THE ROLE OF ARABIDOPSIS ACTIN DEPOLYMERIZING FACTOR 4 IN IMMUNE SIGNALING AND GENE EXPRESSION

By

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A DISSERTATION

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

Cell and Molecular Biology - Doctor of Philosophy

2014

ABSTRACT

THE ROLE OF ARABIDOPSIS ACTIN DEPOLYMERIZING FACTOR 4 IN IMMUNE SIGNALING AND GENE EXPRESSION

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Arabidopsis thaliana actin-depolymerizing factor 4 (AtADF4) is a member of the over 75 characterized actin binding proteins (ABPs) including the 11 ADFs in Arabidopsis. As an ADF, AtADF4 has been shown to possess many of the biochemical and cellular functions associated with its role in the modification and regulation of the actin cytoskeleton. The collective works of numerous studies over the past few decades have demonstrated that ADFs from both plants and animals bind, sever, and/or depolymerize the aging pointed ends of filamentous actin. In the present study, I demonstrated that AtADF4 also contributes to a cellular function that has not previously been shown. In brief, the current body of work shows that AtADF4 specifically is required for the resistance of Arabidopsis when infected with *Pseudomonas syringae* pv. *tomato* expressing the bacterial effector AvrPphB, a cysteine protease known to target components of the immune-signaling responsible for recognition of non-self. While it is apparent that AtADF4 is required for resistance to *Pst* AvrPphB, the exact mechanism by which loss of AtADF4 (*adf4*) results in enhanced susceptibility remains largely unknown. Plant immunity is often achieved through recognition of bacterial effectors by a cognate resistance gene (R-gene). The R-gene of Arabidopsis that confers resistance to *Pst* AvrPphB is *resistance to Pseudomonas syringae-5* (*RPS5*). Analysis of the expression of known R-genes of Arabidopsis in *adf4* revealed a significant reduction in the expression of *RPS5* while expression of other R-genes was not affected. Mitogen-

activated protein kinase (MAPK) activation was examined in *adf4* for the ability to recognize non-self through the pattern recognition receptor flagellin-sensitive 2 (FLS2) in the presence of AvrPphB. It was found that MAPK activation was reduced specifically in the *adf4*, while MAPK-signaling was not affected in the wild-type Col-0 or the *rps5* mutant. The reduction of MAPK activation in *adf4* but not *rps5* suggests that in addition to regulating the expression of *RPS5*, AtADF4 also plays a role in FLS2-MAPK signaling in the presence of AvrPphB. The loss of resistance to *Pst* AvrPphB could be alleviated in *adf4* when complemented with the serine-6 phosphorylation mimic AtADF4^{S6D}. Although phosphorylation of serine-6 of plant ADFs is often associated with a reduced affinity for the actin cytoskeleton and is considered the inactive form of ADF, phosphorylation of serine-6 is in fact required for the immune-related functions of AtADF4. Establishment of the correlation of AtADF4 phosphorylation at serine-6 and resistance led to the examination of AtADF4 for additional unique biochemical features that may be related to immunity. Comparison of AtADF4 with its closest homologue AtADF1 revealed potential motifs of AtADF4 that may contribute to immune signaling, including phosphorylation of an additional residue, tyrosine-53. Interestingly, other recent examples of ADFs being required for immunity support these predictions. Taken together, the data presented herein identify the components of the immune response to which AtADF4 is associated, including the regulation of gene expression and recognition of non-self. These results provide a foundation for further defining the biochemical properties of AtADF4's role(s) in immune signaling.

I would like to dedicate this dissertation to my husband, Joe and my son, Xavier.
You two have given me the motivation to be my best.

ACKNOWLEDGEMENTS

I would like to thank Brad Day for all of the help and encouragement throughout my entire PhD career. When I joined the lab I knew nothing about the physiology of plants, as was evident when I asked about “soil containment units”, but now I feel confident in considering myself a plant molecular biologist. Aside from teaching me scientific principles, Brad patiently guided me through the process of scientific writing, for which I am grateful. I would also like to thank all of the Day lab members, past and present, for dealing with my crazy, it takes a special group of people to handle me when I’m off on a wild tangent. I am happy to take a part of all of the Day lab members with me in my future endeavors. I would specifically like to thank Dr. Miaoying Tian for her initial work on the AtADF4 project.

I would also like to thank my committee members; Dr. Sheng Yang He, Dr. Beronda Montgomery, Dr. Federica Brandizzi, and Dr. Karen Friderici, for helping keep me on track and hearing out my ideas and making great suggestions for my research.

I want to thank the National Science Foundation (IOS-1021044), for supporting the actin project and allowing for collaborations with Dr. Jeff Chang and Dr. Chris Staiger.

I want to thank my parents for continuously supporting me to get my PhD and even though they may not have understood what I was talking about when I called to tell them about a result or miss-step taking the time to listen and encourage me.

I also want to thank my husband, who with his amazing family kept me grounded throughout this entire process. I could not have imagined that when I started my PhD I would get married, get a dog and have a child along the way. Thank you for not letting me forget about life, and showing me how to love life as much as I love science.

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KEY TO ABBREVIATIONS

AtADF4	*Arabidopsis thaliana* Actin depolymerizing factor 4
AvrPphB	*Pseudomonas syringae* bacterial effector
RPS5	Resistance to *Pseudomonas syringae*-5
PAMP	Pathogen associated molecular pattern
PTI	(PAMP)-triggered immunity
ETI	Effector triggered immunity
FLS2	Flagellin sensitive-2
FRK1	Flg22-induced receptor like kinase 1

CHAPTER 1

Assembly, regulation, and pathogen targeting of the plant actin cytoskeleton

Alex Corrion; Michigan State University Graduate program in Plant Pathology; contributed the image of the actin cytoskeleton used in Figure 1.3.

Abstract

Actin is required for numerous eukaryotic processes, inducing development, movement, gene expression, signal transduction, and response to stress. In recent years, studies in plants and animals have identified and characterized the role of the actin cytoskeleton in each of these processes, demonstrating both the requirement of, and potential for actin as a key component of cellular signaling. Collectively, these studies have demonstrated that the activity and organization of the actin cytoskeleton underpins the function of numerous cellular processes, providing further strong support for the hypothesis that the actin cytoskeleton functions as a key cellular hub. In recent years, advances in genomics and cell biology have enabled the elucidation of the mechanisms that drive the dynamic changes in host cytoskeletal architecture. For example, quantitative cell biology-based approaches of living cells during development, pathogen infection, and cell movement have not only helped define the critical cellular processes that are required for signaling, but have enabled the discovery of environmental (biotic and abiotic) stimuli that influence host cytoskeletal dynamics. In this chapter I will highlight key advances that have enabled a better understanding of the regulation and activity of the eukaryotic actin cytoskeleton, focusing on the role of actin as a signaling and surveillance platform.

Introduction

The eukaryotic actin cytoskeleton is a dynamic network whose activity is governed by spatial and organizational changes in monomeric globular (G)- and filamentous (F)-actin (Day *et al.*, 2011). As a tightly regulated component of cell architecture and signaling – with more than 200 actin binding proteins (ABPs) described in mammals, and nearly 75 in plants – actin has been demonstrated to be required for the activity and function of a diverse suite of cellular processes. In brief, these can include cell elongation and division (Barrero *et al.*, 2002), polarity and movement (Blanchoin *et al.*, 2014), endocytosis and vesicle trafficking (Robertson *et al.*, 2009; Johnson *et al.*, 2012; Mooren *et al.*, 2012), gene expression (Percipalle, 2013), and immunity (Tian *et al.*, 2009; Day *et al.*, 2011; Porter *et al.*, 2012; Henty-Ridilla *et al.*, 2014). Underpinning each of these processes is the expression, regulation, and activity of the ABP superfamily, collectively required for a wide variety of actin remodeling processes, including nucleation, polymerization and elongation, cross-linking and branching, and depolymerization (Winder & Ayscough, 2005; Uribe & Jay, 2009; Figure 1.1).

Given its ubiquity, stochastic behavior, and functional association with numerous cellular processes, the actin cytoskeleton can be viewed as the ideal surveillance platform. In this review, we focus on the assembly, regulation, and activity of key cellular processes in plants whose functions rely on the dynamism of the eukaryotic actin cytoskeleton. Additionally, as a means to describe the regulation of these processes in response to external and developmental stimuli, we will highlight a growing body of

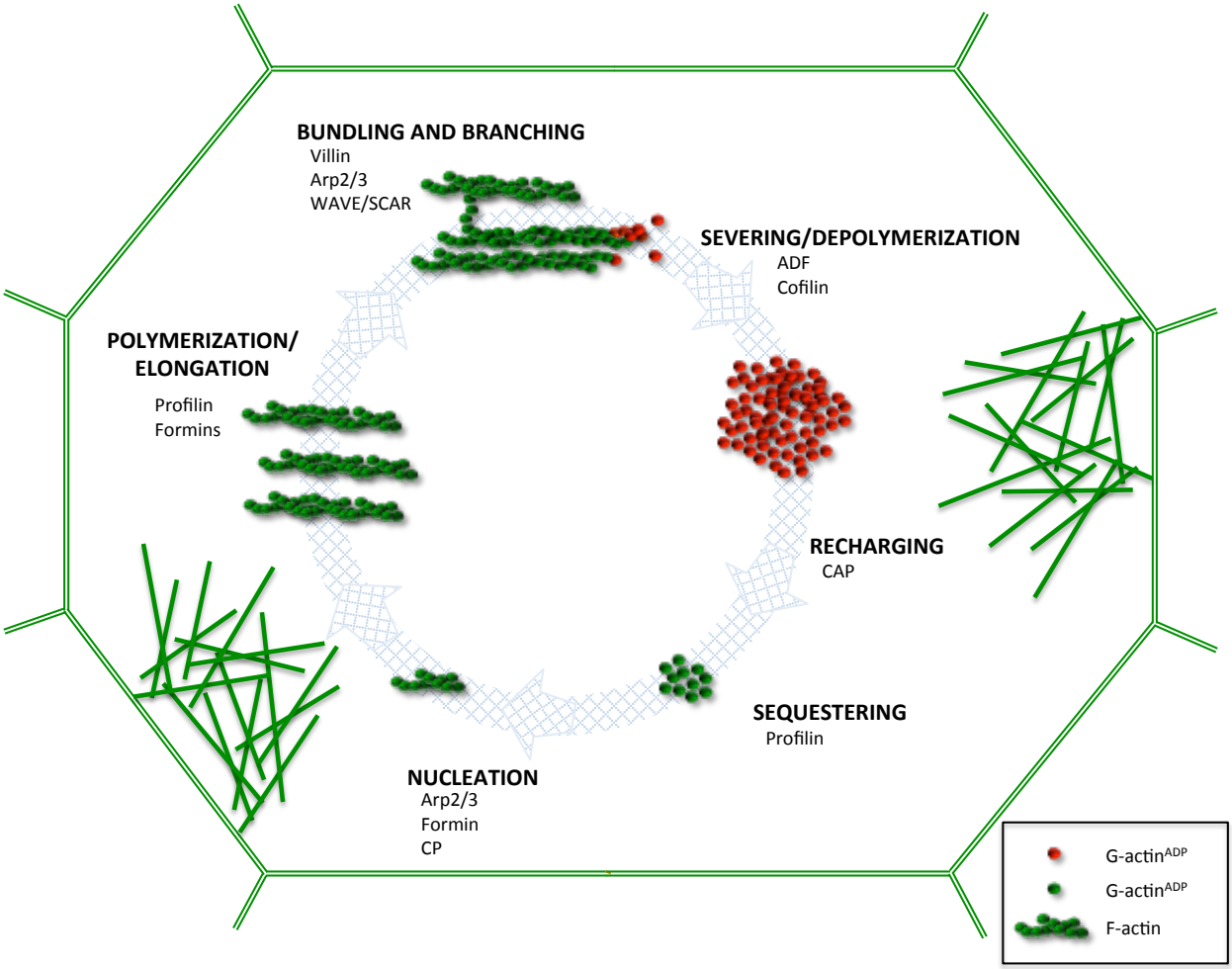


Figure 1.1. Schematic of actin remodeling in the plant cell. Illustration of the basic actin cycling that occurs in the plant cell including some of the ~75 actin binding proteins. Free globular (G-) actin is initially sequestered by profilin in order to both prevent spontaneous nucleation and elongation, and to incorporate G-actin into filamentous F-actin in a controlled manner. Nucleation of G-actin is aided by actin nucleators including: Arp2/3, formins, and capping proteins (CPs). Elongation of F-actin occurs at the barbed end, and is achieved through the actions of both formins and profilin. F-actin can then be bundled and/or branched through the accessory proteins: vilins and Arp2/3. The aging pointed end of F-actin is then severed or depolymerized by ADFs allowing for recharging of ADP to ATP by cyclase associated protein (CAP) for eventual re-incorporation into growing F-actin.

literature that has collectively demonstrated active targeting of the actin cytoskeleton by pathogens in plants. It is the ultimate aim of this review to illustrate that through multiple points of converging function and regulation, actin is an important component of

immunity, defined by both its role as a signaling platform, as well as a key target of immune subversion by pathogens.

Assembly and regulation of the actin cytoskeleton

The primary building block of the eukaryotic actin cytoskeleton is G-actin, a 42 kDa ATP-binding protein capable of undergoing spontaneous self-assembly – a process by which G-actin is added to the barbed ends of existing F-actin filaments (Day *et al*., 2011; Figure 1.1). In Arabidopsis there are 10 actin genes, eight of which are expressed (Meagher *et* al., 1999; Kandasamy *et* al., 2002). The actin genes can be divided into two classes, vegetative and reproductive, and ectopic expression of reproductive actin variants within vegetative tissues can lead to morphological defects in plant development (Kandasamy *et* al., 2002). Actin filament assembly begins with the formation of a homo-/hetero-trimer complex, a multi-step process referred to as actin nucleation. Energetically, this is the most expensive step in F-actin formation, and is influenced by a multitude of factors, including 1) the availability of filament ends, 2) the size of the G-actin pool, 3) the nucleotide-loaded state of the G-actin monomers, and 4) the spatial and temporal expression of ABPs. In both plants and mammals, each of these four steps have been extensively characterized (Day *et al*., 2011; Mullins & Hansen, 2013; Lee & Dominguez, 2010; Hussey *et al*., 2006), and in short, have been shown to be regulated by the activity of a multi-protein complex referred to as the actin-related 2/3 (Arp2/3) complex (Campellone & Welch, 2010; Mathur *et al*., 2003).

Additional actin nucleators have also been identified, and include formin (Chesarone *et al.*, 2010), capping protein (CP; Huang *et al.*, 2003), and gelsolin (Silacci *et al.*, 2004).

Once nucleation is initiated, trimeric-actin seeds F-actin maturation through a process known as elongation, a process that requires the addition of ^{ATP}G-actin to the barbed plus end of either newly nucleated actin-trimer or to a preformed severed F-actin strand (Day *et al*., 2011; Figure 1.1). As the filament matures, ATP hydrolysis, coupled to the activity of actin depolymerizing factor (ADF) proteins, drives the depolymerization of the filament at the pointed ^{ADP}F-actin end. This processes – referred to as "treadmilling" – results in the directional remodeling of actin through the precise control of both balance and direction of F-actin formation. In plants, G-actin availability is regulated by three ABPs: profilin (PRF), an adenylate cyclase-associated protein (CAP), and ADF (Bugyi & Carlier, 2010; Figure 1.1). As illustrated, PRF, whose activity is responsible not only for the prevention of spontaneous nucleation, but also the addition of ^{ATP}G-actin to the barbed end of F-actin, drives F-actin formation. It is noteworthy that in plants, PRF only binds ^{ATP}G-actin, while the process of nucleotide exchange (i.e., recharging the of ^{ADP}G-actin monomers) from the pointed ends of F-actin, is performed by CAP (Barrero *et al.*, 2002). In mammalian systems, however, PRF performs both G-actin sequestration and nucleotide exchange (Porta & Borgstahl, 2012). Further illustrating the complexity and tight regulation of this process, as well as the explosive rates of expansion of the cytoskeletal network, filament elongation occurs from both nucleated actin, as well as from available preformed actin filaments, which again can be generated through the severing activities of multiple additional ABPs, including villin/gelsolin (Ono, 2007).

Once nucleated, F-actin exists within the cell in one of two forms: the first, a fine and highly dynamic singular filament structure, and the second, a thick bundle of multiple filaments arranged in a stable yet stochastic state (Thomas, 2012). It is hypothesized that these fine filamentous structures serve as substrates for further integration into actin bundles, which have been demonstrated to function in organelle movement and in cellular trafficking via the activity of myosin motors (Akkerman *et al.*, 2011; Day *et al.*, 2011; Thomas, 2012).

Actin dependent cytosolic-plasma membrane connectivity: preformed connections as targets and barriers of pathogenesis

The actin cytoskeleton is required for numerous cellular processes, ranging from cell membrane-associated dynamics (e.g., receptor activation and attenuation; Beck *et al.*, 2012), to the regulated delivery, as well as secretion, of signals within and from the cell. We argue that additionally, one of actin's most important roles is as a dynamic interface between the cell and the environment. In this role actin has been described as the ideal surveillance mechanism (Staiger *et al.*, 2009; Lee & Dominguez, 2010; Day *et al.*, 2011; Smethurst *et al.*, 2013), linking the extracellular matrix of mammalian cells and apoplast of plants to numerous intracellular processes, such as organelle movement and gene transcription. Through its function as a surveillance mechanism, actin has been demonstrated to regulate a plethora of cellular signaling pathways, including those required for response to injury, infection, development, and environment. It is therefore

not surprising that the actin cytoskeleton, while connecting many facets of cellular signaling, form and function, would be an ideal target of pathogens.

Passport control: hijacking endomembrane transport

In contrast to mammalian cells where endomembrane organization is dependent upon microtubules, the plant endomembrane system is largely dependent upon actin and myosin (Brandizzi & Wasteneys, 2013). Indeed, recent studies have demonstrated that plant myosin co-fractionates with the endoplasmic reticulum (ER; Yokota *et al.*, 2011), and that disruption of either the actin cytoskeleton through chemical treatment or utilization of a myosin tail over expressing line, which competes with intact myosin in plants, leads to a disruption in ER integrity (Sparkes *et al.*, 2009). Similarly, actin also plays a role in Golgi body motility and functions in the trans-Golgi network (TGN; Brandizzi & Wasteneys, 2013). Interestingly, Akkerman *et al*. (2011) demonstrated a distinct difference in the motility of Golgi depending upon the state of F-actin i.e. bundled cortical actin and a more free moving singular microfilament.

While our understanding of the interplay between host endomembrane dynamics and pathogen invasion is limited, there are two recent reports that highlight the importance of actin-dependent hijacking of the ER by the plant enveloped virus *Tomato spotted wilt tospovirus* (TSVW; Feng *et al.*, 2013; Ribeiro *et al.*, 2013; Figure 1.2.a; Table 1.1). As an infectious agent, the TSWV membrane envelope is predominantly formed by two viral glycoproteins, Gc and Gn (Ribeiro *et al.*, 2013). In addition to the membrane

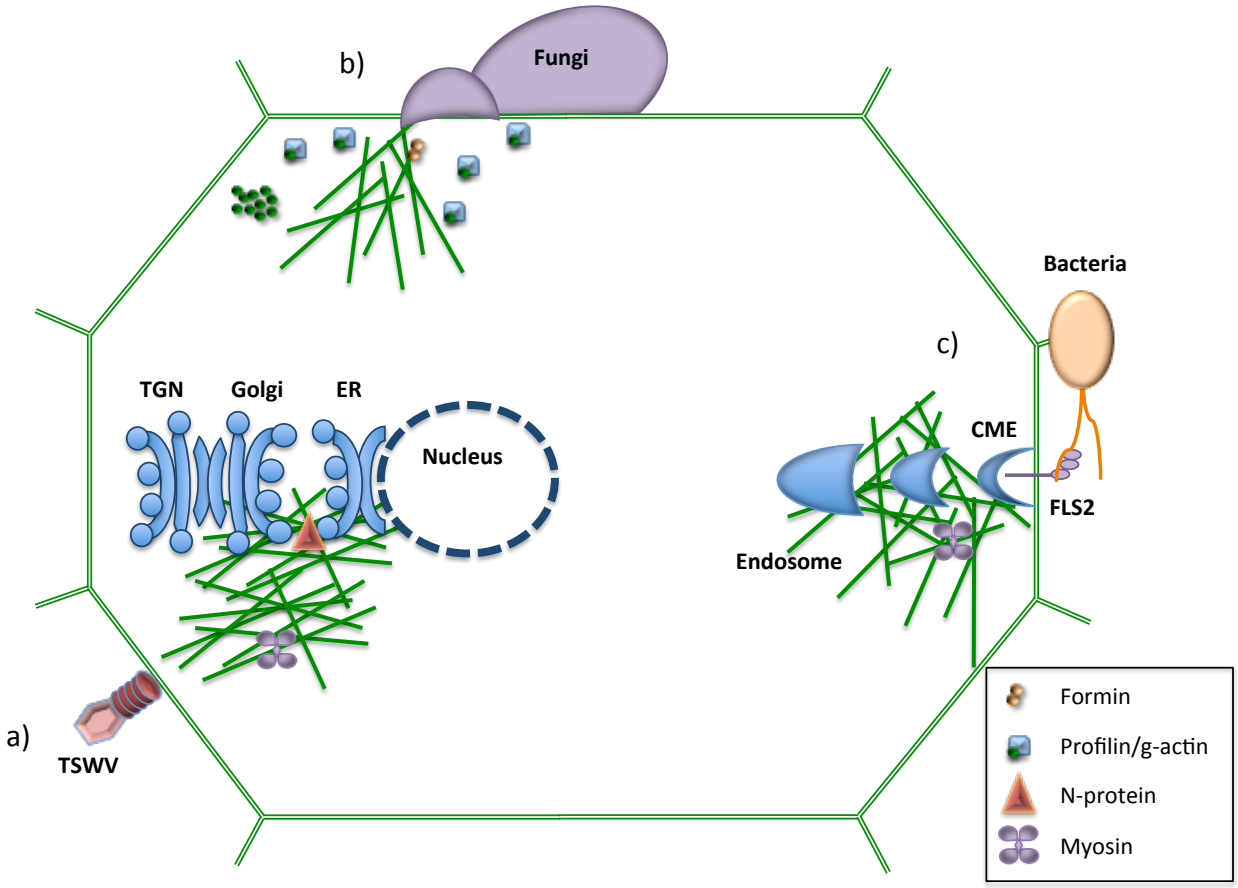


Figure 1.2. Examples of preformed cellular functions of the actin cytoskeleton utilized in defense signaling and targeted by pathogens. (a.) Actin-dependent intracellular movement of the *Tomato spotted virus wilt tospovirus* (TSVW) N-protein. N-protein of TSVW forms inclusion bodies that then associate with the endoplasmic reticulum (ER) and are trafficked through the endomembrane system in an actin and myosin dependent manner. (b.) Involvement of the actin cytoskeleton in the formation of the cell wall apposition (CWA), a defense related formation of anti-fungal compounds at the site of fungal penetration. Fungal penetration also signals the recruitment of actin filaments towards the penetration site. (c.) The actin cytoskeleton and myosin play key roles in the clathrin-mediated endocytosis (CME) of pattern recognition receptors including FLS2, which recognizes bacterial flagellin. Inhibition of either myosin or the actin cytoskeleton results in improper internalization of and endomembrane trafficking of FLS2.

envelope, TSWV also synthesizes a spherical viral particle consisting of ribonucleoproteins, where the single stranded genomic RNA is in tight association with the nucleoprotein (N-protein; Feng *et al.*, 2013). This work demonstrated that N-protein

Pathogen/elicitor	Type of virulence factor	Effect on host	Reference
Tomato spotted wilt tospovirus	N-protein	Targets actin and myosin to alter endomembrane trafficking.	Feng *et al.*, 2013
Pseudomona syringae pv *tomato* DC3000	Whole pathogen	Alter actin dynamics by increasing actin density.	Henty-Ridilla *et al.*, 2013
Agrobacterium tumefaciens	Whole pathogen	Alter actin dynamics by increasing actin density.	Henty-Ridilla *et al.*, 2013
Magnaporthe grisae	Whole pathogen	Alter actin dynamics by increasing actin density.	Henty-Ridilla *et al.*, 2013
Flagellin/flg22	PAMP/peptide ligand	Alter actin dynamics by increasing actin density.	Henty-Ridilla *et al.*, 2013
Chitin	PAMP	Alter actin dynamics by increasing actin density.	Henty-Ridilla *et al.*, 2013
Ef-Tu/elf26	PAMP/peptide ligand	Alter actin dynamics by increasing actin density. In the absence of Arabidopsis ADF4 this increased density was not observed.	Henty-Ridilla *et al.*, 2014
HopW1	Bacterial effector	Alters actin dynamics and disrupts endocytosis and cellular trafficking in an actin dependent manner.	Kang *et al.*, 2014
AvrPphB	Bacterial effector	Reduces flg22/FLS2 related MAPK signaling in the absence of Arabidopsis ADF4.	Porter *et al.*, 2012
VD toxin	Toxin	Disrupts the actin cytoskeleton.	Yuan *et al.*, 2006
ToxA	Toxin	Causes cell death when internalized, possibly through actin dependent CME.	Manning & Ciuffetti 2005

Table 1.1. Pathogen virulence factors that specifically target the host cytoskeleton, actin, and/or actin binding proteins.

Table 1.1. (cont'd) Pathogen associated molecular pattern (PAMP); Actin depolymerizing factor 4 (ADF4); Mitogen associate protein kinase (MAPK); Clathrin mediated endocytosis (CME).

forms cytoplasmic inclusion bodies that associate with and are trafficked along the host ER in an actin- and myosin-dependent manner. Interestingly, they determined that this intracellular trafficking, while actin-dependent, functions independently of microtubules. In parallel, Ribero *et al*. (2013) came to a similar conclusion, showing that N-protein trafficking was actin-dependent and microtubule-independent, while further demonstrating that actin was not required for the assembly of the viral glycoproteins with N-protein. Taken together, these two studies demonstrate a role for the actin cytoskeleton in cellular trafficking of the viral proteins, yet not in the formation of the viral complexes. This is noteworthy, as while there are many examples of enveloped viruses in the animal kingdom, few have been identified to infect plants. Thus, TSVW represents an exciting foundation, and case study, for the further analysis of actin-endomembrane dynamics and function during host-virus interactions.

Plasma membrane – cell wall connectivity

In addition to the cellular link of the actin cytoskeleton and the endomembrane system, there is growing evidence of the involvement of two ABPs, formins and profilins, in actin-plasma membrane (PM)-cell wall connectivity (van Gisbergen & Bezanilla, 2013; Sun *et al.*, 2013; Figure 1.2.b). Formins posses certain biochemical features that would make them ideal for connecting the plant cytoskeleton to the plasma membrane; for example some classes of formins contain a predicted trans-membrane domain, a signal peptide, and a proline-rich peptide that is hypothesized to interact with proteins within

the cell wall; others still have a phosphate and tension homolog (PTEN)-like domain that catalyzes and binds phosphoinisotides in the PM (van Gisbergen & Bezanilla, 2013). As a direct link between the host cytoskeletal network and the plasma membrane, profilin has been shown to have biochemical properties that would allow for its direct or indirect interaction with the PM (Sun *et al.*, 2013). In addition to binding actin, profilin can interact with proline-rich peptides of itself and other proteins including formins, which in turn interact with the PM, as well as bind phosphoinisotides directly in the PM (Sun *et al.*, 2013).

The actin cytoskeleton has been shown to play a key role in the formation of cell wall apposition (CWA), the accumulation of anti-fungal compounds, and resistance to penetration by fungi (Hardham *et al.*, 2007; Kobayashi & Kobayashi, 2013). Indeed, the involvement of actin in resistance to penetration is even apparent when the plant is micro-wounded as a mimic of failed penetration, where treatment with actin polymerization inhibitor cytochalasin A eliminated the observed penetration resistance (Kobayashi & Kobayashi, 2013). A study in cultured parsley cells infected with the oomycete *Phytophthora infestans* revealed pathogen-induced rearrangement of the actin cytoskeleton and ABPs. They found that upon attachment and penetration of the plant cell by the pathogen, the actin cytoskeleton oriented itself towards the area on infection (Schutz *et al.*, 2006). Additionally, this group demonstrated that the ABP profilin was also found to locate to the infection site (Schutz *et* al., 2006; Figure 1.2.b). This accumulation of profilin to the PM and reorientation of the actin cytoskeleton during

oomycete infection further supports the potential for profilin to connect the actin cytoskeleton and PM.

Pathogen perception and receptor dynamics

Another interesting example of the link between cellular membranes and the function of the actin cytoskeleton is the process of clathrin-mediated endocytosis (CME). Originally defined in yeast (Kaksonen *et al.*, 2003), a growing body of literature in other systems, including animals and plants, has demonstrated a requirement for several ABPs, including Arp2/3, CP, and ADFs, for the function of endocytosis (Galletta *et al.*, 2010). Recent work using mammalian models has described a similar function for CME-actin cooperation, demonstrating a function for actin as both a filamentous network that not only links endocytosis and the plasma membrane, but also as a mechanical process that can alter membrane shape, inducing membrane curvature, hypothesized to be an early key step in CME (Galletta *et al.*, 2010; Figure 1.2.c).

In plants, a recent study by Beck *et al*. (2012) dissected the process of the endocytosis of the immune-related pattern recognition receptor (PRR) flagellin sensing 2 (FLS2), which recognizes the pathogen associate molecular pattern (PAMP) flagellin or the 22 amino acid peptide flg22, further demonstrating the correlation between the actin cytoskeleton and endocytosis (Figure 1.2.c). The authors utilized a series of inhibitors in order to determine to what degree the actin cytoskeleton is involved in endocytosis and endomembrane trafficking. They found that in contrast to their previous study, treatment

with the actin depolymerization inhibitor latrunculin B (LatB) did not inhibit internalization of FLS2, but instead LatB impaired the trafficking of the FLS2 endosome, while the myosin inhibitor, 2,3-butanedione monoxime, inhibited FLS2 endocytosis. Furthermore, this study demonstrated that use of endosidin 1, an inhibitor of receptor-mediated endocytosis, both reduced the motility of FLS2 endosomes as well as stabilized actin filaments. Taken together this study suggests a synergistic function for myosin and the actin cytoskeleton in the internalization and endomembrane trafficking of FLS2 (Beck *et al.*, 2012).

Actin and guard cells movement: controlling entry to the apoplast

The actin cytoskeleton has also been implicated in having a role in Arabidopsis guard cell architecture (Higaki *et al.*, 2010). Higaki and colleagues (2010) utilized confocal microscopy techniques and cluster analysis to quantitatively analyze cytoskeletal orientation, as well as actin filament bundling (skewness) and percent occupancy (density), during diurnal cycles. This group found that the actin cytoskeleton has a radial orientation when stomata are open, and that actin is transiently bundled during the stomata opening process, but these bundled actin structures dissolve once the stomata is opened. Furthermore, it was determined that during the light portion of the day heavy bundles are present continuously while the stomata remained closed. Taken together these results suggest a correlation between actin bundling and stomata movement (Higaki *et al.*, 2010). A recent study has examined the actin orientation of the crop plant grapevine during leaf infection with various plant pathogens (Guan *et al.*, 2014). This

group treated grapevine leaves with 3 pathogens; *Erwinia amylovora*, *Agrobacterium tumefaciens*, and *Agrobacterium vitis*, representing true pathogen, non-host pathogen and host pathogen that does not infect through the leaves respectively, and measured changes in the actin cytoskeleton of the guard cells. They determined that there was no change in the skewness of the guard cells inoculated with *Erwinia amylovora*, but there were greater than 50% reductions in the skewness of the guard cells inoculated with either *Agrobacterium tumefaciens* or *Agrobacterium vitis* (Guan *et al.*, 2014). These observed changes in the skewness of actin filaments within the guard cells of grapevine may be due to elicitors of the pathogens, and suggest a role for the actin cytoskeleton as an output of pathogen-dependent guard cell re-orientation.

Involvement of actin cytoskeleton in immunological signaling: a dynamic target of pathogens

Numerous parallels exist between immune signaling in plants and animals (Ausubel, 2005). Broadly, these include the signaling of resistance *via* receptor-ligand interactions (Chisholm *et al.*, 2006; Chtarbanova & Imler, 2011), the activation of MAPK cascades (Rodriguez *et al.*, 2010; Whelan *et al.*, 2011), and the transcriptional reprogramming of cellular processes associated with cell death and defense (Pandey & Somssich, 2009). The immune systems of both plants and animals are among the best-defined examples of biological platforms that function as cell surveillance mechanisms. Indeed, much like the dynamism of the eukaryotic actin cytoskeleton, immune signaling is tightly regulated, highly responsive, and is seamlessly integrated with numerous signaling

cascades. In this regard, it is not surprising that the actin cytoskeleton is required for the function and regulation of immunity.

