabE1 family members. $REI1_{464-880}$ interacts with RAB-E1a, -E1b, -E1e and -E1d-GS and weakly interacts with RAB-E1d-Q74L-GS (Figure 3-3). $REI1_{464-880}$ does not interact with RAB-E1d-S29N-GS.

A ~200 amino acid fragment from the C-terminal half of REI2 (At1g75370) was recovered from a Y2H screen with RAB-E1d-Q74L-GS. REI2 is annotated as a membrane-localized SEC14p-like phosphotidyl inositol transfer protein (PITP) (Swarbreck et al. 2007). The SEC14-like proteins in Arabidopsis, mammals and yeast are predicted to maintain the lipid composition of membrane compartments and are components of vesicle trafficking (Mousley et al. 2007). $REI2_{494-613}$ was cloned and interacts with RAB-E1a, -E1b, -E1e, -E1d-GS and

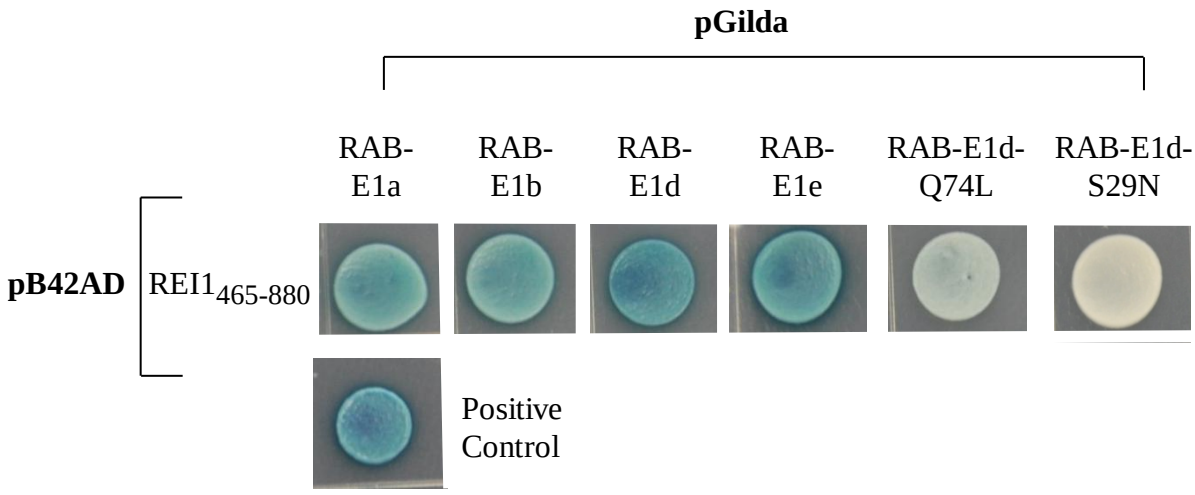

Figure 3-3. $REI1_{465-880}$ interacts with wild type RAB-E1a, -E1b, -E1d and –E1e in the yeast two-hybrid system.

$REI1_{465-880}$ has a weak interaction with RAB-E1d-Q74L and does not interact the RAB-E1d-S29N. RAB-E1d was expressed from the pGilda vector and $REI1_{465-880}$ was expressed from pB42AD. The postive control expresses pB42AD-T and pLexA53. Blue color indicates protein-protein interaction. The RABE1d proteins have two C-terminal cysteine residues substituted to glycine and serine to prevent prenylation.

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RAB-E1d-Q74L-GS (Figure 3-4). $REI2_{494-613}$ does not interact with RAB-E1d-S29N-GS. This is an interaction pattern typical of authentic small GTPase interactors (Grosshans et al. 2006). Due to its predicted function in vesicle trafficking, REI2 was analyzed further for evidence of a functional interaction with RabE1.

rei2 plants are not altered in their response to *Pst* DC3000 infection

T-DNA insertion lines were acquired for *REI2* (At1g75370). Genomic PCR revealed that lines Salk_114805 and Salk_047863 were homozygous for the T-DNA insertion and RT-PCR detected no full-length mRNA (Figure 3-5a). The T-DNA insertion lines displayed normal growth and development and did not display reduced size, a phenotype of plants with suppressed expression of three of the *RabE1* family genes (Bray Speth et al. 2009).

