**Running head:** *Arabidopsis* actin depolymerizing factor

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**Research area:** Plants interacting with other organisms
Arabidopsis actin depolymerizing factor AtADF4 mediates defense signal transduction triggered by the Pseudomonas syringae effector AvrPphB

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ABSTRACT

The actin cytoskeleton has been implicated in plant defenses against pathogenic fungi and oomycetes with limited, indirect evidence. To date, there are no reports linking actin with resistance against phytopathogenic bacteria. The dynamic behavior of actin filaments is regulated by a diverse array of actin-binding proteins, among which is the Actin Depolymerizing Factor (ADF) family of proteins. Here, we demonstrate that actin dynamics play a role in the activation of gene-for-gene resistance in *Arabidopsis thaliana* following inoculation with the phytopathogenic bacterium *Pseudomonas syringae* pv. *tomato*. Using a reverse genetic approach we explored the roles of *Arabidopsis* ADFs in plant defenses. *AtADF4* was identified as being specifically required for resistance triggered by the effector AvrPphB, but not AvrRpt2 or AvrB. Recombinant *AtADF4* bound to monomeric actin (G-actin) with a marked preference for the ADP-loaded form, and inhibited the rate of nucleotide exchange on G-actin, indicating that *AtADF4* is a *bona fide* actin depolymerizing factor. Exogenous application of the actin disrupting agent cytochalasin D partially rescued the *Atadf4* mutant in the AvrPphB-mediated hypersensitive response, demonstrating that *AtADF4* mediates defense signaling through modification of the actin cytoskeleton. Unlike the mechanism by which the actin cytoskeleton confers resistance against fungi and oomycetes, *AtADF4* is not involved in resistance against pathogen entry. Collectively, this study identifies *AtADF4* as a novel component of the plant defense signaling pathway, and provides strong evidence for actin dynamics as a primary component that orchestrates plant defenses against *P. syringae*. 
INTRODUCTION

The actin cytoskeleton has been implicated in plant defenses against pathogenic fungi and oomycetes (Hardham et al., 2007). Evidence largely comes from studies using actin cytoskeleton-disrupting agents, such as cytochalasins. Treatments with a variety of cytochalasins were shown to increase the penetration rate of both adapted and non-adapted pathogens in multiple plant-pathogen systems, thereby implicating the actin cytoskeleton as having a role in basal defenses and non-host resistance (Kobayashi et al., 1997; Yun et al., 2003; Shimada et al., 2006; Miklis et al., 2007). The actin cytoskeleton may also play a role in race-specific resistance (Skalamera and Heath, 1998). To date, no reports linking actin dynamics with resistance against phytopathogenic bacteria have been published.

While the actin cytoskeleton as a virulence target of plant pathogens has not been documented, it was well characterized in mammalian pathosystems, particularly in studies investigating macrophage interactions with the pathogenic bacterium *Yersinia pestis* (Mattoo et al., 2007). *Yersinia* spp. delivers a suite of effectors into the target host cell, and at least four of them (YopE, YpkA/YopO, YopT and YopH) are involved in rearrangement of the actin cytoskeleton (Aepfelbacher and Heesemann, 2001). YopT, a cysteine protease, targets a plasma membrane-localized Rho GTPase in affected phagocytes (Aepfelbacher and Heesemann, 2001). Cleavage of the GTPase by YopT releases the prenylated protein from the plasma membrane and disrupts the actin cytoskeleton, effectively shutting down phagocytosis, preventing elimination of the pathogen (Iriarte and Cornelis, 1998; Shao et al., 2002). Similarly, microbial pathogens also usurp host processes for the benefit of infection, disease and death. *Listeria* spp. hijacks the host’s cytoskeleton to move around inside the infected cell through the induction of directed polymerization of actin (Pistor et al., 1994). *Salmonella* injects into host cells several actin-binding proteins (SipA & SipC) as well as other regulators of actin dynamics to enhance phagocytic uptake and intracellular propagation (Galan and Zhou, 2000). In short, by either preventing polymerization, or by promoting it, pathogens have evolved strategies to modify the host actin cytoskeleton for purposes of evading detection or eliciting disease and death.
Dynamic actin cytoskeleton rearrangements are regulated by a pool of actin-binding proteins, which sense environmental changes and modulate the cytoskeleton through various biochemical activities (Hussey et al., 2006; Staiger and Blanchoin, 2006). Among the proteins which regulate these dynamic processes are the Actin Depolymerizing Factor (ADF) family of proteins (Maciver and Hussey, 2002). In general, ADFs bind both monomeric (G-) and filamentous (F-) actin to increase actin dynamics. They function by severing F-actin to generate more ends for polymerization, and by increasing the dissociation rate of actin monomers from the pointed ends (Maciver, 1998; Maciver and Hussey, 2002). Plant ADFs play roles in pollen tube growth (Chen et al., 2003), root formation (Thomas and Schiefelbein, 2002) and cold acclimation (Ouellet et al., 2001). There is also one report linking ADFs with plant defenses (Miklis et al., 2007). In that study, ectopic expression of barley HvADF3 and several isovariants of Arabidopsis ADFs in barley epidermal cells was shown to compromise penetration resistance to powdery mildew fungi (Miklis et al., 2007).