Stochastic dynamism of basal immunity and the actin cytoskeleton

In plants, immune responses are typically classified based on the characterization of two primary nodes of defense signaling: PAPM-triggered immunity (PTI) and effector-triggered immunity (ETI; Chisholm *et al.*, 2006). In PTI, perception and activation is mediated by extracellular recognition of PAMPs (e.g., flagellin, LPS, chitin) by plasma membrane-localized PRRs. Binding of PAMPs by PRRs initiates downstream signaling, including the activation of the MAPK signaling cascade, the generation of reactive oxygen species (ROS), and transcriptional reprogramming of pathogen-responsive genes (Zhang & Zhou, 2010; Figure 1.3.a). In total, PTI responses appear to be highly conserved in plants and animals, both in terms of the mode of activation (e.g., receptor-ligand interactions), as well as with respect to regulation (e.g., MAPK signaling). Several recent studies have demonstrated the importance of actin – and ABPs – as a component of PTI signaling cascades (Porter *et al.*, 2012; Henty-Ridilla *et al.*, 2013; Henty-Ridilla *et al.*, 2014). This is of particular interest, as recent data demonstrate an increase in density of the actin cytoskeleton in *Arabidopsis thaliana* cotyledons inoculated with a myriad of plant pathogens; *Pseudomonas syringae* pv. *tomato* DC3000, *Pseudomonas syringae* pv. *phaseolicola*, *Agrobacterium tumefaciens,* or *Magnaporthe grisea,* as well as the purified PAMPs flg22 and chitin (Henty-Ridilla *et al*., 2013; Figure 1.3b; Table 1.1). Additionally, the authors demonstrated an enhanced

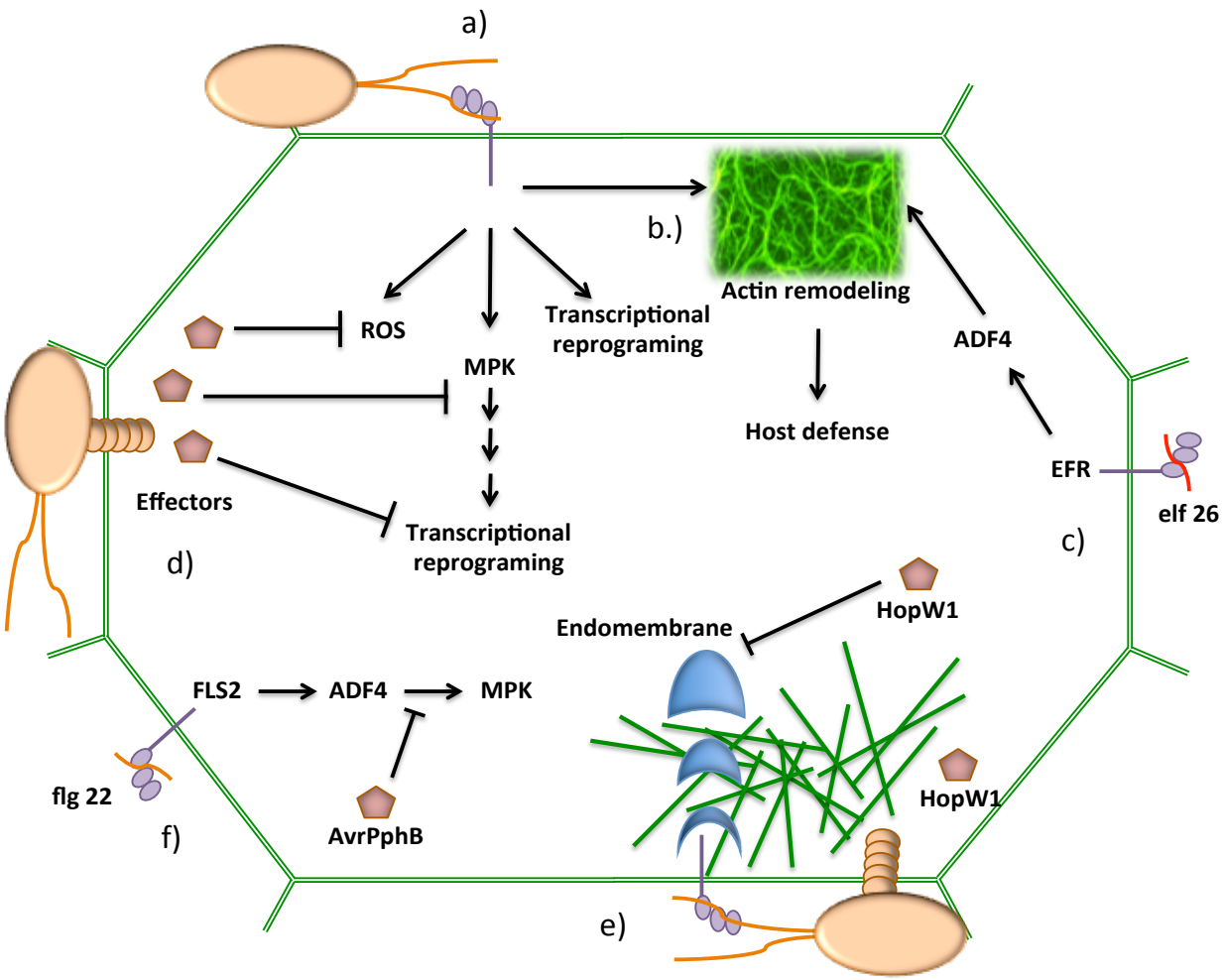


Figure 1.3. Direct targeting of the actin cytoskeleton by pathogens to enhance virulence. (a.). Examples of pathogen associated molecular pattern (PAMP)-triggered immunity (PTI). Recognition of conserved PAMPs results in a multitude of cellular signaling, including; formation of reactive oxygen species (ROS), mitogen associated protein kinase (MPK) activation, transcriptional reprograming, and (b.) actin remodeling. PTI responses aid in the basal resistance of plants to pathogens. (c.) The actin depolymerization factor 4 (ADF4) of Arabidopsis has been demonstrated to play a role in the actin remodeling associated with the PTI response of the pattern recognition receptor (PRR) EFR, that recognized the peptide elf26. (d.) Pathogenic effectors are secreted into the host cell in order to target components of the PTI response that will ultimately reduce the resistance of the plant cell. (e.) The bacterial effector HopW1 specifically targets actin and alters the endomembrane trafficking associated with resistance through the actions of both actin and myosin. (f.) Arabidopsis ADF4 has also been demonstrated to play a role in MPK activation by the ligand flg22 through the PRR FLS2 specifically when the bacterial effector AvrPphB is present.

virulence of *Pseudomonas syringae* pv. *tomato* DC3000 in mature plants whose actin

cytoskeleton was disrupted with the pharmacological agent LatB (Henty-Ridilla *et al*., 2013). A second study implicated the importance of the ABP ADF4 in PTI responses to the PAMP elf26 (Henty-Ridilla *et al*., 2014; Figure 1.3c; Table 1.1). In this study, dark grown hypocotyls of *Arabidopsis* were used to examine the changes in actin dynamism in the *adf4* mutant as compared to wild-type plants. It was determined that wild-type density increased with elf26 and that this increase in density was not observed in the *adf4* mutant (Henty-Ridilla *et al*., 2014; Figure 1.3.c). Additionally, an increase in actin filament length, filament lifetime and a decrease in severing frequency were also observed in the wild-type hypocotyls treated with elf26. These observations were phenocopied in the *adf4* mutant and furthermore, no change of these outputs was measured in the *adf4* mutant with elf26 treatment (Henty-Ridilla *et al*., 2014). These collective works open the door to additional studies aimed at defining the role of actin as a senor and activator of numerous signal transduction cascades.

Effectors effects on actin and immunity

To subvert the host PTI response, pathogens deploy secreted effector proteins (Figure 1.3.d) whose collective activities function to abrogate the host immune responses by perturbing host cellular processes. To prevent this, hosts have evolved mechanisms to recognize and respond to pathogen secreted effector proteins. This process, ETI, has been best-described as an enhanced PTI-like response that is initiated via the direct or indirect recognition of pathogen effectors by host resistance (R) proteins (Chisholm *et al.*, 2006). Numerous virulence targets of pathogen effectors identified thus far are

components of PTI signaling pathways – the hypothesis being that targeting of PTI components can lead to increased growth of the pathogen (Zhang *et al.*, 2010; Zhang & Zhou, 2010; Figure 1.3.d).

As virulence molecules, effectors from bacterial pathogens of plants induce a wide range of cellular changes in their host(s), including alterations in transcription, perturbations in cellular trafficking, as well as the enzymatic targeting of host proteins required for immunity. Thus, it is not surprising that bacterial effectors have been identified which target the host actin cytoskeleton for the purpose of blocking immune signaling. Recently, an effector from *Pseudomonas syringae* pv. *maculicola*, HopW1, was shown to disrupt the actin cytoskeleton, and through this activity, enhance pathogen virulence (Kang *et al.*, 2014; Figure 1.3.e; Table 1.1). In this study, Kang *et al*., 2014 found that the effector HopW1 directly interacts with actin both *in vitro* and *in vivo*. *In vitro* HopW1 bound to and disrupted actin remodeling, as demonstrated both through sedimentation assays as well as through confocal visualization techniques. Experiments *in vivo* mirrored the *in vitro* results with reduced bundling of actin as well as enhanced bacterial growth *in planta* when infected with *Pseudomonas syringae* HopW1 similar to reduced bundling and enhanced growth when treated with the pharmacological agent LatB (Kang *et al*., 2014; Figure 1.3.e). Further analysis of the effects of HopW1 on plant function demonstrated an inhibition of endocytosis and trafficking to the vacuole, which again can be reproduced with LatB treatment (Kang *et al*., 2014; Figure 1.3.e; Table 1.1). Taken together these data suggest HopW1 disrupts

the actin cytoskeleton, which in turn perturb cellular functions including endocytosis and cellular trafficking to enhance pathogenic virulence.

As a final demonstration of the connection between pathogen effectors, the host actin cytoskeleton, and the numerous homeostatic processes in plants that require the activity of actin for their function, the work of Tian *et al*. (2009) and myself provides insight into the intimate relationships that underpin the link(s) between cytoskeletal dynamics and immune signaling. The functional analysis of ADF4 has not only shown that actin depolymerization is important for immunity, but through a series of complementary genetic and cell biology-based approaches, has shown that compromised immune signaling in the *adf4* mutant is the result of a drastic reduction in the expression of the mRNA encoding the resistance protein RPS5. As a mechanism supporting this function, my work (Porter *et al*., 2012; Chapter 2) defined that phospho-regulation of ADF4 influences association with actin as well as correlates with the expression of *RPS5*. In addition to the actin-binding activity of ADF4, I also demonstrated that the loss of *RPS5* mRNA expression did not fully explain the parallel reduction in MAPK signaling also observed in the *adf4* mutant. It was only in the presence of the bacterial effector AvrPphB that a reduction in MAPK signaling was observed (Figure 1.3.f; Table 1.1). Because this loss was not observed in the *rps5* mutant in the presence of AvrPphB, these data support a role for ADF4 in the activation of MAPK signaling. Taken together, these findings offer a unique example of a multi-layered interaction of a bacterial effector targeting both PTI and ETI in an actin-dependent manner. Recently, these works were supported by the observations of Fu *et*

al., (2013) in the crop plant *Triticum aestivum* (wheat) where silencing of the three copies of TaADF7 resulted in an enhanced susceptibility to *Puccinia striiformis* f. sp. *tritici*.

Toxic targeting of the actin cytoskeleton

One of the best-characterized virulence mechanisms of pathogens is the production, delivery, and site of action of a suite of host-specific toxins. As a class of highly conserved diffusible compounds, toxins serve many functions during infection, including roles as long-range signaling molecules, extracellular triggers of host cell lysis, and internalized inducers of programed cell death. Pathogens of plants, particularly fungi, have been shown to perturb the homeostatic function of the host actin cytoskeleton through the delivery of strain-specific elicitors and toxins, presumably as a mechanism to alter defense signaling, including host-derived secretion of anti-fungal compounds. In most cases described thus far, these toxins (Table 1.1) have been shown to either mimic the biochemical activities of eukaryotic ABPs, or more broadly, disrupt the structure/function of the microfilaments themselves. To date, two well-established examples of toxin-specific targeting of the host actin cytoskeleton by plant pathogens have been described. In the first, Yuan *et al*. (2006) showed that treatment of Arabidopsis suspension-cultured cells with the toxin from *Verticillium dahlia*, VD toxin, induces a dose-dependent response to the broader organization of the cytoskeleton. At low concentrations, the overall actin filament structure was disrupted; however, changes to microtubules were not detected. Conversely, at high concentrations of VD toxin, both

actin and microtubule structures were disrupted, suggesting a convergent point of targeting by V. *dahlia*, and moreover, implicating both the actin cytoskeleton and the microtubule network as virulence targets of fungal pathogens.

Lastly, ToxA, from the necrotrophic fungus *Pyrenophora tritici-repentis* has been shown to elicit cell death if expressed in either sensitive or insensitive wheat mesophyll cells, yet is only actively translocated into the cytoplasm of the sensitive wheat cells (Manning & Ciuffetti, 2005; Table 1.1). What is most striking about ToxA is that it contains an RGD tripeptide sequence (i.e., *Arginine-Glycine-Aspartic acid*), which is required for its function (Meinhardt *et al.*, 2002). This is interesting as the RGD motif is most commonly associated with the function of mammalian integrins, required for their actin-dependent association with the extracellular matrix. In plants, it was recently demonstrated that the immune signaling regulator, non-race specific disease resistance-1 (NDR1), is an integrin-like protein that plays a role in cell wall-plasma membrane adhesion through the function of an NGD-like (i.e., *Asparagine-Glycine-Aspartic acid*) motif (Knepper *et al.*, 2011). As described above CME is known to involve the actin cytoskeleton, thus, it is tempting to hypothesize that actin plays a role in the internalization of the ToxA protein through a yet to be identified extracellular receptor (e.g., integrin-like protein). In total, this is a nice example of cellular mimicry, whereby the structure-function activity of a fungal toxin can mimic the endogenous behavior of a cell wall-plasma membrane process, thereby driving changes in host actin cytoskeletal dynamics for the purpose of promoting pathogen infection.

Pathogen targeting of the eukaryotic cytoskeleton in mammalian systems

Numerous parallels exist between immune signaling in plants and animals (Ausubel, 2005). Broadly, these include the signaling of resistance *via* receptor-ligand interactions (Chisholm *et al.*, 2006; Chtarbanova & Imler, 2011), the activation of MAPK cascades (Rodriguez *et al.*, 2010; Whelan *et al.*, 2011), and the transcriptional reprogramming of cellular processes associated with cell death and defense (Pandey & Somssich, 2009). The specific targeting of the actin cytoskeleton to promote pathogen virulence is another shared component of plant- and mammalian- pathogen interactions. In this section I will highlight some key examples of perturbation of the mammalian actin cytoskeleton by pathogens.

Akin to what has been observed in plants, bacterial toxins have been identified which can directly modify actin of mammalian cells, as is the case of the *Clostridium botulinium* C2 toxin, an actin-ADP-ribosylating exotoxin that causes ADP-ribosylation of arginine-177 of actin, leading to the inhibition of actin polymerization (Aktories, 2011; Aktories *et al.*, 2011). Similarly, a second toxin from *C. botulinium*, C3 toxin, while not directly targeting actin, instead targets the Rho-family of GTPases, altering cytoskeletal dynamics, and ultimately phagocytosis (Visvikis *et al.*, 2010). Interestingly, this virulence activity is similar to that of YopT, a type III effector from *Yersinia pestis*, described below. In addition to activities associated with the physical disruption of host actin cytoskeletal dynamics, toxins have also been identified which usurp, or mimic, the endogenous function of ABPs. For example, the Tc toxin from *Photorhabdus*

luminescens was shown to impact actin cytoskeletal architecture by inducing ADP-ribosylation of actin, initiating unregulated polymerization, ultimately leading to the formation of actin aggregates (Aktories *et al.*, 2011). Thus, as noted, regardless of the specific toxin targeting the actin cytoskeleton, whether direct or indirect, the primary function of these toxins are to disrupt actin cytoskeletal dynamism, thereby hindering the cell's ability to respond to pathogenesis.

Mammalian pathogens, like those of plants, secrete effectors directly into the host cells to alter cellular processes for their own advantage. Identification of secreted effectors targeting the actin cytoskeleton has been established in mammalian systems for a relatively long time. Among the best-described examples of this are the numerous functional analyses of the suite of effectors from *Yersinia pestis*, which have been shown to block the activation of phagocytosis (Shao, 2008). In total, three host Rho-GTPases are targeted by the secreted effectors (i.e., YopT, YopE, and YpkA) from *Y. pestis*: RhoA, Rac and Cdc42. Similar to the toxins described above, once delivered into the host cells, these effectors inhibit the function of the key regulators of phagocytosis, resulting in the inhibition of pathogen uptake, which in turn permits the proliferation of *Y. pestis* within its host.

Salmonella enterica modulates the actin cytoskeleton and additionally the endomembrane system of epithelial cells to cause infection and promote proliferation (McGhie *et al.*, 2009; Haglund & Welch, 2011). The infection cycle of *Salmonella* begins with entry in the host cells. *Salmonella* delivers six secreted proteins, known as the SPI-

1 class of effectors (SopE, SopE2, SptP, SipA, SopB, and SipC), in a well-orchestrated, temporal manner to gain entry into epithelial cells through a process referred to as cellular ruffling (McGhie *et al.*, 2009; Haglund & Welch, 2011). Once inside the cell, the bacterium utilizes effector release to create a protected niche to allow for pathogen replication. Through this process of regulated effector delivery and hijacking of host processes, *Salmonella* manipulates its host to allow for the formation and maturation of the *Salmonella*-containing vacuole (SCV), utilizing the preformed endosomal maturation pathway as well as components of the exocytic pathway (Ramos-Morales, 2012). To persist, it is crucial that the SCV does not encounter the same fate as that of the phagosome or lysosome. To avoid the endosomal pathway, thereby evading host immunity, *Salmonella* secretes the type III effector SifA to form *Salmonella*-induced filaments (Sifs) that protrude from the SCV, thereby driving SCV motility along microtubules *via* kinesin interactions with the host protein SKIP (SifA and kinesin-interacting protein; Boucrot *et al.*, 2005).

Similar to the activity of secreted effectors from *Salmonella*, *Listeria* also utilizes subterfuge of the actin cytoskeleton to gain cellular entry. *Listeria* utilizes a surface bacterial invasion protein InlA to hijack actin dependent internalization via interactions with E-cadherin (Pizarro-Cerda & Cossart, 2009). As a second, perhaps more striking example of pathogen targeting of the host actin cytoskeleton is the case of how *Listeria* infection spreads within animal cells. *Listeria* utilizes yet another surface protein, ActA, as a mimic of N-WASP, resulting in the activation of the Arp2/3 nucleating factor to encourage the rapid formation of F-actin “rocket-tails”, filamentous actin structures used

by the pathogen for motility and infection of neighboring cells (Pizarro-Cerda & Cossart, 2009).

Actin's role in nuclear reprograming: a potential for pathogens to gain control of host gene expression

The movement of actin, including both the induction of changes in filament architecture as well as the (re) distribution of actin and ABPs within the cell, not only illustrates the dynamism of the cytoskeleton, but also the breadth of cellular engagement. Until recently, it was not widely accepted that actin was purposefully present in the nucleus (i.e. it was assumed that actin was present in the nucleus either due to sample prep contamination or simple passive diffusion) let alone possessed any physiologically relative functions. Actin was first observed in isolated nuclear fractions from *Xenopus laevis* in 1977 (Clark & Merriam, 1977), and since this time, the proposed function(s) of actin within the nucleus has been a point of discussion (Bettinger *et al.*, 2004). In the following section we aim to review some of the main questions that have arisen since this initial observation in 1977, as well as the new question that is of most interest to us regarding the potential of pathogens to target nuclear actin.

What role(s) does actin play in the nucleus?

After the initial observation of actins presence in the nucleus, researchers sought to demonstrate that actin played an active role in nuclear processes. It was found that the

microinjection of actin antibodies directly into the nuclei of salamander oocytes resulted in cessation of RNA synthesis from the lampbrush chromosomes (Scheer *et al.*, 1984). It was later determined through a multitude of studies reviewed in (Miyamoto & Gurdon, 2012; Percipalle, 2013) that actin plays a role in transcription by all 3 of the RNA polymerases. More specifically it has been demonstrated that actin plays a key part in enhancing the assembly of protein complexes required for RNA polymerase function. In addition to aiding in the functionality of RNA polymerases, actin has also been found in association with chromatin remodeling complexes (Olave *et al.*, 2002; Kapoor & Shen, 2014; Figure 1.4.b). Kandasamy *et al*. (2010) demonstrated the localization of multiple vegetative class actin variants within the plant nucleus. Additionally, within this subclass of actin it was demonstrated that Act 7 had a different localization pattern inside the nucleus from that of Act 2 or Act 8 (Kandasamy *et al.*, 2010). These differing sub-nuclear localizations of different actins may suggest that there are unique nuclear roles for Act 7 that differ from that of Act 2 or Act 8. Future work will hopefully shed light on the functional importance of these differing sub-nuclear localizations of various actins as visualization techniques improve.

Are ABPs nuclear localized, and if so, what are their nuclear functions?

In mammals, actin is hypothesized to be shuttled into the nucleus by ADF/Cofilin (AC), one of few members of the ABP superfamily that contain a nuclear localization signal. In humans, the import/translocation of AC-actin into the nucleus has been shown to require the function of importin-9, while the favored hypothesis is that the export of actin

is mediated by PRF, through the activity of exportin-6 (Wada *et al.*, 1998; Dopie *et al.*, 2012; Figure 1.4.a). In plants, the import/export control of actin and ABPs into and out of the nucleus is unclear; however, it has been demonstrated that like mammalian systems, plant nuclei contain ABPs, specifically ADF1-4 and profilin (Kandasamy *et al.*, 2010; Porter *et al.*, 2012).

In addition to the active nuclear import/export of actin, ABPs themselves have been identified to have direct interactions with genes as well as the nuclear machinery (Percipalle, 2013; Miyamoto & Gurdon, 2012). While direct interactions have been observed in mammalian systems, plant research has currently revealed indirect alterations in gene expression due to either loss of ABPs or alterations in cytoskeletal dynamics (Porter *et al*., 2012; Burgos-Rivera *et al.*, 2008; Moes *et al*., 2013). It has been demonstrated that knocking out ADF9 in *Arabidopsis thaliana* results in reduced expression of *flowering locus C* (*FLC*; Burgos-Rivera *et al*., 2008; Figure 1.4.b). Chromatin Immunoprecipitation (ChIP) revealed a reduction in histone H3 lysine 4 trimethylation and histone H3 lysine 9 and 14 acetylation of the *FLC* promoter, which is associated with reduced expression. I will show that my *Arabidopsis thaliana* ADF of interest, ADF4, is associated with the expression of the resistance protein *RPS5* (Porter *et al*., 2012; Chapter 2).

In *Nicotiana tabacum* the LIM protein WLIM2, which is predicted to occupy both the cytosol and nucleus as well as bind actin, was recently shown to interact with the *Arabidopsis thaliana* histone H4A748 (Moes *et al*., 2013). Additionally, it was confirmed

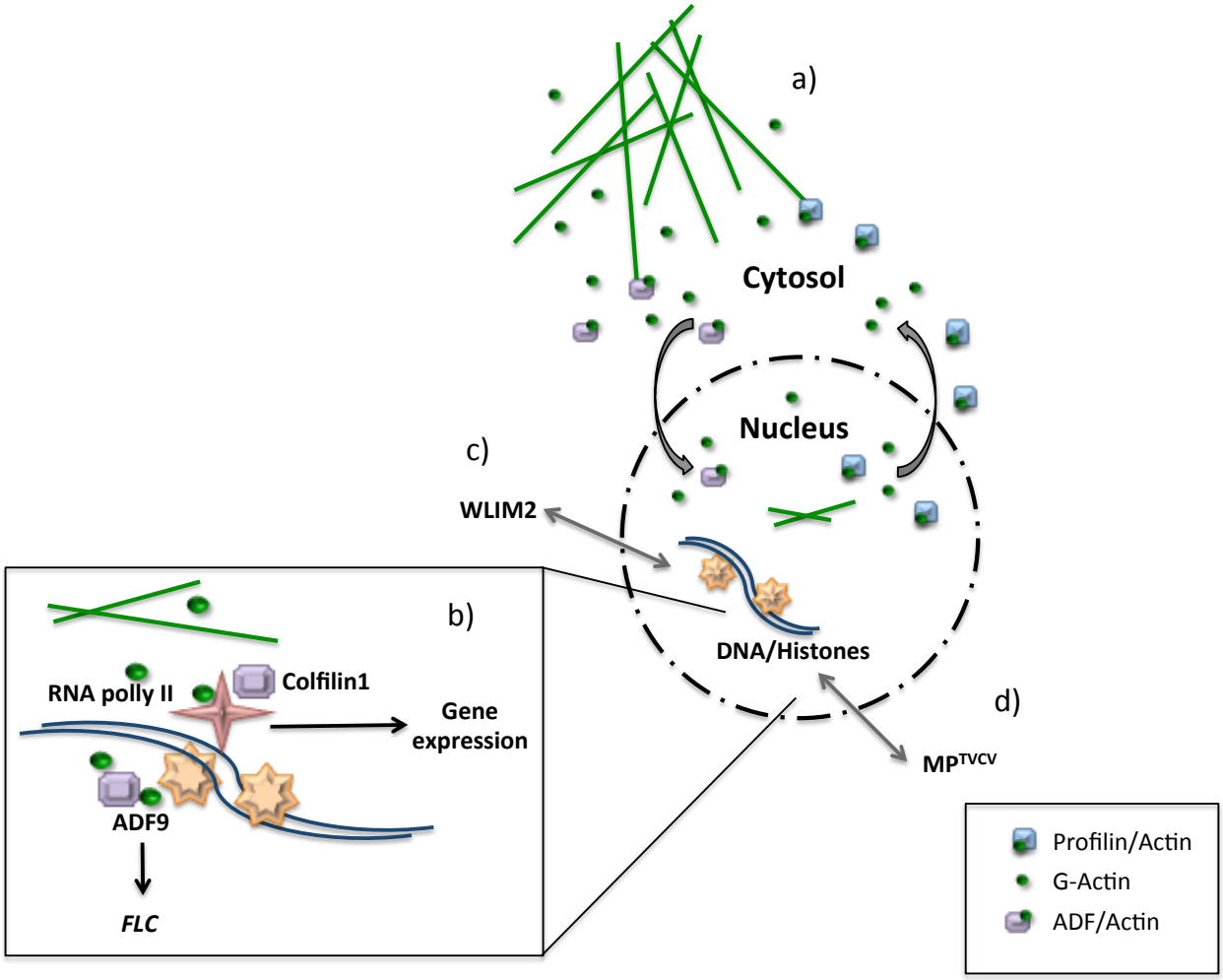


Figure 1.4. Nuclear involvement of the actin cytoskeleton in gene expression and it's targeting by plant pathogens. (a.) Proposed translocation of actin into and out of the nucleus by the actin binding proteins; actin depolymerizing factors (ADFs) and profilin, as demonstrated in mammalian systems. (b.) Sub-nuclear functions of monomeric globular (G-) actin, filamentous (F-) actin and ADFs in gene transcription. G- and F- actin, as well as Cofilin1 have been determined to play a role in gene expression in mammalian systems. Arabidopsis ADF9 has been demonstrated to be required for expression of the *flowering locus C* (*FLC*) in a histone modification dependent manner. (c.) The *Nicotiana tabaccum* LIM protein WLIM2 associated with both actin and histone H4A748. Additionally WLIM2 has subcellular localization patterns in the cytosol and nucleus. (d.) *Turnip Vein Clearing Virus* (TVCV) movement protein (MP^{TVCV}) posses a strong nuclear localization signal and interacts with F-actin. Visualization of MP^{TVCV} resulted in visualization of F-actin structures within the nucleus of plants as well as co-localization of MP^{TVCV} with histone H2B.

that WLIM2 did indeed localize to the cytosol and nucleus as well as bind to, and bundle, actin filaments. Interestingly, treatment of cells with LatB resulted in increased

nuclear occupancy of WLIM2, suggesting cross-talk between cytoskeletal dynamics and the nucleus (Moes *et* al., 2013; Figure 1.4.c).

How prevalent is F-actin in the nucleus and what is the role of F-actin within the nucleus?

This has been a major question from the earliest studies that identified the formation of actin rods in *Dictyostelium* is response to stress (Fukui, 1978). A recent study in mammalian systems found that cofilin-1 is in a complex with actin and phosphorylated polymerase II (Obrdlik & Percipalle, 2011). Additionally, they found that cofilin-1 was in association with transcribed regions of genes, and this occupancy is affected by the polymerization of actin. Knocking down cofilin-1 resulted in reduction in gene expression and a loss of actin and RNA polymerase II from the transcribed regions, as well as an increase in the accumulation of F-actin foci in the nucleus (Obrdlik & Percipalle, 2011; Figure 1.4.b). It has been suggested that the high levels of profilin and cofilin in the nucleus, as well as additional ABPs, allow for the utilization of both G- and F-actin in the nuclear remodeling machinery (Miyamoto & Gurdon, 2012).

There are many obstacles to the visualization of F-actin within both plant and mammalian cells, although there have been many breakthroughs in recent years. A few of these concerns were presented in Kandasamy *et al*. (2010) in which actin rods were visualized in *Arabidopsis thaliana* by attaching a strong NLS to the C-terminus of Act 7. The authors worried that the addition of the NLS may have caused the actin to

aggregate in the nucleus so that the overexpression needed to visualize these structures in the nucleus may have disrupted the ability of the actin to be turned over in the nucleus by ABPs (Kandasamy *et al.*, 2010). Some advances in the visualization of F-actin in the nucleus are being developed that do not appear to require the over expression of actin nor seem to affect the natural remodeling of the actin cytoskeleton (Belin & Mullins, 2013; Grosse & Vartiainen, 2013). Recently, Levy *et al*. (2013) utilized TagRFP-UtrCH, a protein that contains the calponin-binding domain of UthCH and TagRFP, to visualize F-actin in the nuclei of *N. benthamiana*. The advantage of utilizing the calponin-binding domain is that it is reported to label F-actin without affecting actin's dynamism.

Is there any pathogen that targets nuclear actin or ABPs to enhance virulence?

A recent review by (Deslandes & Rivas, 2011), suggested that the plant nucleus could be the next major area of study in plant immunity research, including transport of macromolecules into and out of the nucleus and regulation of gene expression. Given the shuttling of actin and ABPs into and out of the nucleus, as well as the involvement of these components in gene transcription, we feel that actin and its components should not be overlooked when endeavoring into this research. Indeed, I have demonstrated a requirement for Arabidopsis ADF4 for the proper expression of the resistance gene *RPS5*, and ultimately, resistance to *P. syringae* expressing the cysteine protease AvrPphB (Chapter 2). Furthermore, I determined that expression of *RPS5* was not only dependent upon the presence of ADF4, but also the phosphorylation status of ADF4.

These data provide preliminary insight into the potential mechanisms by which expression of resistance genes may be regulated by ABPs in a post-translational dependent manner and could be targeted by plant pathogens to enhance virulence.

In support of ADFs playing a role in both resistance and nuclear function a group working in rice has recently identified a pathway involving the direct interaction of rice ADF with a lectin receptor-like kinase, OsleRK, and expression of α-amylase, required for seed germination and expression of defense genes (Cheng *et al.*, 2013). Specifically, knock down of either OsleRK or rice ADF resulted in reduced expression of the resistance genes *PR1a*, a pathogen-related gene, *LOX*, encoding a lipoxygenase and *CHS*, which encodes a peroxidase. In addition to this alteration of gene expression both mutants also exhibited enhanced susceptibility to multiple pathogens including the bacterium *Xanthomonas oryzae* pv. *oryzae* and the fungi *Magnaporthe grisea* (Cheng *et al*., 2013; Figure 1.4.b).

Another recent study examined the movement protein (MP) of the tobamovirus *Turnip Vein Clearing Virus* (TVCV: MP^{TVCV}) and found that in addition to its expected localization to endoplasmic reticulum and plasmodesmata MP^{TVCV} was located in the plant nucleus in association with F-actin within the nucleus (Levy *et al.*, 2013; Figure 1.4.d). Within the nucleus MP^{TVCV} did not co-localize with nucleoli or Cajal bodies, but instead co-localized with histone H2B. It was further determined that MP^{TVCV} posses a strong NLS signal that is required for proper infection by TVCV. Taken together these

data suggest that MP^{TVCV} may directly alter nuclear actin dynamics to alter the expression of genes in order to enhance virulence.

Final Thoughts

As highlighted above, the plant actin cytoskeleton is ubiquitous, dynamic, and highly regulated, requiring the activity of more than 75 ABPs for its assembly and function. In addition to the basic processes that regulate the filament architecture and organization, actin cytoskeletal dynamics are intimately governed by a suite of host processes that require its function, including those associated with growth and development, movement and organization, and response to stimuli. In recent years, advances in genomics and cell biology have further enhanced our understanding of the processes governing, and governed by, the actin cytoskeleton. From these collective studies, it is evident that we have only begun to scratch the surface of our understanding of the *hows* and *whys* regarding the extent of the role of the actin cytoskeleton in plant biology. Of particular interest is the role of actin as a surveillance mechanism, continually sensing the cell for perturbations, including both chemical and physical changes in the intracellular and extracellular environment. As a central component of actin's role as a surveillance platform, the localization, including changes in the subcellular concentration of actin and various ABPs, is noteworthy. To begin to address this knowledge gap, studies using plant - pathogen cell models have demonstrated that changes in ABP localization within the cell serves not only as a stimulus for reorientation of actin filament architecture, but also as a trigger that initiates the induction of processes including changes in signal

transduction pathways and gene expression. To this end, the role of actin in the nucleus represents largely unexplored areas of research, possibly holding the answers to areas of biology beyond the dynamics of actin assembly, and the realm of actin as a mediator of gene activation and cellular homeostasis.

CHAPTER 2

Arabidopsis Actin-Depolymerizing Factor-4 Links Pathogen Perception, Defense Activation and Transcription to Cytoskeletal Dynamics.

This research was originally published in *PLoS Pathogens*.
Porter K, Shimono M, Tian M, Bay B. 2012. Arabidopsis Actin-Depolymerizing Factor-4 links pathogen perception, defense activation and transcription to cytoskeletal dynamics. *PLoS Pathog* 8: e1003006. Minor edits have been made in formatting this chapter and addressing committee concerns.

The confocal images of the co-localization of ADF4_S6A and ADF4_S6D were performed by co-author Masaki Shimono; Department of Plant Pathology at Michigan State University, while the image analysis was performed by me, Katie Porter. The plant lines *adf4*/35S:ADF4S6A and *adf4*/35S:ADF4S6A were constructed by co-author Miayoing Tian while in the Department of Plant Pathology at Michigan State University.

Abstract

The primary role of Actin-Depolymerizing Factors (ADFs) is to sever filamentous actin, generating pointed ends, which in turn are incorporated into newly formed filaments, thus supporting stochastic actin dynamics. Arabidopsis ADF4 was recently shown to be required for the activation of resistance in Arabidopsis following infection with the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) expressing the effector protein AvrPphB. Herein, we demonstrate that the expression of *RPS5*, the cognate resistance protein of AvrPphB, was dramatically reduced in the *adf4* mutant, suggesting a link between actin cytoskeletal dynamics and the transcriptional regulation of R-protein activation. By examining the PTI (PAMP Triggered Immunity) response in the *adf4* mutant when challenged with *Pst* expressing AvrPphB, we observed a significant reduction in the expression of the PTI-specific target gene *FRK1* (Flg22-Induced Receptor Kinase 1). These data are in agreement with recent observations demonstrating a requirement for RPS5 in PTI-signaling in the presence of AvrPphB. Furthermore, MAPK (Mitogen-Activated Protein Kinase)-signaling was significantly reduced in the *adf4* mutant, while no such reduction was observed in the *rps5-1* point mutation under similar conditions. Isoelectric focusing confirmed phosphorylation of ADF4 at serine-6, and additional *in planta* analyses of ADF4's role in immune signaling demonstrates that nuclear localization is phosphorylation independent, while localization to the actin cytoskeleton is linked to ADF4 phosphorylation. Taken together, these data suggest a novel role for ADF4 in controlling

gene-for-gene resistance activation, as well as MAPK-signaling, *via* the coordinated regulation of actin cytoskeletal dynamics and *R*-gene transcription.

Author Summary

The activation and regulation of the plant immune system requires the coordinated function of numerous pre-formed and inducible cellular responses. Following pathogen perception, plants not only activate specific defense-associated signaling, such as resistance (*R*) genes, but also redirect basic cellular machinery to support innate immune signaling. Within each of these processes, the actin cytoskeleton has been demonstrated to play a significant role in structural-based defense signaling in plants in response to pathogen infection. Most notably, the actin cytoskeleton of plants has been shown to play a role in structural-based defense signaling following fungal pathogen infection. Recent work from our laboratory has demonstrated that the actin cytoskeleton of Arabidopsis mediates defense signaling following perception of the phytopathogenic bacterium *Pseudomonas syringae*. Using a combination of genetic and cell biology-based approaches, we found that ADF4, a regulator of actin cytoskeletal dynamics, is required for the specific activation of *R*-gene-mediated signaling. By analyzing the activation of signaling following pathogen perception, we have identified substantial crosstalk between recognition of pathogen virulence factors (e.g., effector proteins) and the regulation of *R*-gene transcription. In total, our work highlights the intimate relationship between basic cellular processes and the perception and activation of defense signaling following pathogen infection.

Introduction

The actin cytoskeleton is an essential, dynamic component of eukaryotic cells, involved in numerous processes including growth and development, cellular organization and organelle movement, and abiotic and biotic stress signaling (Day *et al.*, 2011). Underpinning these processes in plants is a tightly regulated genetic and biochemical mechanism driven by the function of more than 70 actin-binding proteins (ABPs), which through their coordinated activity, regulates the balance of free globular (G)-actin versus filamentous (F)-actin, of which nearly 95% is unpolymerized in plants (Gibbon *et al.*, 1999; Snowman *et al.*, 2002). As a consequence of this large pool of free G-actin, the potential exists for explosive rates of polymerization following elicitation by a broad range of external stimuli, including pathogen infection (Day *et al.*, 2011). Among the numerous ABPs in plants responsible for modulating the balance of G- to F-actin, one subclass, Actin-Depolymerizing Factors (ADFs), both sever and disassemble F-actin. In addition to thier primary role in modulating host cytoskeletal architecture, a role for ADFs in defense signaling following pathogen infection is emerging (Miklis *et al.*, 2007; Clément *et al.*, 2009; Tian *et al.*, 2009).