The *rei2* plants displayed normal symptom development and bacterial growth following inoculation of *Pst* DC3000, compared to WT Col-0 plant (Figure 3-5b). The peptide flg22 derived from bacterial flagellin activates PTI-associated defense responses, including trafficking-dependent defense responses such as formation of papillae in the plant cell wall (Felix et al. 1999). REI2 is an interactor of the RabE1 family, which are regulators of vesicular trafficking. To determine whether the flg22-mediated PTI may be compromised in *rei2* plants, I pre-treated Col-0 and *rei2* plants with flg22 and examined PTI in these plants. However, flg22 pre-treatment resulted in similarly lower levels of *Pst* DC3000 on 2 days post inoculation in Col-0, *rei2-1,* and *rei2-2* plants, indicating that PTI is not compromised in the *rei2* mutant plants (Figure 3-5b).

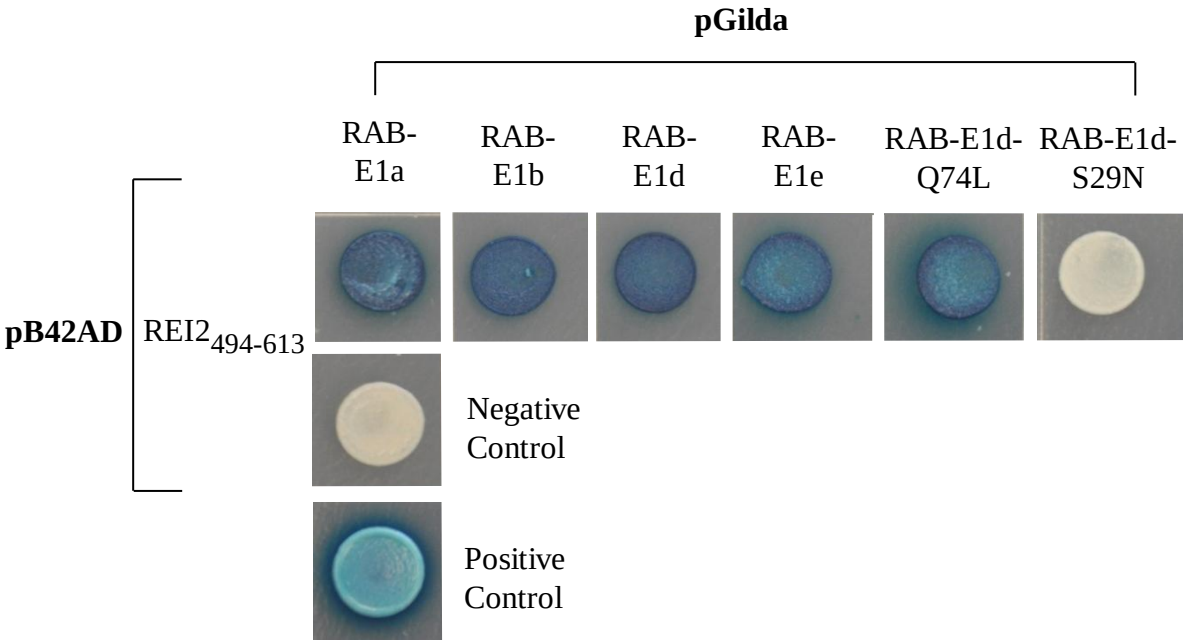

Figure 3-4. **REI2 $_{494-613}$ interacts with wild type RAB-E1a, -E1b, -E1d, –E1e and RAB-E1d-Q74L but not RAB-E1d-S29N in the yeast two-hybrid system**.

RabE1 proteins are expressed from the pGilda vector and REI2 $_{494-613}$ was expressed from pB42AD. As negative control, pB42AD:: REI2 $_{494-613}$ expressed in yeast with the empty pGilda vector. The postive control expresses pB42AD-T and pLexA53. Blue color indicates protein-protein interaction. The RAB-E1d proteins have two C-terminal cysteine residues substituted to glycine and serine to prevent prenylation.