The Arabidopsis thaliana-Pseudomonas syringae interaction provides an ideal model plant-pathogen system to study plant defense signaling. Like Yersinia spp., P. syringae delivers effector proteins into the host cells via the type III secretion system, and relies on these proteins for pathogenesis (Alfano and Collmer, 2004). However, once these proteins (Avr) are recognized either directly or indirectly by plant resistance (R) proteins, plant immune responses are activated (Jones and Dangl, 2006). Exciting progress has been made towards understanding the indirect recognition of several pairs of Avr-R proteins; the best examples include AvrB/AvrRPM1-RPM1, AvrRpt2-RPS2 and AvrPphB-RPS5. During activation of defense mediated by AvrB/AvrRPM1-RPM1 and AvrRpt2-RPS2, the phosphorylation or elimination of a third protein, RIN4, is essential (Mackey et al., 2002; Axtell and Staskawicz, 2003). In the case of AvrPphB-RPS5 recognition, AvrPphB, the cysteine protease of the same family as YopT (Shao et al., 2002), cleaves a plant protein kinase PBS1, inducing a conformational change in RPS5, which in turn leads to the activation of resistance (Ade et al., 2007). Although these studies have greatly enhanced our understanding of how pathogen effectors initiate plant defense responses, the ultimate signaling processes associated with the activation of
resistance remain largely unknown due to the limited number of genetic loci identified in these pathways. In the current work, we hypothesize that actin-binding proteins play a role during plant-bacteria interactions based on the functional and structural similarity between AvrPphB and YopT.

There are 11 ADFs in the *Arabidopsis* genome (Ruzicka et al., 2007). We utilized a reverse genetic approach to identify the putative roles these proteins play in plant resistance against the bacterial pathogen *P. syringae* pv. tomato. AtADF4 was identified as a novel signaling component in AvrPphB-RPS5 mediated defense signal transduction pathway. Loss of AtADF4 confers on *Arabidopsis* enhanced susceptibility to *P. syringae* expressing AvrPphB. Further subcellular localization and biochemical analyses, as well as pharmacological studies, suggest that AtADF4 functions as a *bona fide* actin depolymerizing factor through modifying the actin cytoskeleton. Unlike the documented mechanism by which the actin cytoskeleton plays roles in resistance against fungi and oomycetes, the resistance against phytobacteria *P. syringae* mediated by AtADF4 is not involved in hindering pathogen entry.

**RESULTS**

*Atadf4* Knockout Mutant Specifically Compromises AvrPphB-mediated Resistance against *Pseudomonas syringae* pv. tomato.

To identify a role for the *Arabidopsis* ADFs in plant defenses we obtained and characterized 14 T-DNA insertion lines corresponding to AtADF1, AtADF2, AtADF3, AtADF4, AtADF5 and AtADF9 (Supplemental Table S1). Four lines (Salk_144459, Salk_139265, Garlic_823_A11.b.1b.Lb3Fa and Salk_056064) were confirmed to be null mutants and were named *Atadf1, Atadf3, Atadf4* and *Atadf9* (Fig. 1b, Supplemental Fig. S1). Homozygous mutant plants were dip inoculated with the *P. syringae* pv. tomato (*Pst*) DC3000 virulent strain, as well as three avirulent strains expressing AvrRpt2, AvrB and AvrPphB. Multiple independent experiments showed that *Atadf1, Atadf3, Atadf4* and *Atadf9* responded similar to wild-type (WT) Col-0 upon inoculation with the virulent strain, as well as strains expressing AvrRpt2 and AvrB (Fig. 1c, data not shown). However, in response to inoculation with *Pst* expressing AvrPphB, the *Atadf4* mutant,
which contains a T-DNA insertion at the second exon (Fig. 1a), was strikingly more susceptible than WT and the other Atadf mutant plants (Fig. 1c). Quantitative analysis of bacterial growth revealed that infected leaves of Atadf4 supported a significantly larger bacterial population than WT plants infected with Pst expressing AvrPphB (Fig. 1d).

Since the hypersensitive response (HR) is typically associated with gene-for-gene resistance, we further tested the Atadf mutant lines for induction of the HR in response to inoculation with Pst strains described above. As expected, leaves from WT plants developed the HR upon infiltration with three avirulent strains after 18-22 hours, yet not after inoculation with the virulent strain (Fig. 1e). There was no difference between the four Atadf mutant lines and WT plants in response to the virulent strain, and strains expressing AvrRpt2 and AvrB (Fig. 1e, data not shown). However, the Atadf4 mutant specifically suppressed the HR mediated by Pst expressing AvrPphB (Fig. 1e).