The initiation of innate immune signaling in plants relies on multiple pre-formed and inducible processes to surveil, respond, and activate defense signaling following pathogen perception (Chisholm *et al.*, 2006; Knepper & Day, 2010). In total, these responses can be cataloged based on two primary nodes of defense signaling: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-

triggered immunity (ETI) (Chisholm *et al.*, 2006). In the case of PTI, perception and activation is typically mediated by extracellular plasma membrane-localized pattern recognition receptors (PRRs), which are responsible for the recognition of conserved pathogen motifs (i.e., PAMPs; e.g., flagellin, LPS, chitin). Recognition of PAMPs by PRRs initiates downstream signaling, including the activation of the Mitogen-Activated Protein Kinase (MAPK) signaling cascade, the generation of reactive oxygen species, and transcription of pathogen-responsive genes (Zhang & Zhou, 2010). Arguably the best-characterized example of PTI signaling in plants is the activation of signaling associated with FLS2 (Flagellin Sensitive-2), a receptor-like kinase containing a serine/threonine kinase, which recognizes flagellin as well as the 22-amino acid peptide flg22 *via* the extracellular leucine rich repeat (LRR) domain (Gomez-Gomez *et al.*, 1999; Gomez-Gomez & Boller, 2000). Activation of FLS2 by flg22 results in the association of FLS2 with BAK1 (BRI1-associated receptor kinase), as well as the phosphorylation of both FLS2 and BAK1 (Chinchilla *et al.*, 2007). FLS2 ligand binding and association with BAK1 has been shown to activate the MAPK signaling pathway resulting in dual phosphorylation of conserved tyrosine and threonine residues of Arabidopsis (*Arabidopsis thaliana*) MAP kinases MPK3/6 (Rodriguez *et al.*, 2010), which in turn leads to transcription of PTI-related genes including *FRK1* (Flg22-induced receptor kinase 1; (Asai *et al.*, 2002)). The expression of *FRK1*, however, is believed to be both MAPK dependent and independent (Asai *et al.*, 2002).

As a counter to the activation of PTI, many plant pathogens deploy secreted effector proteins, which induce a host response (e.g., ETI) - an enhanced PTI-like response, as

well as a more robust, programmed cell death-like, response known as the hypersensitive response (HR) that is initiated *via* the direct or indirect recognition of pathogen effectors by host resistance (R) proteins (Chisholm *et al.*, 2006). As expected, numerous virulence targets of pathogen effectors identified thus far are components of PTI signaling pathways – with the hypothesis being that targeting PTI-components can lead to increased virulence of the pathogen (Zhang *et al.*, 2010; Zhang & Zhou, 2010). Among the best-characterized signaling pathways leading to the activation of ETI, as well as a mechanistic example of the functional overlap between PTI and ETI, is the recognition of the bacterial effector protein AvrPphB by the Arabidopsis resistance protein RPS5 (resistance to *Pseudomonas syringae*-5) (Chisholm *et al.*, 2006). RPS5 is a member of the coiled-coil (CC) nucleotide-binding-site (NBS) LRR R-gene family, required for recognition of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) expressing the cysteine protease effector protein AvrPphB (Warren *et al.*, 1999; Ade *et al.*, 2007). RPS5-mediated resistance signaling is dependent upon AvrPphB cleavage of the receptor-like cytoplasmic kinase (RLCK) AvrPphB-Susceptible 1 (PBS1), which in turn results in the activation of ETI (Shao *et al*., 2003). Recently, it has been suggested that the virulence target of AvrPphB may in fact be another RLCK, the PTI component BIK1 (*Botrytis*-induced kinase; (Zhang *et al.*, 2010)). This hypothesis is based on the observation that not only does AvrPphB cleave BIK1, as well as other RLCKs, including PBL1 (PBS1-like 1), but also that cleavage in the absence of RPS5 results in a significant reduction in PTI responses. It should be noted, that while the *bik1/pbl1* double mutant does have significant reductions in many PTI responses, *bik1/pbl1* does

not exhibit reduced MPK3/6 phosphorylation upon flg22 stimulation (Zhang *et al.*, 2010; Feng *et al.*, 2012).

In the current study, we report the identification of a reduction in the expression and accumulation of *RPS5* mRNA in the absence of ADF4. In total, our data demonstrate that this reduction results in the down-regulation of PTI-signaling in the presence of the bacterial effector AvrPphB. Additionally, we demonstrate this reduction in PTI-signaling is due in part to an ADF4-dependent abrogation of the MPK3/6 branch of the MAPK pathway. From the standpoint of cellular dynamics and the activation of ETI, expression of *RPS5* was restored in an ADF4 phosphorylation-dependent manner, demonstrating a link between ADF4 phosphorylation, activity (e.g., F-actin binding), *RPS5* mRNA accumulation and subsequent resistance signaling. In addition to elucidating the signaling cascade from perception through MAPK activation, we identified a link between reduced actin cytoskeleton co-localization of ADF4 and the activation of RPS5-mediated resistance in a phosphorylation-dependent manner. In total, the work presented herein represents the first identification of linkage between the actin cytoskeleton, the dynamic control of ADF4, and the regulation of a resistance gene transcription.

Results

ADF4 is required for *RPS5* expression

Previous work has shown that Arabidopsis Actin-Depolymerizing Factor-4 (ADF4) is required for resistance to *Pst* AvrPphB, however, the biochemical and genetic mechanism(s) associated with activation were largely undefined (Tian *et al.*, 2009). To elucidate the signaling cascade leading from the recognition of AvrPphB to the activation of resistance, we first investigated the expression of the resistance (*R*) gene (i.e., *RPS5*) required for the recognition of AvrPphB. As shown in Figure 2.1A, we found a significant reduction (~250-fold) in the accumulation of *RPS5* mRNA in the *adf4* mutant compared to wild-type Col-0. It was further determined that there is no significant alteration in the expression of *ADF4* in Col-0 during the course of infection with *Pst* AvrPphB (Figure 2.2). To address the possibility of positional effects in the *adf4* T-DNA SALK line, Tian et al. (Tian *et al.*, 2009) demonstrated that complementation of the *adf4* mutant with native promoter-driven *ADF4* restored resistance to *Pst* AvrPphB. Similarly, these lines also showed a restoration in mRNA expression of *RPS5* (Figure 2.1B). The expression of *RPS5* in a second *ADF* mutant, *adf3,* was not altered (Figure 2.1B), confirming that the loss of resistance is specific to *ADF4*, as previously reported (Tian *et al.*, 2009). To confirm that the loss of RPS5-mediated resistance in the *adf4* mutant is specific to RPS5, we transformed the *adf4* mutant with a RPS5-sYFP (*adf4*/35S:RPS5-sYFP; (Qi *et al.*, 2012)) to uncouple *RPS5* expression from native regulation. As shown in Figure 2.3, *RPS5* mRNA (Figure 2.3A) and HR-induced cell

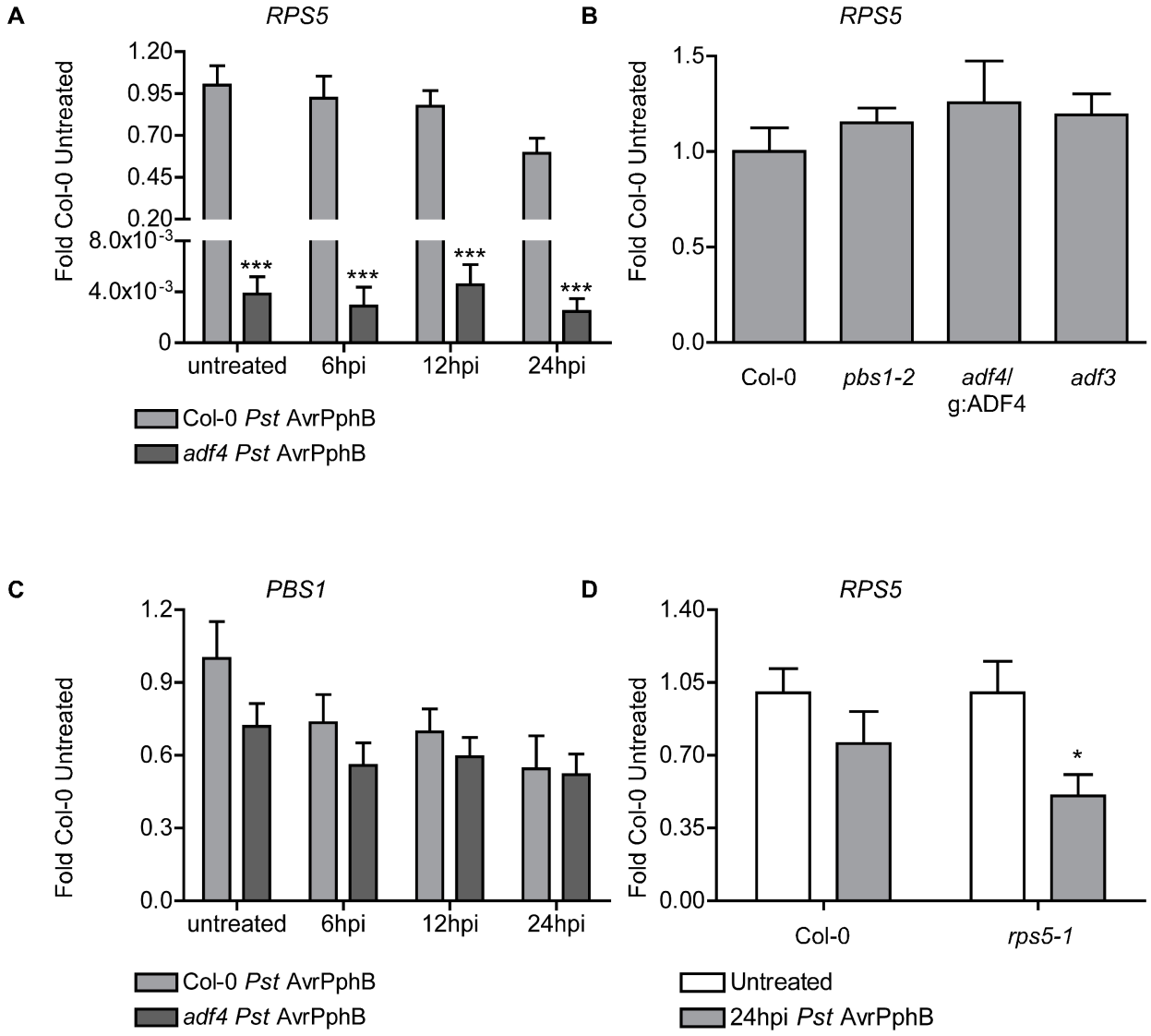


Figure 2.1. ADF4 is required for *RPS5* mRNA accumulation and resistance to *Pseudomonas syringae* expressing the cysteine protease effector AvrPphB. Time-course of mRNA accumulation of (A) *RPS5* and (C) *PBS1* in Col-0 and *adf4* mutant plants following dip inoculation with *Pst* AvrPphB. (B) Expression levels of *RPS5* in Col-0, *pbs1*, *adf4*/g:ADF4, and *adf3*. (D) *RPS5* mRNA accumulation in Col-0 and *rps5-1*, comparing each to their basal untreated levels at 24 hpi with *Pst* AvrPphB. Error bars represent mean ± SEM from two technical replicates of two independent biological repeats (n = 4). Statistical significance was determined using two-way ANOVA as compared to Col-0, with Bonferroni post test, where *p<0.05 and ***p<0.001. hpi = hours post inoculation.

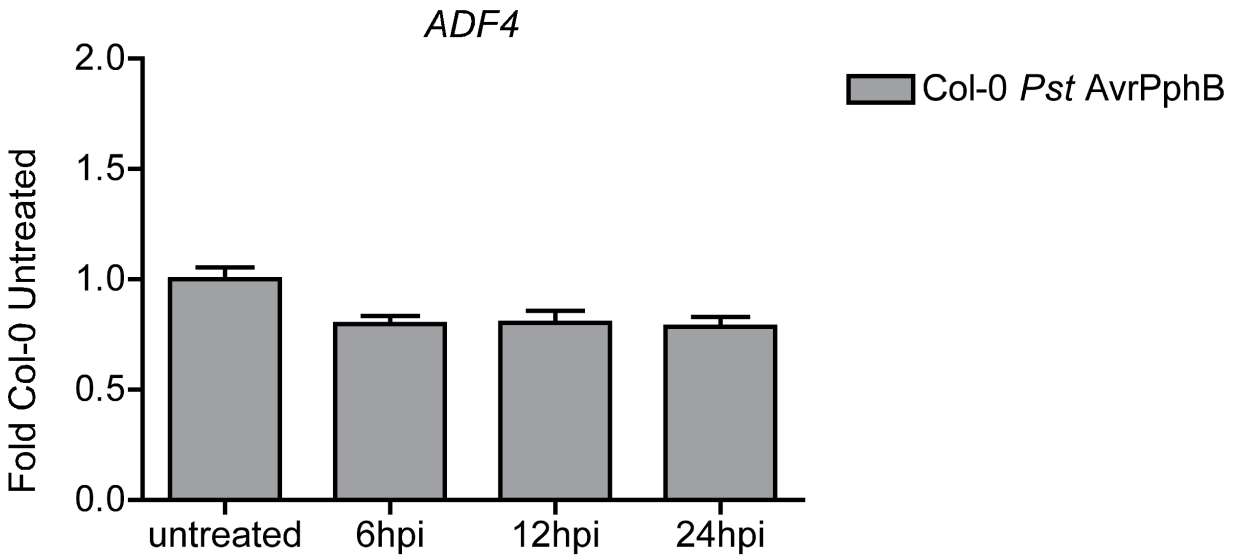
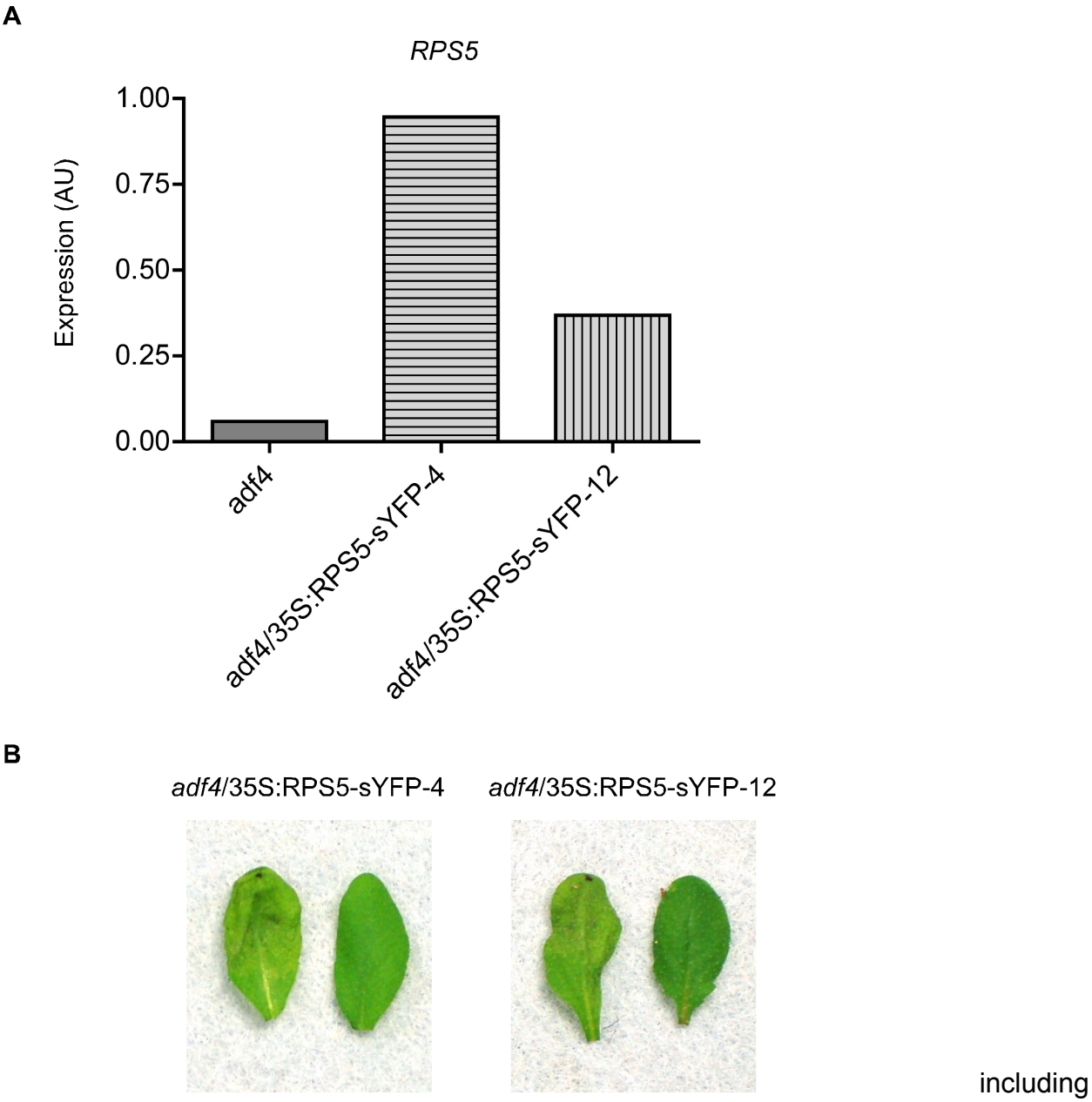


Figure 2.2. *ADF4* expression does not change during the course of infection with *Pseudomonas syringae* expressing AvrPphB. The expression levels of *ADF4* in Col-0, over time, when inoculated with *Pseudomonas syringae* expressing AvrPphB (*Pst* AvrPphB). Error bars represent mean ± SEM from two technical replicates of two independent biological replicates (n = 4). hpi = hours post inoculation. An unpaired student t-test with a 95% confidence interval was performed to determine if change over time was significant, where p>0.05 is considered not significant.

death following AvrPphB recognition (Figure 2.3B) was restored. Taken together, these data demonstrate a direct and specific requirement of ADF4 for RPS5-mediated resistance.

To determine the specificity of the ADF4-*RPS5* genetic interaction, we investigated if the mRNA expression of additional Arabidopsis *R*-genes are altered in the *adf4* mutant. To this end, we examined the expression of *RPS2* (Kunkel *et al.*, 1993), *RPM1* (Grant *et al.*, 1995), *RPS4* (Gassmann *et al.*, 1999) and *RPS6* (Kim *et al.*, 2009). As an additional measure, we monitored the mRNA accumulation of *NDR1* (non race-specific disease resistance-1; (Century *et al.*, 1997; Knepper *et al.*, 2011a; Knepper *et al.*, 2011b)), a required component of most CC-NB-LRR defense signaling pathways in Arabidopsis,



including

Figure 2.3. Expression of 35S:RPS5-sYFP in *adf4* recovers the Hypersensitive Response. (A) *RPS5* expression in two *adf4* mutant-complemented lines expressing 35S:RPS5-sYFP, *adf4*/35S:RPS5-sYFP-4 and *adf4*/35S:RPS5-sYFP-12. (B) Hypersensitive Response (HR) in *adf4*/35S:RPS5-sYFP-4 and *adf4*/35S:RPS5-sYFP-12 when challenged with *Pseudomonas syringae* expressing AvrPphB (*Pst* AvrPphB; left) and untreated (right).

RPS5. As shown in Figure 2.4, we did not observe a reduction in the resting levels of these mRNAs in the *adf4* mutant. To confirm that increased susceptibility and the loss

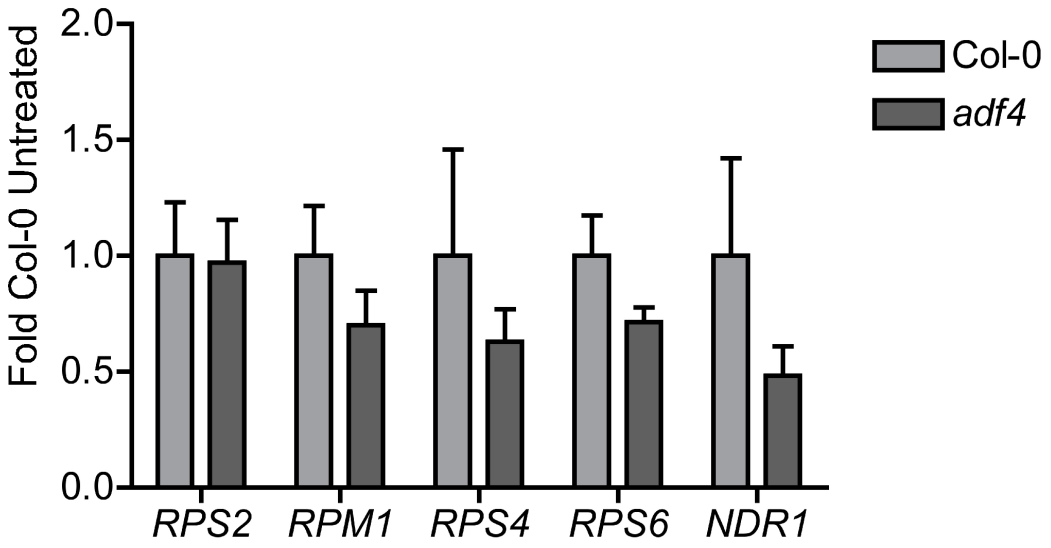


Figure 2.4. The *adf4* mutant does not have altered expression of other resistance genes. The mRNA expression levels of *RPS2, RPM1, RPS4, RPS6* and *NDR1* in Col-0 and *adf4*. Error bars represent mean ± SEM from two technical replicates of two independent biological replicates (n = 4). hpi = hours post inoculation.

of the HR in the *adf4* mutant is due to altered expression of *RPS5* (i.e., mRNA reduction) and not a reduction in the expression of the AvrPphB cleavage target PBS1 (Warren *et al.*, 1999; Swiderski & Innes, 2001; Innes, 2003; Shao *et al.*, 2003b; Ade *et al.*, 2007), the expression of *PBS1* mRNA was also measured. As shown in Figure 2.1C, we did not detect a significant difference between *PBS1* expression in the *adf4* mutant and Col-0. Additionally, there was no alteration of *RPS5* mRNA expression in the functional PBS1 mutant, *pbs1-2* (Swiderski & Innes, 2001; Figure 2.1B).

Our data present a role for ADF4 in the expression of *RPS5*, but not for the expression of *PBS1*, suggesting the loss of ETI in the *adf4* mutant may be a direct result of reduced *RPS5* expression (Figure 2.1A, Figure 2.1C). However, whether a role for AvrPphB in the down-regulation of *RPS5* expression exists is unknown. In order to address this

question, we measured the expression of *RPS5* in both Col-0 and the RPS5 point-mutant, *rps5-1*, generated by EMS and thus while inactive is expressed at wild-type levels and therefore is able to be quantitated by qRT-PCR; the rationale being that if AvrPphB negatively regulates the expression of *RPS5*, its expression should be reduced in the absence of the activation of ETI (Warren *et al*., 1999). In support of this hypothesis, as shown in Figure 2.1D, we observed a significant reduction in *RPS5* expression in *rps5-1* at 24 hpi following inoculation with *Pst AvrPphB*.

The virulence activity of AvrPphB blocks MAPK signaling in *adf4*

Based on our observations above, we hypothesize that absence of RPS5-derived ETI in *adf4* is most likely due to the reduced expression of *RPS5*. Based on this, and given the significant overlap in signaling of ETI and PTI, particularly with regard to AvrPphB activity (Lu *et al.*, 2010; Zhang *et al.*, 2010; Zhang & Zhou, 2010), we asked if PTI signaling is affected in the *adf4* mutant. To address this question, we first monitored the activation of *FRK1* expression, a transcriptional marker for FLS2 activation (Asai *et al.*, 2002), in wild-type (WT) Col-0, *adf4* and *rps5-1*. As shown in Figure 2.5A, when Col-0, *adf4* and *rps5-1* plants were treated with flg22, no significant changes in *FRK1* mRNA expression were observed, and mock infiltration did little to activate *FRK1* (Figure 2.5A, Figure 2.5B). As a second, complementary analysis of the fidelity of PTI-based signaling responses in the *adf4* mutant, we also monitored root growth inhibition in the presence of flg22 (Chinchilla, 2007; same as in the methods section). As shown in Figure 2.6, we did not observe a significant difference in root growth in *adf4* in the presence of flg22 as

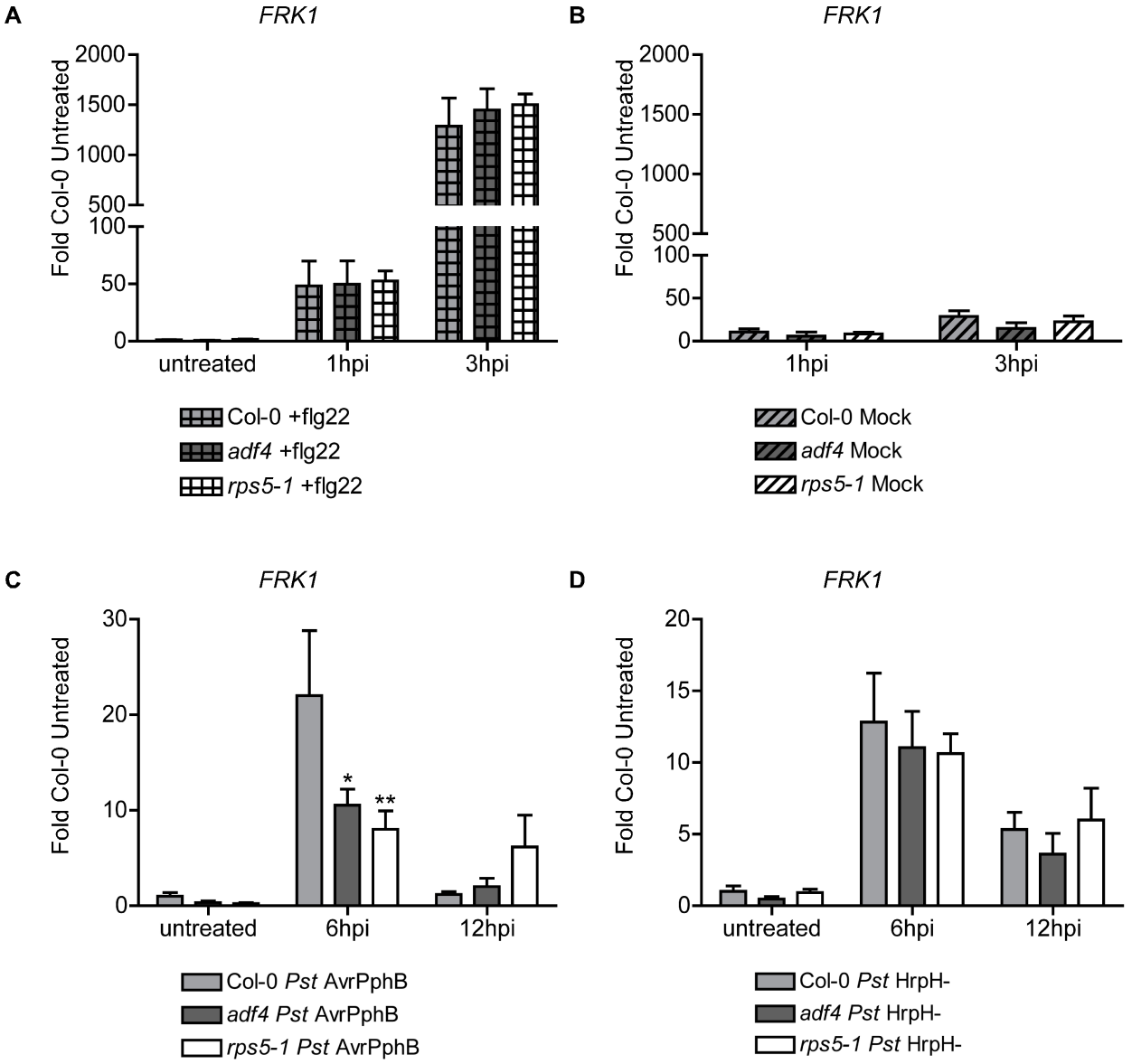


Figure 2.5. Flg22-induced receptor kinase 1 expression in the *adf4* mutant is reduced when the effector protein AvrPphB is expressed *in planta*. Relative expression levels of *FRK1* mRNA in Col-0, *adf4*, and *rps5-1* plants when treated with (A) 10 μM flg22, (B) mock inoculated with $MgCl_2$ by hand infiltration (C) *Pst AvrPphB*, or (D) the *hrpH*$^-$ (*Pst hrpH*$^-$). Error bars represent mean ± SEM from two technical replicates of two independent biological repeats (n = 4). Statistical significance was determined using two-way ANOVA, as compared to Col-0, with Bonferroni post test where *p<0.05 and **p<0.005. hpi = hours post-inoculation.

compared to Col-0. In total, these data demonstrate that flg22-induced PTI-signaling is functional in both the *rps5-1* and *adf4* mutants. As an additional measure to ensure that the technique employed in Figure 2.5A and B did not have an adverse effects on *RPS5*

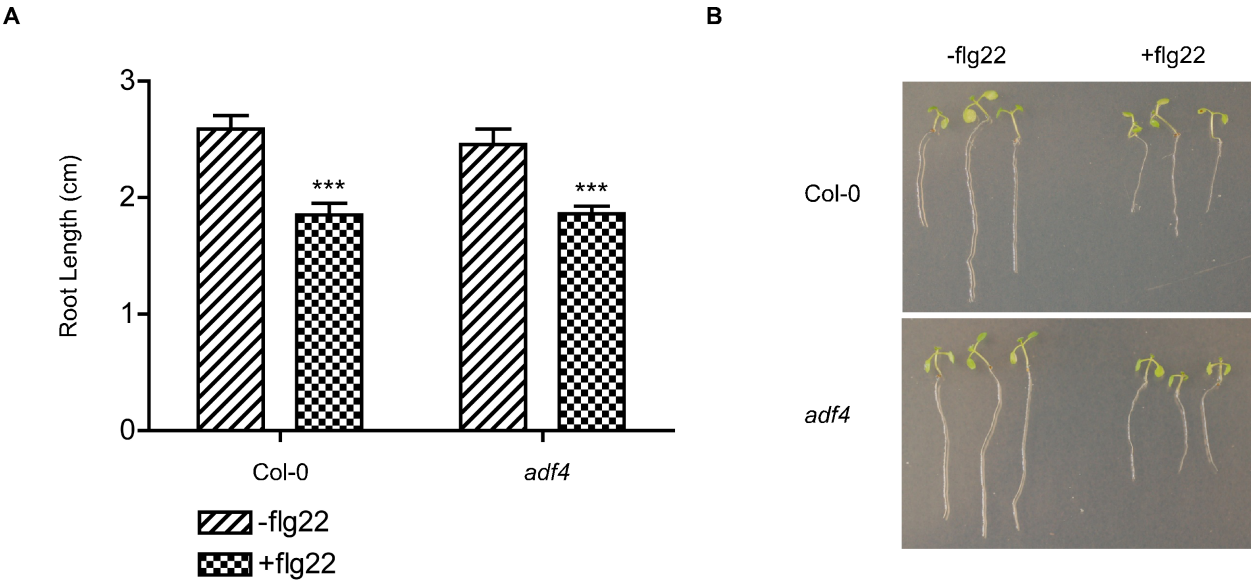


Figure 2.6. *adf4* mutants are sensitive to fl22 in root length assay. (A) Graphical representation of root lengths of Col-0 and *adf4* grown 10 days in the presence (+flg22) or absence (-flg22) of 10 nM flg22. Error bars represent mean ± SEM from two independent biological replicates (n = 32-46). Statistical significance was determined using two-way ANOVA, with Bonferroni post test, where ***p<0.001. (B) Col-0 and *adf4* seedlings grown for 10 days ± 10 nM flg22.

mRNA expression in either Col-0 or *adf4, RPS5* mRNA was monitored following hand-infiltration with either flg22 or mock (i.e., buffer alone). As shown in Figure 2.7A, we observed that flg22-induced expressional changes of *RPS5* mRNA was similar to that of mock, thus assuring the observed activation of *FRK1* in Col-0 and *adf4* (Figure 2.5A) can be attributed specifically to flg22, and is independent of the infiltration technique (Figure 2.5B), or changes in *RPS5* expression (Figure 2.7A).

Recent work from Zhang et al. (2010) suggests that *FRK1* mRNA accumulation is reduced in the *rps5-1* mutant following flg22 treatment of protoplasts expressing AvrPphB. This raises the question of the relationship between the activation of PTI-signaling in parallel with the activation of ETI. To investigate the downstream signaling

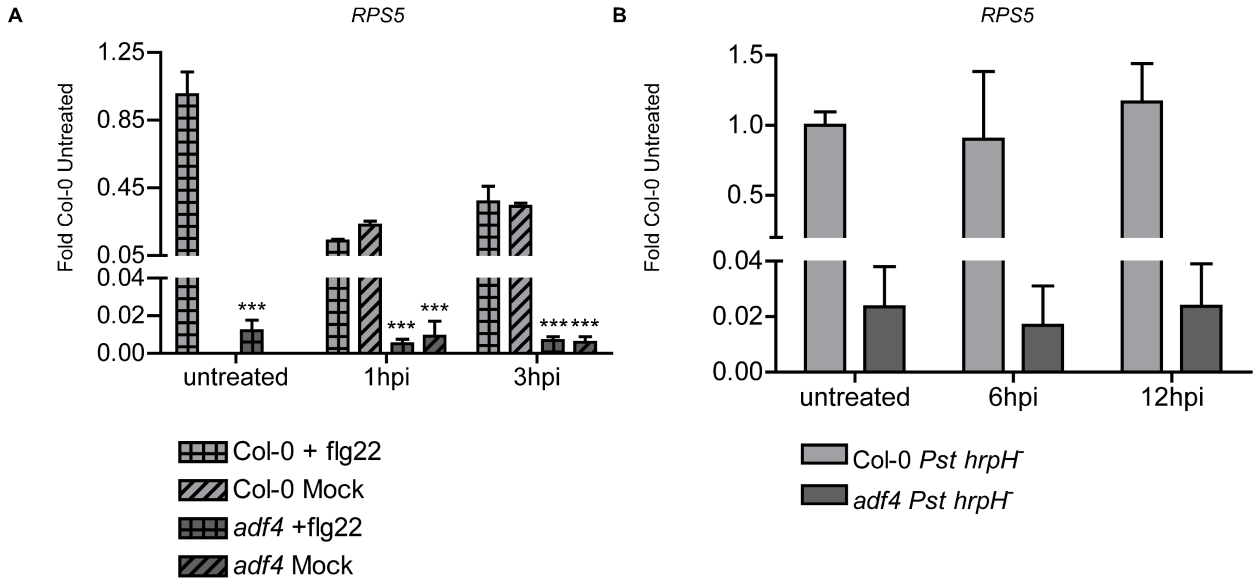


Figure 2.7. Expression of *RPS5* mRNA is not affected by treatment with flg22, or by inoculation with the *hrpH*⁻ mutant of *Pseudomonas syringae*. Real-time PCR analysis of *RPS5* mRNA accumulation in Col-0 and *adf4* following (A) flg22 treatment, mock inoculation or (B) dip-inoculation with the *hrpH*⁻ mutant of *Pseudomonas syringae* (*Pst hrpH*⁻). Expression was determined by qRT-PCR, utilizing amplification of *UBQ10* as an endogenous control. Error bars, representing mean ± SEM, were calculated from two technical replicates of two independent biological repeats (n = 4). Statistical significance was determined using two-way ANOVA as compared to Col-0, with Bonferroni post test, where ***p<0.001. hpi = hours post inoculation.

response(s) associated with the activation of RPS5-mediated resistance, we measured the expression of *FRK1* mRNA accumulation in Col-0, *adf4*, and *rps5-1* when inoculated with *Pst* AvrPphB. As shown in Figure 2.5C, we observed a significant decrease in *FRK1* mRNA expression in both the *adf4* and *rps5-1* mutants, as compared to Col-0, at 6 hpi with *Pst AvrPphB*. Coupled with the results of Zhang et al. (Zhang *et al.*, 2010), this would suggest that the *adf4* mutant has a decreased level of RPS5. In support of this, we did not detect a significant difference between *FRK1* expression in the *adf4* and *rps5-1* mutants when inoculated with flg22 (Figure 2.5A), demonstrating that the mutants had equivalent signaling potential following to FLS2 activation, and that

ultimately, the reduction in *FRK1* expression is a direct result of a loss in ETI, most likely due to a reduction in *RPS5* mRNA expression and accumulation (Figure 2.1A).