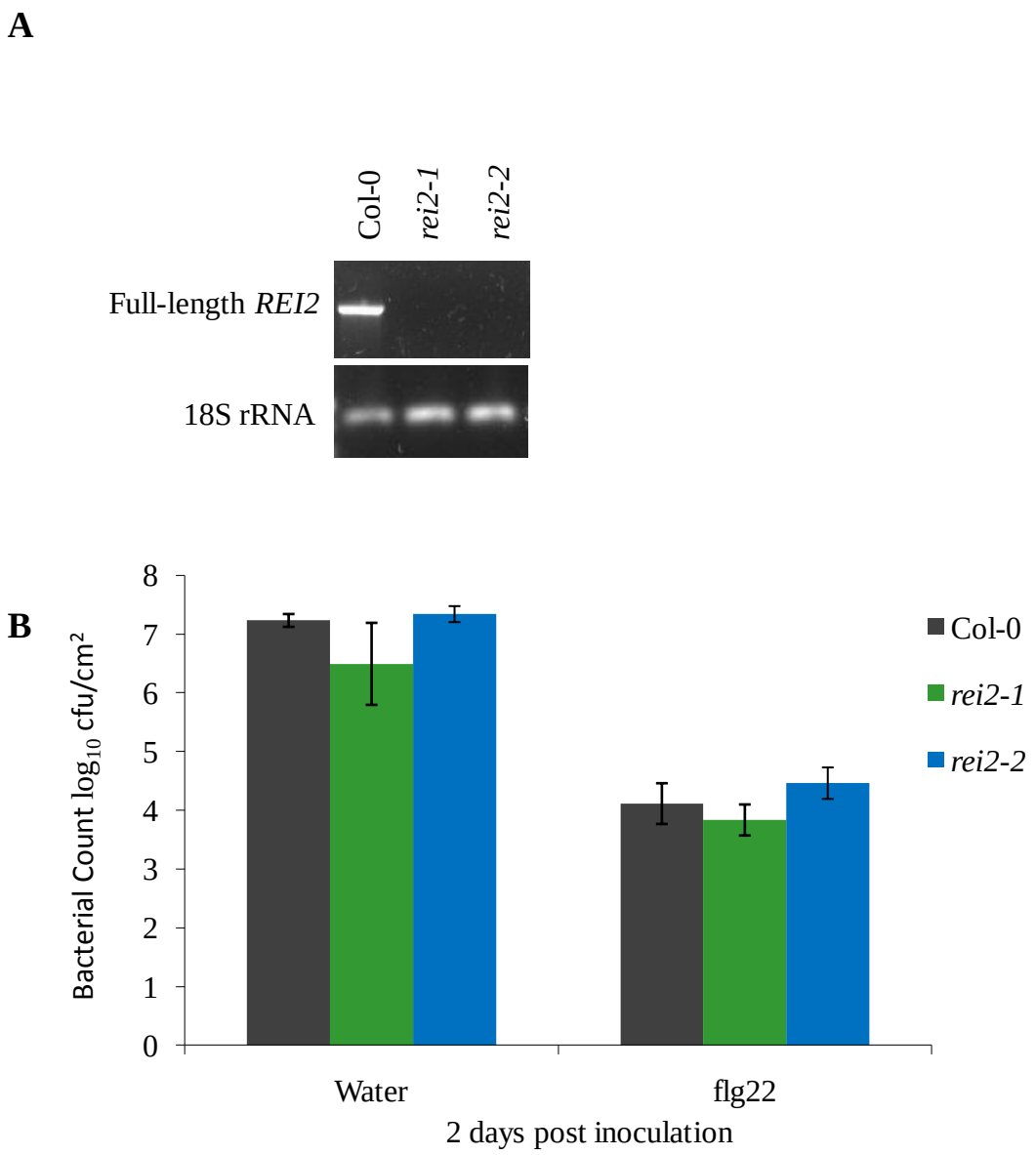

Figure 3-5. flg22-triggered resistance to *Pst* DC3000 in *rei2* plants

A. RT-PCR analysis of the *REI2* transcript in Col-0 leaves. There is no full length *REI2* transcript in T-DNA insertion lines Salk_114805 *(rei2-1*) and Salk_047863 (*rei2-2*). RT-PCR product of 18S rRNA from the same RNA samples is shown as a loading control. B. Col-0 plant were treated with flg22 or water 24 hours prior to inoculation with 10^6 CFU/ml *Pst* DC3000. The number of bacteria in infected leaves was determined two days after inoculation. Bars indicate standard errors (n=4). There was no significant difference (P value >0.05) between *rei2-1* or *rei2-2* and Col-0 for each treatment at day 2.

DISCUSSION

Although quite well characterized in yeast and mammals, the role of Rab GTPases in plants has only recently begun to be elucidated (Zheng et al. 2005; Camacho et al. 2009; Bray Speth et al. 2009; Kwon et al. 2010). At the time this project was initiated, there were no published data on RabE1 GTPases and evidence was growing for the role of secretion in resistance to plant pathogenes. As a predicted regulator of polarized secretion, RabE1 was an attractive target for further study.