Infection of plants by Pst involves antagonistic cross-talk between salicylic acid (SA) and jasmonic acid (JA)-dependent signaling pathways, and the plant susceptibility is associated with induction of JA responsive genes and concomitant repression of SA responsive pathogenesis-related (PR) genes (Zhao et al., 2003). We tested the gene expression of PRI and PDF1.2, marker genes for the SA and JA pathways respectively, during the infection time course after WT and Atadf4 mutant were dip-inoculated with Pst expressing AvrPphB. Consistent with the compromised resistance phenotype of Atadf4 mutant in response to Pst expressing AvrPphB, PRI gene expression of Atadf4 was delayed and reduced compared with WT (Fig. 2a), while PDF1.2 expression was highly elevated (Fig. 2b). The induction of JA signaling pathway in Atadf4 mutant was independently confirmed with microarray data collected at 24-h post-dip inoculation. Among 23 differentially expressed JA-responsive genes, 22 of them were upregulated in Atadf4 mutant compared with WT (M. Tian and B. Day, unpublished data).

**Silencing of Four AtADFs Uncouples the AvrPphB-triggered HR from Resistance**

To obtain additional evidence that Arabidopsis ADFs are required for AvrPphB-mediated resistance, and to determine whether the Arabidopsis ADFs play redundant roles in resistance mediated by other effectors, we generated a gene silencing construct AtADF1-4Ri, which simultaneously targets all four subclass I ADFs, AtADF1 through
AtADF4 (Ruzicka et al., 2007). Four independent homozygous lines were tested by quantitative RT-PCR to determine the expression level of each of the AtADF genes. The gene expression of these four genes in all four transgenic AtADF1-4Ri lines was reduced when compared with WT plants (Fig. 3a). HR tests were performed as described above. As we observed with the Atadf4 T-DNA insertion mutant, all four knockdown lines specifically suppressed the HR mediated by AvrPphB, but not other effectors (Fig. 3b, data not shown), suggesting that AtADF(s) are required for the AvrPphB-mediated HR. We further investigated the disease resistance phenotype(s) of these lines by dip inoculation. Interestingly, silencing of the four AtADFs did not result in a detectable loss of resistance. All four AtADF1-4Ri lines exhibited resistant phenotypes similar to that of WT plants, and none of them supported significantly more bacterial growth than WT plants for all strains tested (data not shown).

**AtADF4 Gene Complements the Atadf4 Knockout Mutant**

Although the experiments above suggest that AtADF4 is likely involved in AvrPphB-mediated resistance, they do not determine whether AtADF4 itself is required for defense signaling. To test this hypothesis, we transformed the homozygous Atadf4 mutant plants with a construct expressing AtADF4 genomic DNA fused with a C-terminal T7 tag, driven by the AtADF4 native promoter. T3 plants from two independent homozygous transgenic lines did not exhibit the disease phenotype as observed with the Atadf4 mutant following inoculation with *Pst* expressing AvrPphB (Fig. 4a). Further measurements of bacterial growth were consistent with the resistant phenotype, as these two lines supported bacterial populations equivalent to those observed in WT plants (Fig. 4b). These data strongly support our finding that AtADF4 is able to restore the resistance compromised in the Atadf4 mutant. Similarly, using the HR as a second test for the activation of resistance, AtADF4-complemented lines showed a restoration in the activation of the HR (Fig. 4c). The integrity of the transgenic lines was also tested; results of these analyses are shown in Supplemental Fig. S2. Taken together, the complementation experiments provide strong evidence that AtADF4 is an essential signaling component of the AvrPphB-mediated resistance transduction pathway.
**AtADF4 is Localized on the Actin Cytoskeleton**

To determine the subcellular localization of AtADF4, a DsRed-AtADF4 fusion protein was transiently expressed in *Nicotiana benthamiana* and protein localization was determined using confocal microscopy. In contrast to DsRed alone (Fig. 5a), DsRed-AtADF4 is localized in a filamentous pattern (Fig. 5b), suggesting that AtADF4 is associated with the cytoskeleton. To further confirm that AtADF4 is localized along actin filaments, we co-expressed DsRed-AtADF4 with GFP-labeled ABD2, the second actin-binding domain of *Arabidopsis* Fimbrin 1, which was developed as a reporter of the actin cytoskeleton (Wang et al., 2004). As expected, the green fluorescence of ABD2-GFP substantially overlapped with the red fluorescence of DsRed-AtADF4 (Fig. 5f, g, h), yet not with DsRed alone (Fig. 5c, d, e), demonstrating that AtADF4 is localized on the actin cytoskeleton. Note that sometimes the actin cytoskeleton appears slightly perturbed (Fig. 5f, g, h), which is not surprising given that AtADF1 overexpression has previously been shown to reduce the extent of actin bundles in *Arabidopsis* cells (Dong et al., 2001).