It is possible that our observations described above could be an indirect result of cross-talk of PTI response signaling pathways in *adf4* and *rps5-1* in the presence of *Pst*. To test this, *FRK1* mRNA expression in Col-0, *adf4* and *rps5-1* following inoculation with the type three secretion system (T3SS) mutant *Pst hrpH*$^{-}$ was assessed to differentiate PTI from ETI in the *ADF4-RPS5* signaling node. As shown in 2.2D, we detected no difference in *FRK1* mRNA expression between Col-0, *adf4* or *rps5-1*. Additionally, *RPS5* mRNA expression following *Pst hrpH*$^{-}$ inoculation (Figure 2.7B) and elf18-induced PTI-signaling in Col-0 and *adf4* (Figure 2.8) further supports these observations. When challenged with *Pst* expressing the catalytically inactive AvrPphB-C98S isoform (Ade *et al*., 2007; Shao *et al*., 2003), both WT Col-0 and the *adf4* mutant showed increased expression levels of *FRK1* mRNA, in agreement with previously published data (Zhang *et al.*, 2010; Figure 2.9A). A loss of induction of the HR in Col-0, *adf4* and *rps5-1* when challenged by *Pst*-AvrPphB-C98S variant (Shao *et al.*, 2003a) confirms the catalytic inactivity of AvrPphB-C98S (Figure 2.9B).

At this point, we reasoned that altered *FRK1* expression in both the *rps5-1* and *adf4* mutants is due to a specific block in the MAPK signal cascade, most likely a function of the virulence activity of AvrPphB in the absence of ETI. To examine MAPK activation in the presence of both flg22 and AvrPphB, in the absence of pathogen, Col-0, *adf4* and *rps5-1* plants were transformed with an estradiol-inducible AvrPphB construct (i.e., Col-

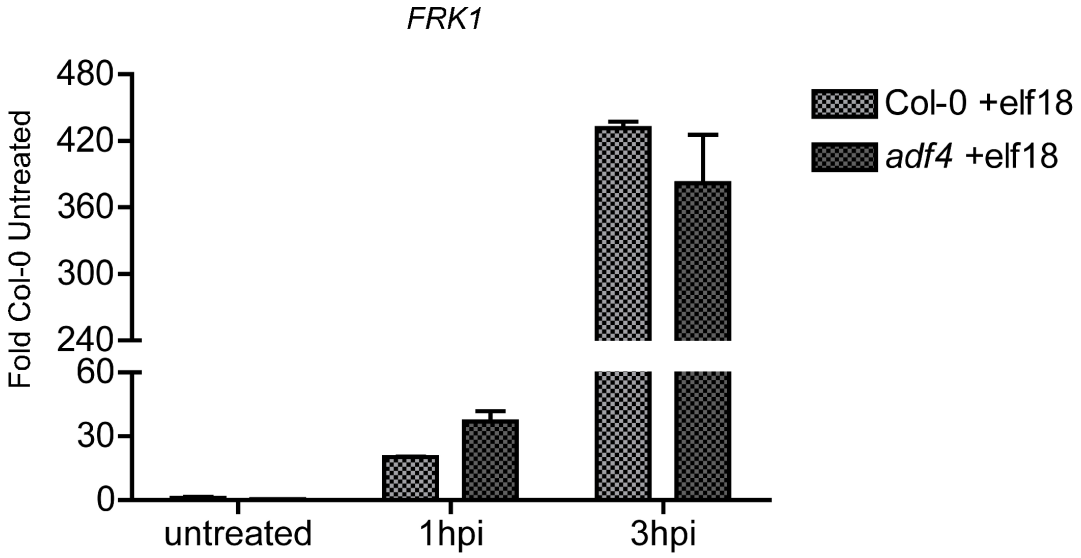


Figure 2.8. Both Col-0 and *adf4* have induced *FRK1* expression when treated with elf18. Relative expression levels of *FRK1* in Col-0 and *adf4* mutant plants, hand infiltrated with elf18. All expression values were determined by qRT-PCR, with amplification of *UBQ10* as an endogenous control. Error bars, representing mean ± SEM, are representative of two technical replicates of one biological repeat (n = 2). hpi = hours post inoculation.

0/pER8:AvrPphB, *adf4*/pER8:AvrPphB and *rps5-1*/pER8:AvrPphB) to enable us to monitor the interplay between flg22 perception (i.e., PTI) and AvrPphB (i.e., ETI). As shown in Figure 2.10A and Figure 2.10C, when phosphorylation of both MPK3 and MPK6 was measured in response to flg22, a significant reduction in *adf4*/pER8:AvrPphB was observed as compared to Col-0 at 10 minutes; this reduction was not observed in *adf4*, and Col-0/pER8:AvrPphB. Interestingly, no significant reduction of MPK3 and MPK6 was observed in the *rps5-1*/pER8:AvrPphB 10 minutes after flg22 treatment (Figure 2.10B and Figure 2.10C). This observation suggests a potential combinatory role for ADF4 in both the expression of *RPS5* (Figure 2.1A), resulting in reduced PTI-signaling (Figure 2.5C), as well as in the proper regulation of MAPK-signaling in the presence of AvrPphB (Figure 2.10A and Figure 2.10C). Estradiol induction of *AvrPphB* is shown in Figure 2.11.

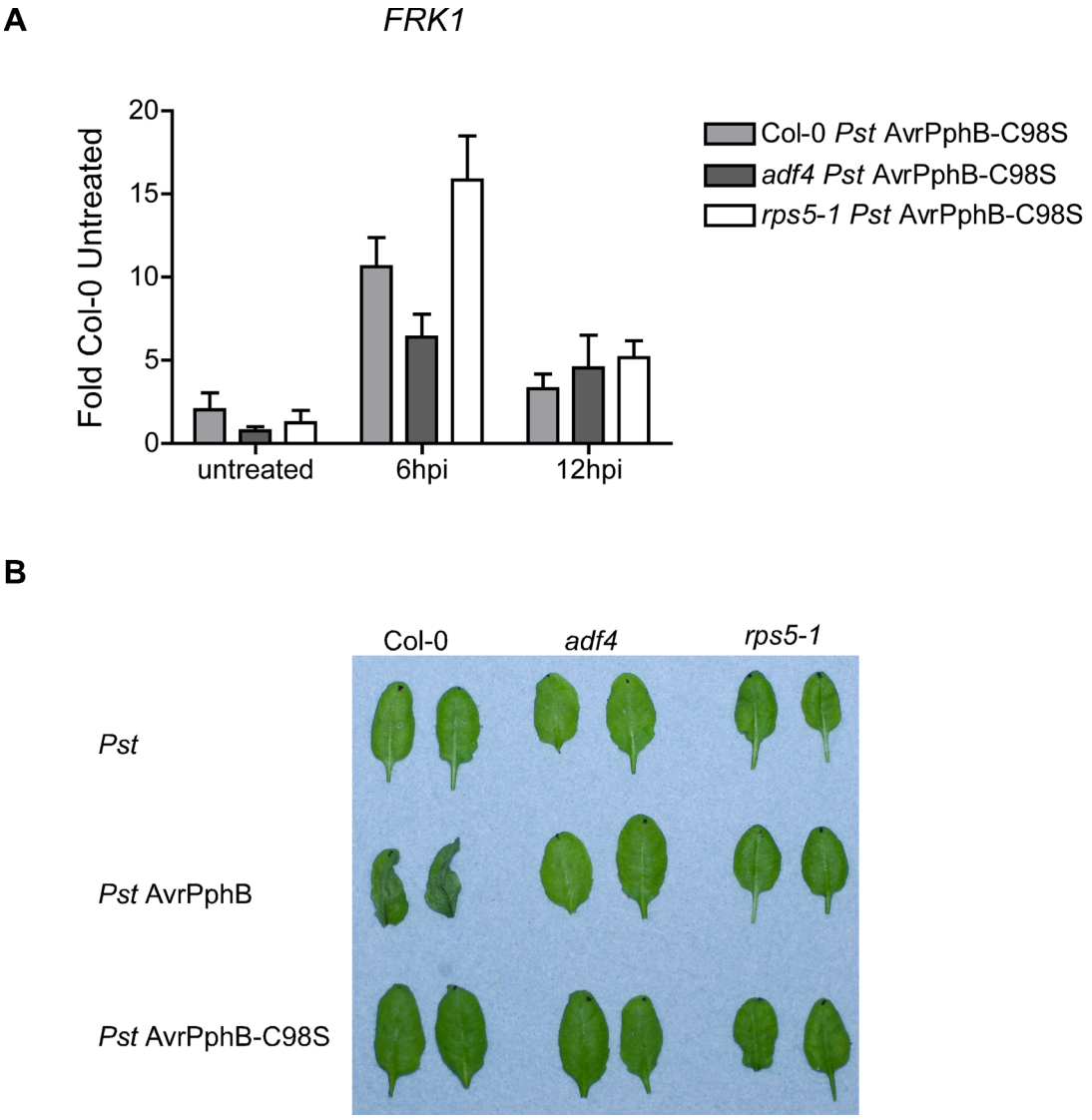


Figure 2.9. Increased *FRK1* expression in Col-0 and *adf4* when challenged by *Pst* AvrPphB-C98S, and HR phenotypes in Col-0, *adf4*, and *rps5-1*. (A) The expression levels of *FRK1* in Col-0, *adf4* and *rps5-1* following dip-inoculation with *Pseudomonas syringae* expression the AvrPphB catalytic mutant C98S (*Pst* AvrPphB-C98S). All expression values were determined by qRT-PCR, with amplification of *UBQ10* as an endogenous control. Error bars, representing mean ± SEM, are representative of two technical replicates of three biological replicates (n = 6). hpi = hours post inoculation. (B) HR phenotypes in Col-0, *adf4* and *rps5-1* when hand inoculated with *Pst* AvrPphB-C98S.

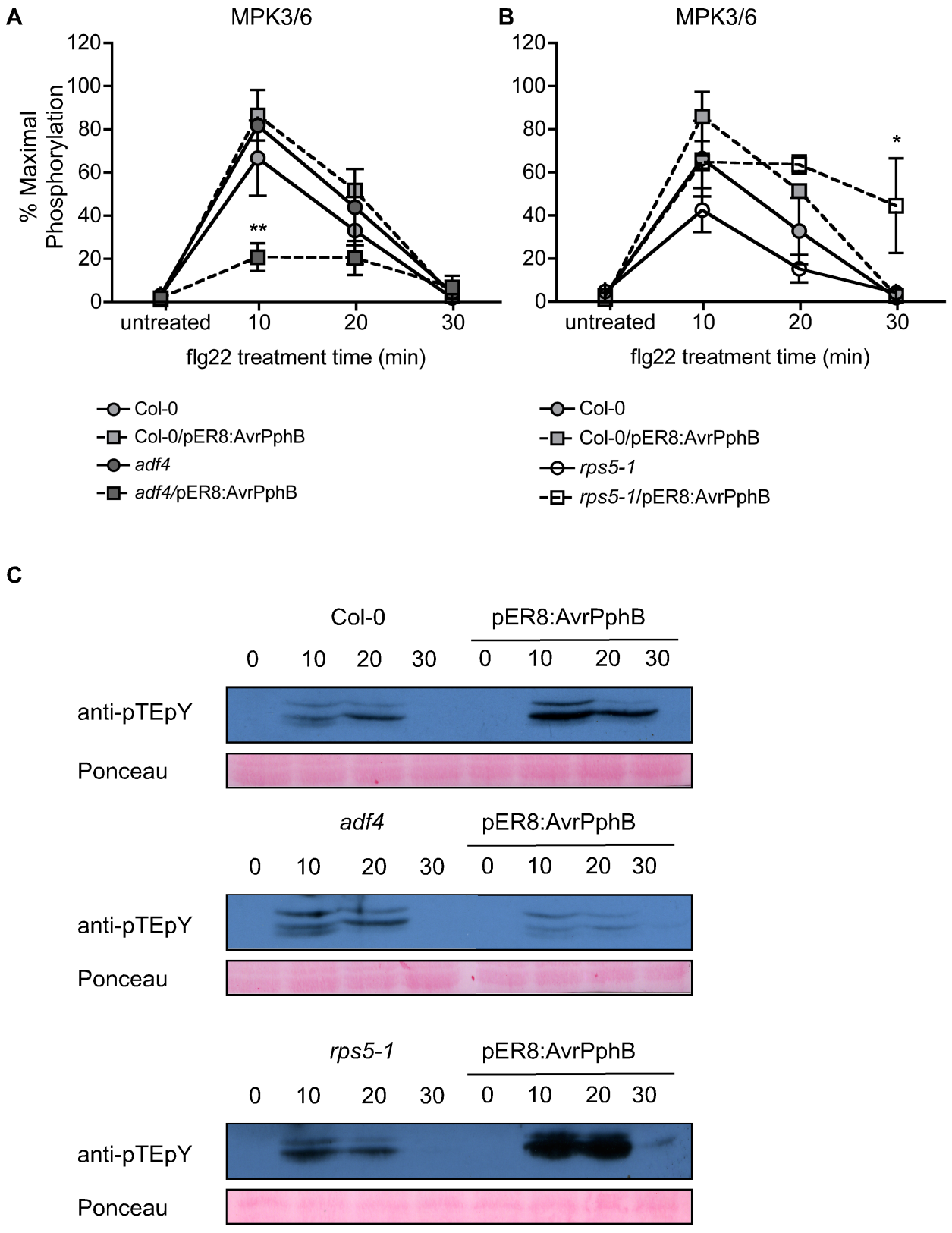


Figure 2.10. Mitogen Activated Protein Kinase (MAPK) phosphorylation is reduced in the *adf4* mutant in the presence of AvrPphB.

Figure 2.10 (con'd). Mitogen Activated Protein Kinase (MAPK) phosphorylation is reduced in the *adf4* mutant in the presence of AvrPphB. (A) Percent maximal phosphorylation of the MPK3/6 TEY motif in Col-0 and the *adf4* mutant, +/- *AvrPphB*, followed by 1 μM flg22 treatment. (B) Percent maximal phosphorylation of the MPK3/6 TEY motif in Col-0 and the *rps5-1* mutant, +/- *AvrPphB*, followed by 1 μM flg22 treatment. *AvrPphB* expression was induced at 48 h pre-treatment with 100 μM estradiol in Col-0, *adf4* and *rps5-1* mutant plants containing an estradiol-inducible *AvrPphB* transgene (pER8:*AvrPphB*). Statistical significance was determined using two-way ANOVA as compared to Col-0 untreated, with Bonferroni post test, where *p<0.05, **p<0.005, n = 3. (C) Western blot analysis of MPK3/6 TEY phosphorylation.

Phosphorylated ADF4 is required for *RPS5* expression and subsequent activation of resistance

ADF4-mediated actin depolymerization is regulated in large part by the phosphorylation status of ADF. Indeed, previous work has demonstrated that mammalian cofilin/ADF activity is regulated by phosphorylation at serine-3, and that de/phosphorylation at this residue is responsible for the regulating the activation of actin depolymerization (Yang *et al.*, 1998). In plants, a direct correlation between the phosphorylation status of ADF and its function has not been demonstrated; however, ADF4 function is presumed to be regulated in a manner similar to that of mammalian cofilin (Yang *et al.*, 1998; Allwood *et al.*, 2002; Shvetsov *et al.*, 2009). Herein, we demonstrate for the first time that Arabidopsis ADF4 is indeed phosphorylated at serine-6, and that the phosphorylation status directly correlates with its activity and function of actin cytoskeletal dynamics. ADF4 and the phospho-null ADF4_S6A (i.e., serine-6 to alanine) plant lines were generated by expressing T7:ADF4 and T7:ADF4_S6A in the *adf4* mutant under the control of a constitutive promoter (*adf4*/35S:ADF4 and *adf4*/35S:ADF4_S6A). As shown in Figure 2.12A, after 2D isoelectric focusing (IEF) and SDS PAGE, native ADF4 shows

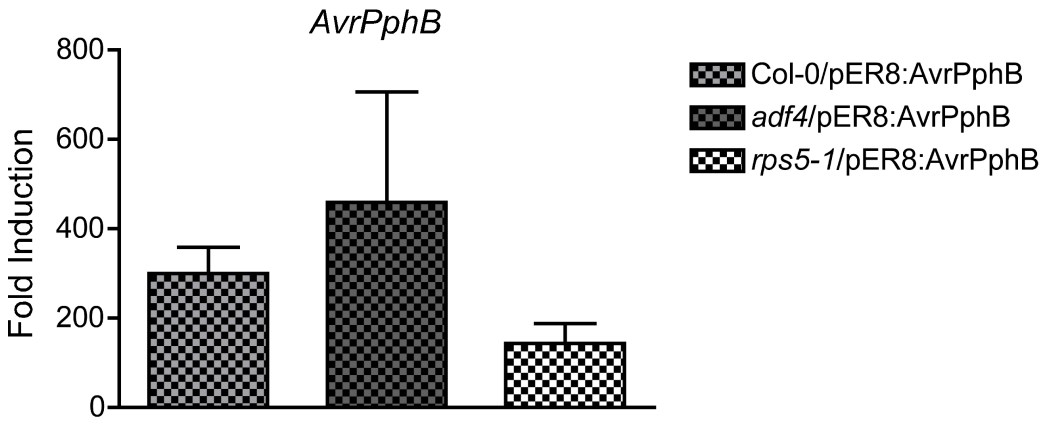


Figure 2.11. Estradiol-inducible expression of *avrPphB* in Col-0, *adf4* and *rps5-1*. Induction of *avrPphB* expression in Col-0, *adf4* and *rps5-1* plants containing the estradiol-inducible *avrPphB* construct pER8:AvrPphB following 48 h pre-treatment with 100 μM estradiol. Expression values were determined by quantitative real-time PCR (qRT-PCR), with amplification of *UBQ10* as an endogenous control. Error bars, representing mean ± SEM, are representative two technical replicates of one biological repeat (n = 2).

a differential IEF profile than the phospho-null ADF4_S6A. In order to determine if phosphorylation of ADF4 affects *RPS5* expression, an additional phosphorylation isoform line was generated: a phospho-mimic isotype, reflecting a serine to aspartic acid change at amino acid position 6 (i.e., S6D) expressed in the *adf4* mutant background (*adf4*/35S:ADF4_S6D). As shown in Figure 2.12B, the phosphomimetic isoform, *adf4*/35S:ADF4_S6D, restored *RPS5* mRNA expression, while the phospho-null isoform, *adf4*/35S:ADF4_S6A, did not. A second independent transgenic Arabidopsis line expressing the ADF4 phosphorylation mutants were generated and tested for *RPS5* expression to ensure that altered mRNA expression was not due to a positional transgene insertion effect (Figure 2.13A).

To confirm that the ADF4 phosphomimetic constructs were functional in their ability to restore resistance in the *adf4* mutant, the induction of HR and disease phenotypes, as

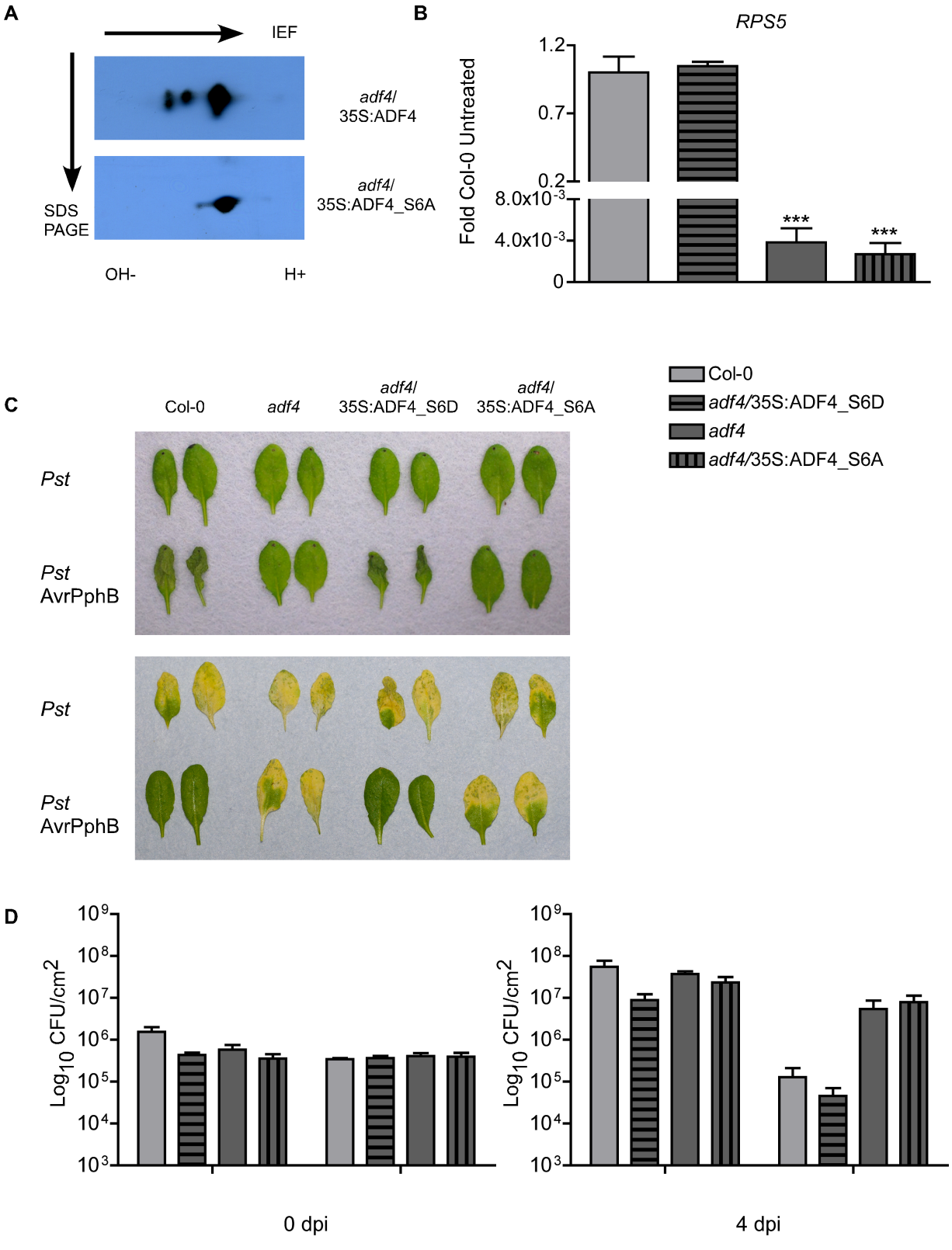


Figure 2.12. Phosphorylation of ADF4 is required for *RPS5* mRNA expression.

Figure 2.12 (cont'd). Phosphorylation of ADF4 is required for *RPS5* mRNA expression. (A) Western blot of isoelectric focusing (IEF) and SDS PAGE analysis of wild type ADF4 (upper) and phospho-null ADF4_S6A (lower). Arrows indicate direction of IEF and SDS PAGE. (B) The relative expression levels of *RPS5* were determined by qRT-PCR. (C) HR phenotypes at 22 hours after bacterial infiltration (upper), disease phenotypes at 4 dpi (lower). (D) Enumeration of bacterial growth at 0 and 4 dpi. HR and bacterial population experiments were repeated at least 3 times. Error bars, representing mean ± SEM, were calculated from two (A; n = 4) or three (D; n = 9) technical replicates of two independent biological repeats. Statistical significance was determined using two-way ANOVA, comparing *adf4* to Col-0, with Bonferroni post test, where *p<0.05; ***p<0.001. hpi = hours post inoculation; dpi = days post inoculation.

well as bacterial growth were assessed to determine the relationship between ADF4 phosphorylation and resistance activation through AvrPphB-RPS5. As shown in Figure 2.12C and 2.4D, inoculation of *adf4* mutant plants expressing the phosphomimetic (ADF4_S6D) with *Pst* AvrPphB restored the WT Col-0 resistance phenotype, both in terms of HR (Figure 2.12C, top panel), disease symptoms (Figure 2.12C, lower panel), and bacterial growth at 4 dpi (Figure 2.12D). Conversely, inoculation of the phospho-null-expressing plants (i.e., *adf4*/35S:ADF4_S6A) with *Pst* AvrPphB resulted in the absence of HR (Figure 2.12C, top panel), the development of disease symptoms (Figure 2.12C, lower panel), and an increased growth of the pathogen (Figure 2.12D), similar to that observed in the *adf4* mutant. As a control, to correlate transgene expression levels with our observations, the relative expression levels of both ADF4_S6A and ADF4_S6D were assessed by western blot to confirm that the observed restoration of *RPS5* with the phosphomimetic isoform was in fact due to the phosphorylation status and not an artifact of expression (Figure 2.13B). In total, our data confirms a restoration in resistance, as well as supports the hypothesis that phosphorylated ADF4 is required for resistance to *Pst* AvrPphB. Similarly, and in agreement our phosphorylation data, expression of *FRK1* following *Pst* AvrPphB

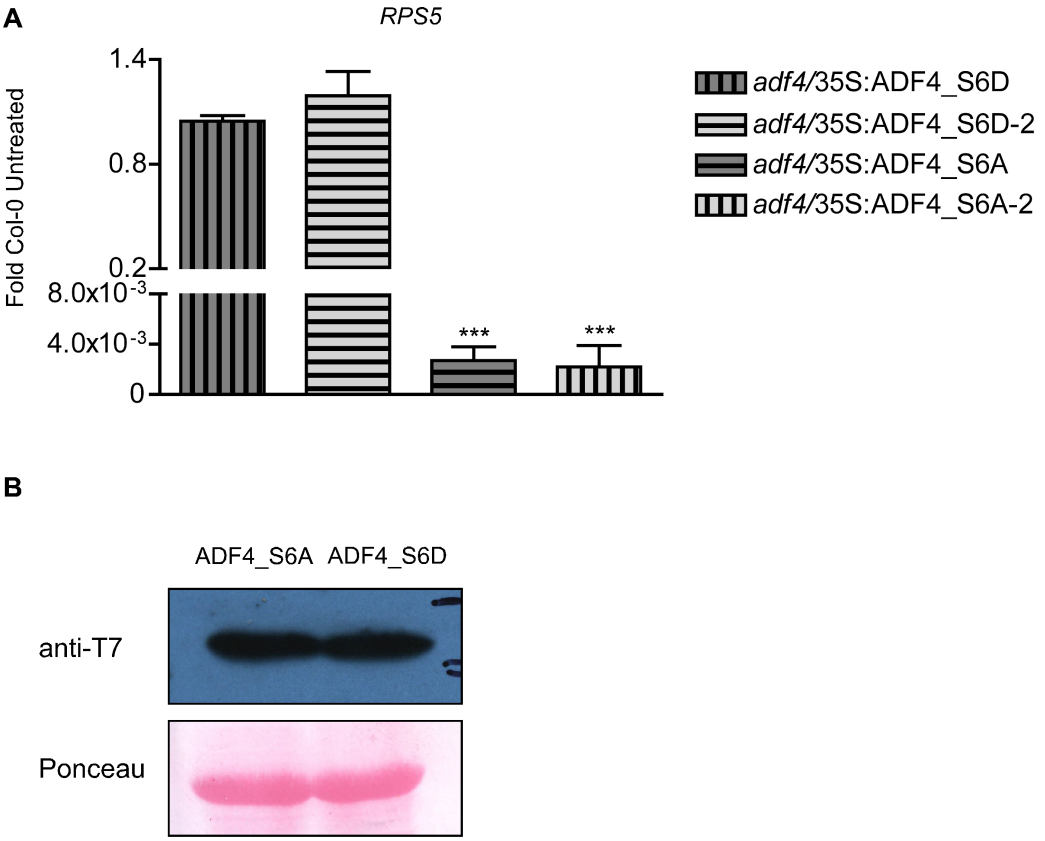


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Figure 2.13. *RPS5* mRNA expression in additional *adf4*/35S:ADF4_S6A and *adf4*/35S:ADF4_S6D lines confirm observed *RPS5* expression is not due to positional effects of the transgene nor disproportionate levels of protein levels of protein expression. (A) The expression level of *RPS5* in a second set of *adf4*/35S:ADF4_S6A (*adf4*/35S:ADF4_S6A-2) and *adf4*/35S:ADF4_S6D (*adf4*/35S:ADF4_S6D-2) transgenic lines, as compared to the first line shown in Figure 4A. All expression values were determined by quantitative real-time PCR (qRT-PCR), with amplification of *UBQ10* as an endogenous control. Error bars, representing mean ± SEM, are representative of two technical replicates of one biological repeat (n = 2). hpi = hours post inoculation. (B) Relative protein levels of ADF4_S6A and ADF4_S6D in *adf4*/35S:ADF4_S6A and *adf4*/35S:ADF4_S6D as determined by western blot when probed with anti-T7-HRP. Ponceau blot is shown to demonstrate equal loading.

noculation in the *adf4*/35S:ADF4_S6D mutant was similar to that observed in Col-0, whereas the *adf4*/35S:ADF4_S6A plants had an *FRK1* expression pattern similar to the *adf4* mutant (Figure 2.14).

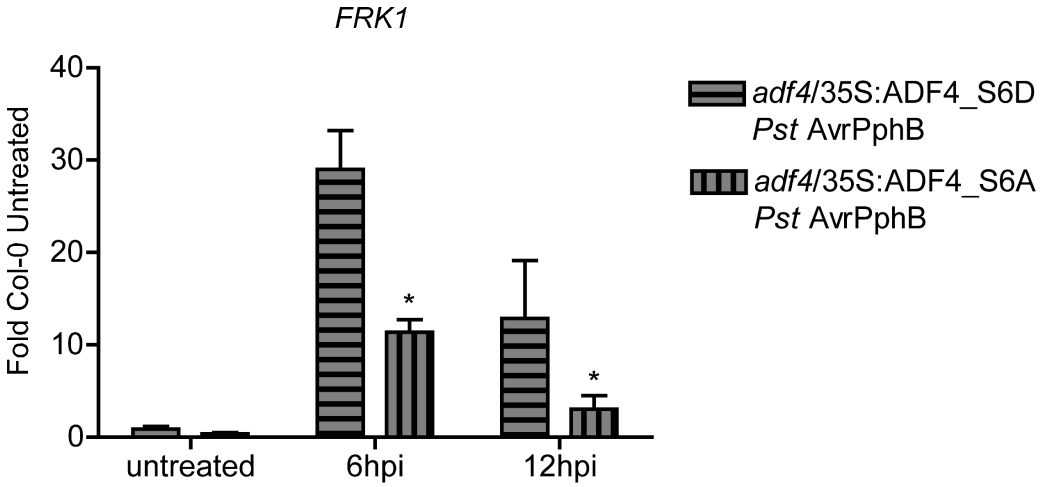


Figure 2.14. *FRK1* expression in *adf4*/35S:ADF4_S6A and *adf4*/35S:ADF4_S6D lines confirm link between *RPS5* expression and *FRK1* in the presence of *Pseudomonas syringae* expressing AvrPphB. Relative expression levels of *FRK1* mRNA following dip-inoculation with *Pseudomonas syringae* expressing AvrPphB (*Pst* AvrPphB) in *adf4*/35S:ADF4_S6A and *adf4*/35S:ADF4_S6D determined by quantitative real-time PCR (qRT-PCR), with amplification of *UBQ10* as an endogenous control. Error bars, representing mean ± SEM, are representative of two technical replicates of two independent biological replicates (n = 4). Statistical significance was determined using two-way ANOVA as compared to Col-0, with Bonferroni post test, where *p<0.05. hpi = hours post inoculation.

Phosphorylation of ADF4 reduces its co-localization with F-actin, but does not influence nuclear targeting

As shown above, phosphorylated ADF4 is required for the accumulation of *RPS5* mRNA, as well as for resistance signaling in response to *Pst* AvrPphB (Figure 2.12). Previous work has demonstrated the potential for nuclear localization of ADFs, supportive of a role for actin and ADFs in regulating gene transcription (Burgos-Rivera *et al.*, 2008; Kandasamy *et al.*, 2010; Meagher *et al.*, 2010). To this end, we sought to determine if translocation of ADF4 into the nucleus is dependent upon the phosphorylation status of ADF4. As shown in Figure 2.15A, we found that ADF4,

ADF4_S6A and ADF4_S6D are all present in the nucleus. These data would suggest that perturbation of *RPS5* expression in the *adf4*/35S:ADF4_S6A plants is not due to an inability of phospho-null ADF4 to enter the nucleus. However, the phospho-null ADF_S6A (ds-Red_ADF4) does show an increased co-localization with the actin cytoskeleton (filamentous Actin Binding Domain 2-GFP; fABD2-GFP), as well as the formation of filamentous like structures in the ADF4_S6A panel (Figure 2.15B). Conversely, phosphomimetic ADF4_S6D is more diffuse within the cytosol and has reduced co-localization with the actin cytoskeleton (Figure 2.15B).

To confirm our observations of a phosphorylation-specific alternation in the co-localization of our ADF4 isoforms (i.e., S6A *versus* S6D) with the actin cytoskeleton, we next performed a red-green analysis on the collected images, calculating the overlap coefficients, according to Manders (R). In short, this analysis will determine the actual overlap of the red/green signals in our collected images (Zinchuk & Grossenbacher-Zinchuk, 2011), providing an *in vivo* quantification of the co-localization of ADF4 with the actin cytoskeleton. As shown in Figure 2.15C, both ADF4_S6A and ADF4_S6D were found to have a significant R-value, 0.697 ± 0.009 and 0.701 ± 0.009 respectively, with significant differences in co-localization of ADF4_S6A and ADF4_S6D based on co-localization coefficients m_1 and m_2. For a red-green pairing, such as was performed in our analysis, m_1 refers to the fraction of red pixels co-localized with green pixels, while m_2 is the fraction of green pixels co-localized with red pixels. The m_1 values for ADF4_S6A and ADF4_S6D are 0.604 ± 0.032 and 0.485 ± 0.033 respectively, while the m_2 values are 0.250 ± 0.028 and 0.353 ± 0.030 (Figure 2.15C). The co-localization

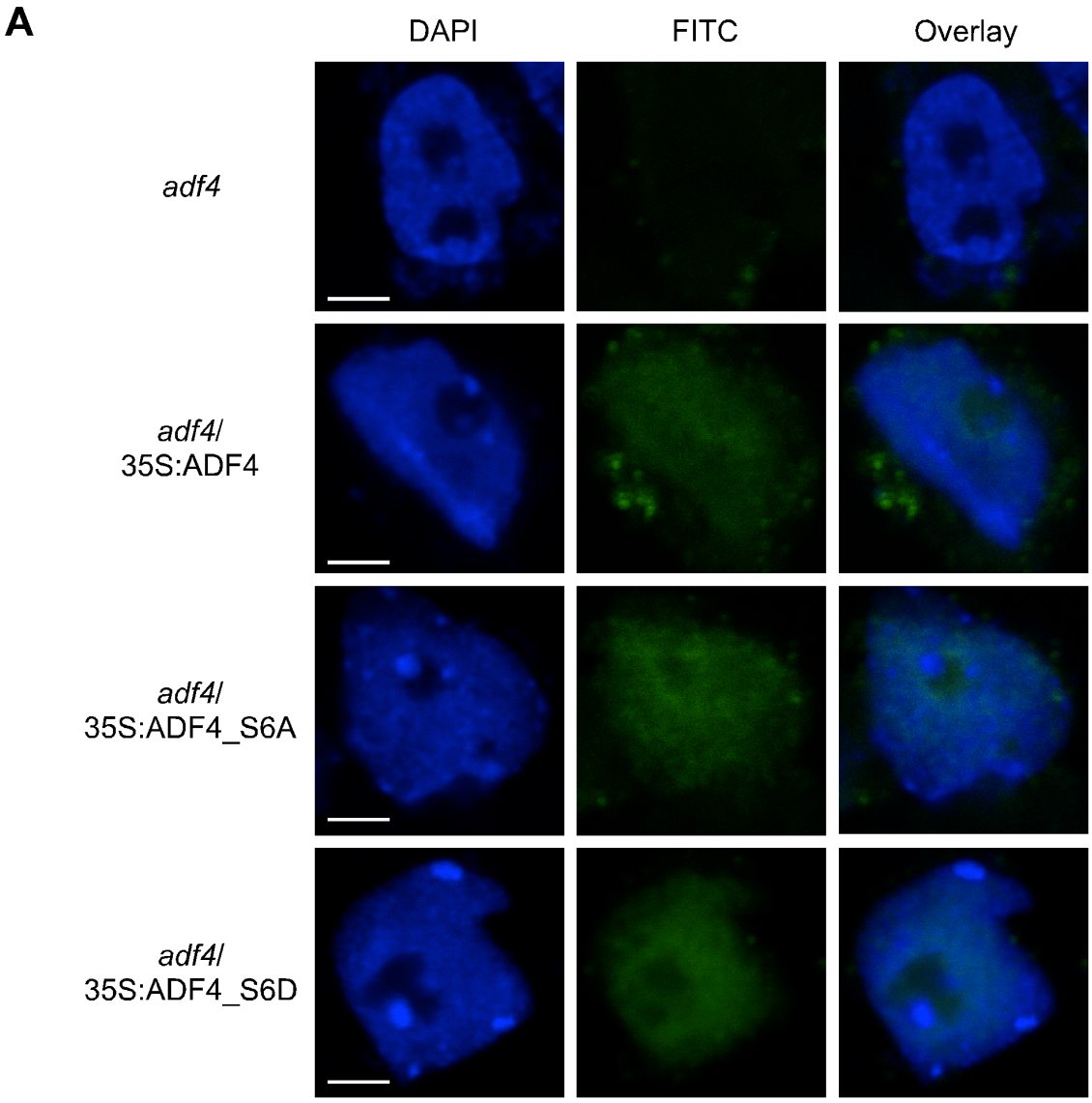


Figure 2.15. Confocal microscopy demonstrates phosphorylation of ADF4 affects cytoskeletal localization, but not nuclear localization.