The RabE1 family, but no other Arabidopsis Rab GTPases tested, interacted with AvrPto, indicating specificity for this family of GTPases (Bray Speth et al. 2009). Additionally, tomato Rab8, an orthologue of the RabE1 family, was also identified as a Y2H interactor of AvrPto (Bogdanove and Martin, 2000). I found that AvrPto interacts with wild-type and the GTP-bound forms of RAB-E1d, but not the GDP-bound form. Rab GTPases are frequently targets of mammalian pathogens, resulting in both activation and inactivation of the GTPases (Barbieri et al. 2002). The specific interaction of AvrPto with the active form of Arabidopsis RabE1 proteins suggests that the Rab family of GTPases may be common targets of mammalian and plant pathogens. However, further experiments are needed to critically assess the role of RabE1 GTPases in *Pst* DC3000 pathogenesis.

At present the other components of RabE1-regulated vesicle trafficking are poorly understood. Therefore, I sought to identify the potential interactors of RAB-E1. I utilized the Y2H system to identify such interactors and discovered two Y2H interactors, REI1 and REI2. Based on sequence similarity to known proteins, REI1 is predicted to be a receptor-like kinase and REI2 is predicted to be a member of the SEC14p-like superfamily. Both are predicted to be membrane-localized proteins, which is where active-state RabE1 GTPases are located.

Unfortunately, both *REI1* and *REI2* are from multi-gene families and, like the *RabE1* family, individual gene knock-outs may be compensated for by closely related members. REI1 shares 77% amino acid identity with the closest receptor-like kinase family protein (At5g39000) and REI2 shares 71% amino acid identity to similar another SEC14p-like phosphotidylinositol transfer family protein (At1g19650). I identified two T-DNA insertion lines lacking full-length *REI2* transcript. *rei2* plants responded to *Pst* DC3000 and flg22 activation of PTI in a manner similar to Col-0, indicating normal immune response.

Because I was unable to observe any mutant phenotypes in *rei2* mutant plants, this project was suspended. I chose instead to focus my effort on investigating the potential substrates the Arf GEF MIN7, an Arabidopsis protein degraded in the presence of the *Pst* DC3000 effector HopM1 (Chapter 2).

ACKNOWLEDGEMENTS

I gratefully acknowledge Drs. Paula Hauck and Elena Bray Speth for initiating this project. Dr. Hauck provided the AvrPto yeast two-hybrid construct. Dr. Speth designed the primers for *RabE1* family RT-PCR. Dr. Sheng Yang He generated the RAB-E1d-Q74L and -S29N mutants. Andy Scollon performed the RT-PCR for *arlb1-1* and *arb1-2* T-DNA insertion lines. I would like to thank Drs. Jonathan Jones and Jeff Dangl for providing the yeast two-hybrid cDNA libraries.

Table 3-1. Primers for RT-PCR of RabE1 GTPases from Arabidopsis leaf RNA.

Gene (At Locus)	Primer Sequence
RAB-E1a (At3g53610)	F: 5'-CCGACGATCTATCTTCCCCGAGTAG-3' R: 5'-GACAGGCGTCGTGGACCC-3’
RAB-E1b (At5g59840)	F: 5'-CCAACAAGGTCTCTTCTCTTCTC-3' R: 5'-CAACTTTGGAGCCTTTTGGGAC-3'
*RAB-E1*c (At3g46060)	F: 5'-GTCGTCCGCCATAACCTTC-3' R: 5'-CACTTCACCCCCAAACTTTTTTCG-3'
RAB-E1d (At5g03520)	F: 5'-GTTTCTGACGATGGCGGTTGC-3' R: 5'-CAGCAAGCTGACTTCTCGGCTG-3'
RAB-E1e (At3g09900)	F: 5'-GGCTGTCTCCGGCGAGAAG-3', R: 5'-CATAGGACGATCCCTTGAATGATGC-3
*ACT8 (*At1g49240)	F: 5'-GCTTCATCGGCCGTTGCATTTC-3' R: 5'-GATCCCGTCATGGAAACGATGTCTC-3'

Table 3-2. Arabidopsis proteins recovered from yeast two-hybrid screens with RabE1 GTPases. The last column indicates the number of times cDNA was recovered in the Y2H screen.

