A FUNCTIONAL GENOMIC AND GENETIC ANALYSIS OF SUBCLASS III ACTIN DEPOLYMERIZING FACTOR (ADF) PROTEINS IN *ARABIDOPSIS THALIANA*

by

LORI MARIE KING

(Under the Direction of Richard B. Meagher)

ABSTRACT

The Actin Depolymerizing Factor (ADF)/Cofilin family of proteins are essential actin-binding proteins found in all eukaryotes. ADF proteins modulate actin filament dynamics by severing monomers from the pointed end of the filaments. The *Arabidopsis thaliana* genome encodes 11 ADF proteins, which group into 4 subclasses. Mutants of the two ADF proteins in Subclass III, ADF5 and ADF9, have opposite flowering-time phenotypes: *adf5*-1 flowers with more rosette leaves than wild-type plants grown under the same conditions (16h light/8h night photoperiod), while *adf9-1* flowers with fewer rosette leaves than wild-type. Also, qRT-PCR assays show that the transcript levels of the central repressor of flowering in Arabidopsis, Flowering Locus C (FLC), are up (fold-change: 4±0.2) in *adf5-1* and down (fold change: 5±0.5) in *adf9-1*. My objective in this study was to determine how ADF5 and ADF9 proteins can change gene expression.

Using a microarray analysis, I determined that ADF5 and ADF9 change the expression of approximately 7% and 12% respectively (false discovery rate of 0.1) of the genes expressed in the tissues (shoot) and at the time (2-4 leaves visible) I tested. Using genetic analyses focused on suppression studies, I show that the change in FLC

expression is actin- dependent. Treatment with the actin filament disruptor Cytochalasin D is sufficient to increase *FLC* expression, suggesting that actin-cytoskeletal dynamics play a regulatory role in *FLC* expression. I overexpressed a wild-type polymerizable form of ACTIN2 in *adf9-1* and showed that early flowering and the down-regulation of *FLC* is suppressed in this mutant by increasing the actin monomeric pool.

INDEX WORDS: Actin, Actin Binding Proteins, Actin Depolymerizing Factor, ADF, Cytoskeletal Dynamics, DNA Microarray, FLC, Gene Transcription, Transcriptome Analysis

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DEDICATION

I dedicate my dissertation to my beloved grandmother, Nellie Dunn, who passed away 5 November 2009. I wish she had been here, in happiness and good health, to see me finish. I love you and miss you, Grandma, and I carry the memory of you in my heart every day.

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CHAPTER 1

Introduction and Literature Review

The Actin Cytoskeleton

In all eukaryotes, actin has a monomeric (G-actin) and a polymeric (F-actin) form. Each actin molecule can bind ATP, which it then hydrolyzes to ADP sometime after it polymerizes. Polymerization can occur spontaneously, so ATP hydrolysis is not required for polymerization, but does speed up the process. The difference between monomer addition to the filament and ATP hydrolysis is what gives filamentous actin its polarity (the plus or barbed end of the filament polymerizes more quickly than the minus or pointed end or the filament). The concentration of actin in most cells is kept above the critical concentration required for spontaneous polymerization; however, without actin-binding proteins actin filaments would be disorganized (Schmidt and Hall 1998, Staiger 2000).

Therefore, the actin cytoskeleton is actually a dynamic network of proteins made up of actin polymers and actin-binding proteins. These actin-binding proteins are responsible for the tight regulation of the spatio-temporal organization of F-actin important to such vital cellular functions as cell division and expansion, cell motility, membrane dynamics, and the tip growth of root hairs and pollen tubes (Schmidt and Hall 1998, Staiger 2000). One of these actin-binding proteins is Actin Depolymerizing Factor (ADF).