Figure 2.15 (cont'd)

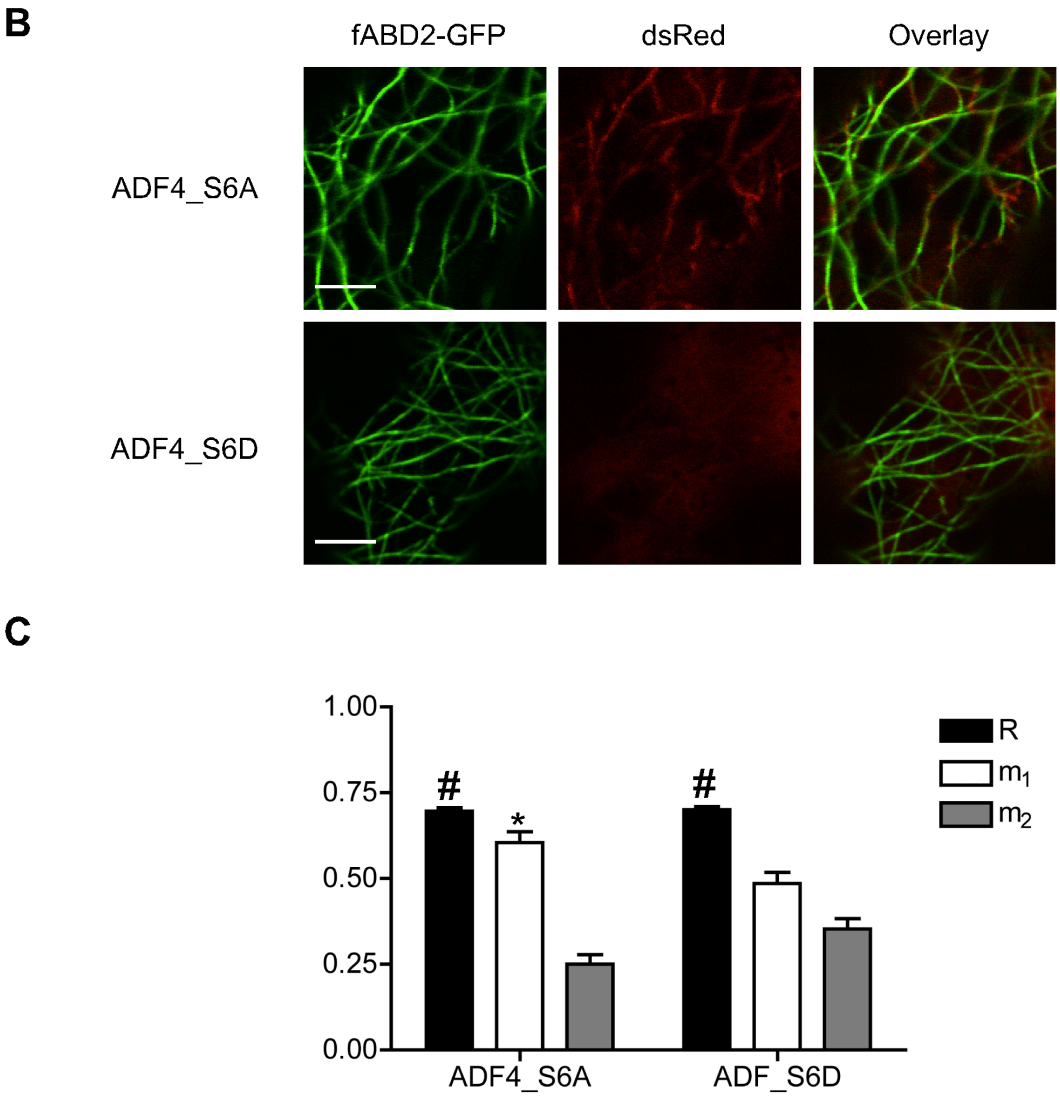


Figure 2.15 (cont'd). Confocal microscopy demonstrates phosphorylation of ADF4 affects cytoskeletal localization, but not nuclear localization. (A) Laser-scanning confocal microscopy of *adf4*, *adf4*/35S:ADF4, *adf4*/35S:ADF4_S6A and *adf4*/35S:ADF4_S6D isolated nuclei; DAPI stained nuclei (blue), immunochemistry FITC (green), and overlay. Bar = 2 μm. (B) Images of transiently expressed fABD2-GFP (green), dsRed- ADF4 _S6A/_S6D (red), and overlay in *Nicotiana benthamiana* taken by laser-scanning confocal microscopy. Bar = 5 μm. (C) Graphical representation of the overlay coefficient according to Manders (R) and the co-localization coefficients m_1 and m_2. Error bars, representing mean ± SEM, were calculated from two biological repeats (n = 40). Overlap coefficient (R) is considered to be co-localized when #R = 0.6 to 1.0, and co-localization coefficients indicate co-localization when *m_1>0.5 and *m_2>0.5.

coefficients suggest a significant co-localization of ADF_S6A with fABD2, but not for ADF4_S6D. In total, these observations are in agreement with previous reports of phosphorylated cofilin having reduced binding to both G- and F-actin (Bamburg & Bernstein, 2010).

Discussion

Understanding the mechanism(s) of pathogen effector recognition, as well as elucidating the putative virulence function(s) of these secreted proteins, provides the foundation for our understanding of innate immune signaling in plants (Knepper & Day, 2010). Using a combination of cell biology, biochemical, and genetics-based approaches, we show that ADF4 is required for the specific activation of RPS5-mediated resistance. In both plants and animals, the actin cytoskeletal network plays a broad role in numerous cellular processes, including cell organization, growth, development and response to external stimuli, including pathogen infection. Herein, we propose a mechanism through which the expression of the *R*-gene *RPS5* is under the control of the actin binding protein ADF4, in a phosphorylation dependent manner, independent of nuclear localization, which subsequently affects co-localization with actin, suggesting a possible cytoskeletal role in gene transcription (Figure 2.16).

In animal cells, a complex signaling network involving Rho-GTPase activation, actin cytoskeletal dynamics, and the interplay between pathogen virulence has been extensively characterized (Day *et al.*, 2011). In plants, however, the elucidation of the

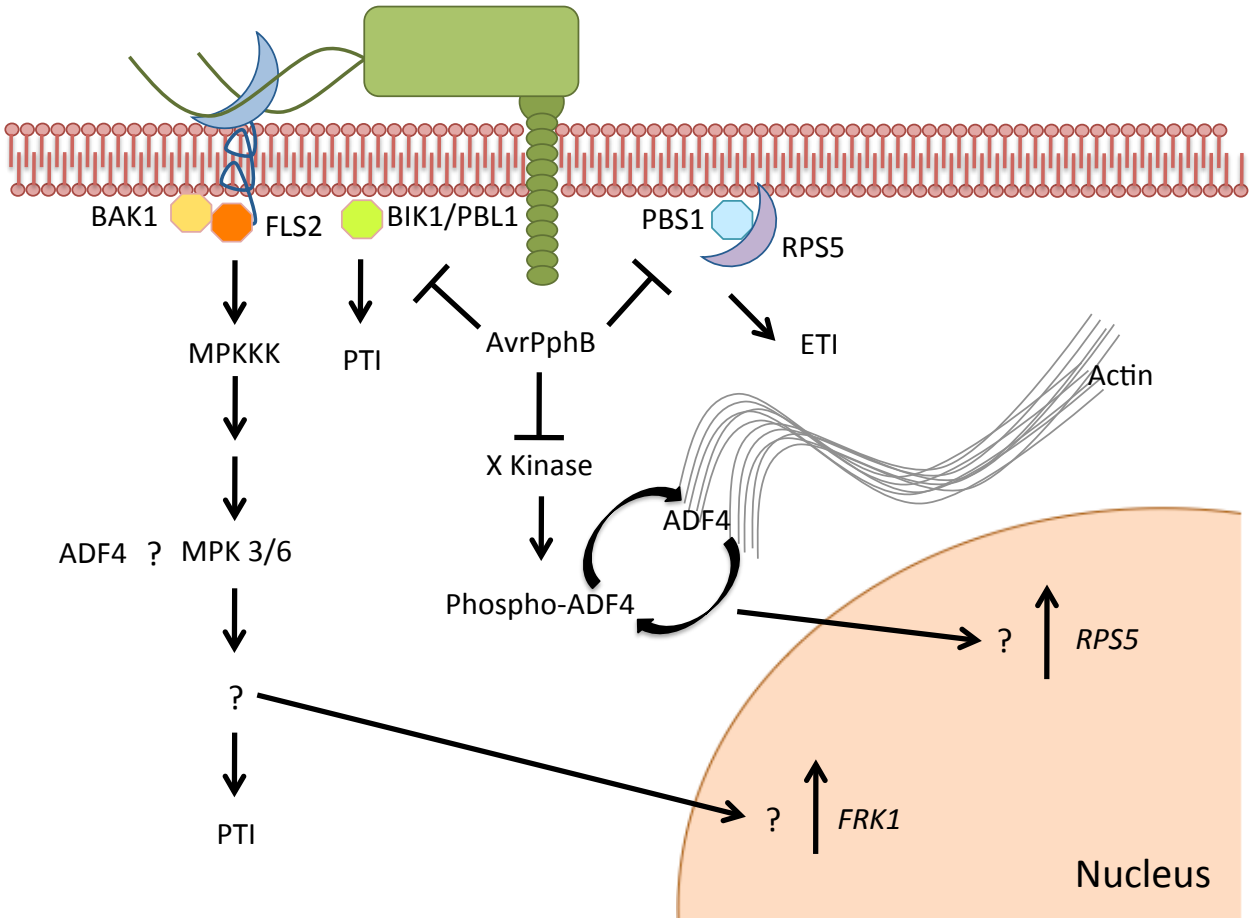


Figure 2.16. Proposed model illustrating the virulence and avirulence function of the bacterial cysteine protease AvrPphB through an ADF4-dependent mechanism. Following delivery of AvrPphB into the plant cells by *Pst* via the T3SS, AvrPphB targets multiple innate immune signaling pathways, including: 1) PTI, *via* the cleavage of BIK1 kinase; 2) ETI, *via* the cleavage of the kinase PBS1, a guardee of the resistance protein RPS5. We propose a potential role for AvrPphB in the modulation of actin cytoskeletal dynamics *via* the targeting of an unknown kinase responsible for the phosphorylation of ADF4 that ultimately results in reduced expression of *RPS5*, as well as specific down-regulation of MAP kinase signaling. ADF4 translocation into the nucleus is independent of phosphorylation status, however, F-actin co-localization and *RPS5* gene expression are dependent upon the phosphorylation of ADF4.

genetic link between pathogen virulence and the regulation of actin cytoskeletal dynamics has only recently been described (Clément *et al.*, 2009; Tian *et al.*, 2009). In plant-pathogen interactions, the effects of modulation to the host actin cytoskeleton have been best characterized using a combination of pharmacological and cell biology-based approaches to monitor focal orientation of F-actin filaments to the site of infection

during fungal pathogenesis (Takemoto & Hardham, 2004; Takemoto *et al.*, 2006; Hardham *et al.*, 2007; Miklis *et al.*, 2007; Hardham *et al.*, 2008). As a first step towards elucidating the mechanism of activation of RPS5-mediated resistance, we examined the expression levels of Arabidopsis genes associated with resistance to *Pst* AvrPphB. We observed a marked reduction in mRNA levels of the *R*-gene *RPS5*, while the protein kinase PBS1 was not affected (Figure 2.1B, Figure 2.1C). Additionally, the mRNA levels of *R*-genes unrelated to the recognition of AvrPphB were not affected in the *adf4* mutant (Figure 2.3B). From these data, we conclude that ADF4 is specifically required for the expression of *RPS5* and subsequent resistance to *Pst* AvrPphB.

The initiation of resistance signaling in plants following pathogen infection engages a multitude of processes, including PRR activation (Chinchilla *et al.*, 2007), MAPK signaling (Asai *et al.*, 2002) and transcriptional reprogramming (Pandey & Somssich, 2009). In the current study, our observation of a reduction in PTI-signaling in the *adf4* mutant supports our hypothesis that *RPS5* mRNA levels correlate with reduced levels of RPS5 protein. In support of this, we observed a reduction in *FRK1* transcript accumulation in the presence of AvrPphB in both the *adf4* and *rps5-1* mutants. This observation is in agreement with recent reports, including a study demonstrating a physical interaction between FLS2 and RPS5, which would suggest that PTI and ETI signaling is more interdependent than previously hypothesized (Qi *et al.*, 2011). Subsequent analysis of upstream MAPK components partially attributed diminished *FRK1* mRNA levels to a reduced activation of MPK3/6. Herein, we did not detect a significant reduction in flg22-induced phosphorylation of MPK3/6 in either Col-

0/pER8:AvrPphB or *rps5-1*/pER8:AvrPphB; however, in *adf4*/pER8:AvrPphB plants, a significant reduction in MPK3/6 phosphorylation following flg22 treatment was observed (Figure 2.10). MAPK signaling is often primarily associated with PTI (i.e. flagellin activation of the FLS2 receptor); however, many reports have demonstrated the necessity of these components for ETI. For example, in tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*) the requirement of MAPK signaling-components for AvrPto- and N-mediated ETI has been well documented (Ekengren *et al.*, 2003; Jin *et al.*, 2003; Oh & Martin, 2011). Our data would suggest that in the case of AvrPphB, R-Avr activation does not specifically induce MPK3/6 within 48 hours of estradiol-induced expression of AvrPphB (Figure 2.10B). Furthermore, the absence of perturbation to MPK3/6 in the *rps5-1*/pER8:AvrPphB suggest that while it appears recognition is important for aspects of PTI-signaling i.e. *FRK1* mRNA expression (Figure 2.5C), MAPK-signaling specifically is independent of the need for recognition (Figure 2.10B).

One possible explanation for reduced MAPK-signaling in the absence of ADF4 reflects the virulence activity of AvrPphB. Indeed, recent work has demonstrated a physical interaction between BIK1 and the FLS2 receptor upon ligand activation – an association that is required for the activation of PTI-signaling (Zhang *et al.*, 2010). As a mechanism linking with the virulence activity of AvrPphB with both PTI and ETI, cleavage of BIK1 by AvrPphB results in reduced PTI-signaling in the absence of recognition (i.e. the *rps5-1* mutant). Our observation of a reduction in MPK3/6 phosphorylation in *adf4*, but not Col-0 nor *rps5-1*, would suggest an additional role for ADF4 in regulation of MAPK-signaling, while the reduced *FRK1* in *adf4* and *rps5-1* as compared to Col-0, supports

the aforementioned potential virulence activity of AvrPphB, as well as a possible role for recognition (i.e. ETI) in the protection/recovery of the targeted PTI-signaling pathway. Although the mechanism(s) utilized by Arabidopsis to preserve the integrity of the MAPK- and PTI-signaling pathway are not yet fully understood, it is possible that ETI-induced SA accumulation, which has been demonstrated to prime and enhance accumulation of MPK3/6, can be partially responsible for the recovery of MAPK signaling in Col-0 (Beckers *et al.*, 2009). Another possible contribution to the reduction in PTI-signaling associated with loss of ETI is the aforementioned direct association of FLS2 with RPS5 (Qi *et al.*, 2011).

In plants, ADF localization is intimately associated with actin reorganization (Jiang *et al.*, 1997). At present, a full understanding of how translocation of ADFs into the nucleus occurs has not been defined (Bamburg, 1999); moreover, the precise function within the nucleus is unclear (Kandasamy *et al.*, 2010). The current hypothesis is the translocation of ADFs, as well as other ABPs, into the nucleus may serve a chaperone function (Bamburg & Bernstein, 2010). In support of this, actin, as well as several actin-binding proteins (including ADFs), has recently been shown to be present in the nuclei of Arabidopsis (Kandasamy *et al.*, 2010). This data support the hypothesis that in addition to actin, ABPs and actin-related proteins (ARPs) may have specific functions within the nucleus, including chromatin assembly and remodeling, as well as participation in various steps of RNA transcription and processing (Castano *et al.*, 2010; Kandasamy *et al.*, 2010). It is quite possible that ADF4 either facilitates nuclear translocation of specific actin isoforms required for processes related to the expression of *RPS5*, or, ADF4 itself

is required for gene expression (i.e., transcription), as has been demonstrated to be the case for other ARPs. Mechanistically, however, it is unclear how ADF proteins are translocated into the nucleus. Plant ADFs do not have a conserved nuclear localization signal sequence, as is found in the vertebrate ADFs/cofilins; however, plant ADFs do have two regions with basic amino acids which are similar to domains in other plant proteins that function together as a nuclear localization signal (NLS; Shieh *et al.*, 1993). To date, the function of these domains has not been explored. Our data, as well as a recent study by Kandasamy et al. (2010), suggests that these two regions of basic amino acids may be both sufficient for translocation to the nucleus, which is not affected by the phosphorylation status of ADF4 at serine-6 (Figure 2.15).

In the current study, we demonstrate that ADF4 phosphorylation influences both actin cytoskeletal localization, and ultimately, *RPS5* mRNA expression (Figure 2.12, Figure 2.15). In total, our data provide *prima facie* evidence for an actin-based regulatory mechanism controlling *R*-gene expression, and further support the emerging hypothesis that there are critical physiological roles for phosphorylated ADFs in plants (Bamburg & Bernstein, 2010). Phosphorylation of cofilin, the predominant ADF found in animal cells, is regulated in part through the action of LIM kinase (Bernard, 2007), and results in a reduced affinity of cofilin for F-actin. To this end, ADF phosphorylation has commonly been viewed as an inactivation mechanism, however, recent data suggest that this is not the case (Bamburg & Bernstein, 2010). In plant-pathogen interactions, numerous defense-associated processes are regulated by kinase phosphorylation (Shao *et al.*, 2003a; Zhang *et al.*, 2010; Chung *et al.*, 2011; Liu *et al.*, 2011). Conversely, the

regulatory mechanisms controlling the phosphorylation, and subsequent regulation of actin dynamics, have not been well established, nor has the crosstalk between ADF regulation and innate immune signaling been fully defined. One obvious disconnect in the link between innate immune signaling and kinase activity in plants and animals is that plants do not have a kinase family homologous to mammalian LIM kinases (Bernard, 2007), and thus, ADF phosphorylation is likely mediated by the activity of additional kinase(s), such as calcium dependent protein kinases (Allwood *et al.*, 2002). One interesting hypothesis in support of the work described herein is that the kinase responsible for the phosphorylation of ADF4 may be a virulence target of AvrPphB. This hypothesis is supported in part by Figure 2.1D, in which *RPS5* expression is significantly reduced in the *rps5-1* point mutant following inoculation with *Pst* AvrPphB. Additionally, the observed requirement of ADF4 for MAPK-signaling in the presence of AvrPphB (Figure 2.10A) lends support for the idea of ADF4, or the kinases required for its regulation as potential virulence targets. In this regard, such a mechanism would further solidify a link between the virulence function and activity of AvrPphB and the role of the actin cytoskeleton in controlling *RPS5* transcription and disease signaling.

Methods and Materials

Plant growth, transformation, and bacterial growth assays

Arabidopsis plants were grown in a BioChambers walk-in growth chamber (model FLX-37; Winnipeg, Manitoba, Canada) at 20 °C under a 12-hour light/12-hour dark cycle, with 60% relative humidity and a light intensity of 100 μmol photons $m^{-2}s^{-1}$.

Transformation of Arabidopsis, as well as selection of transformants, was performed as described by Clough and Bent (Clough & Bent, 1998).

Pseudomonas syringae pv. tomato DC3000 (*Pst*) strains were grown as previously described (Tian *et al.*, 2009). Four-week-old plants were used for bacterial inoculations. For growth assays and qRT-PCR analyses, whole plants were dip inoculated into bacterial suspensions of 3 x 10^8 colony-forming units (cfu) mL^{-1} in 10 mM $MgCl_2$ containing 0.1% Silwet L-77. Three 0.7 cm diameter leaf disks were collected from three plants for bacterial growth assays, as previously described (Tian *et al.*, 2009). The hypersensitive response (HR) was analyzed by hand infiltrating bacterial suspension in 10 mM $MgCl_2$ at 5 x 10^7 cfu mL^{-1} and scoring leaves for tissue collapse 20 to 24 hours post inoculation.

flg22 infiltration was performed at a concentration of 1-10 μM in 10 mM $MgCl_2$, as previously described (Knepper *et al.*, 2011b). Col-0 and *adf4* plants were grown upright on plates containing MS media for 10 days ± 10 nM flg22 in a GC8-2H growth chamber (Environmental Growth Chambers LTD., Winnipeg, Manitoba, Canada) at 20 °C under a 12-hour light/12-hour dark cycle, with 60% relative humidity and a light intensity of 120 μmol photons $m^{-2}s^{-1}$. Analysis of flg22 inhibition of root growth was performed as previously described (Chinchilla *et al.*, 2007).

Plasmid construction

The native promoter driven pMD1-g:*ADF4* (g:*ADF4*) was constructed as described in Tian et al. (Tian *et al.*, 2009). Primer sequences 5´-GCGGTCGACATGGCTAATGCTGCGTCAGGAATGG-3´ (forward ADF4), 5´-GCGGTCGACATGGCTAATGCTGCGGCAGGAATGG-3´ (forward ADF4_S6A), 5´-GCGGTCGACATGGCTAATGCTGCGGACGGAATGG-3´ (forward ADF4_S6D) and 5´-GCGGTCGACATGGCTAATGCTGCGTCAGGAATGG -3´ (reverse for all 3) were used to add *Sal*I restriction enzyme sites (underlined) for cloning *ADF4* and its phospho-mutants into pMD1:35S:T7 (Knepper *et al.*, 2011b).

Nuclei isolation and immunocytochemistry

Nuclei isolations were conducted as described in Kandasamy et al. (2010). Approximately 1g of 2- to 3-week old *adf4*/35S:ADF4, _S6A, and _S6D Arabidopsis seedlings, grown upright on MS medium plates were used for each nuclear extraction. The isolated nuclei were fixed on chrome alum slides, permeabilized, and incubated with primary antibody T7-monoclonal (EMD Chemicals, Gibbstown, NJ, USA), secondary anti-mouse IgG-FITC (Sigma-Aldrich) and DAPI (Sigma-Aldrich) before imaging (Kandasamy *et al.*, 2010).

Laser-scanning confocal microscopy and co-localization analysis

Isolated nuclei and transiently expressed dsRed-ADF4 constructs, and fABD2-GFP generated using *Agrobacterium tumefaciens*-mediated transient expression in *Nicotiana benthamiana*, were imaged using laser confocal scanning microscopy using a 60x/1.42 PlanApo N objective on an Olympus FV1000 (Olympus America Inc, Center Valley, PA), as described in Tian et al. (2011). Co-localization was performed utilizing FluoView FV1000 (System Analysis Software, Olympus). An area of each image was selected for analysis containing < 50 % fABD2-GFP occupancy in order to examine true co-localization and not artificial co-localization due to over abundance of fABD2-GFP. Thresholds were set manually to account for background, and overlap coefficient according to Manders (R), and co-localization coefficients m1 and m2 were generated by the FV1000-ASW. Co-localization coefficient equations used can be found in Table 2.1.

RNA isolation and qRT-PCR analysis

Total RNA was extracted from leaves using the PrepEase Plant RNA Spin kit (USB Affymetrix, Santa Clara, CA, USA). First-strand cDNA was synthesized from 1 μg total RNA using the First-Strand cDNA Synthesis kit (USB Affymetrix). Primers used for quantitative real-time PCR (qRT-PCR) are listed in Table 2.2. qRT-PCR was performed using the Mastercycler ep Realplex system (Eppendorf AG, Hamburg, Germany), as previously described (Knepper *et al.*, 2011b), using the Hot Start SYBR Master mix 2X

Overlap coefficient according to Manders (R)	$R = \frac{\sum_i S1_i \cdot S2_i}{\sqrt{\sum_i (S1_i)^2 \cdot \sum_i (S2_i)^2}}$
Co-localization coefficient m_1	$m_1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i}$
Co-localization coefficient m_2	$m_2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$

Table 2.1 Microscopy overlay equations.

(USB Affymetrix). Ubiquitin (*UBQ10*) was used as an endogenous control for amplification. Fold Col-0 was determined using the following equation: (relative expression)/(relative expression of Col-0 untreated), where "relative expression" = $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{gene\ of\ interest} - Ct_{UBQ10}$.

Statistical analysis

All data were analyzed using GRAPHPAD PRISM Software (San Diego, California, USA). Values are represented as mean ±SEM. All statistical analysis was performed using two-way ANOVA, followed by the Bonferroni post-test as compared to Col-0. In Figure 2.5C, a two-way ANOVA, followed by the Bonferroni post-test was performed in order to determine if there is a significant difference between *rps5-1* and *adf4*. In Figure 2.2, an unpaired student t-test with a 95% confidence interval was performed to determine if change over time was significant. P values ≤ 0.05 are considered significant, where *p<0.05; **p<0.01 and ***p<0.005.

Locus Tag	Gene	Forward Primer	Reverse Primer
AT1G12220	*RPS5*	GTTGTCATGGTCTAAAGACATTTG	GTACAAATCCAATGATCACTAACCA
AT5G13160	*PBS1*	TCAATGTGCATCCAAGAACAGGCG	AATTTACTTCCCGAGCCACCTCCA
AT4G26090	*RPS2*	GGCGGAGAGAAGAGGACATA	CAGCTTCGTCCCTCTAGACC
AT3G07040	*RPM1*	TCGCGGAGAAGGGAGTGTGGA	GAAGCTTGCCTTGGCCGCCT
AT4G05320	*UBQ10*	AGAAGTTCAATGTTTCGTTTCATGTAA	TTACGAATCCGAGGGAGCCATTG
AT2G19190	*FRK1*	CGGTCAGATTTCAACAGTTGTC	AATAGCAGGTTGGCCTGTAATC
AT5G45250	*RPS4*	CCTAACATTATGGGCATCATCA	CCGCCTTCACAATTTCATTGA
AT5G46470	*RPS6*	GGTCAATTCAACTACGATCACG	GTTATCCAGGGATGGGACAT
AT3G20600	*NDR1*	CGGTTTTACGAGCGGTTTTG	CCAACTTCAACCCCATACCTC
AT3G45640	*MPK3*	TGACGTTTGACCCCAACAGA	CTGTTCCTCATCCAGAGGCTG
AT2G43790	*MPK6*	CCGACAGTGCATCCTTTAGCT	TGGGCCAATGCGTCTAAAAC

Table 2.2 List of primers.

Immunoblot analysis

Western blot analysis of phosho-MPK3/6 was performed using 40 µg total protein, utilizing anti-pTEpY (Cell Signaling Technology, Danvers, MA, USA), while analysis of *adf4*/35S:ADF4_S6A and *adf4*/35S:ADF4_S6D was prerformed using 20 µg total protein, utilizing anti-T7-HRP (EMD Chemicals, Gibbstown, NJ, USA), as previously described (Knepper *et al.*, 2011a).

2D IEF was performed on 500mg of total lysate from *adf4*/35S:ADF4 and

adf4/35S:ADF4_S6A. The lysates were precipitated using chloroform:methanol (1:4) and reconstituted in Urea buffer (7 M Urea, 2 M Thiourea, 2% CHAPS, 2% ASA-14, 50 mM DTT, 0.2% Biolyte ampholytes and 0.1% bromophenol blue). Isoelectric focusing was conducted according to manufacturing guidelines at the proteomics core at Michigan State University Research Technology Support Facility (Bio-Rad). Immunoblot analysis was performed as above.

Acknowledgements

We would like to thank Doug Whitten (MSU RTSF) for assisting with the 2D IEF, Melinda Frame and Elizabeth Savory for confocal microscopy assistance, Caitlin Thireault for assisting with screening of *adf4*/35S:RPS5-sYFP lines, and Alyssa Burkhardt and Jeff Chang for critical reading of the manuscript. Special thanks to Roger Innes for providing the AvrPphB C98S catalytic mutant and the RPS5-sYFP construct.

CHAPTER 3

In silico comparison of *Arabidopsis thaliana* actin depolymerizing factors AtADF1 and AtADF4 identify subtle biochemical features that support their distinct cellular functions.

The AtADF1 and AtADF4 structural modeling was performed by Leann Buhrow; Cell and Molecular Biology graduate program at Michigan State University. The floral dip transformation and maintenance of *Arabidopsis thaliana adf4* mutant transformation plant lines described within were performed with the aid of Jessica Schein; Cell and Molecular Biology graduate program at Michigan State University.

Abstract

Plant actin depolymerizing factors (ADFs) are a conserved family of proteins represented by multiple distinct members. This is in contrast to other eukaryotes; e.g., yeast that have only one ADF and humans that possess three ADFs. The plant ADF family is a large and ancient clade, and the possession of multiple members within the family is estimated to have occurred prior to the divergence of monocots and dicots. Given that plants have such a multitude of ADFs, it would not be unexpected that some of the ADFs may have adopted additional cellular functions in addition to the regulation of the actin cytoskeleton. One such example is that of Arabidopsis ADF4 (AtADF4), which, as I have shown in the last chapter, plays a role in immune signaling and the regulation of gene expression. Specifically, AtADF4 is required for resistance to *Pseudomonas syringae* containing the bacterial effector AvrPphB (*Pst* AvrPphB) and the expression of the resistance gene *Resistance to Pseudomonas syringae-5*. Interestingly, AtADF4 possesses high sequence identity to Arabidopsis ADF1 (AtADF1), the mutant of which, *adf1*, is resistant to *Pst* AvrPphB. Due to this high sequence identity, and yet differing immunity phenotypes, I have chosen to utilize computational *in silico* analysis to explore the differential amino acid residues between AtADF1 and AtADF4. In this chapter I outline my findings of these comparisons and propose a suite of AtADF1 and AtADF4 chimeric proteins, as well as single point mutations, which will allow me to gain insight into the important amino acid residue(s) that contribute to the unique cellular roles of AtADF4.

Introduction

Actin Depolymerizing Factors (ADFs) represent a large and highly conserved class of actin binding proteins (ABPs) whose function – analogous to the mammalian ADF/cofilin family of proteins – is the regulation of the stochastic eukaryotic actin cytoskeleton (Day *et al.*, 2011; Bamburg & Bernstein, 2010). At a fundamental level, ADFs alter the organization and dynamics of the actin cytoskeleton by depolymerizing pointed filamentous (F-) actin in to globular (G-) actin monomers, which in turn, can be acted upon by additional ABPs, in order to be reincorporated into the barbed ends of growing actin filaments. Collectively, this process of filament assembly and disassembly is modeled as a process referred to as treadmilling (McGough *et al.*, 1997; Bugyi & Carlier, 2010; Day *et al.*, 2011). Although the mechanism is not well understood, plant ADFs have been identified to, in addition to their better characterized function of depolymerizing actin, sever actin filaments and act to assist in the bundling of multiple actin filaments to form stable actin cables (Tholl *et al.*, 2011; Henty *et al.*, 2011).

While lower eukaryotes, such as yeast, have as few as one ADF, higher eukaryotes tend to have more than one; for example, mammals have 3 ADF/Cofilin proteins, whereas the model plant *Arabidopsis thaliana* has 11 expressed ADFs. The 11 Arabidopsis ADFs (AtADFs) can be divided into four subclasses based on amino acid sequence alignments, suggesting that plant ADFs may have a variety of functions (Poukkula *et al.*, 2011; Ruzicka *et al.*, 2007). The differences between developmental, and tissue-specific expression of the four subclasses AtADFs, suggest that these

proteins have a wide assortment of cellular and tissue-specific functions. This is best exemplified by the differing expression and subcellular localization of AtADF subclass I and AtADF subclass II. The AtADFs in subclass I, including AtADF1, AtADF2, AtADF3 and AtADF4 are expressed in a vegetative pattern throughout the whole plant excluding pollen during all developmental stages, and at the subcellular level are expressed both in the cytosol and nucleus (Ruzicka *et al.*, 2007; Kandasamy *et al.*, 2010; Porter *et al.*, 2012). AtADF subclass II, containing AtADF7, AtADF8, AtADF10 and AtADF11, unlike subclass I, are only expressed in the cytosol and can be divided into two smaller clades dependent upon their tissue specific expression patterns within the plant. The first clade containing AtADF7 and AtADF10, which are expressed in a reproductive pattern, are specifically expressed in pollen and pollen tubes while the second clade containing AtADF8 and AtADF11 are expressed in certain cells of the root system and at the tips of fast growing cells (Ruzicka *et al.*, 2007).

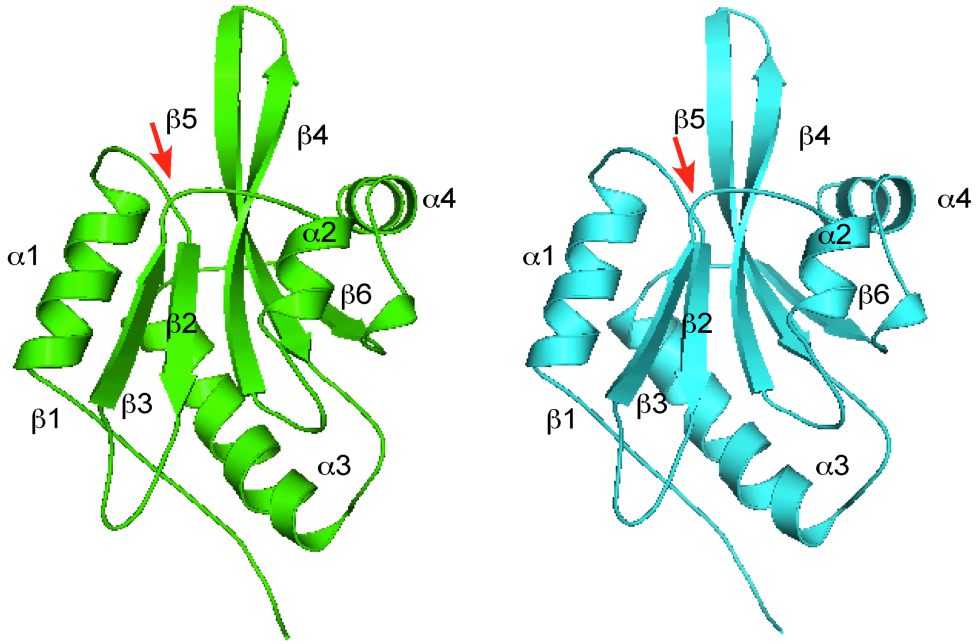
As an experimental demonstration of the tissue- and developmental- specific roles and interactions of the various subclasses of ADFs, it was determined that the ectopic over-expression of Arabidopsis reproductive actin AtAct1 in vegetative tissue would cause morphological defects in the plant. Such defects include dwarfism, which is alleviated by ectopic over expression of either subclass II members, AtADF7 or AtADF8, but not AtADF9, a member of subclass III (Kandasamy *et al.*, 2007). Interestingly, when the ADFs of rice, *Oryza sativa* L. (OsADFs), are grouped in phylogeny based upon amino acid sequence similarities with AtADFs, four subclasses are also observed. The grouping of rice ADFs results in an apparent division based upon vegetative and

reproductive OsADFs (Feng *et al.*, 2006). Taken together, these amino acid sequence phylogenies and expression pattern clustering of rice and Arabidopsis ADFs suggest that the multi-protein, and most likely multi-functional nature of plant ADF families existed before monocots and dicots diverged (Feng *et al.*, 2006; Ruzicka *et al.*, 2007).