**AtADF4 Shows a Marked Preference for ADP-G-actin**

ADFs from diverse organisms generally share the ability to bind G-actin, with higher affinity for ADP-G-actin versus ATP-G-actin (Carlier *et al*., 1999). For example, AtADF1 shows ~100-fold higher affinity for ADP-G-actin when compared with ATP-loaded monomer (Carlier *et al*., 1997; Chaudhry *et al*., 2007). To test the activities of AtADF4, we purified recombinant AtADF4 protein fused with an N-terminal FLAG tag. The protein, which migrated as a single ~17 kDa polypeptide on SDS-PAGE gels (Fig. 6a), was used for G-actin binding assays, as well as for nucleotide exchange experiments described below. AtADF1, expressed and purified from *E. coli* as previously described (Carlier *et al*., 1997; Chaudhry *et al*., 2007), was used as a control. The affinity of AtADF4 for ATP- and ADP-G-actin was tested with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD)-labeled actin. A dose-dependent quenching of fluorescence for 0.2 μM NBD-G-actin was observed in the presence of both *Arabidopsis* ADF isoforms. As shown in Fig. 6b, the quenching of NBD-G-actin fluorescence was maximal when 12-18 μM of AtADF1 was added to ATP-G-actin, whereas AtADF4 failed to reach saturation at these concentrations. In marked contrast, the quenching of NBD fluorescence on ADP-G-
actin was maximal when ADF1 or ADF4 concentrations were above 2 µM (Fig. 6c). From several such experiments, average $K_d$ values (± SD, $[n]$) of 37 ± 9 µM (3) for AtADF4 binding to ATP-G-actin and 0.104 ± 0.04 µM (4) for AtADF4 binding to ADP-G-actin were determined. AtADF1 gave average $K_d$ values (± SD, $[n]$) of 16 ± 3 µM (4) for ATP-actin and 0.216 ± 0.112 µM (4) for ADP-actin, in agreement with published data (Carlier et al., 1997; Chaudhry et al., 2007). Therefore, AtADF4 has ~ 355-fold higher affinity for ADP-G-actin when compared with the ATP-loaded form.

**AtADF4 Inhibits the Rate of Nucleotide Exchange on G-actin**

Nucleotide exchange analysis using 1 µM ATP-G-actin in the presence or absence of AtADFs, under both physiological and low salt conditions, was performed. Under low salt conditions, the rate of nucleotide exchange in the presence of 2.5 µM and 5 µM AtADF4 was significantly lower than for ATP-G-actin alone (Fig. 7a). Nucleotide exchange in the presence of 1 µM AtADF1 was used as a positive control. However, with a physiological ionic strength buffer, no inhibition was observed even in the presence of 10 µM AtADF4 or AtADF1 (data not shown). Given the weak binding affinity of AtADF4 for ATP-G-actin, we decided to monitor nucleotide exchange on 1 µM ADP-G-actin under physiological salt conditions. In agreement with previously published findings using other ADF proteins (Ouellet et al., 2001; Chaudhry et al., 2007), AtADF4 markedly inhibited the rate of nucleotide exchange in a concentration-dependent manner (Fig. 7b). Thus, AtADF4 shows two conserved biochemical features of eukaryotic ADF proteins—the ability to bind monomeric actin with a marked preference for the ADP-loaded form and the ability to inhibit nucleotide exchange on monomers. Preliminary results indicate that AtADF4 also binds filamentous actin and induces severing (data not shown).

**Cytochalasin D Partially Rescues Atadf4 Mutant in the AvrPphB-mediated HR**

To gain insight into AtADF4’s role in transducing defense signaling through its action on the actin cytoskeleton, we co-infiltrated cytochalasin D with *Pst* DC3000 expressing AvrPphB into leaves of WT and the *Atadf4* mutant plants and measured the effects on induction of the HR. Cytochalasin D was applied at varying concentrations in
combination with and without co-inoculation with \( Pst \). As expected, application of cytochalasin D alone did not result in tissue collapse, nor did increasing the concentration of cytochalasin D in co-inoculation experiments with bacterial suspensions affect the induction of the HR in WT plants (Table 1). Interestingly, exogenously applied cytochalasin D restored a significant percentage of leaves from the \( \text{Atadf4} \) mutant to generate the HR (Table 1). The average proportion of leaves developing an HR was 49.6%, 58.3% and 35% for concentrations of 2.5, 5 and 10 \( \mu \text{M} \) cytochalasin D, respectively. This result strongly supports the hypothesis that \( \text{AtADF4} \) transduces defense signaling through modification of the actin cytoskeleton.

**AtADF4 is not involved in Resistance against Bacterial Entry**

So far, the documented mechanism for actin cytoskeleton-based resistance is to hinder pathogen penetration (Hardham et al., 2007; Miklis et al., 2007). To determine if \( \text{AtADF4} \) is also involved in penetration resistance, we tested the plant resistance of \( \text{Atadf} \) mutants after manually infiltrating \( Pst \) DC3000 strains into the extracellular space of the plants. Repeated experiments showed that \( \text{Atadf1, Atadf3 and Atadf4} \) responded similar to WT plants upon inoculation with the virulent strain, as well as strains expressing AvrRpt2 and AvrB (Fig. 8). However, in response to inoculation with \( Pst \) expressing AvrPphB, the \( \text{Atadf4} \) mutant supported a significantly larger bacterial population than WT and other \( \text{Atadf} \) mutant plants (Fig. 8). This is consistent with the result obtained by dip inoculation (Fig. 1d), suggesting that \( \text{AtADF4} \) is not involved in resistance against bacterial entry.