The Actin Depolymerizing Factor (ADF)/Cofilin (AC) family of proteins

The Actin Depolymerizing Factor (ADF)/Cofilin (AC) proteins are a family of actin-binding proteins with representative homologs throughout eukaryotes. ACs are small, monomeric proteins from 13-19 kDa, with several highly conserved domains, including a conserved N-terminal serine residue (S3 in vertebrates, generally S6 in plants), and, in mammals, a strong Nuclear Localization Sequence (NLS). I have indicated these conserved domains on the crystal structure of ADF1 from *Arabidopsis thaliana* in **Figure 1.1**. The AC class of actin-binding proteins binds to both G-actin monomers and F-actin polymers, and its function is to modulate actin filament dynamics in a concentration-dependent way.

The function and regulation of ACs

Active ACs bind with the highest affinity to actin-ADP subunits, so they bind at the pointed end of filaments where the actin subunits have "aged" by hydrolyzing their bound ATP, leaving actin-ADP subunits (Bamburg 1999). **Figure 1.2** shows an illustration of the basic AC function. When the AC/actin ratio is low, ACs bind to filaments and induce a conformational change that twists monomers off the filament (Bamburg and Bernstein 2010, Staiger *et al.* 2010). This severing function provides filament ends for polymerization and more actin monomers for growth at the barbed or fast-growing end of the filament; therefore ACs are said to modulate actin filament dynamics. And, as mentioned above, actin filament dynamics are important for many vital cellular functions. However, when AC/actin ratios are high, the filaments become saturated with AC proteins, so pieces of the larger filament twist off but are stabilized in the twisted conformation as bundles or rods and can serve as nucleation sites for actin in plants (Andrianantoandro and Pollard 2006, Bamburg and Bernstein 2010, Staiger *et al.* 2010).

These functions of ACs require that they are active for actin binding. Their activity is regulated in several ways: pH, phosphorylation at the conserved serine residue, and phosphoinositide binding. Changes in pH do not inactivate ACs for actin binding; rather, changes in pH affect what form of actin ACs bind. For instance, ACs preferentially bind F-actin at acidic pH (i.e. pH 6.0) but bind to G-actin at alkaline pH (pH 7.4 or above) (Allwood *et al.* 2002, Gungabissoon *et al.* 1998). Therefore, most of the depolymerization activity occurs at higher pH, while very little occurs at lower pH (Yeoh *et al.* 2002).

Phosphorylation on the conserved serine residue, in contrast, inactivates ACs for actin binding. In mammalian systems, LIM kinases are responsible for this phosphorylation, but LIM kinases are not found in plants. In plant systems, the kinase responsible has yet to be definitely identified, but Allwood *et al.* found that a calmodulin-like domain protein kinase (CDPK) phosphorylated ADF3 from *Zea mays* (ZmADF3), so the kinase responsible for phosphorylating plant ADFs is likely to be from this class of kinases unique to plants and protists (Allwood *et al.* 2001). Although, Chen *et al.* have shown that Rac1from tobacco (NtRac1), a Rho-related Rac/Rop GTPase is also important for phosphorylation of NtADF1 (Chen *et al.* 2002).

ACs are also able to bind to phosphoinositides (phosphatidylinositol phosphate or PIP and phosphatidlyinositol (4,5)-bisphosphate or PIP_2), which, like phosphorylation, inactivates them for actin binding. Usually, ACs bind PIP_2 with higher affinity than PIP, so PIP_2 is seen to inhibit AC activity slightly more than PIP. For instance, for ZmADF3, PIP reduced its activity by 83% while PIP_2 reduced its activity by 98%(Gungabissoon *et al.* 1998). AC binding with PIP_2 inhibits the activity of phospholipase C, thus negatively regulating calcium-dependent signaling cascades, probably by competitively binding to PIP_2 (Staiger *et al.* 2010).

Because their activity is regulated by PIP_2 binding and Rho-family small GTPases, ACs

may serve as sensors and transducers of signaling cascades that cause actin cytoskletal reorganization in response to extrinsic or intrinsic stimuli (Allwood *et al.* 2001, Staiger 2000). And in the case of animal cells, ACs are known to facilitate changes in the actin cytoskeleton that can affect transcription of downstream target genes.