ADF proteins possess a 13-19kDa domain known as the actin depolymerizing factor homology domain (ADF-H domain), a region of the protein required for F-actin disassembly that in most true ADFs, represents nearly the entire protein (Bamburg & Bernstein, 2010; Poukkula *et al.*, 2011). As a primary driver for ADF/Cofilin function, the ADF-H domain is required for binding F- and G- actin; however, in a few rare instances, the ADF-H domain does not enable proteins to bind both forms of actin, but instead binds either F- or G- actin, or neither (Poukkula *et al.*, 2011). A characterized example of the latter is that of mammalian glia maturation factor, which does not interact directly with actin, but instead binds Arp2/3 (Poukkula *et al.*, 2011).

Structurally, the ADF-H domain consists of 5 mixed internal β-strands, of which the two most carboxyl-terminal strands are parallel, surrounded by 4 α-helicies, 2 on each face of the domain (Poukkula *et al.*, 2011). In the case of AtADF1, and predicted for AtADF4, there are 6 β-strands, only 5 of which are internal (Poukkula *et al.*, 2011; Dong *et al.*, 2013; Figure 3.1A). While the crystal structure of both the N-terminal ADF-H domain of mammalian gelsolin, an ABP that contains 6 ADF-H domains and severs actin, and the C-terminal ADF-H domain of twinfilin, a two ADF-H domain containing protein from yeast and drosophila, have been solved in complex with bound actin, this is not the

A



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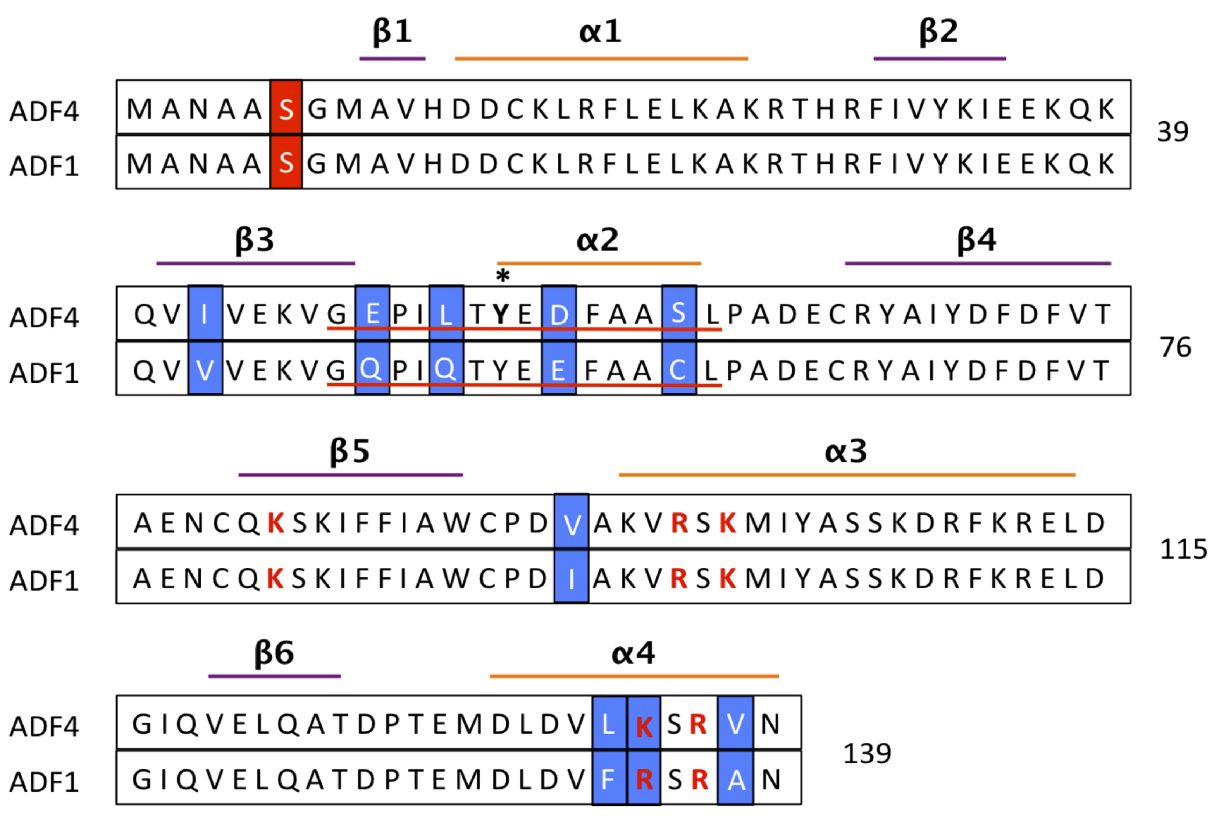

Figure 3.1. Structural comparison and sequence alignment of AtADF1 and AtADF4.

Figure 3.1 (cont'd). Structural comparison and sequence alignment of AtADF1 and AtADF4. A.) Side by side comparison of the AtADF1 (left) and AtADF4 (right) structural models. The β-strands and α-helices are numbered and the red arrows indicate the region within the two structures with the largest predicted difference in amino acid sequences. B.) Sequence alignments of AtADF4 and AtADF1. The β-strands are indicated by purple lines, while the α-helices are indicated by orange lines. Differing amino acids are highlighted in blue. The phosphorylatable serine residue is highlighted in red, while the tyrosine-53 residue, predicted to be phosphorylated in AtADF4, is bolded and is denoted by an asterisk. The region of swapped amino acids in the formation of the chimeric proteins is underlined in red.

case for Arabidopsis ADFs (Burtnick *et al.*, 2004; Paavilainen *et al.*, 2008). Although the structure of the ADF-actin interaction of plants has not been solved, experimental evidence exists whereby site directed mutagenesis of AtADF1 has identified that two residues within α3, arginine-98 and lysine-100, are important for both F- and G- actin binding (Dong *et al.*, 2013; Figure 3.1B). These findings are in agreement with what has been previously observed in the site directed mutagenesis studies of mammalian ADF/cofilin and Drosophila twinfilin, both of which studies determined that in addition to α3, the unstructured N-terminus as well as the loop area prior to the C-terminal α-helix are also involved in G-actin binding (Paavilainen *et al.*, 2008; Poukkula *et al.*, 2011). A second apparent function of these two residues within the α3 is the proper depolymerization of actin, as the AtADF1^{R98AK100A} mutant has a severely reduced ability to depolymerize F-actin *in vitro* (Dong *et al.*, 2013). This work, as well as the work of others has further identified residues important for F-actin binding, but not G-actin binding; specifically, these studies showed that lysine-82 of β5 and both arginine-135 and arginine-137 of α4 all play critical roles in the binding of ADF1 to F-actin (Ono, 2007; Lappalainen *et al.*, 1997; Dong *et al.*, 2013; Figure 3.1B).

In addition to the importance of the above identified specific residues for AtADFs binding to and depolymerizing actin, ATP/ADP loading, as well as monomeric or polymeric actin interactions have also been shown to affect interactions between ADFs and actin (Tian *et al.*, 2009; Carlier *et al.*, 1997; Day *et al.*, 2011). The stochastic cycling of actin from monomeric G-actin to filamentous F-actin and back again begins with ATP loaded G-actin (G-actin^{ATP}), which can spontaneously from heterotrimeric complexes in a concentration dependent manner, through a process known as actin nucleation (Day *et al.*, 2011; Campellone & Welch, 2010). This initial actin nucleation however is kinetically expensive and therefore, is often aided by the ABP Arp2/3, which mimics dimeric actin allowing for more energetically favorable actin nucleation (Day *et al.*, 2011; Mathur *et al.*, 2003). Once this initial step is achieved additional G-actin^{ATP} are incorporated into the pointed end and F-actin elongates and matures, hydrolyzing actin^{ATP} to actin^{ADP-Pi} and eventually to actin^{ADP} (Day *et al.*, 2011; Bugyi & Carlier, 2010). Upon dissociation of Pi from actin^{ADP-Pi} the barbed end of F-actin becomes less structurally secure and more susceptible to dissociation to monomeric actin^{ADP} (Poukkula *et al.*, 2011; Day *et al.*, 2011). Cofilin itself has been demonstrated to enhance the dissociation of Pi from actin^{ADP-Pi} thus increasing the recruitment of additional cofilin to further promote actin depolymerization (Poukkula *et al.*, 2011; Blanchoin *et al.*, 2000). When interacting with F-actin, ADFs preferentially bind F-actin^{ADP} and promote actin disassembly either by severing F-actin or through the depolymerization of single G-actin^{ADP} by altering the twist of the F-actin polymer, thus destabilizing the barbed end (McGough *et al.*, 1997; Carlier *et al.*, 1997; Henty *et al.*, 2011). The resulting free G-actin^{ADP} is then recharged with ATP, typically via the activity

of cyclase-associated protein (CAP), and sequestered by profilin (PRF) until it is re-incorporated into F-actin (Day *et al.*, 2011; Barrero, 2002; Sun *et al.*, 2013).

Given the substantial influence ADFs have on the organization of the actin cytoskeleton, it is important that the cell possesses the biochemical mechanism(s) required for the regulation of ADF/cofilin actin binding, severing, and depolymerization activity. In short, the primary mechanism for this regulation is the phosphorylation at a N-terminal serine residue (Ressad *et al.*, 1998; Smertenko *et al.*, 1998). While it is unclear if phosphorylation of serine directly inhibits the ability of ADF to depolymerize actin, it has been demonstrated that phosphorylation does significantly reduce ADFs' ability to interact with F- and G-actin (Ressad *et al.*, 1998; Smertenko *et al.*, 1998; Porter *et al.*, 2012). In plants, it has been demonstrated that calmodulin-like domain protein kinases are capable of phosphorylating ADFs of both Arabidopsis, AtADF1 and maize, ZmADF3 (Dong & Hong, 2013; Allwood *et al.*, 2001). Whether serine is the only residue of plant ADFs that can/is being phosphorylated is still a point of interest (Dong & Hong, 2013; Porter *et al.*, 2012).

My work outlined in the last chapter, as well as the work of others have suggested non-canonical roles for AtADF4 in immune signaling and gene expression (Chapter 2; Tian *et al.*, 2009; Porter *et al.*, 2012; Henty-Ridilla *et al.*, 2014). In addition to what I have demonstrated in Chapter 2, it has been reported that AtADF4 is required for the dynamic reorganization of the actin cytoskeleton observed during recognition of pathogen associated molecular patterns (PAMPs), specifically elongation factor-Tu

(Henty-Ridilla *et al*., 2014). Moreover, this finding is in further support that AtADF4 and the actin cytoskeleton are key components of the basal immune response referred to as PAMP-triggered immunity (PTI; Chisolm *et* al., 2006; Henty-Ridilla *et al*., 2014). I have also demonstrated that AtADF4 also plays a role in the second phase of plant immunity; often referred to as effector-triggered immunity (ETI; Chisholm *et al*., 2006). Specifically, I have shown that AtADF4 is required for the expression of *Resistance to Pseudomonas syringae-5* (*RPS5*), the resistance protein that recognizes the bacterial effector AvrPphB (Chapter 2; Porter *et al*., 2012). My finding were in addition to work done previously in our lab, that when challenged with *Pseudomonas syringae* containing AvrPphB (*Pst* AvrPphB), the AtADF4 mutant (*adf4*) has reduced expression of *Pathogen related protein 1* (*PR1*), a resistance related gene, and also a loss of the hypersensitive response (HR), a phenotype suggestive of a resistant interaction in Arabidopsis (Tian *et al*., 2009).

In addition to the characterization of the disease phenotypes for the *adf4* mutant, Tian *et al*., (2009) examined two other class I ADF mutants for their disease phenotypes to *Pst* AvrPphB. They demonstrated that both the AtADF1 mutant (*adf1*) and AtADF3 mutant (*adf3*) are resistant *Pst* AvrPphB, while the *adf4* mutant plant is susceptible to *Pst* AvrPphB (Tian *et al*., 2009). These findings are of interest due to the close relation of AtADF1 and AtADF4 (Ruzicka *et al*., 2007). In this chapter, I utilize the high sequence identity of AtADF1 and AtADF4 to identify biochemical features of AtADF4 that may account for the unique immunity related roles of AtADF4 through an *in silico* approach.

The work presented herein will allow for additional, well-focused *in planta* follow up experiments.

RESULTS

Arabidopsis actin depolymerizing factor-4 and -1 are highly homologous, yet the mutant plants *adf1* and *adf4* have differing disease phenotypes

As described above, the *adf4* mutant is susceptible to *Pst* AvrPphB, while the *adf1* mutant is resistant (Tian *et* al., 2009). The differences in the resistance phenotypes of *adf1* and *adf4* to *Pst* AvrPphB, is due in part to the inability of *adf4* to express *RPS5*, and therefore recognize AvrPphB; while *adf1* should properly express *RPS5* (Porter *et al*., 2012). In order to confirm this, we examined mutant *adf1* and compared the expression of *RPS5* to wild type Col-0 as well as the *adf4* mutant (Figure 3.2). As shown in Figure 3.2, the *adf1* mutant expresses *RPS5* at near wild-type levels, which allows the *adf1* mutant plant to recognize AvrPphB and therefore the mutant is resistant to *Pst* AvrPphB.

These results are interesting given that AtADF1 and AtADF4 are 97% homologous with a 93.5% amino acid sequence identity, as determined by NCBI Blast and *CLUSTAL Omega* (Figure 3.1B). In order to better understand what implications the nine differing amino acids between AtADF1 and AtADF4 would have on the structure of the proteins, models were generated of both AtADF1 and AtADF4 (Figure 3.1A). As shown by the red arrows, the free loop between β3 and α2 appears to have the largest region of

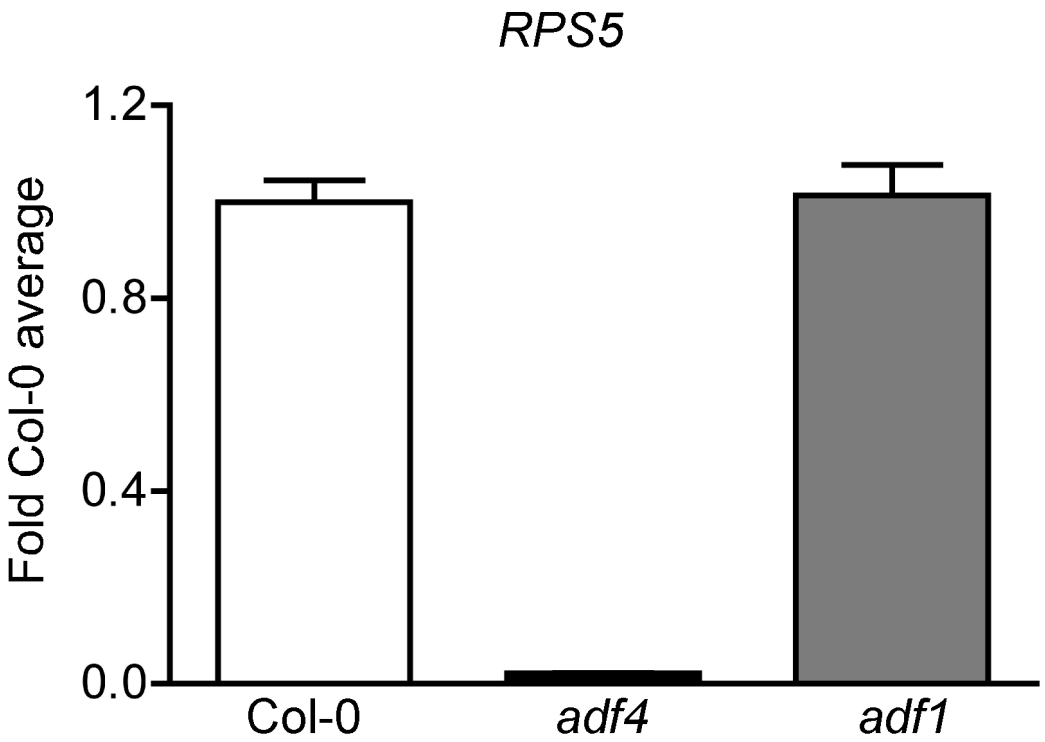

Figure 3.2. Expression of *resistance to Pseudomonas syringae-5* in AtADF1 mutant (*adf1*) and AtADF4 mutant (*adf4*) as compared to wild-type Col-0. The relative expression levels of *RPS5* were determined by qRT-PCR.

structural difference. This result is logical, given that four of the nine amino acid differences are located within this region of AtADF1 ad AtADF4 (Figure 3.1B).

In silico analysis of differing amino acids in AtADF1 and AtADF4 that may account for the unique cellular function of AtADF4

We decided to use the high amino acid sequence identity of AtADF1 and AtADF4 to our advantage in deciphering their differing cellular functions. Initial *in silico* analysis of the amino acid sequences of AtADF1 and AtADF4 revealed two amino acids that were unfavored when changed in the AtADF4 with the amino acids from AtADF1. Both SIFT

Amino acids AtADF1 to AtADF4	**SIFT score**	**Amino acids** AtADF4 to AtADF1	**SIFT score**	**BLOSUME 62 value**
V42I	0.98	**I42V**	1.00	3
Q48E	1.00	**E48Q**	0.72	2
Q51L	1.00	**L51Q**	0.46	-2
E55D	0.78	**D55E**	1.00	2
C59S	1.00	**S59C**	0.40	-1
I94V	1.00	**V94I**	0.98	3
F134L	1.00	**L134F**	0.49	0
R135K	1.00	**K135R**	0.90	2
A138V	1.00	**V138A**	0.78	0

Table 3.1. Amino acid substitutions of AtADF1 and AtADF4. This table displays the SIFT score values of amino acid substitutions where either AtADF1 or AtADF4 is proposed to have the nine amino acids of the other in substitution for their own. All SIFT substitutions are predicted to be tolerated, as only scores <0.05 are not tolerated. Additionally, BLOSUM62 values are shown which examine the favorability of individual amino acid substitutions in the without regard to the surrounding sequences. Positive BLOSUM62 values are favorable, while negative values are less favorable.

analysis and the BLOSUM62 amino acid substitution table, summarized in Table 3.1, suggest that the substitution of AtADF4 leucine-51 for AtADF1 glutamine-51 and AtADF4 serine-59 for AtADF1 cysteine-59 are the least favorable (Ng, 2003; Henikoff & Henikoff, 1992). This information as well as the relatively dense region of differing amino acids between β3 and α2 resulting in an alteration of the free loop, led me to decide to swap the four amino acids between glycine-47 and leucine-60, as indicated by the red underline in Figure 3.1B, to generate AtADF1 and AtADF4 chimeric proteins to test the effect of multiple amino acid substitutions at once (Figure 3.1A; 3.1B). The resulting AtADF1$^{Q48E,\ Q51L,\ E55D,\ C59S}$ and AtADF4$^{E48Q,\ L51Q,\ D55E,\ S59C}$ chimeric proteins will then be used to complement the *adf4* mutant to determine the importance of the substituted amino acids in the expression of *RPS5* and resistance to *Pst* AvrPphB

Construct name	Express *RPS5*	Resistant
AtADF1$^{Q48E, Q51L, E55D, C59S}$	Yes	Yes
AtADF1$^{S6A, Q48E, Q51L, E55D, C59S}$	No	No
AtADF1$^{S6D, Q48E, Q51L, E55D, C59S}$	Yes	Yes
AtADF4$^{E48Q, L51Q, D55E, S59C}$	No	No
AtADF4$^{S6A, E48Q, L51Q, D55E, S59C}$	No	No
AtADF4$^{S6D, E48Q, L51Q, D55E, S59C}$	No	No
AtADF1^{Q51L}	Yes	Yes
AtADF1$^{S6A, Q51L}$	No	No
AtADF1$^{S6D, Q51L}$	Yes	Yes
AtADF4^{L51Q}	No	No
AtADF4$^{S6A, L51Q}$	No	No
AtADF4$^{S6D, L51Q}$	No	No

Table 3.2. List of protein constructs and their predicted ability to complement the *adf4* mutant for expression of *RPS5* and resistance to *Pst* AvrPphB.

(Table 3.2). It is expected that complementation of *adf4* with 35S:T7-AtADF1$^{Q48E, Q51L, E55D, C59S}$ will restore *RPS5* expression, while complementation with 35S:T7-AtADF4$^{E48Q, L51Q, D55E, S59C}$ will not. Additionally, due to the very low SIFT score of the substitution of glutamine for leucine at position 51 I have also developed individual amino acid substitution constructs to examine the impact of this specific substitution within the above described region (Table 3.2). If the SIFT predictive software is correct in the identification of this amino acid substitution as the least favorable, the 35S:T7-AtADF1^{Q51L} construct may indeed be capable and sufficient to restore both *RPS5* expression and resistance to *Pst* AvrPphB in the *adf4* mutant. Conversely, the substitution of leucine to glutamine at position 51 may be the minimal required change to AtADF4 to alter its ability to participate in the expression of *RPS5* and subsequent

resistance to *Pst* AvrPphB, thus the 35S:T7-AtADF4^{L51Q} complementation of *adf4* would not result in restoration to wild-type function with regard to immunity.

Phosphorylation prediction software reveals potential differing secondary phosphorylation site(s) between AtADF1 and AtADF4

AtADF4 does not only have to be present for resistance to *Pst* AvrPphB and expression of *RPS5*, but must also be phosphorylated at serine-6 (Tian *et al*., 2009; Porter *et al*., 2012). To confirm the necessity of the phosphorylation of serine-6, as well as the importance of the four amino acids of the AtADF1 and AtADF4 chimeras, I have developed serine-6 phosphorylation mutants of the chimeric proteins (Table 3.2). I believe that similar to what was observed in Chapter 2, the phosphorylation mimic 35S:T7-AtADF1$^{S6D\ Q48E,\ Q51L,\ E55D,\ C59S}$ will complement the *adf4* mutant and restore *RPS5* expression, because this chimeric phosphomimic will possess both the mimicked phosphorylation at serine-6 and the swapped four amino acids expected to be required for *RPS5* expression. Additionally, neither the 35S:T7-AtADF4$^{S6D,\ E48Q,\ L51Q,\ D55E,\ S59C}$ nor the two phosphorylation null mutants; 35S:T7-AtADF1$^{S6A,\ Q48E,\ Q51L,\ E55D,\ C59S}$, and 35S:T7-AtADF4$^{S6A,\ E48Q,\ L51Q,\ D55E,\ S59C}$, should complement the *adf4* mutant. Taken together these complements should confirm the importance of one or more of the substituted amino acids, as well as reiterate the requirement of phosphorylation of serine-6 for the AtADF4 dependent expression of *RPS5*.

While the impact of the phosphorylation of serine-6 of plant ADFs has been well documented as a cellular mechanism to regulate the affinity of ADFs for actin, and therefore their ability to depolymerize F-actin^{ADP}, as well as the apparent requirement for AtADF4 to be phosphorylated at serine-6 for proper gene expression, phosphorylation of additional amino acids is less apparent (Porter *et al*., 2012; Dong & Hong, 2013; Ressad *et al*., 1998; Smertenko *et al.*, 1998; Allwood *et al*., 2001). The recent report from Dong & Hong (2013) demonstrated that AtADF1 is phosphorylated at serine-6 by the calmodulin-like domain protein kinase (CDPK6). This report is useful in that, with the additional works in maize, it demonstrates the importance of calcium signaling and calmodulin-like protein kinases in the regulation of ADFs by phosphorylation, and ultimately the remodeling of the actin cytoskeleton. This report was not able to definitively determine that serine-6 is the only phosphorylation site of AtADF1 (Dong & Hong, 2013; Allwood *et al*., 2001; Smertenko *et al.*, 1998). Additionally, as I demonstrated in Chapter 2, serine-6 is confirmed to be a phosphorylatable residue of AtADF4 by 2D electrophoresis; however, there are additional isoelectric focused points on the 2D gel in both AtADF4 and the phospho-null AtADF4S6A (Chapter 2; Porter *et al*., 2012).

In order to identify alternative phosphorylated residues of AtADF1 and AtADF4, NetPhos 2.0 server software was used to predict the phosphorylation potential of each residue (Blom *et al.*, 1999; Table 3.3). It should be noted, that while NetPhos 2.0 server software does not predict the phosphorylation of serine-6 in either AtADF1 or AtADF4, the software also fails to predict phosphorylation of serine-3 in mammalian cofilin1,

Potential phosphorylation site	AtADF1 Score	AtADF1 Predicted	AtADF4 Score	AtADF4 Predicted
S6	0.018	No	0.018	No
T26	0.139	No	0.139	No
Y32	0.036	No	0.036	No
T52	0.936	Yes	0.805	Yes
Y53	0.336	No	0.629	Yes*
S59	N/A	N/A	0.290	No
Y67	0.915	Yes	0.915	Yes
Y70	0.013	No	0.013	No
T76	0.010	No	0.010	No
S83	0.329	No	0.329	No
S99	0.091	No	0.016	No
Y103	0.899	Yes	0.899	Yes
S105	0.995	Yes	0.995	Yes
S106	0.454	No	0.454	No
T124	0.015	No	0.015	No
T127	0.222	No	0.222	No
S136	0.008	No	0.049	No

Table 3.3. Predicted phosphorylation residues of AtADF1 and AtADF4. NetPhos 2.0 software was used to analyze the potential of all the serine, tyrosine and threonine to be phosphorylated within AtADF4 and AtADF1. A score >0.5 indicates a predicted phosphorylation potential. The N/A for AtADF1 at serine-59 is because AtADF1 has a cysteine at position 59.

which is a well-established phosphorylated residue of cofilin1 (Ressad *et al.*, 1998; Table 3.3; Table 3.4). Of interest is the potential phosphorylation of tyrosine-67 and -103, which are residues identical to those in maize found to be important for G- and F-actin binding (Jiang *et al.*, 1997). Specifically, it was demonstrated that mutation of tyrosine-103 and alanine-104 to phenylalanine and glycine respectively resulted in a reduced affinity for both F- and G- actin; while mutation of tyrosine-67 and -70 to phenylalanine prevented binding to F-actin completely with no effect on G-actin affinity (Jiang *et al*., 1997).

Potential phosphorylation site	Score	Predicted
S3	0.02	No
S8	0.482	No
S23	0.996	Yes
S24	0.997	Yes
T25	0.959	Yes
S41	0.003	No
T63	0.917	Yes
Y68	0.868	Yes
T70	0.214	No
Y82	0.309	No
Y85	0.086	No
T88	0.016	No
Y89	0.791	Yes
T91	0.232	No
S94	0.997	Yes
S108	0.005	No
S113	0.023	No
S119	0.996	Yes
Y117	0.899	Yes
S120	0.150	No
T129	0.024	No
Y140	0.627	Yes
T148	0.597	Yes
S156	0.017	No
S160	0.854	Yes

Table 3.4. Cofilin 1 phosphorylation prediction. NetPhos 2.0 software was used to analyze the potential of all the serine, tyrosine and threonine to be phosphorylated within Cofilin 1. A score >0.5 indicates a predicted phosphorylation potential.

The aforementioned residues should be considered for additional analysis, however, what is most interesting to the current study is the difference in the potential of phosphorylation of tyrosine-53 (Table 3.3). It appears that AtADF4 tyrosine-53 is capable of being phosphorylated while the exact same residue in AtADF1 is not predicted to possess a phosphorylation potential (Table 3.3). This finding is given further credence because the tyrosine-53 is located within the region of amino acids that

was originally swapped in the construction of AtADF1 and AtADF4 chimers, due to the clustering of four different amino acids (Figure 3.1). In order to test the involvement of tyrosine-53 phosphorylation in AtADF4's regulation of gene expression, various tyrosine-53 to phenylalanine-53 mutants were constructed utilizing Quick change PCR (Table 3.5). If phosphorylation of tyrosine is required for expression of *RPS5*, it is expected that the 35S:T7-AtADF4^{Y53F} will not complement the *adf4* mutant's loss of *RPS5* expression as 35S:T7-AtADF4 has in previous works (Table 3.5; Porter *et al*., 2012). Additionally, Quick change PCR was performed on the chimeric proteins as well to determine the contribution of the amino acids from AtADF4, as well as the contribution of phosphorylation of tyrosin-53 will play in immunity (Table 3.5).

Within the focus of the above AtADF1 and AtADF4 chimeric proteins with regard to tyrosine-phosphorylation I next sought to examine the role of individual amino acids that differ between AtADF1 and AtADF4 and how they would effect the aforementioned phosphorylation of tyrosine. To achieve this, I ran the NetPhos 2.0 software on AtADF1 with single insertions of AtADF4 amino acid residues within the swapped region and determined how each amino acid substitution would effect the phosphorylation potential of tyrosine-53. I found that while neither the substitution of glutamic acid-48, leucine-51, nor cysteine-59 would change the potential of phosphorylation at tyrosine-53, substitution of aspartic acid at position 55 would allow for the predicted phosphorylation of tyrosine-53 in AtADF1. Because of these findings I have created a single point mutation construct, AtADF1^{E55D}, to test the requirement of aspartic acid at position 55 for the phosphorylation of tyrosine-53 (Table 3.5). Additionally, this construct will be

Construct name	Express *RPS5*	Resistant
AtADF4^{Y53F}	No	No
AtADF1$^{Q48E, Q51L, Y53F, E55D, C59S}$	No	No
AtADF4$^{E48Q, L51Q, Y53, D55E, S59C}$	No	No
AtADF1^{E55D}	Yes	Yes

Table 3.5. List of phosphorylation-related protein constructs and their predicted ability to complement the *adf4* mutant for expression of *RPS5* and resistance to *Pst* AvrPphB.

used to examine its ability to complement the *adf4* mutant for resistance to *Pst* AvrPphB and expression of *RPS5*. This construct is very interesting in that it has the potential to address three facets of AtADF4's role in immunity. Complementation of *adf4* with 35S:T7-AtADF1^{E55D} would identify the minimal amino acid substitution required for the immune function of AtADF4, while simultaneously confirming the necessity of tyrosine-53 phosphorylation and the dependence of aspartic acid-55 for said phosphorylation.

Comparison of AtADF4 and AtADF1 with other plant ADFs

Recent work using other plant species, including *Triticum aestivum* (wheat) and *Oryza sativa* (rice), has identified potential roles for ADFs in immune signaling and resistance (Cheng *et al.*, 2013; Fu *et al.*, 2014). In rice, for example, the OsADF mutant displayed enhanced susceptibility to both the fungal pathogen *Magnaporthe grisea* and the bacterial pathogen *Xanthomonas oryzae* pv *oryzae* (Cheng *et al*., 2013). A reduction in the expression of defense genes including *PR1a*, was also observed, which relates to what was found in the *adf4* mutant of Arabidopsis (Tian *et al*., 2009; Cheng *et al*., 2013). Fu *et al*., (2014) found that silencing the tree copies of TaADF7 in wheat resulted in enhanced susceptibility to the fungal pathogen *Puccinia striiformis* f. sp. *trici* (Fu *et al*.,

2014). Interestingly, similar to what was observed in Tian *et* al., 2009, both HR and expression of *PR1* were also reduced in the wheat TaADF7 mutant (Tian *et al*., 2009; Fu *et al*., 2014).

Based on the above functions of ADFs in broader immune signaling pathways, I decided to use *CLUSTAL Omega* alignment software to compare the sequences of AtADF1 and AtADF4 with the wheat TaADF7 sequence, including AtADF3 as a control for the differences among class I AtADFs (Figure 3.3). The *adf3* mutant also expresses *RPS5* at wild type levels, as well is resistant to *Pst* AvrPphB (Tian *et* al., 2009; Porter *et al*., 2012). Surprisingly, TaADF7 shared all of the AtADF4 amino acid residues within the swapped region used in the creation of the chimeric proteins (Figure 3.3). Additionally, AtADF4 was most similar with TaADF7 as compared to the other AtADFs with 79.86% sequence identity. AtADF3 does possess aspartic acid at position 55, however AtADF3 does not have tyrosine at position 53, but instead has a histone (Figure 3.3).

In an attempt to rule out the possibility that the immunity phenotype in wheat TaADF7 mutant was not due to a different function of TaADF7 with regard to actin-binding or modification of the actin cytoskeleton *via* bundling or severing I sought to identify studies that found plant ADFs known to preform these functions and have a reduced ability to depolymerize actin. It should be noted that both AtADF1 and AtADF4 have been demonstrated to both sever and depolymerize F-actin (Tian *et al*., 2009; Henty *et al*., 2011). These findings, along with other recent data, suggest that severing may be a

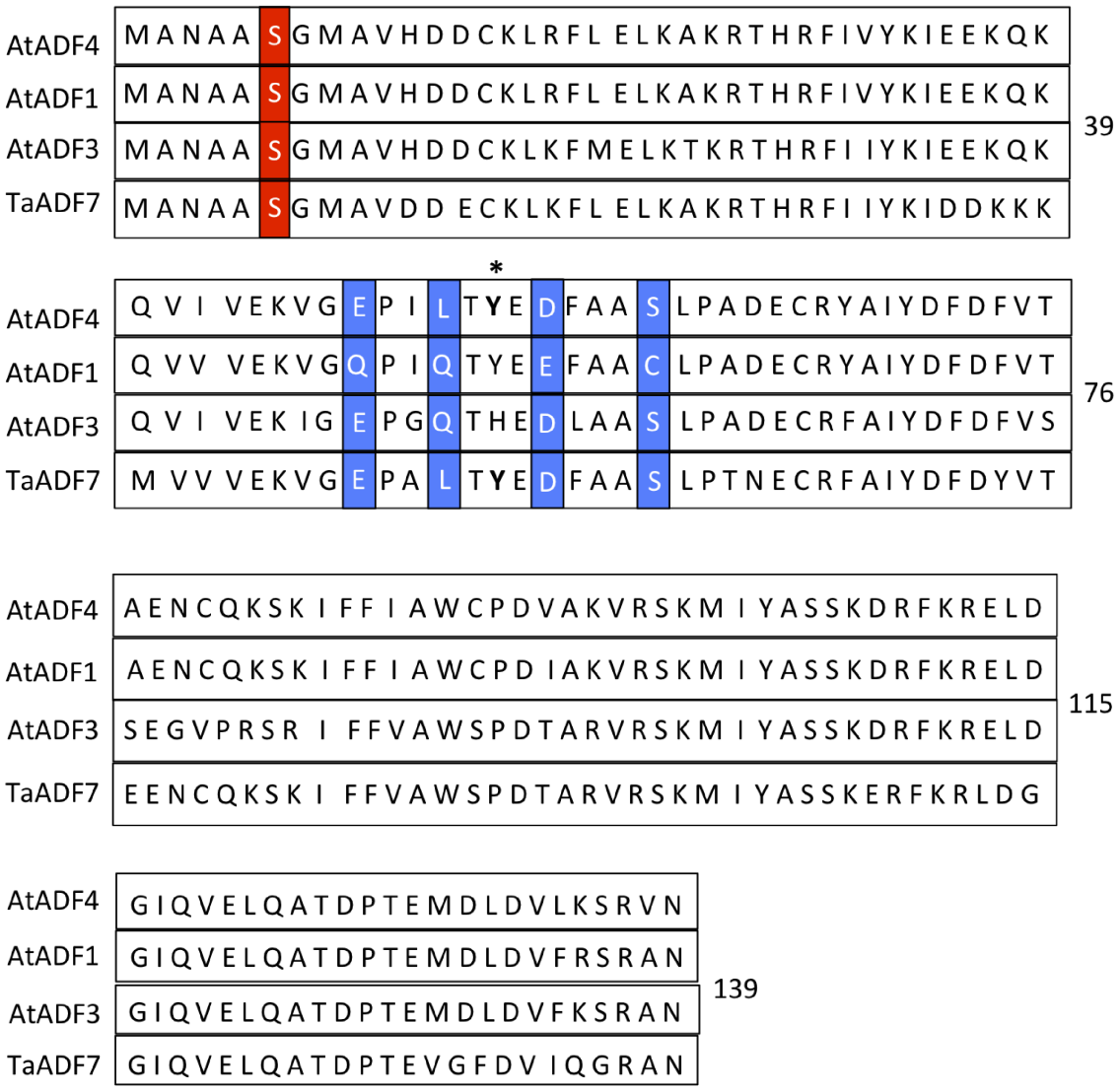

Figure 3.3 Sequence alignments of AtADF1, AtADF3, AtADF4 and TaADF7. Differing amino acids are highlighted in blue. The phosphorylatable serine residue is highlighted in red, while the tyrosine-53 residue, which is predicted to have the potential to be phosphorylated in AtADF4 and TaADF7, is bolded and is denoted by an asterisk.

key mechanism of ADFs for filament turnover (Henty *et al*., 2011; Andrianantoandro & Pollard, 2006; Roland et al., 2008). With regard to plant ADFs with an enhanced ability to bundle F-actin, a recent study by Tholl *et al*. (2011) found that AtADF9 does not depolymerize, but instead acts to stabilize and bundles F-actin. This study focused on

functional data as well as potential changes in the secondary structure of AtADF9, specifically in the F-actin binding pocket (Tholl *et al*., 2011).