**DISCUSSION**

The involvement of the actin cytoskeleton in plant resistance against pathogenic fungi and oomycetes is largely based on two lines of indirect evidence. First, studies using actin cytoskeleton-disrupting agents or the ectopic expression of ADFs show that plant resistance is compromised following pathogen inoculation (Kobayashi et al., 1997; Yun et al., 2003; Shimada et al., 2006; Miklis et al., 2007). Second, cytological studies have shown that the cytoplasm and nucleus are re-localized directly beneath the infection sites by the actin cytoskeleton machinery (Takemoto et al., 2003; Takemoto and
In the present study, we determined that AtADF4 is required for AvrPphB-mediated resistance against the phytopathogenic bacterium *P. syringae* pv. *tomato*. Subcellular localization and biochemical analyses demonstrate that AtADF4 is a *bona fide* actin-binding protein possessing activities consistent with previously characterized ADFs. Further pharmacological studies suggest that AtADF4 mediates defense signaling via modification of the actin cytoskeleton. Our data also suggest that AtADF4 is not involved in resistance against bacterial entry. In total, this study provides strong evidence that the actin cytoskeleton plays an important role in the plant defenses against phytopathogenic bacterium *P. syringae*, with a distinct mechanism from the one by which the actin cytoskeleton confers resistance against fungi and oomycetes.

Although the AtADF1-4Ri gene silencing lines suppressed the AvrPphB-mediated HR, they retained the disease resistance phenotype. This finding is intriguing. First, it provides another piece of evidence that the HR can be uncoupled from resistance. This is consistent with previous studies showing that the HR is not always required for gene-for-gene resistance. Examples include the *Arabidopsis ndr1* and *dnd1* mutants. The *dnd1* mutant confers gene-for-gene disease resistance in the absence of HR (Yu *et al.*, 1998). In the case of the *ndr1* mutant plants, while they compromise disease resistance triggered by AvrRPM1 and AvrB, they still exhibit HR-like lesions in response to high doses of bacterial inoculation (Century *et al.*, 1995). Second, it suggests that AtADF4 might function in a dose-dependent manner to amplify the defense signal. Based on the hypothesis described by Jones and Dangl (2006), effective resistance, or the HR, is achieved only when the amplitude of the defense signal reaches a certain threshold; it is hypothesized that the threshold for eliciting HR is higher than that for eliciting the effective resistance. In the AtADF knockdown lines, it is possible that the residual transcript (and protein) levels are sufficient to sustain disease resistance, yet insufficient to amplify the signal to attain the threshold for eliciting the HR.

One interesting question raised from our study is that of the functional specificity of AtADF isovariants. While AtADF4 was found to be required for AvrPphB-mediated resistance, it seems that AtADF1, AtADF3 and AtADF9 individually are dispensable.
Ruzicka et al. (2007) classified the Arabidopsis ADFs into four subclasses. AtADF9 belongs to subclass III, whereas AtADF1, AtADF3 and AtADF4 belong to subclass I. The expression patterns of AtADF1 and AtADF3 were found to be similar to that observed for AtADF4; all are strongly expressed in most tissues and organs, with the exception of pollen (Ruzicka et al., 2007). From this, we can likely rule out the possibility that tissue-specific expression accounts for the differential phenotypes we observed in Atadf1, Atadf3 and Atadf4 mutant lines upon the infection with P. syringae expressing AvrPphB. Further studies investigating the differential gene expression patterns in response to pathogen inoculation, the biochemical activities as well as posttranslational regulation may reveal the mechanisms determining this specificity.

The mechanism by which AtADF4 mediates AvrPphB-triggered resistance remains unknown. The simplest hypothesis is that AtADF4 is involved in the proper localization of proteins, a process in which the actin cytoskeleton is thought to play essential roles (Stamnes, 2002). During the infection of plants by pathogens, a wide range of effector proteins and/or cognate resistance proteins are collectively targeted (or relocalized) to various host cellular compartments, including the apoplast, plasma membrane, cytoplasm and nucleus (Alfano and Collmer, 2004; Kamoun, 2007). In support of a role for the actin cytoskeleton in this process of dynamic protein re-localization following pathogen perception and defense activation, a recent study on tobacco mosaic virus effector p50 and its tobacco N receptor suggests that pathogen elicitor recognition defense signaling involves complex multi-step processes at multiple subcellular compartments (Burch-Smith et al., 2007). This study indicates that the recognition of p50 by N protein likely occurs in the cytoplasm, and subsequent signaling is initiated by the activated N protein within the nucleus. Another example of dynamic protein re-localization processes associated with defense signaling in plants involves the immune receptor MLA10 and the effector AVR\textsubscript{A10} from the powdery mildew fungus Blumeria graminis f sp. hordei (Shen et al., 2007). Although the exact mechanisms remain unknown, it appears that the initiation of effector-triggered immunity involves active intracellular protein transport. In the case of AvrPphB and RPS5, the initial recognition is believed to take place at the plasma membrane (Nimchuk et al., 2000; Holt
et al., 2005). Whether the activation of defense signaling requires re-localization of RPS5 to a second cellular compartment is not known, but remains a possibility. Thus, AtADF4-mediated rearrangement of the actin cytoskeleton might be responsible for the transport of AvrPphB and RPS5 within the plasma membrane, or alternatively, away from the plasma membrane following the initial recognition events. Further localization studies of AvrPphB and RPS5 in both WT and Atadf4 mutant plants may shed light on this possibility.