Actin cytoskletal dynamics in gene expression

Serum response factor (SRF) is a MADS-box transcription factor upstream of many of the immediate-early genes responsible for cell cycle control, apoptosis, cell growth, and cell differentiation in animal cells. SRF binds to so-called CArG box promoter elements (consensus sequence $CC[A/T]_2A[A/T]_3GG$) in the promoters of target genes, and some of the downstream targets of SRF are cytoskeletal components (Sun *et al.* 2006). SRF is an exclusively nuclear protein; however, actin cytoskeltal dynamics is one of the factors that controls expression of SRF target genes. Evidence now shows that a cofactor of SRF, MAL, binds G-actin in the cytoplasm through RPEL motifs (Guettler *et al.* 2008). Activation of signaling cascades by serum stimulation ultimately causes actin polymerization, which removes G-actin from the monomeric actin pool. As actin monomers are polymerized, MAL is released where it translocates into the nucleus, possibly with the help of another actin-binding protein, STARS, binds to and activates SRF poised on CArG boxes, thereby leading to transcription of target genes (Posern and Treisman 2006, Zheng *et al.* 2009).

Actin-binding proteins and drugs that can affect actin treadmilling also affect MAL:SRF-dependent gene transcription. For instance, overexpression of profilin (an actin-binding protein that stimulates actin polymerization) or treatment with Cytochalasin D (a drug that binds actin filaments and inhibits polymerization) activates MAL:SRF-dependent gene transcription, probably by depleting the G-actin pool (Posern and Treisman 2006). Sotiropolous *et al*. show in

cell culture that mutation of the phosphorylatable serine (S3) of cofilin to alanine (making cofilin constitutively inactive for actin binding) inhibits the ability of LIM kinase-1 to activate SRF-controlled reporter genes, which suggests that, as one would expect, ACs may have the opposite effect of profilin on MAL:SRF-dependent gene transcription (Sotiropoulos *et al.* 1999). Likewise, treatment with Latrunculin B (LatB) disrupts polymerization, but does not affect the actin monomer pool, so it inhibits MAL:SRF-dependent gene transcription (Posern and Treisman 2006).

The effects of cytoskeletal dynamics on MAL:SRF-dependent gene transcription has been the most extensively studied system, but other examples of the effects of actin cytoskeletal dynamics on gene transcription exist. For instance, PREP2 is a TALE class of homeodomain-containing transcription factors that colocalizes in the cytoplasm with the actin and tubulin cytoskeletons, and drug-induced disruption of either the actin (with CytD) or tubulin cytoskeletons causes PREP2 to translocate to the nucleus (Haller *et al.* 2004). Also, cofilin inhibits transcription of glucorticoid receptor (GR) target genes. GR is held in the cytoplasm by interaction with hsp90, which anchors it to the actin cytoskeleton, where it is activated by glucocorticoid. Activation causes GR to release from the cytoplasm and translocate to the nucleus. Overexpression of cofilin inhibits transcription of GR target genes, possibly because the increased G-actin pool has two inhibitory effects. First, the increased G-actin pool induces the known GR inhibitor c-Jun, and second, the increase in actin treadmilling may release GR before it has been activated by glucocorticoid, thereby saturating nuclear GR binding sites with inactive GR (Ruegg *et al.* 2004).