I decided to use this information and the sequence of AtADF9 in combination with what is known about important residues for actin binding to determine if TaADF7 shared any similarities with the differing amino acids of AtADF9 that may account for its bundling activities (Tholl *et al*., 2011; Ono, 2007; Lappalainen *et al.*, 1997; Dong *et al.*, 2013). Initially, I used *CLUSTAL omega* to perform sequence alignments between AtADF1, AtADF4, AtADF9 and TaADF7; I have highlighted the regions that I will examine further with respect to actin binding (Figure 3.4). Next the SIFT amino acid substitution software was utilized to examine the differing amino acids between AtADF1 and AtADF9 because these are the two proteins that were examined for function differences in actin binding in the original manuscript (Table 3.6). It was determined that there are two unfavorable substitutions predicted by SIFT software, the arginine-82 to methionine and asparagine-139 to lysine (Table 3.6). These findings are interesting because arginine-82 has been demonstrated to be important for F-actin binding, and furthermore has a BLOSUM 62 value of -1, indicating an unfavorable substitution (Ono 2007; Lappalainen *et al*., 1997; Table 3.6). Asparagine-139 has not been demonstrated to be required for F-actin binding, but is close to arginine-137 and has a SIFT score of 0.00, which is predicted to be deleterious to the function of the protein (Table 3.6). It should be noted that three of the five amino acids demonstrated to have a role in actin binding in AtADF1 are completely unchanged in AtADF9, which may allow for binding of AtADF9 to F-actin, but alter the interaction such that instead of depolymerizing F-actin,

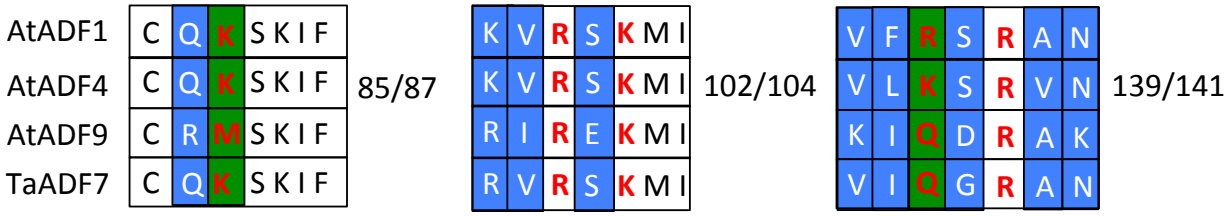

Figure 3.4 Sequence alignments of actin binding regions of AtADF1, AtADF4, AtADF9 and TaADF7. Differing amino acids not demonstrated to be required for actin binding are highlighted in blue, while amino acids required for actin binding are highlighted in green. The residues required for actin binding are denoted in red text. The amino acid position numbering is different because AtADF9 has 141 amino acids, while AtADF1, AtADF4 and TaADF7 have 139 amino acids.

AtADF9 instead bundles F-actin. Taken together with the functional data from Tholl *et al*. (2011), the amino acids identified above were used to compare AtADF4 and TaADF7 to AtADF9 (Table 3.6).

Similar to what was found in the AtADF1 comparison to AtADF9, both AtADF4 and TaADF7 possess an arginine at position 82, while AtADF9 has a methionine, resulting in a lower SIFT score and -1 BLOSUM62 score (Table 3.6). Additionally, both AtADF4 and TaADF7 have asparagine-139 as compared to lysine-141 of AtADF9 resulting in a SIFT score of 0.00, which again is predicted to be deleterious to protein function (Table 3.6). Interestingly, the valine-138 to alanine-140 substitution between AtADF4 and AtADF9 also has a SIFT score of 0.00, while this same substitution between AtADF4 and either AtADF1 or TaADF7 results in a SIFT score of 0.78 (Table 3.1; Table 3.6). This output is most likely due to the SIFT parameters, which take into account neighboring amino acids in its comparison (Ng, 2003). The low SIFT score seen is this substitution between AtADF4 and AtADF9 is most likely due to the additional differing amino acid at position 139 (Table 3.6). A unique shared residue of TaADF7 and AtADF9

Amino acids AtADF1 to AtADF9	SIFT score	Amino acids AtADF4 to AtADF9	SIFT score	Amino acids TaADF7 to AtADF9	SIFT score	Amino acids AtADF4 to TaADF7	SIFT score
Q81R	1.00	**Q81R**	1.00	**Q81R**	1.00	**-**	-
K82M	**0.17**	**K82M**	**0.17**	**K82M**	**0.17**	**-**	-
K96R	0.89	**K96R**	0.88	**-**	-	**K96R**	0.90
V97I	0.97	**V97I**	0.96	**V97I**	0.97	**-**	-
S99E	1.00	**S99E**	1.00	**S99E**	1.00	**-**	-
V133K	1.00	**V133K**	1.00	**V133K**	1.00	**-**	-
F134I	1.00	**L134I**	0.90	**-**	-	**L134I**	0.90
R135Q	0.81	**K135Q**	0.72	**-**	**-**	**K135Q**	0.73
S136D	1.00	**S136D**	1.00	**G136D**	1.00	**S136G**	0.92
-	-	**V138A**	**0.00**	**-**	-	**V138A**	0.78
N139K	**0.00**	**N139K**	**0.00**	**N139K**	**0.00**	**-**	-

Table 3.6. Amino acid substitutions of AtADF1, AtADF4, TaADF7 and AtADF9 around regions predicted to be involved in actin binding. This table displays the SIFT score values of amino acid. SIFT substitutions with scores <0.05 are not predicted to be tolerated. The bolded SIFT values are either low and should be considered or are below 0.05 and therefore not tolerated. The amino acids and SIFT scores in red text are those amino acids that have been demonstrated to have a role in actin binding.

is glutamine-135, however there appears to be little negative impact of substitution of either glutamine or lysine for the arginine in AtADF1 (Table 3.1; Table 3.6). Lastly, when AtADF4 and TaADF7 are compared within the region of interest there are only five differing amino acids, none of which are predicted to negatively impact protein function (Table 3.6). Taken together, these data suggest that in addition to the four shared amino acids between AtADF4 and TaADF7 within our initial region of interest surrounding tyrosine-53 (Figure 3.3), AtADF4 and TaADF7 most likely have the ability to interact with actin in a similar manner (Figure 3.4; Table 3.6). TaADF7 is most similar to AtADF4 with respect to the regions thought to be involved in actin binding, where AtADF4 has been experimentally shown to interacts with actin like AtADF1 (Henty *et al*., 2011). Furthermore, TaADF7 also share differing amino acids as compared to AtADF9,

which has been functionally demonstrated to interact with actin in a manner that is antagonistic to AtADF1 (Tholl *et al*., 2011).

When AtADF4 was compared to all 11 of the rice ADFs by *CLUSTAL omega* software, additional patterns emerged (Figure 3.5). Rice OsADF7, which was recently re-annotated, possesses glutamic acid-48, leucine-51, tyrosine-53, aspartic acid-55 and cysteine-59 and has an 81.29% sequence identity to AtADF4 (Figure 3.5). Interestingly, OsADF7 and TaADF7 have a 92.81% sequence identity. Additionally, nine rice OsADFs have an aspartic acid aligning with AtADF4 aspartic acid-55 and nine have tyrosine aligning to tyrosine-53 (Figure 3.5). Taken together these alignments highlight an interesting region of plant ADFs that may give insight into additional cellular functions of some of the members of the large family of ADFs found in plants.

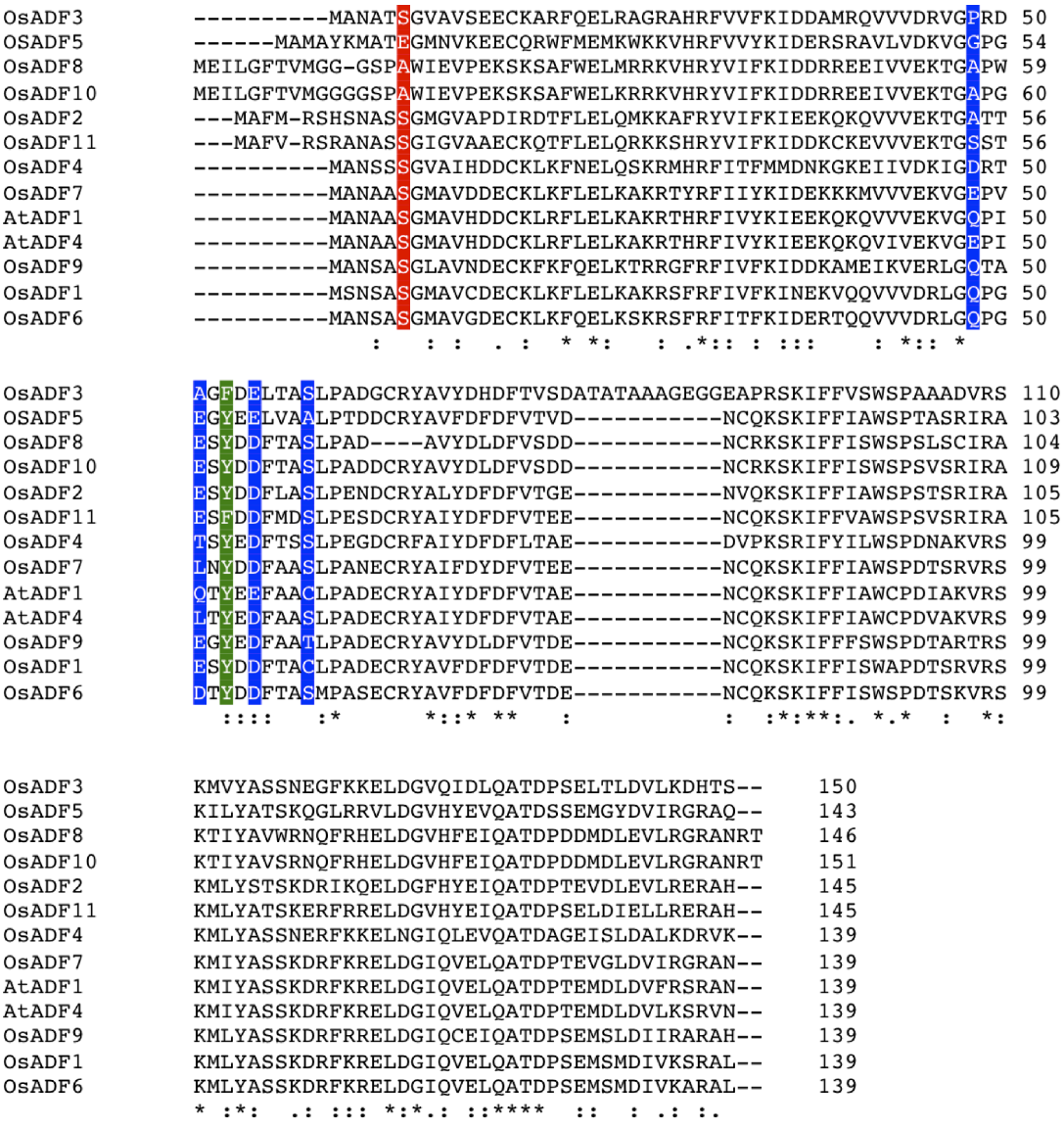

Figure 3.5. Alignment of 11 rice Actin depolymerizing factors (OsADFs) and AtADF1 and AtADF4. Differing amino acids within the region swapped in the formation of the chimeric proteins are highlighted in blue. The phosphorylatable serine residue is highlighted in red, while the tyrosine-53 residue, predicted to be phosphorylated in AtADF4 and TaADF7, highlighted in green.

Discussion

AtADF4 has been demonstrated to be an important component of defense-signaling in Arabidopsis protection against the phytopathogen *Pseudomonas syringae* AvrPphB (*Pst* AvrPphB; Porter *et al*., 2012; Tian *et al*., 2009). Previous studies have identified that this importance extends to the expression of the pathogen-related gene *PR1* and the resistance gene (R-gene) *resistance to Pseudomonas syringae-5* (*RPS5*), as well as the activation of immune-signaling pathways in the presence of the bacterial effector AvrPphB (Tian et al., 2009; Chapter 2; Porter *et al*., 2012). Additionally, AtADF4 is required for defense specific remodeling of the actin cytoskeleton upon recognition of pathogen associated molecular patterns (PAMPs) and subsequent PAMP-triggered immunity (PTI; Henty-Ridilla *et al*., 2014).

With regard to the biochemical properties of AtADF4 that allow for its observed defense-related properties, I have demonstrated in the previous chapter that AtADF4 can indeed be phosphorylated at the predicted serine-6 residue, and furthermore, that this phosphorylation was required for the appropriate expression of *RPS5* and subsequent resistance to *Pst* AvrPphB (Chapter 2; Porter *et al*., 2012). This finding was unexpected, as concurrent works demonstrated that, as is the case for other ADFs, the phosphorylation of serine-6 is often associated with a loss of activity, through the reduced affinity of the ADF for the actin cytoskeleton (Carlier *et al.*, 1997; Ouellet *et al.*, 2001; Porter *et al*., 2012). The phosphorylation of serine-6 as an activation of gene

expression is suggestive of a role for AtADF4 that is less tightly correlated with the actin cytoskeleton, and may in fact be independent of the well-established functions of ADFs.

In the current study, I attempted to identify additional biochemical features of AtADF4 that may explain the divergent role of AtADF4 in immune-signaling by utilizing *in silico* protein analysis techniques. This undertaking was initialized with the comparison of AtADF4 to its closest Arabidopsis homologue AtADF1. It was determined that AtADF1 and AtADF4 are 97% homologous with 93.5% sequence identity (Figure 3.1). Furthermore, it was determined that in addition to the observed resistance phenotype to *Pst* AvrPphB, the AtADF1 mutant (*adf1*) also properly expresses *RPS5* (Figure 3.2).

There must however be some motif and/or amino acid differences between AtADF4 and AtADF1 that can explain the differences in susceptibility to *Pst* AvrPphB. In depth computational examination of the sequences, differing amino acids and projected protein structures of AtADF4 and AtADF1 identified a region containing four amino acid differences with the greatest likelihood of inducing changes in structure and/or function between the two proteins (Figure 3.1; Table 3.1). Although these differing amino acids are not directly implicated in known β5 and α4 regions of the proteins for binding filamentous (F-actin), the affected α2 and free loop are distal to these regions, and therefore there could be some differences in F-actin association, although whatever subtle difference may be present, it does not seem to alter the depolymerization or severing functions of AtADF4 (Figure 3.1; Tian *et* al., 2009; Henty *et* al., 2011; Carlier *et al*., 1997; Ono *et* al., 2007; Lappalainen *et* al., 1997; Tholl *et al*., 2011). More

importantly, these residues may indeed affect the resistance to *Pst* AvrPphB. In order to test this possibility, I developed chimeric proteins for complementation of the *adf4* mutant to test this hypothesis. Specifically, I believe that the complementation of *adf4* with AtADF1 containing the substitution of the four differing amino acids in this region (AtADF1$^{Q48E,\ Q51L,\ E55D,\ C59S}$) will allow for complementation of *RPS5* expression and subsequent resistance to *Pst* AvrPphB based on my computational analysis of the potential impact of these amino acids. Furthermore, based on the aforementioned predictive software, substitution of leucine-51 for glutamine in AtADF1 may be the minimal substitution required for complementation.

An important control of AtADF4 mediated resistance to *Pst* AvrPphB is the aforementioned phosphorylation of serine-6 (Chapter 2; Porter *et al*., 2012). While there are a considerable number of amino acids within AtADF4 and AtADF1 that are predicted to possess the potential for phosphorylation, there is only one differing residue between the two, tyrosine-53 (Table 3.3). Because I have focused my work primarily on the biochemical differences between AtADF4 and AtADF1 I chose to create a suite of point mutations that would allow us to definitively determine if tyrosine-53 is indeed phosphorylated in AtADF4 (Table 3.5). Furthermore, I created an additional point mutation to address the predicted importance of aspartic acid-55 in the phosphorylation of tyrosine-53. Taken together this set of experiments will allow me to 1) determine if tyrosine-53 is phosphorylated in AtADF4, 2) demonstrate to what extent aspartic acid-55 has on this phosphorylation and, 3) examine the importance of this phosphorylation in the immune-signaling cellular function of AtADF4.

It should be noted, that with respect to amino acids substitutions leucine-51 of AtADF4 appears to have the greatest impact, while aspartic acid-55 of AtADF4 may regulate the phosphorylation of tyrosine-53. Because of this neither AtADF1^{Q51L} nor AtADF1^{E55D} may be sufficient to complement the *adf4* for immune-related function and in fact AtADF1$^{Q51L, E55D}$ may be the minimal substitutions required to restore this function.

The overall goal of this chapter was to identify unique biochemical features of AtADF4 that allowed for its role in defense signaling. While ultimately, the complementation of the *adf4* mutant with the constructs outlined in this chapter is required to determine the minimal substitution(s) of AtADF4 amino acids in AtADF1, the computational analyses performed herein will dramatically reduce the number of constructs needed for this determination. Furthermore, I hypothesize that due to the relatively large size of the ADF family in plants, some members could adopt additional cellular functions outside their well-described role in cytoskeleton regulation. Recent publications support this hypothesis, in that there is an observed susceptibility in other plant species, including rice and wheat, where an ADF has been silenced or knocked-out (Cheng *et al*., 2013; Fu *et al*., 2014). My *in silico* examination of specific residues and motifs of AtADF4 as compared to other plant ADFs determined to play a role in resistance has highlighted a potential immunity-related region of these ADFs. Taken together, I believe that the observed unique cellular function of AtADF4 in immune-related signaling is conserved in plants and has existed since the expansion of the ADF family.

Methods and Materials

Arabidopsis thaliana ADF protein sequence alignment and ADF4 homology model construction

The *A. thaliana* genome was searched for sequences similar to the ADF1 amino acid sequence using *BLASTP*. Eleven identified ADF protein sequences were aligned using *CLUSTAL Omega* (Sievers *et al.*, 2011) with five MBED-like Clustering Guide-Tree and five HMM iterations. The three-dimensional protein structure of *A. thaliana* ADF4 was predicted using SWISS-MODEL (Arnold *et al.*, 2006; Kiefer *et al.*, 2009) and the *A. thaliana* ADF1 crystal structure (PDB ID: 1F7S; (Bowman *et al.*, 2000)) or a *A. thaliana* ADF1 molecular dynamic (MD) snapshot (Tholl *et al.*, 2011) as template structures. The MD structure was selected as an additional template as the amino and carboxyl termini structures and confirmations were included. These templates had greater than 93% sequence identity with resultant homology structures having E-values greater than 3×10^{-68} and Q-mean scores less than -2.5. The SWISS-MODEL Anolea and Qmean quality metrics identified a loop region, residues G47-E54, as having high-energy backbone and side chain conformations. This loop region had no steric overlap or neighboring charged groups with the remainder of the protein. It is possible that another confirmation of this mobile region would be more stable, but no modification of the alignment to the ADF1 template or side chain rotation improved energy values.

Prediction of tolerance of amino acid substitutions and phosphorylatable residues

Prediction of the tolerance of amino acid substitutions within was performed using the publically available SIFT software (http://sift.jcvi.org; (Ng, 2003)). The analysis was run on AtADF1 with regard to the nine amino acids of AtADF4 that were different and conversely, with AtADF4 with the nine amino acids that were different in AtADF1. Any SIFT score > 0.05 is considered to be a tolerated amino acid substitution. Examination of the tolerance of individual amino acid substitutions without the influence of the neighboring amino acids was performed with the BLOSUM62 chart (Henikoff & Henikoff, 1992). Amino acid substitutions with positive scores are more likely to occur, while negative scores indicate less favorable substitutions.

Plasmid construction and cloning

The AtADF1$^{Q48E,\ Q51L,\ E55D,\ C59S}$ and AtADF4$^{E48Q,\ L51Q,\ D55E,\ S59C}$ plasmids were synthesized by Life Technologies Gene Art AG (www.lifetechnologies.com) in the pENTR 221 vector backbone. The pENTR 221 constructs were sub-cloned into the binary vector pMDT7 as described in Chapter 2. All primers used for cloning are listed in Table 3.7.

Plant growth and Arabidopsis transformation

Arabidopsis plants were grown in a BioChambers walk-in growth chamber (model FLX-37; Winnipeg, Manitoba, Canada) at 20 °C under a 12-hour light/12-hour dark cycle, with 60% relative humidity and a light intensity of 100 μmol photons $m^{-2}s^{-1}$. Transformation of Arabidopsis, as well as selection of transformants, was performed as described by Clough & Bent, (1998).

RNA extraction and qRT-PCR

Total RNA was extracted from leaves using the PrepEase Plant RNA Spin kit (USB Affymetrix, Santa Clara, CA, USA). First-strand cDNA was synthesized from 1 μg total RNA using the First-Strand cDNA Synthesis kit (USB Affymetrix). Primers, specifically, *RPS5* and *UBI10*, used for quantitative real-time PCR (qRT-PCR) are listed in Table 2.2 in Chapter 2. qRT-PCR was performed using the Mastercycler ep Realplex system (Eppendorf AG, Hamburg, Germany), as previously described(Chapter 2), using the Hot Start SYBR Master mix 2X (USB Affymetrix). Ubiquitin (*UBQ10*) was used as an endogenous control for amplification. Fold Col-0 was determined using the following equation: (relative expression)/(relative expression of Col-0 untreated), where "relative expression" = $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{gene\ of\ interest} - Ct_{UBQ10}$.

Primer name	Primer sequence 5' to 3'	Utility
SalI ADF4 F	GCGGTCGACATGGCTAATGCTGCGTCAGGAATGG	pMDT7 cloning
SalI ADF4 S6A F	GCGGTCGACATGGCTAATGCTGCGGCAGGAATGG	pMDT7 cloning
SalI ADF4 S6D F	GCGGTCGACATGGCTAATGCTGCGGACGGAATGG	pMDT7 cloning
ADF4 R SalI	GCGGTCGACTTAGTTGACGCGGCTTTTCAAAAC	pMDT7 cloning
SalI ADF1 F	GCGGTCGACATGATGGCGAACGCGGCATCTGGAATGG	pMDT7 cloning
SalI ADF1 S6A F	GCGGTCGACATGATGGCGAACGCGGCAGCTGGAATGG	pMDT7 cloning
SalI ADF1 S6D F	GCGGTCGACATGATGGCGAACGCGGCAGATGGAATGG	pMDT7 cloning
ADF1 R SalI	GCGGTCGACTTAGTTGGCACGGCTCCTGAAAAC	pMDT7 cloning
ADF4 L51Q A	CTGCAAAGTCCTCGTAAGTTTGAATAGGTTCACCAACTTTC	Quick change
ADF4 L51Q S	GAAAGTTGGTGAACCTATTCAAACTTACGAGGACTTTGCAG	Quick change
ADF1 Q51L A	CAAACTCCTCGTAAGTTAGGATCGGTTGACCAACT	Quick change
ADF1 Q51L S	AGTTGGTCAACCGATCCTAACTTACGAGGAGTTTG	Quick change
ADF4 Y53F A	CTGCAAAGTCCTCGAAAGTTAGAATAGGTTCACCAACTT	Quick change
ADF4 Y53F S	AAGTTGGTGAACCTATTCTAACTTTCGAGGACTTTGCAG	Quick change
ADF1 Y53F A	TGCAAACTCCTCGAAAGTTTGGATCGGTTGACCAA	Quick change
ADF1 Y53F S	TTGGTCAACCGATCCAAACTTTCGAGGAGTTTGCA	Quick change
ADF1 D55E A	GATCCAAACTTACGAGGATTTTGCAGCATGTCTTCCA	Quick change
ADF1 D55E S	TGGAAGACATGCTGCAAAATCCTCGTAAGTTTGGATC	Quick change

Table 3.7. List of primers used for cloning.

Quick change PCR

An adapted version of the Quik Change Site Directed Mutagenesis kit instruction manual was used to preform the Quick change PCRs (Stratagene catalog # 200518). In short, Quick change primers were designed using the Agilent Technologies Quik Change Primer Design software ((www.genomics.agilent.com/primerDesignProgram.jsp); Table 3.7). PCR reaction

contained: 1 unit Pfu Turbo DNA polymerase (Agilent), 1:10 dilution of 10X PCR reaction buffer (Agilent), 300ng sense and antisense Quick change primer (Invitrogen), 100nM dNTPs (Denville), and 50ng of ds DNA plasmid. Recommended thermocycler reactions were performed followed by digestion of parental plasmid with 1 unit Dnp1 (New England Biosciences) at 37ºC for 1 hour followed by heat inactivation and subcloning into *E. coli* DH5α (Invitrogen).

CHAPTER 4

Conclusions and Future directions

The actin 2 data presented in this chapter was performed by myself, and is part of an ongoing collaboration with Dr. Jeff Chang and Allison Creason at Oregon State University.

Conclusions

Arabidopsis actin-depolymerizing factors (AtADFs) are actin binding proteins (ABPs) with well-characterized biochemical functions to depolymerize and/or sever actin filaments contributing to the overall regulation of cytoskeletal dynamics (Carlier *et al.*, 1997; McGough *et al.*, 1997; Ruzicka *et al.*, 2007). As such, much of the research on AtADFs and other ABPs has focused primarily on their interactions with and on the actin cytoskeleton. My research pursuits, however, have centered on the requirement of a specific AtADF, AtADF4, for the defense responses of Arabidopsis to the phytopathogen *Pseudomonas syringae* expressing the bacterial effector AvrPphB (*Pst* AvrPphB; Tian *et al.*, 2009). The purpose of the dissertation project was to identify the immune pathways of Arabidopsis lacking ADF4 (*adf4*) that are compromised during the compatible interaction with *Pst* AvrPphB, and ultimately, to determine the role of AtADF4 is in these defense-related pathways. Additionally, I wanted to examine unique biochemical properties of AtADF4 that would allow for the divergence and expansion of cellular functions to include disease signaling. The hypothesis being that AtADF4 possesses specific biochemical attributes that allow for non-canonical cellular functions related to immune signaling that may be directly related to, or independent of its pre-established interactions with the actin cytoskeleton or a fine-tuned balance of both. To this end, I have established, in Chapter 2 of this thesis, a role for AtADF4 in expression of the resistance gene (R-gene) *resistance to Pseudomonas syringae-5* (*RPS5*), and immune signaling related to the recognition of pathogen associated molecular patterns (PAMPs). Additionally, I have utilized *in silico* protein analysis tools, in Chapter 3, to

identified biochemical features and specific amino acids that may allow for the unique cellular functions of AtADF4. These findings have contributed to the expansion of the field of study of actin and ABPs past the singular role in cellular architecture, towards gene expression, surveillance, and signal transduction.

My findings in Chapter 2 explored further our lab's initial findings that identified a unique and unexpected feature of one of the Arabidopsis ABPs, AtADF4, in the defense response to *Pst* AvrPphB (Tian *et al*., 2009). Briefly, it was determined that the *adf4* mutant plant specifically is susceptible to *Pst* AvrPphB, but maintains resistance to *Pst* expressing other bacterial effectors, including ArvRpt2 and AvrB (Tian *et al*., 2009). However the mechanism by which this compatible interaction occurred was not well understood. Due to the unique gene-for-gene defense response of plants that ultimately results in effector-triggered immunity (ETI), I initially measured the expression of the R-gene required for recognition of AvrPphB, *RPS5* and determined that the *adf4* mutant has significantly reduced expression of *RPS5* (Figure 2.1A), while the expression other well-characterized R-genes is not affected (Figure 2.4). Furthermore, complementation of *adf4* with AtADF4 under the control of the native promoter allows for restoration of the expression of *RPS5* (Figure 2.1B). These findings are in support of my hypothesis that AtADF4 has additional cellular functions including regulating the expression of the R-gene *RPS5*, that contribute to its role in the host defense response.

Although ETI is effective in activating a robust immune response that is often associated with conferring resistance to a specific pathogen, it is not the only immune response of

plants. Upon initial recognition of non-self PAMPs through pattern recognition receptors (PRRs) the plant cell activates PAMP-triggered immunity (PTI), which includes many of the same pathways of ETI. I found that *adf4* was indeed sensitive to flg22, a 22-amino acid peptide of the PAMP flagellin (Figure 2.6), and responded appropriately with activation of the *flg22-induced receptor-like kinase 1* (*FRK1*; Figure 2.5A), which suggests intact signaling by flagellin-sensitive 2 (FLS2), the PRR for flg22 and flagellin. The activation of *FRK1* was, however, significantly reduced when *adf4* was challenged with *Pst* AvrPphB (Figure 2.5C).

Initially, it was believed that the reduction of *FRK1* when challenged with *Pst* AvrPphB was due to a reduction in the activation of the mitogen-activated protein kinase (MAPK) signaling pathway due the loss of *RPS5* expression in the *adf4* mutant. This hypothesis was supported by the mirrored reduction in *FRK1* in the RPS5 point mutant (*rps5*) when challenged similarly with *Pst* AvrPphB (Figure 2.5C). However, a more in depth molecular examination of the activation of the MAPK signaling pathway, wherein AvrPphB was expressed *in planta* in the absence of the pathogen, and MAPK was activated by flg22, revealed that MAPK signaling was reduced distinctively in the *adf4* mutant and not the *rps5* mutant (Figure 2.10). These findings are of interest in two aspects. First, it supports our hypothesis that AtADF4 is required for *RPS5* expression and subsequent accumulation of RPS5 protein, in that the *adf4* and *rps5* have nearly identical reduction in *FRK1* expression when challenged by *Pst* AvrPphB. Secondly, it suggests a specialized role for AtADF4 in the PTI-related MAPK activation in the presence of AvrPphB.

Beyond identifying the requirement of AtADF4 for both ETI- and PTI- responses to *Pst* AvrPphB through the expression of *RPS5* and the proper activation of FLS2-driven MAPK signaling pathway, respectively, I sought to identify the individual biochemical aspects of AtADF4 that regulate and facilitate these functions of immunity. Because of the well-established phosphorylation of serine-6 in other plant ADFs (Allwood *et al.*, 2001; Smertenko *et al.*, 1998; Ouellet *et al.*, 2001), including AtADF1 (Carlier *et al.*, 1997; Ressad *et al.*, 1998; Dong & Hong, 2013), I chose to examine the impact of serine-6 phosphorylation on the ability of AtADF4 to complement *adf4* with regard to *RPS5* gene expression and disease response to *Pst* AvrPphB. First I established, using 2-D gel electrophoresis and the AtADF4^{S6A} phospho-null complement, that serine-6 was indeed a residue capable of being phosphorylated in AtADF4 (Figure 2.12A). Furthermore, I established that, as previous works had identified of other plant ADFs (Carlier *et al.*, 1997), that the phospho-null AtADF4^{S6A} co-localized to the actin cytoskeleton with a higher affinity than the phospho-mimic AtADF4^{S6D} (Figure 2.15B; C). Interestingly, the phospho-mimic AtADF4^{S6D} complemented the disease resistance and *RPS5* expression of the *adf4* mutant, while the phospho-null AtADF4^{S6A} did not (Figure 2.12B; C; D). Although this finding was unexpected, it did support my original hypothesis that AtADF4 may function as a component of the immune response in plants in a less actin-centric manner.

To identify additional biochemical aspects of AtADF4 that would allow for a role in immune signaling, I chose to utilize an *in silico* approach and examine other members

of AtADFs to see if there were any striking similarities or differences. I found that AtADF4 and AtADF1 have a 97% homology and furthermore a 93.5% sequence identity (Figure 3.1B). Given the surprising level of sequence identity between AtADF1 and AtADF4, yet differing resistance phenotypes of the *adf4* mutant and AtADF1 mutant (*adf1)* to *Pst* AvrPphB (Tian *et al*., 2009; Figure 3.2), I decided to compare AtADF1 and AtADF4 further in order to identify potential differing biochemical features that may contribute to the differences in disease resistance.

Comparison of the amino acid difference between AtADF4 and AtADF1 (Figure 3.1B; Table 3.1) and the potential impacts these substitutions would have on protein structure (Figure 3.1A) revealed a region between β3 and α2 that had the most dense amount of differing amino acids as well as the largest negative impact on amino acid substitution. Based on these findings I proposed a set of chimeric proteins, as well as individual point mutant proteins that could address what effects this region of AtADF4 and AtADF1 would have on disease resistance to *Pst* AvrPphB (Table 3.2). The hypothesis being that if the differing amino acids within this region are of importance to the immunity function of AtADF4 the AtADF1$^{Q48E,\ Q51L,\ E55D,\ C59S}$ construct, and perhaps even the single amino acid substitution construct AtADF1^{Q51L}, will complement *adf4* mutant. This would further support my original hypothesis that there are unique biochemical features of AtADF4 that allow for its faculty in immune signaling.

Both AtADF1 and AtADF4 are phosphorylated at serine-6, which affects their interactions with actin (Carlier *et al.*, 1997; Porter *et al.*, 2012). However, it is not known

if there are additional phosphorylation sites on either. Predictive software was used to identify potential phosphorylatable resides of AtADF4 and AtADF1 (Table 3.3). AtADF4 is predicted to have a single amino acid with differing phosphorylation potential not predicted to be phosphorylated in AtADF1, tyrosine-53 (Table 3.3). Further computational analysis demonstrated that the amino acid responsible for the differing phosphorylation potential of tyrosine-53 in AtADF4 is aspartic acid-55. The creation of AtADF4^{Y53F}, and AtADF1^{E55D} complements of *adf4* will allow us to determine if tyrosine-53 phosphorylation is required for the immunity roles of AtADF4; and if so, if the substitution of a single amino acid from AtADF4 into AtADF1, that allows for this phosphorylation, is enough to complement the resistance of *adf4* to *Pst* AvrPphB (Table 3.5). All together, my in depth *in silico* biochemical comparison of AtADF4 and AtADF4 has given me insight into the unique features of AtADF4 that allow for its exceptional role in defense signaling.

Future Directions

The dissertation research described herein has illuminated unique properties of AtADF4 that contribute to disease signaling and resistance to *Pst* AvrPphB, providing support for the hypothesis that domain-specific features of ADFs – specifically, AtADF4 - may be required for its part in immune signaling. These works have answered many questions, and have also introduced many more with respect to the unique functions of AtADF4 and the actin cytoskeleton in response to disease, as well as the cellular functions of AtADF4 itself. In this section of my thesis I would like to outline the future directions of

the AtADF4 project and what I believe will be gained from completion of these future directions. There are three distinct directions that this project should go in order to completely examine AtADF4's role in immunity.

First, in continuation of my *in silico* identification of important residues and motifs of AtADF4, the *in vivo* complementation of the *adf4* mutant with the proposed chimeric and/or single amino acid substitution constructs should be completed. These plants can then be used to determine the importance of the identified residues as well as predicted phosphorylation of tyrosine-53 in expression of *RPS5* and resistance to *Pst* AvrPphB. Additionally, works in other plant species have also demonstrated a role for ADFs in immune signaling, as outlined in Chapter 3, further support my initial findings and proposed region of interest in AtADF4 (Cheng *et al.*, 2013; Fu *et al.*, 2014; Figure 3.3; Figure 3.5). Given the importance of the proposed region of AtADF4 and its conservation in additional plant lines, an interesting question arose: *Would ADFs of other plant species with this motif complement the Arabidopsis adf4 mutant for resistance to Pst ArvPphB*? Exploring cross-species complementation would definitively demonstrate the expansion of certain members of the ADF family in plants to adopt additional diverse cellular functions in immune signaling.