An alternate hypothesis is that AtADF4 is directly involved in defense signal transduction. Although the mechanism is not fully understood, there is evidence showing that actin depolymerization itself may serve as a signal transducer. Studies on human B-cell receptor (BCR) signal transduction have shown that actin depolymerization enhances BCR-induced transcription factor activation. This finding suggests that by blocking actin depolymerization, BCR signaling is inhibited (Hao and August, 2005). A recent study also reported that depolymerization of the actin cytoskeleton in tobacco plants induces the expression of defense-related genes PR1 and PR2 (Kobayashi and Kobayashi, 2007). Similarly, actin depolymerization mediated by AtADF4 might directly transduce downstream defense signaling.

MATERIALS AND METHODS

Plants, Growth Conditions, and Arabidopsis Transformation

*Arabidopsis thaliana* and *Nicotiana benthamiana* plants were grown at 20 °C under 14-h-light/10-h-dark cycle. *Arabidopsis* T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (ABRC). *Arabidopsis* transformation and selection of transformants were carried out as described by Clough and Bent (1998).

Pathogens, Inoculation and Measurement of Bacterial Growth

*Pseudomonas syringae* pv. tomato DC3000 strains containing pVSP61 (empty vector) or AvrRpt2 or AvrB or AvrPphB (in the vector pVSP61) were described previously (Kunkel et al., 1993; Simonich and Innes, 1995). 4-week-old plants were used for bacteria infection. Dip inoculation was performed by dipping the whole plants into
bacterial suspensions of $3 \times 10^8$ CFU/ml as described by Kunkel et al. (1993). Hand infiltrations were conducted with bacteria resuspended in 10 mM MgCl$_2$ to $5 \times 10^7$ CFU/ml for testing the HR or to $10^5$ CFU/ml for measurement of bacterial growth. Before inoculation, 3 leaves of similar size from each plant were marked for analysis. To analyze the HR, leaves were scored for tissue collapse 20-24 hours after inoculation. For measurement of bacterial growth, three leaf discs with a diameter of 0.7 cm were collected from 3 plants and placed into a single tube, serving as one replicate. Following bacteria recovery, serial dilution and plating were performed as described previously (Tornero and Dangl, 2001) with minor modifications. Instead of 2 μl drops, we plated 5 μl drops from each dilution on the plate for bacterial colony counting.

**Plasmid Construction**

Plasmid pFLAG-AtADF4 was constructed by cloning PCR-amplified AtADF4 protein encoding sequence into HindIII and KpnI sites of pFLAG-ATS (Sigma-Aldrich, St. Louis, MO), a vector that allows secreted expression of N-terminally FLAG-tagged proteins in *E. coli*. The primers (F: 5’-GCGAAGCTTatggctaatgctgcgtcaggaatgg-3’) and (R: 5’-GCGGGTACCttagttgacgcggcttttcaaaac-3’) were used to amplify the fragment. The gene-specific sequence is in lower case and the introduced restriction sites are underlined. pGDR-AtADF4 was constructed by cloning AtADF4 protein encoding sequence into BgIII and SalI sites of pGDR (Goodin et al., 2002). The primers used contain the same gene-specific sequence as above. To construct pMD1-gAtADF4, a genomic DNA fragment starting from 665bp upstream of the AtADF4 start codon (e.g., ATG) to the stop codon TAA was cloned into the binary vector pMD1(Li et al., 1997), which was digested with HindIII and XbaI to remove 35S promoter. Primers (F: 5’-GCGAAGCTTacatcttgtcttcacataatgaaaac-3’) and (R: 5’-GCGTCTAGAttaACCCATTTGTTGACCACCTGTCATTGAAGCCATgttgacgcggctttcaaaacatcaagatcc-3’) were used to amplify this fragment. The gene-specific sequence is in lower case and the introduced restriction sites are underlined. T7 epitope tag sequence was added immediately preceding the stop codon TAA and is shown in bold. The plasmid AtADF1-4Ri for silencing AtADF1 through AtADF4 was constructed as
illustrated in Supplemental Fig. S3 and its design is based on previously published methods (Pawloski et al., 2006).

RNA Isolation and RT-PCR Analysis

Total RNA from leaves was extracted using RNeasy Plant Mini Kit (Qiagen, Valencia, CA) and treated with DNA-free™ (Ambion, Austin, TX) to remove contaminating DNA. First-strand cDNA was synthesized from 1 μg of total RNA using superscript™ III reverse transcriptase (Invitrogen, Carlsbad, CA). The primers for amplifying AtADFs from T-DNA insertional mutants are listed in Table S1. The expression of AtADF genes was controlled with Arabidopsis β-tubulin gene (NCBI accession number AY059075) using primers (F: 5’-GTCCAGTGTCTGTGATATTGCACC-3’) and (R: 5’-TTACGAATCCGAGGGAGCCATTG-3’). Quantitative RT-PCR was performed on an Mastercycler ep realplex real-time PCR system as previously described (Ruzicka et al., 2007) using ubiquitin gene UBQ10 primers (Lai et al., 2004) as the endogenous control. The primers used for AtADFs, PRI (At2g14610) and PDF1.2 (At5g44420) are listed in Supplemental Table S2.