At Locus	Annotated Function	
At5g38990 (*REI1*)	Receptor-like protein kinase	2
At1G75370 (*REI2*)	SEC14 domain containing protein; Phosphoinositol transfer protein	1
At1g67950	RNA recognition motif-containing protein	3
At4g17720	RNA recognition motif-containing protein	1
At5g16840	RNA recognition motif-containing protein	1
At3g11730	RabD1	1
At2g30860	Glutathione-S-transferase	1
At3g26650	Glyceraldehyde 3-phosphate dehydrogenase	1
At1g78080	AP2 domain-containing transcription factor (RAP2.4)	1

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Chapter 4

Conclusions and Future Directions

Plant pathogens have long been infecting plants, and in the course of co-evolution with plants, pathogens have developed a suite of virulence factors to subvert the host cellular systems (Lewis et al. 2009). Beyond their importance in understanding plant-pathogen interactions, these pathogen molecules can be used to illuminate the fundamental workings of the plant cell. One set of pathogen virulence factors, the type three secretion system effectors (T3SE), is secreted into the cells of the host plant and has been shown to compromise plant defense systems. My interest was directed to the plant vesicle trafficking system by the work on two effectors of the plant pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000): HopM1 and AvrPto. HopM1 mediates the degradation of Arabidopsis protein MIN7 which has a SEC7 domain and was predicted to be an ADP-ribosylation factor (ARF) guanine nucleotide exchange factor (GEF) (Nomura et al. 2006). The RabE1 family of Arabidopsis GTPases interacts with the effector AvrPto (Bray Speth et al. 2009), and RAB GTPases regulate several steps in vesicle trafficking (Stenmark, 2009).

I was able to demonstrate that, in the presence of GST::MIN7$_{556\text{-}772}$, the region predicted to contain the SEC7 domain, the Arabidopsis ARF GTPase Δ17ARF-A1c binds [^{35}S]GTPγS at least three-fold more than in the presence of buffer or GST alone. So far, this activity has been demonstrated for only two other Arabidopsis ARF GEFs, GNOM and BIG2 (Steinmann et al. 1999; Nielsen et al. 2006; Anders et al. 2008). I observed that MIN7::DsRed co-localizes, at least partially, with five Arabidopsis ARFs from the four different ARF families in *N. benthamiana* leaf cells. My data show that BTH hypersensitivity, as seen in *min7, pen1* and *bip2* plants (Wang et al. 2005; Kalde et al. 2007), can be disassociated from defects in BTH-induced defense in *min7* plants. Additionally, I identified two yeast two-hybrid interactors for the RAB-E1 GTPases of Arabidopsis, and showed that in yeast AvrPto preferentially interacts

with wild type RAB-E1d and RAB-E1d-Q74L, which are predicted to be the active, effector binding form. In contrast, AvrPto does not interact with RAB-E1d-S29N, which is predicted to be in the inactive state (Bray Speth et al. 2009).

The next step towards understanding the function of MIN7 in plant growth, development and pathogenesis would be to determine whether other Arabidopsis ARF GTPases are substrates of MIN7 in the *in vitro* GTP/GDP exchange assay. I observed a relatively low GEF activity (threefold increase in GTPγS binding by Δ17ARF-A1c) of GST::MIN7-SEC7$_{556-772}$. Testing additional ARFs would clarify whether the relatively low binding is due to assay conditions or whether ARF-A1c is not a preferred target of MIN7. Of the 17 *ARF*s and *ARL*s in Arabidopsis, ten *ARF*s and seven *ARL*s have expression levels detectable by RT-PCR in leaf tissue, the site of *Pst* DC3000 infection. I have cloned the remaining nine *ARF*s and seven *ARL*s in pET42a. All clones produced soluble proteins in *E. coli* (data not shown). If none of the remaining ARFs or ARLs has a higher level of GTPγS-binding activity than ARF-A1c in the presence of MIN7-SEC7$_{556-772}$, the portion of MIN7 used for the assay may need to be modified (e.g., including other domains).

I reasoned that *in vivo* co-localization studies of MIN7 and ARFs/ARLs could provide clues to which ARFs/ARLs would be *in vivo* substrates of MIN7. Previous studies have shown that MIN7 is localized in the trans-Golgi network/early endosome (TGN/EE) and two Arabidopsis ARFs, ARF-A1c and ARF-B1a, are localized to the TGN (Pimpl et al. 2000; Xu and Scheres 2005; Stefano et al. 2006; Matheson et al. 2007; Matheson et al. 2008). My research now shows that five Arabidopsis ARFs from the four ARF families are partially co-localized with MIN7. Due to the apparently indiscriminate co-localization of MIN7::DsRed with all of the ARF::GFPs tested, simple co-localization of ARFs and MIN7 may not be informative regarding