I encountered another question during the comparison of AtADF1 and AtADF4: *How similar are the biochemical functions of AtADF1 and AtADF4 to act upon actin, and what effects would the proposed amino acid substitutions have on these functions*? As exemplified by the antagonistic functions of AtADF1 and AtADF9, discussed in

Chapter3, not all ADFs in plants appear to interact with actin in the same way (Tholl *et al.*, 2011). While I have demonstrated the differences between AtADF1 and AtADF4 should not be as severe, prior examination of AtADF4 biochemically suggests there are subtle differences. It has been shown that AtADF4 has a markedly increased affinity for G-actin^{ATP} as compared to AtADF1, while little to no change was observed in affinity for G-actin^{ADP} (Tian *et al*., 2009). Additionally, knocking out AtADF4 does result in changes to the organization of the actin cytoskeleton *in planta*, confirming our hypothesis that functional redundancy of AtADFs cannot be assumed (Henty *et al.*, 2011). An in-depth biochemical analysis of AtADF1, AtADF4, and the chimeric proteins, with regard to their interactions with and upon the actin cytoskeleton, will give further insight into the role(s) of the specific regions of AtADF4 and AtADF1.

Second, due to the alterations of gene expression in the *adf4* mutant, and other plant species with ADF knock-outs, including the re-occurrence of reduced *PR1* expression, many questions have arose about the specificity and mechanism of AtADF4's importance in gene expression. An initial question being: *Are the AtADF4-regulated genes specifically immune-related genes*? Although we have examined the expression of genes related to immune signaling in the *adf4* mutant, because differences in resistance exist when plants are challenged with biotic stresses, this does not mean that the only genes AtADF4 regulates are defense-related. A critical next step in this project would be examining the global changes in gene expression within the *adf4* mutant compared to the wild-type Arabidopsis Col-0, through RNA sequencing techniques. The samples for this RNA-sequencing analysis should include both resting state plants and

plants that have been challenged with *Pst* AvrPphB. This analysis would result in determining if AtADF4 regulates the expression of genes in a defense-related pattern or regulates multiple genes related to many cellular functions, as wells as identify additional potential gaps in the immune-signaling of *adf4* due to loss of transcriptional reprograming within specific immune-related signaling pathways.

Within the scope of AtADF4 and it's role in gene expression I pose another question: *To what degree does actin play a role in the AtADF4 dependent regulation of gene expression*? As discussed in Chapter 1, the past few decades have seen numerous advances towards understanding the role and activity of actin and the actin cytoskeleton within the nucleus (Chapter1; Pederson & Aebi, 2002; Bettinger *et al.*, 2004). In 2010 Kandasamy *et al*. identified the presence of the 3 vegetative forms of actin – ACT2, ACT8, and ACT7 – within the nuclei of plant cells, although actin itself posses no true nuclear localization signal (NLS: Kandasamy *et al.*, 2010). These findings confirm that what is believed in other systems with regard to actin having a nuclear function is a possibility in plants as well. Of particular interest to our work, it has been determined that the nuclear import of actin is facilitated by cofilin, the predominant mammalian ADF, in a Ran-dependent manner through interactions with nuclear importin 9 (Dopie *et al.*, 2012; Figure 1.4). Additional work in this area has demonstrated that profilin, an actin binding protein that functions in the polymerization of G-actin into F-actin filaments, facilitates the export nuclear actin through association with exportin-6 (Stuven *et al.*, 2003; Dopie *et al.*, 2012; Figure 1.4). This mechanism is supported in plants by the observation of each of these ABPs (i.e., ADF and Profilin), including specifically

AtADF4, is localized within the nucleus (Kandasamy *et al.*, 2007; Kandasamy *et al.*, 2010; Figure 2.15A).

Based upon the above information, that vegetative actins and AtADF4 are present in the nucleus and that the presence of actin within in the nucleus may be dependent upon ADFs, I wondered if perhaps the expression of *RPS5* is altered in actin mutants (Kandasamy *et al.*, 2007; Kandasamy *et al.*, 2010; Figure 2.15A). In collaboration with Dr. Jeff Chang's laboratory at Oregon State University, who identified a disease phenotype of the AtActin2 over expression mutant (Act2OE2) when challenged with *Pst* AvrPphB, I identified alteration of *RPS5* expression in not only the Act2OE2 plant line, but also the AtActin2 mutant *act2* (Figure 4.1). Specifically, the Act2OE2 line has a highly significant loss in *RPS5* expression and the *act2* mutant also has a significant reduction in *RPS5* expression (Figure 4.1). Interestingly, it seem that either the over expression of, or the loss of AtActin2 results in alteration of RPS5 expression, suggesting that it may be the balance of specific isoforms of actin that regulate gene expression. These findings support the hypothesis that the reduction in *RPS5* expression in the *adf4* mutant may be related to AtADF4's interaction with and possible import of actin into the nucleus.

AtADF4 nuclear localization mutants (i.e. AtADF4-NLS and AtADF4-NES; Figure 4.2) can be used to investigate the necessity of AtADF4 in the nucleus, as well as its proposed role in the delivery of actin into the nucleus. If the differences in gene expression only require AtActin2, and not AtADF4 directly, then AtADF4 with the strong

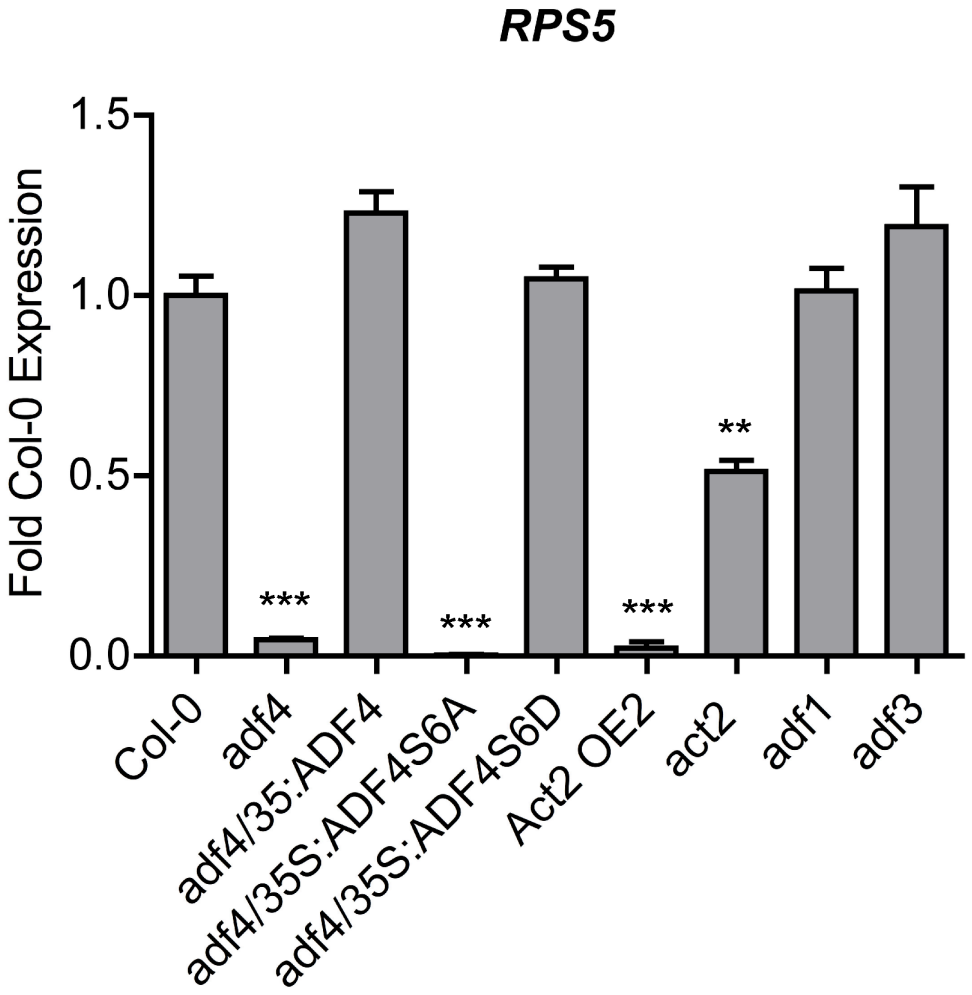

Figure 4.1. *RPS5* mRNA expression is reduced in the Act2 OE2 line and *act2* mutant. mRNA expression of *RPS5* in the Actin2 overexpression line Act2 OE2 and *act2* mutant plants. Expression values are normalized to wild-type Col-0. The other mutant lines; *adf4*, *adf4*/35S:ADF4, *adf4*/35S:ADF4S6A, *adf4*/35S:ADF4S6D, *adf1*, and *adf3*, previously shown are included for comparison purpose. Error bars represent mean ± SEM from three independent biological repeats. Statistical significance was determined using two-way ANOVA as compared to Col-0, with Bonferroni post test, where **p<0.005 and ***p<0.0005.

nuclear export signal, AtADF4-NES, should still be able to deliver AtActin2 to the nucleus before its export, thus complementing the *adf4* mutant gene expression deficiencies. However, if the AtADF4-NES does not complement gene expression in *adf4* then we would predict that AtADF4 is required to not only for translocation of actin

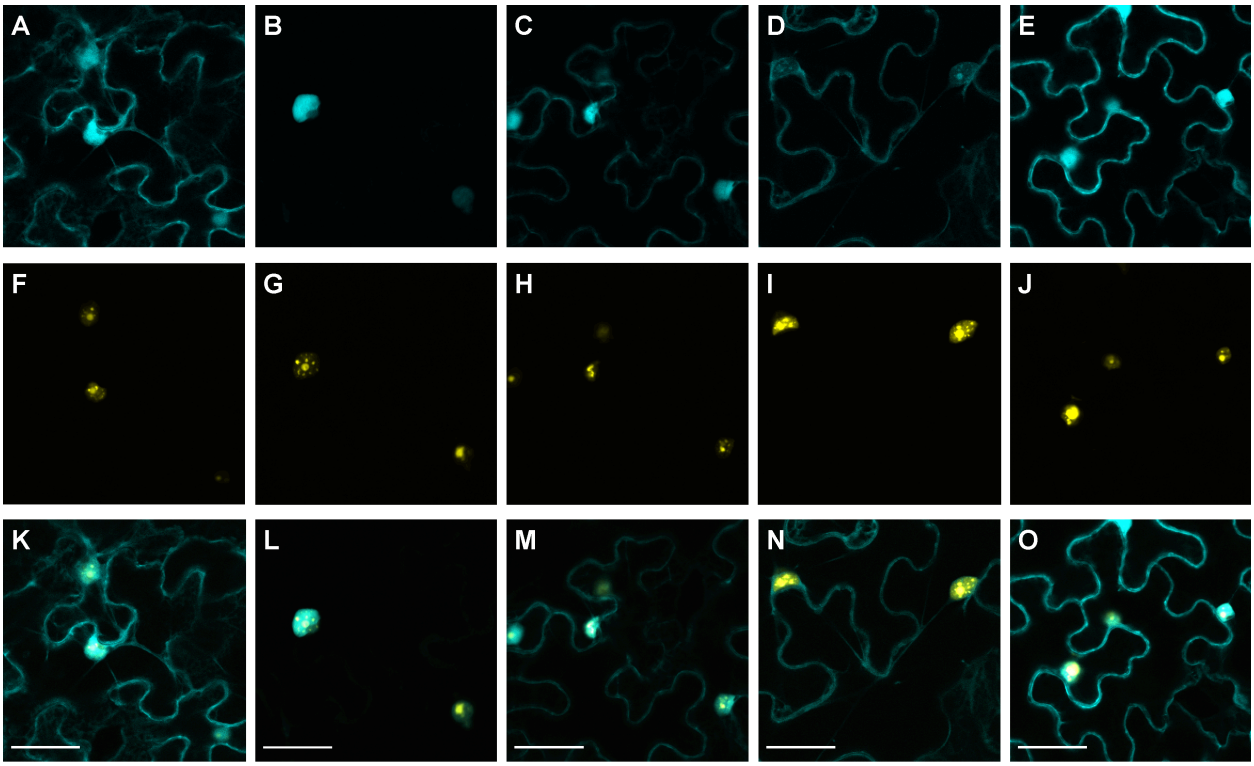

Figure 4.2. Nuclear localization mutants transiently expressed in *Nicotiana benthamiana.* Localization of (A) 35S:ADF4-cCFP, (B) 35S:ADF4-NLS-cCFP, (C) 35S:ADF4-nls-cCFP, (D) 35S:ADF4-NES-cCFP and (E) 35S:ADF4-nes-cCFP when expressed in *Nicotiana benthamiana*. AtFib-YFP (F-J) is co-expressed to indicate location of nuclei in ADF4-cCFP constructs in panels (A-E). Overlay (K-O) of ADF4cCFP constructs (A-E) with AtFib (F-J).

into the nucleus, but also play a role itself within the nucleus. This nuclear function may be independent of, or in collaboration with actin.

The presence of ABPs and actin within the nucleus has led researchers to further examine the possibility that not only is actin actively imported into and exported out of the nucleus, but that it may also exist in various forms including monomeric G-actin as well as filamentous F-actin. At present, however, it is not known if nuclear polymeric actin assumes the same structural configuration as F-actin found in the cytosol (Pederson & Aebi, 2002; Belin & Mullins, 2013; Grosse & Vartiainen, 2013; Kapoor & Shen, 2014). An interesting question is: *Does AtADF4 effect the organization of nuclear*

actin, as it does within the cytosol? This question is a particularly tricky one to answer as imaging of F-actin in the nucleus of plants and other systems is in its infancy. However, recent studies demonstrate the possibility of imaging nuclear F-actin in plant cells. Kandasamy *et al*. (2010) were able to visualize actin rods in the nucleus when Act 7 was overexpressed and tagged with a putative NLS (Kandasamy *et al.*, 2010). Additionally, a recent report found that the viral movement protein of *Turnip vein clearing virus* contained an NLS required for virulence, and in addition to being present in the nucleus, appeared to co-localize with F-actin that was labeled with nuclei specific TagRFP-UtrCH, a protein that contains TagRFP and the calponin-binding of UtrCH (Levy *et al.*, 2013). This study demonstrated F-actin structures are present in the plant nucleus, can be visualized using a nuclear specific F-actin probe, and may be targeted to enhance a pathogen's virulence (Levy *et al.*, 2013). Utilization of such probes would allow for examination of nuclear actin in presence and absence of AtADF4.

The remaining question is: *What is the mechanism by which AtADF4 is altering gene expression*? There are three main functions within the nucleus that actin has been demonstrated to affect, and are therefore potential mechanisms by which AtADF4 may regulate gene expression. The first is actin's role in the activities of RNA polymerases. Actin has been implicated in being a component of and/or playing a role in multiple phases of gene transcription by all three RNA polymerases (Grosse & Vartiainen, 2013; Percipalle, 2013). There is specific evidence in mammals that cofilin is required for elongation by RNA polymerase II, and is found to interact with actin, RNA polymerase II and DNA, specifically with the transcribed regions of genes (Percipalle, 2013; Obrdlik &

Percipalle). Nuclear immuno-precipitation (IP) followed by mass spectroscopy (MS/MS) of AtADF4-NLS constructs could be utilized to identify specific components of the nucleus that AtADF4 is able to interact with, including perhaps RNA polymerase machinery.

Second, gene expression is influenced by actin not only through it's interactions with the transcriptional machinery (i.e., RNA polymerase II), but through interactions with chromatin modifying and remodeling complexes (Kapoor *et al.* 2013; Belin & Mullins, 2013). In plants, *Arabidopsis* ADF9 has been shown to be required for appropriate expression of *flowering locus C* (FLC) in a histone modification dependent manner (Burgos-Rivera *et al.*, 2008). The aforementioned IPs could in fact identify interactions with chromatin modifying complexes, however there is the chance that the IPs may not work well due to weak interactions with nuclear components. Utilizing chromatin IP-PCR (ChIP PCR) with known hetero- and eu- chromatic targets for the *RPS5* gene could determine if the mechanism of gene regulation by *adf4* is through influences on chromatin modification machinery.

Lastly, the actin cytoskeleton in its various nuclear forms has been demonstrated to be involved in chromatin spatial organization (Dundr *et al.*, 2007). The three-dimensional spatial positioning of regions of chromatin within the interphase nucleus is not random, but instead is a well-orchestrated process that allows for an energetically favorable control of gene expression throughout the nucleus (Cope *et al.*, 2010). Long-range chromatin interactions for instance, allow the sharing of specific transcriptional

machinery, or conversely gene silencing machinery, for multiple genes on the same (*cis*) or different (*trans*) chromosomes (Cope *et al.*, 2010). Both fluorescent *in situ* hybridization and transmission electron microscopy could be used to identify potential differential localization of specific genes within the nucleus, i.e. *RPS5*, or gross morphological differences of the nucleus of the *adf4* mutant that might suggest that AtADF4 plays a role in the overall structural organization of the nucleus.

What may come to light is, that as with the apparent importance of the balance of AtActin2 and AtADF4 in the expression of *RPS5*, the mechanism by which these proteins regulate said expression might be combinatory. A combinatory role in multiple mechanisms of expression may also explain why the loss of AtActin2 does not nearly abolish *RPS5* expression as either loss of AtADF4 or overexpression of AtActin2 does. My findings within my thesis coupled with the proposed future directions with regard to AtADF4 and actin having a role in expression of *RPS5* will contribute to the overall understanding of the role of actin and ABPs in gene expression.

As a final measure to identify what roles AtADF4 is playing in immunity a global cellular approach should be taken. That is to say, the cell as a whole needs to be examined because, as outlined in Chapter 1, the actin cytoskeleton is involved in numerous cellular processes. The endomembrane trafficking system in the *adf4* mutant specifically should be examined due to the identification of actin as a key component of the system as well as recent findings that demonstrated the endocytosis of plasma membrane localized receptors as targets of pathogenesis through the targeting of the actin

cytoskeleton (Brandizzi & Wasteneys, 2013; Beck *et* al., 2012; Kang *et* al., 2014; Chapter 1; Figure 1.2; Figure 1.3). It is possible that the differences in MAPK activation observed in Chapter 3 in the *adf4* mutant in the presence of the bacterial effector AvrPphB are due in part to an alteration of either the positioning of FLS2 at the plasma membrane after *denovo* synthesis, or improper endocytosis of FLS2 after activation (Beck *et* al., 2012; Kang *et* al., 2014; Figure 2.10).

When I began this dissertation project little was known about the molecular role the actin cytoskeleton, let alone an individual ABP, played in defense signaling in plants. Much of the pre-existing literature focused on actin as a potential physical barrier to oomycete and fungal penetration peg formation or as a component of cellular architecture for the movement and trafficking of organelles and other cellular components (reviewed in Day *et al*., 2011; Chapter 1). The publication by Tian *et al*. (2009) demonstrated the requirement of a specific ABP, AtADF4 for resistance to a bacterial pathogen, *Pst* AvrPphB, and introduced the possibility that components of the actin cytoskeleton may have specialized roles in defense signaling. The findings herein demonstrate the requirement for AtADF4 in both PTI and ETI pathways of the immune response to *Pst* AvrPphB, through the expression of the cognate R-gene *RPS5* and the activation of MAPK-signaling in the presence of AvrPphB (Figure 2.1.A; Figure 2.10). In turn, AtADF4 possesses biochemical features that allow for these distinct cellular functions not commonly attributed to ADFs. While AtADF4 is capable of being phosphorylated at serine-6, this phosphorylation, which is commonly associated with the reduced functionality of plant ADFs, is required for AtADF4's role in the expression

of *RPS5*. In depth comparisons of AtADF4 with its closest homologue AtADF1, the latter of which has been shown not to play a role in defense signaling to *Pst* AvrPphB, revealed additional unique features that may contribute to the divergent cellular functions of AtADF4, including the potential phosphorylation of tyrosine-53. In conclusion the work of this dissertation project expand our collective understanding of the roles of ABPs within the cell. AtADF4, and other ABPs of plants, should now be considered, in addition to their influence on the actin cytoskeleton, as critical components of immune signaling and gene expression.

Methods and Materials

Plasmid construction and cloning

All primers used for cloning are listed in Table 4.1. ADF4 was initially cloned into the pENT/D Topo vector using the pENT/D Topo Cloning kit (www.Invitrogen.com), followed by LR clonase reaction (www.invitorgen.com) into pVKH18En6gw-cCFP (Tian *et al*., 2011). Next the pGEM cloning kit was used to add the nuclear localization tag to the ADF4-cCFP construct (www,promega.com). Lastly the ADF4-cCFP localization mutants were cloned into pENT/D and LR clonase was performed to clone the constructs into the pGWB.2 bianary expression vector (Nakagawa *et al*., 2007).

Primer name	Primer sequence 5' to 3'
ADF4 GW F	CACCATGGCTAATGCTGCGTCAGGAATGG
ADF4 no stop R	GTTGACGCGGCTTTTCAAAACATCAAGATCC
Knp1 ADF4 F	ggtacc ATG GCT AAT GCT GCG TCA GGA ATG G
cCFP Stop Xba1 R	tctaga *TTA* CTTGTACAGCTCGTCCAT
cCFP NES Stop Xba1 R	tctaga*TTA*GATATCAAGACCTGCTAATTTCAAGGCTAACT TGTACAGCTCGTCCAT
cCFP nes Stop Xba1 R	tctaga*TTA*AGCATCAGCACCTGCAGCTTTCAAGGCTAACT TGTACAGCTCGTCCAT
cCFP NLS Stop Xba1 R	tctaga*TTA*GGGGTCTTCTACCTTTCTCTTCTTTTTTGGCTT GTACAGCTCGTCCAT
cCFP nls Stop Xba1 R	tctaga*TTA*GGGGTCTTCTACCTTTCTCTTAGTTTTTGGCTT GTACAGCTCGTCCAT

Table 4.1 List of primers. With the exception of the first two primers; the cut sites are indicated by lowercase letters, the stop codon is italicized, while the nuclear locaization tag is normal font, and the cCFP sequence is undelined. Constructs were cloned with cut sites in the case that the bianary vecotor pMD1 would be used for expression in plants.

Plant growth and transient *Nicotiana benthamiana* transformation

Arabidopsis and *Nicotiana benthamiana* plants were grown in a BioChambers walk-in growth chamber (model FLX-37; Winnipeg, Manitoba, Canada) at 20 °C under a 12-hour light/12-hour dark cycle, with 60% relative humidity and a light intensity of 100 μmol photons $m^{-2}s^{-1}$. *Agrobacterium tumefaciens* GV3101 containing the pGWB.2 ADF4-cCFP localization mutants were infiltrated into *Nicotiana benthamiana* leaves for transient expression as described in Tian *et al*., 2009.

RNA extraction and qRT-PCR

Total RNA was extracted from leaves using the PrepEase Plant RNA Spin kit (USB Affymetrix, Santa Clara, CA, USA). First-strand cDNA was synthesized from 1 μg total RNA using the First-Strand cDNA Synthesis kit (USB Affymetrix). *RPS5* primers used for quantitative real-time PCR (qRT-PCR) are listed in Table 2.2. qRT-PCR was performed using the Mastercycler ep Realplex system (Eppendorf AG, Hamburg, Germany), as previously described (Chapter 2), using the Hot Start SYBR Master mix 2X (USB Affymetrix). Ubiquitin (*UBQ10*) was used as an endogenous control for amplification, Table 2.2. Fold Col-0 was determined using the following equation: (relative expression)/(relative expression of Col-0 untreated), where "relative expression" = $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{gene\ of\ interest} - Ct_{UBQ10}$.

APENDIX

APPENDIX

Actin branches out to link pathogen perception and host gene regulation

This Appendix was originally published in *Plant Signaling and Behavior*.
Porter, K and Day, B. 2013. Actin branches out to link pathogen perception to host gene regulation. *Plant Signal Behav*. **8**(3), e23468.

ABSTRACT

Cellular functions of actin, and associated actin binding proteins (ABPs), have been well characterized with respect to their dynamic cytosolic role as components of the complex cytoskeletal network. Currently, research has expanded the role of actin to include functioning within the nucleus as an integral part gene organization and expression. Herein, we describe the requirement of the ABP actin-depolymerizing factor-4 (ADF4) for resistance to *Pseudomonas syringae* DC3000 AvrPphB *via* ADF4's cytosolic and nuclear functions. Significant alterations in the expression of the resistance protein *RPS5* in an ADF4 phosphorylation-dependent manner support both a nuclear function for ADF4, and the potential targeting of the actin cytoskeleton by the bacterial effector AvrPphB.

Introduction

Actin remodeling is required for a multitude of cellular functions in both plants and animals, including growth, development, cell architecture, and response to stress (Day *et al. 2011*). As a ubiquitous network linking extracellular perception to intracellular signaling, the actin cytoskeleton is composed of both filamentous-actin (F-actin) and monomeric globular-actin (G-actin), tightly regulated by the precise interplay of a large group of more than 70 actin-binding proteins (ABPs; Day *et al.*, 2011). In the recent manuscript by Porter et al. the authors demonstrate a cellular function for actin

cytoskeletal dynamics, describing a function which links pathogen perception, gene transcription, and the activation of defense signaling (Porter *et al*., 2012). In this mini-review, we will highlight the significance of this work, which provides the first mechanistic description of actin as a cellular platform for defense signaling in plants following perception of a phytopathogenic bacterium.

ADF4 possesses both classic cellular functions of actin-depolymerizing factors as well as confers resistance to a bacterial pathogen

Among the more than 70 ABPs in plants responsible for the regulation and organization of cytoskeletal dynamics and remodeling, the actin depolymerizing factor (ADF) family fulfills a classic biochemical function to both sever and depolymerize F-actin, functioning in large part as a primary regulator of actin turnover (Ruzicka *et al.* 2007). In Arabidopsis, there are 11 members of the ADF family, further subdivided into 5 subclasses whose function and expression are hypothesized to both differentiate and specify numerous cellular functions. ADF4 is a member of subclass I which includes ADF1, ADF2, and ADF3, each of which are expressed in a wide variety of tissues, as well as within the cell cytoplasm and nucleus (Ruzicka *et al.* 2007; Kandasamy *et al.* 2010; Porter *et al*., 2012).

Biochemically, ADF4 was initially characterized using a reverse genetics approach, identified in a screen of ABP mutants showing enhanced susceptibility to *Pseudomonas syringae* (Tian *et al.*, 2009). Using a complementary series of cell biology and

pharmacological experiments, Tian et al. further defined the actin binding specificity of ADF4, demonstrating that the biochemical function of ADF4 is linked to the ability of the host to activate immune signaling following pathogen infection. In total, this work first described a role for actin cytoskeletal dynamics in the activation of plant defense signaling following perception of *P. syringae*. In a recent publication, the role of ADF4 has been further defined through the application of live cell imaging to monitor actin dynamics (Henty *et al.*, 2011). Taken together, these two studies provide a platform hypothesizing that the cellular function of ADF4 controls – and links – development and defense signaling through modulating the rate of actin turnover. This would suggest that the structural activity of the actin cytoskeleton might serve as a surveillance platform, functioning in large part to both monitor and modulate changes in host cell homeostasis in response to external stimuli.

ADF4 is required for RPS5 gene expression and supports the emerging hypothesis of nuclear functions for ABPS

As noted above, in addition to functioning as an ADF, ADF4 has also been demonstrated to play a key role in immunity to *P. syringae* expressing the bacterial effector AvrPphB (*Pst* AvrPphB; Tian *et al.*, 2009; Porter *et al.*, 2012). AvrPphB is a bacterial effector that upon delivery into the host cell via the type three-secretion system utilizes its cysteine protease activities to cleave host targets including PBS1, PBL1 and BIK1 (Shao *et al.*, 2003; Zhang & Zhou, 2010). While the cleavage of BIK1 and PBL1 result in a dampening of PTI, cleavage of PBS1 leads to activation of ETI though

PBS1's association with the cognate resistant (R)-gene RPS5 (Shao *et al.*, 2003; Zhang & Zhou, 2010). In examining the expression of both, *RPS5* and *PBS1* in wild type Col-0 and the *adf4* mutant it was demonstrated that the *adf4* mutant has a significant reduction in the mRNA levels of *RPS5* and no reduction in *PBS1* (Porter *et al.*, 2012). This observation is in agreement, and furthermore, supports a growing hypothesis that the fluctuation of nuclear actin levels contribute to the activation of gene transcription, in large part through the association of actin with all three RNA polymerases, including chromatin maintenance machinery (Vartiainen *et al.*, 2012). Indeed, the recent work by Porter et al. demonstrating abrogation of *RPS5* expression in the *adf4* mutant coupled with ADF4's presence within the nucleus (Porter *et al.*, 2012), suggests a nuclear role for ADF4 in controlling the activation of defense signaling in plants.

The next step in the current work is to understand the "ins and outs" of the temporal and spatial localization of actin, ABPs, and the dynamics therein. For example, while plant actin has a nuclear export signal, it does not possess a strong nuclear localization signal (Kandasamy *et al.*, 2010). Thus, the precise nature by which actin enters the nucleus remains undefined. However, a recent paper has demonstrated that actin, through interactions with both cofilin and Importin9, is actively translocated into the nucleus, and furthermore, that cofilin/importin9 dependent differential nuclear actin levels ultimately effect transcription efficiency (Dopie *et al.*, 2012). This would support the hypothesis that ABPs themselves are the chaperones that facilitate nuclear localization of G-actin. If this hypothesis proves correct, it would support a model (Figure A.1) whereby ADF4 association with actin may facilitate active nuclear import of

actin, thus regulating expression of *RPS5* through actin dependent assembly and activation of transcription or chromatin modifying machinery.

Phosphorylation of ADF4 regulates its cellular function and reveals a potential new virulence target for *Pseudomonas syringae* DC3000 AvrPphB

To define the mechanism(s) through which the broader function (e.g., actin binding, filament severing, depolymerization) of ADF4 is regulated, Tian et al. investigated the biochemical activity (affinity and depolymerization) of ADF4 (Tian *et al*., 2009). To elucidate the link between (*in vitro*) biochemical function and the regulation of actin cytoskeletal dynamics ultimately leading to immune signaling, Porter et al. (2012) investigated phosphorylation as a likely regulatory processes required for activation and attenuation of signaling. Support for this comes from previous work using the vertebrate homolog ADF/cofilin, where numerous factors have been identified as regulatory steps which alter the biochemical function of cofilin, including most importantly, phosphorylation at Serine-3, binding of phosphatidylinositol 4,5-bisphosphate, and cellular pH (Mizuno, 2013). Indeed, our own work not only showed that ADF4 is phosphorylated at the Serine-6 position, but that this phosphorylation event was required for regulating ADF4 affinity for F-actin, as well as for activation of immune signaling through RPS5 mRNA accumulation and MAPK activation (Porter *et al.*, 2012). Thus, as proposed in Figure A.1, our data support the hypothesis that not only is the actin cytoskeleton a virulence target of *P. syringae* expressing AvrPphB, but that this

function regulates a complex network, linking pathogen perception and virulence to nuclear dynamics and the control of transcription.

FINAL REMARKS

Identifying the ADF4 dependent expression of *RPS5* advances current research into the role of actin within the nucleus, and additionally supporting reports of the actin cytoskeleton as a virulence target of not only mammalian pathogens, but of plant pathogens as well (Tian *et al*., 2009; Day *et al*., 2011; Porter *et al*., 2012).

Acknowledgements

We would like to thank members of the Day lab for critical reading of the manuscript. The work described herein was supported by grants from the Early CAREER award from the National Science Foundation (IOS-0641319) and Arabidopsis 2010 grant from the National Science Foundation (IOS-1021044).

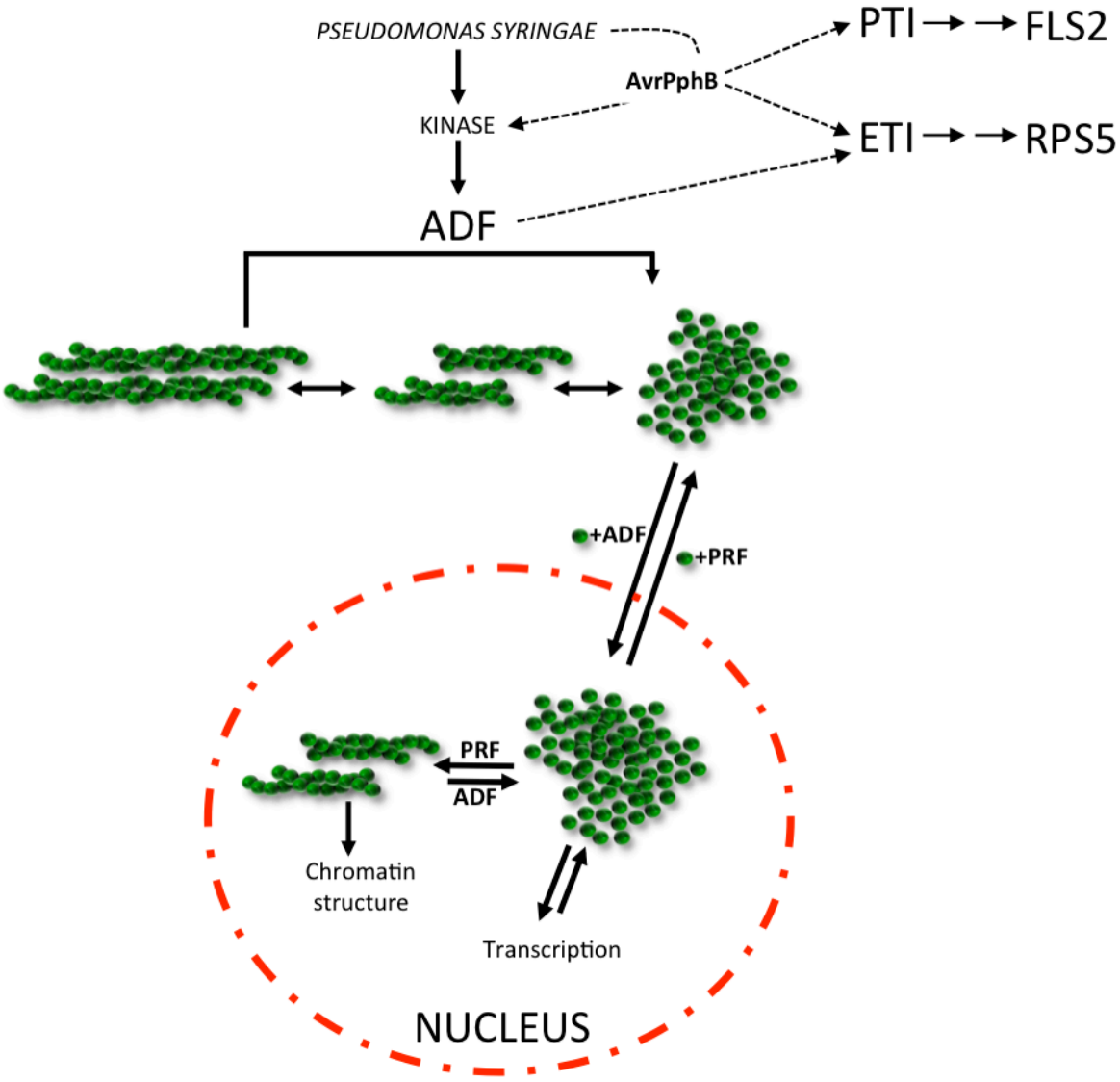

Figure A.1. Working hypothesis for the modulation of host resistance and cell signaling through control of actin cytoskeletal dynamics. The virulence activity of the bacterial cysteine protease AvrPphB targets an unidentified kinase that is responsible for the phosphorylation and subsequent regulation of actin depolymerizing factor-4 (ADF4). As a key regulator in controlling not only actin filament organization, but also as a modulator of the balance of globular (G) and filamentous (F) actin, targeting of ADF4 by pathogens represents a key switch in controlling host cell response. At a transcriptional level, the balance of cytoplasmic *versus* nuclear actin is required for RNA polymerase function and the general organization and maintenance of chromatin architecture. ETI, effector triggered immunity; PTI, pathogen-associated molecular pattern (PAMP)-triggered immunity; ADF, actin depolymerizing factor; PRF, profilin. This figure was inspired by Vartiainen et al. (2012).

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