Transient Protein Expression in N. benthamiana and Laser-scanning Confocal Microscopy

Transient protein expression in N. benthamiana with various plasmids in Agrobacterium tumefaciens GV3101 and confocal microscopy using a LSM Zeiss 510 Meta were performed as previously described (Goodin et al., 2002).

Expression and Purification of Recombinant AtADF4

Expression and purification of recombinant AtADF4 from pFLAG-AtADF4 were conducted as described previously (Tian et al., 2004). Protein concentration was calculated using an extinction coefficient of 14,690 M⁻¹ cm⁻¹ determined with the approach of Gill and von Hippel (1989).

Actin Monomer Binding Assay
The interaction of AtADFs with actin monomers was examined by measuring the fluorescence change of NBD-labeled ATP- and ADP-loaded G-actin in the presence of varying concentrations of AtADFs as described previously (Chaudhry et al., 2007).

Nucleotide Exchange Analysis

The rate of nucleotide exchange on 1 μM ATP-G-actin or ADP-G-actin, in physiological or low salt buffer, was determined by measuring the increase in fluorescence upon incorporation of 1-\(N^\epsilon\)-ethenoadenosine 5’-triphosphate (\(\epsilon\)-ATP; Sigma-Aldrich, St. Louis, MO) as described previously (Chaudhry et al., 2007).

Cytochalasin D Treatments

Cytochalasin D (Calbiochem, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM. Various solutions containing cytochalasin D (Table 1) were prepared by adding appropriate volumes of the stock solution into 10 mM MgCl\(_2\) with or without *P. syringae* pv. tomato (AvrPphB) at 5 × 10\(^7\) CFU/ml. For controls, additional DMSO was added to a final concentration of 0.1% where applicable. The infiltration of leaves and observation of the HR was conducted as described above.

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Table 1. Effects of cytochalasin D on the HR of \textit{Atadf4} mutant triggered by AvrPphB

| Treatments                                | Experiment 1 |          | Experiment 2 |          | Experiment 3 |          |
|-------------------------------------------|--------------|----------|--------------|----------|--------------|----------|
|                                           | Col-0        | \textit{Atadf4} | Col-0        | \textit{Atadf4} | Col-0        | \textit{Atadf4} |
| Pst\textsuperscript{a} DC3000 (Empty vector) | 0/12         | 0/11     | 0/9          | 0/10     | 0/11         | 0/10     |
| Pst\textsuperscript{a} DC3000 (AvrPphB)   | 13/13        | 0/12     | 8/8          | 0/11     | 12/12        | 0/11     |
| 2.5 \( \mu \)M CD                        | n/d          | n/d      | 0/10         | 0/8      | 0/12         | 0/12     |
| 5 \( \mu \)M CD                          | n/d          | n/d      | 0/9          | 0/13     | 0/13         | 0/11     |
| 10 \( \mu \)M CD                         | 0/10         | 0/12     | 0/11         | 0/13     | 0/11         | 0/9      |
| Pst\textsuperscript{a} DC3000 (AvrPphB) + 0.1% DMSO | 12/12        | 0/12     | 8/8          | 0/9      | 10/10        | 0/11     |
| Pst\textsuperscript{a} DC3000 (AvrPphB) + 2.5 \( \mu \)M CD | n/d          | n/d      | 8/8          | 7/13     | 11/11        | 5/11     |
| Pst\textsuperscript{a} DC3000 (AvrPphB) + 5 \( \mu \)M CD | 12/12        | 10/14    | 13/13        | 9/12     | 12/12        | 4/14     |
| Pst\textsuperscript{a} DC3000 (AvrPphB) + 10 \( \mu \)M CD | 11/11        | 5/12     | 12/12        | 3/10     | 11/11        | 5/15     |

\( \text{a, Pst, Pseudomonas syringae pv. tomato; CD, cytochalasin D; n/d, not determined} \)
Figure Legends

Figure 1. *Atadf4* mutant compromises *AvrPphB*-mediated resistance against *Pseudomonas syringae* pv. tomato. (a) Diagram of the *AtADF4* gene carrying a T-DNA insertion in the second exon, starting from the 5′-UTR to the 3′-UTR. Introns and exons are shown in thick lines and boxes, respectively. Shaded boxes represent protein encoding sequence. The numbers indicate the nucleotide position. (b) RT-PCR analysis of *AtADF4* gene expression. Amplification of β-tubulin gene was used as an endogenous control. For resistance analysis, Col-0 and *Atadf* mutant plants were inoculated with *P. syringae* pv. tomato expressing empty vector (EV), AvrRpt2, AvrB and AvrPphB, respectively. (c) Disease phenotypes at 4 days after dip inoculation. (d) Bacterial populations at 0 and 4 days after dip inoculation (dpi). Error bars represent the standard errors calculated from three replications. (e) HR at 22 hours after bacteria infiltration, 2 representative leaves of 12 infiltrated from 4 plants are shown. Experiments in Figure 1 were repeated at least 3 times.