An alternative function? The Nuclear Localization Sequence (NLS)

Expansion during the evolution of the plant ADF gene family allows for the evolution of novel functions. Some ADF variants may therefore serve the well-characterized and essential function of modulating actin dynamics, thus freeing other ADF variants to serve a novel function. At least two lines of evidence suggest that some ADF variants serve a novel function. First, some plant ADF variants have a different level of depolymerizing activity from that of metazoan variants. For instance, ZmADF3 has a similar depolymerizing activity to mammalian ACs, but ADF1 from lily has only 20% of the depolymerizing activity of ZmADF3 (Hussey *et al.* 2002). The decreased depolymerization activity of lily ADF1 is possibly because of functional redundancy with other ADF proteins in the family, yet large gene families also open the possibility of neofunctionalization (Ohta 2003). Second, the plant variants also differ greatly in sequence; for instance, one notices when comparing the Arabidopsis, rice, and poplar sequences shown in **Figure 1.3** that homology within the subclasses is greater than homology within the same species, further raising the possibility that at least plant ADF variants are functionally distinct from one another.

As mentioned previously, mammalian ACs have an NLS. Nuclear localization of ADF proteins has been observed in metazoans (Abe *et al.* 1993, Nebl *et al.* 1996, Ohta *et al.* 1989, Samstag *et al.* 1994, Sanger *et al.* 1980). In vertebrates, nuclear localization of ACs usually accompanies stress. For instance, in both kangaroo rat (Ptk2) and human fibroblast (WI-38) cell lines, treatment with dimethyl sulfoxide (DMSO) causes ACs to translocate into the nucleus, carrying actin with it (Sanger *et al.* 1980). Vertebrate AC proteins contain an NLS with a sequence similar to that of the SV40 large T antigen (Bamburg 1999). The KKRKK motif is

most important in nuclear localization, and mutation of the second of the two basic residues (K31 in the human sequence) at the beginning of the motif prevents the nuclear localization (Iida *et al.* 1992). The first two lysine residues (K30 and K31) of this KKRKK motif, however, are formed from a vertebrate-specific nine residue insertion in mammals (Bowman *et al.* 2000).

No NLS has been confirmed in the plant specific variants of ACs, but ZmADF3 localizes to the nucleus (taking actin with it) during cellular stress, just as the vertebrate variant does (Jiang *et al.* 1997). Ruzicka *et al*. show, using a monoclonal antibody raised against recombinant ADF4 (mADF4a), that Subclass I ADFs from Arabidopsis localize to both the cytoplasm and nucleus (Ruzicka *et al.* 2007). I show in **Chapter 2** that ADF5 from Subclass III similarly localizes to both the cytoplasm and nucleus, and Tholl *et al.* show that ADF9-GFP biolistically transformed into tobacco cells localizes to both actin filaments and the nucleus (Tholl *et al.* 2011).

That ACs often bring actin with them when they redistribute to the nucleus suggests that the function of the NLS in ACs is to help localize actin to the nucleus, where it has important functions in gene transcription, chromatin remodeling, and the formation of heterogeneous nuclear ribonucleoprotein complexes (Zheng *et al.* 2009). Actin serves these important functions in the nucleus, but does not have an NLS. Although the 42 kDa actin monomer is technically small enough to passively diffuse into the nucleus through the nuclear pore complex, actin is rarely seen in the nucleus, possibly because it has two Nuclear Export Sequences (NES) that ensure its cytoplasmic localization under ordinary circumstances (Wada *et al.* 1998).

ADF proteins in *Arabidopsis thaliana*

The genomes of most eukaryotes include only one or two genes for ADF/cofilin. For

instance, mammalian genomes, like those of the mouse and human, have only one ADF and two cofilins, though there are also several cofilin pseudogenes. In contrast, the genomes of many plants encode large families of ADF genes and no cofilin genes (Bamburg 1999). For that reason, I will only use the acronym ADF from here forward. The relevant question when examining gene families is: are the differences in function we observe because of temporal-spatial differences in expression or because of real functional differences in the proteins?

As shown in **Figure 1.3**, the *Arabidopsis thaliana* genome comprises a family of 11 ADF genes, which phylogenetically group into four subclasses with differential expression patterns (Ruzicka *et al.* 2007. Subclass I, for instance, is expressed in all organs and tissues except pollen, and the 4 members of Subclass I have considerable overlap in expression patterns. Subclass II's expression, however, is tip-cell specific, with group IIa expression restricted to mature pollen and group IIb expression restricted to root trichoblasts. I focused my research on Subclass III for several reasons that I will discuss in the next section.