which ARFs are *in vivo* targets of MIN7. An alternative approach would be to determine whether the subcellular localization of MIN7 and the ARFs respond to an external stimulus. When Arabidopsis leaves are infected by *Pst* DC3000(AvrRpt2), MIN7-DsRed is found to accumulate in discrete foci at the cell periphery (Christy Mecey, unpublished). Indentifying ARFs that also localize to these foci in plants infiltrated with *Pst* DC3000(AvrRpt2) may indicate a functional relationship with MIN7. Additionally, because only the GTP-bound form of ARFs is localized in the membrane, whereas GDP-bound ARFs are located in the cytosol (Antonny et al. 1997) the *in vivo* ARF substrates of MIN7 are expected to have a larger, cytosolic pool in *min7* plants than in Col-0 plants. This possibility can be tested by comparing the subcellular locations of ARF::GFPs in Col-0 and *min7* plants.

Another method for identifying the *in vivo* ARF/ARL substrates of ARF GEFs would be to perform a pull-down assay (Cohen et al. 2007). I performed preliminary pull-down experiments to determine the viability of the method. However, I was unable to optimize the conditions due to time constraints. ARF GTPases and GEFs are expected to interact in a transient fashion, and therefore, formaldehyde was used to cross-link proteins before tissue homogenization. I was able to pull-down ARF::6xhistidine::GFPs transiently expressed in *N. benthemiana* leaves using a Ni-NTA affinity resin (data not shown). Unfortunately, MIN7 protein appeared in the resin eluates of all samples in which MIN7 constructs were infiltrated even in those that lacked any ARF-GFP expression. MIN7 or a tobacco protein to which it is cross-linked may non-specifically bind the resin. Optimization of pull-down conditions may include performing the assay by co-immunoprecipitation with anti-GFP antibodies or using Ni-NTA resin with homogenate from tissue treated with brefeldin A (BFA) to lock MIN7 and substrate ARFs in an abortive complex (Peyroche et al. 1996).

Because MIN7 is a host target of the *Pst* DC3000 effector HopM1, any ARF that is the substrate of MIN7 identified by the methods listed above should be tested for its role in pathogenesis. I have identified 18 homozygous T-DNA insertion lines for 13 *ARF* and *ARL* genes. However, whether these lines lack the corresponding *ARF/ARL* transcripts remains to be determined. Once confirmed knock-out lines should be tested for the phenotypes observed in *min7* plants, including a defect in BTH-induced defense, the ability to support growth of ΔCEL bacteria, and altered PIN1 localization (Tanaka et al. 2009; Nomura et al. submitted). Identification of the ARF substrates of MIN7 would represent a significant advance in our understanding of the elusive MIN7-regulated vesicle traffic and its role in plant growth, development, and pathogenesis.

The two RabE1 interactors identified by yeast two hybrid have not been confirmed as interactors *in planta*. There are several approaches that can be taken, including *in vivo* protein pull-down and microscopic methods, such as bimolecular fluorescence complementation (BiFC) or fluorescence resonance energy transfer (FRET). Further analysis of *rei1* and *rei2* mutant plants should be pursued with double or higher-order mutant plants. Such mutants should be monitored for growth and development phenotypes characteristic of *rabE1* co-suppressed plants or for the accumulation of secretion signal-tagged GFP in the cytosol, which was observed in Arabidopsis expressing a dominant-negative mutant of RAB-E1d (Zheng et al. 2005; Bray Speth et al. 2009). In regards to pathogenesis phenotypes, *rei1* and *rei2* mutant plants could be tested for a possible defect in BTH-induced defense responses, which have been seen in the *min7* plants, or for delayed development of papillae (Assaad et al. 2004; Nomura et al. 2006; submitted). To date, few Rab GTPase interactors have been identified in plants. Identification of a novel

interactor with functional association with RabE1 would contribute to a better understanding of the function of the RabE1 family in the plant cell.

Much of the vesicle trafficking system in plants remains poorly understood relative to those of human and yeast cells. However, there is still much to learn in all three systems. I have provided evidence that MIN7 is capable of acting as an ARF GEF *in vitro,* and in the process, I have established protocols and developed the materials needed to identify a substrate ARF for MIN7. Likewise, I have observed that MIN7 may partially co-localize with multiple Arabidopsis ARF GTPases, and the GFP-labeled ARF and ARLs are available for further localization studies. Two potential RabE1 interactors have been identified, which contributes to our current knowledge of Rab GTPase interactors in plants.

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