Figure 2. Relative transcript levels of *PR1* (a) and *PDF1.2* (b) in Col-0 and *Atadf4* mutant during infection time course after dip inoculation with *P. syringae* pv. tomato DC3000 expressing AvrPphB, determined by qRT-PCR, with amplification of *UBQ10* gene as an endogenous control. The transcript level of *PR1* and *PDF1.2* in Col-0 at 0 hour post inoculation (hpi) was set to 1. Similar results were obtained from two biological replicates. Error bars represent standard deviations from 3 technical replicates of one biological replicate.

Figure 3. *AtADF* gene silencing lines suppress the AvrPphB-mediated HR. (a) Relative quantity of transcripts for *AtADF* genes in four independent transgenic lines (S1, S2, S3 and S4), determined by qRT-PCR, with amplification of *UBQ10* gene as an endogenous control. Error bars represent standard deviations from 3 replicates. (b) HR at 22 hours
post inoculation with *P. syringae* pv. tomato expressing AvrPphB, 1 representative leaf of 12 inoculated from 4 plants is shown. Experiments were repeated 3 times.

**Figure 4.** *AtADF4* genomic DNA complements the *Atadf4* mutant for resistance against *Pseudomonas syringae* pv. tomato expressing AvrPphB. (a) Disease phenotypes at 4 days after dip inoculation. (b) Bacterial populations at 0 and 4 days after dip inoculation. Error bars represent the standard errors calculated from three replications. (c) HR at 22 hours after bacteria infiltration, 2 representative leaves of 12 infiltrated from 4 plants are shown. *Atadf4* (*gAtADF4*) #1 and #2 represent two independent transgenic lines. Experiments were repeated 3 times.

**Figure 5.** Laser-scanning confocal micrographs showing fluorescence of leaf cells expressing DsRed alone (a), DsRed-*AtADF4* (b), co-expressing DsRed and ABD2-GFP (c, d, e), and co-expressing DsRed-*AtADF4* and ABD2-GFP (f, g, h). Red channel shows localization of DsRed alone (a, c, e) or DsRed-*AtADF4* (b, f, h). Green channel shows localization of ABD2-GFP (d, g, e, h). (e) Overlay of micrographs of c and d. (h) Overlay of micrographs of f and g. Scale bars represent 20 μm.

**Figure 6.** *AtADF4* binds with higher affinity to ADP-G-actin than to ATP-G-actin. (a) SDS-PAGE gel of the purified recombinant protein *AtADF4* with a N-terminal FLAG tag. The numbers on the left indicate the molecular masses of the marker proteins in kDa. (b) Binding to ATP-G-actin was followed by quenching of NBD-actin fluorescence in the presence of varying amounts of *AtADF1* (squares) and *AtADF4* (circles). This single representative experiment allowed estimation of $K_d$ values of 16 μM and 44 μM for *AtADF1* and *AtADF4*, respectively. (c) The binding of *AtADF1* and *AtADF4* to NBD-labeled ADP-G-actin, from a single representative experiment, gave $K_d$ values of 0.3 μM and 0.08 μM, respectively. a.u. = arbitrary fluorescence units.

**Figure 7.** *AtADF4* inhibits nucleotide exchange on rabbit skeletal muscle actin (RSMA). (a) Nucleotide exchange on 1 μM ATP-G-actin under low ionic strength conditions. (b) Nucleotide exchange on 1 μM ADP-G-actin under physiological ionic conditions. 1 μM
ATP(ADP)-RSMA represents nucleotide exchange in absence of AtADFs. a.u.= arbitrary fluorescence units.

**Figure 8.** Bacterial populations of Col-0 and *Atadf* mutant plants at 0 and 4 days after hand infiltration (dpi) with a $10^5$ CFU/ml of *P. syringae* pv. tomato DC3000 expressing empty vector (EV), AvrRpt2, AvrB and AvrPphB, respectively. Error bars represent the standard errors calculated from three replications. Experiments were repeated twice.
Supplemental Data

**Supplemental Table S1.** Characterization of null mutants from collected *AtADF4* T-DNA insertion lines.

**Supplemental Table S2.** Primer sequences of *AtADF* s, *PRI* and *PDF1.2* used for quantitative RT-PCR.

**Supplemental Figure S1.** RT-PCR analysis of gene expression of *AtADF* genes in wild type Col-0 and *Atadf* mutant plants.

**Supplemental Figure S2.** Molecular characterization of transgenic lines of *Atadf4* complemented with *AtADF4* genomic DNA with T7 tag sequence at the C-terminus.

**Supplemental Figure S3.** Oligonucleotides and the procedure to make a RNAi construct targeting *AtADF1* through *AtADF4* using inverted-repeat PCR (IR-PCR).