Subclass III ADFs in *Arabidopsis thaliana*

The members of Subclass III, ADF5 and ADF9, are considerably different from one another. **Figure 1.5** shows a protein alignment of Arabidopsis ADF5 and ADF9. ADF5 is 143 amino acid residues and ADF9 is 141 amino acid residues. The Subclass III proteins are considered divergent because they are only 80% identical (115 out of 143 residues) in amino acid sequence (Ruzicka *et al.* 2007). Of the 28 residues different between ADF5 and ADF9, exactly 50% are amino acids with similar biochemical properties.

The functionally most significant amino acid changes are likely to be in residues necessary for post-translational modifications important in regulation and residues that form the

actin-binding site. As mentioned previously, a phosphorylatable serine residue is conserved at the N-terminus across 75% of the AC family of proteins. When this serine is phosphorylated, ADF is inactivated for actin binding, probably because the phosphate group blocks actin from the actin-binding site (Bamburg 1999, Staiger *et al.* 2010). In the Arabidopsis Subclass I protein ADF1 this serine is the 6th residue from the N-terminus, but in ADF9 the serine is the 8th residue. In ADF5, the residue is the 10th residue and is changed to a threonine. Two of the amino acid residues at the N-terminus that participate in actin binding are different between ADF5 (M7 and T10) and ADF9 (T5 and S8); however, the rest of the residues thought important for actin binding are identical. The remaining changes seen between the ADF5 and ADF9 amino acid sequences are of unknown significance.

These changes in amino acid sequence, however, may indicate neofunctionalization, at least for ADF9. A recent paper has shown that ADF9 has a different function and pH dependence than ADF1 (Tholl *et al* 2011). In their study, ADF1 induces depolymerization of actin filaments *in vitro* in a concentration-dependent way with greater efficiency at alkaline pH (8.0) than acidic pH (6.0). Contrary to expectation, though, ADF9 did not depolymerize actin filaments; rather, ADF9 promotes actin bundling, and it does so with more efficiency at acidic pH than alkaline pH. An ADF9-GFP construct biolistically transformed into tobacco cells shows that ADF9 can bundle actin filaments *in vivo* as well as *in vitro*, and, as mentioned above, localizes to the both the cytoplasm and the nucleus. Last, ADF1 and ADF9 may be able to compete with one another because ADF9 can counteract the depolymerization effects of ADF1, and ADF1 can counteract the bundling effects of ADF9. Their difference in pH dependence suggests coordination in regulation of their activity that may come into play in cells where they are coexpressed.

In addition to differences in sequence, the Subclass III ADF proteins have significant differences in expression patterns. Their expression overlaps to some extent with that of Subclass I proteins. As mentioned in the previous section, using promoter::GUS fusions, Ruzicka *et al.* showed that, with only minor variation among the four members, Subclass I ADFs are expressed across all the developmental stages and in all the tissues they assayed, except for pollen (Ruzicka *et al.* 2007). Like Subclass I proteins, the two members of Subclass III are broadly expressed in vegetative tissues, but they also have non-overlapping patterns of expression. ADF5 is expressed strongly in cotyledons, emerging leaves, root vascular tissue, and the root tip meristem, but it is expressed weakly in reproductive tissue, adult leaves, and mature roots. ADF9, in contrast, is expressed strongly in young seedlings, apical meristem, the edges of leaves, trichomes, the style, anther/stamen, root vascular tissue, root elongation zone, and root tip meristem.

However, to say that Subclass III ADFs are strongly expressed is only with respect to their own subclass expression levels. Using qRT-PCR, Ruzicka *et al.* showed that the Subclass III ADF genes are expressed at the lowest levels of all the ADF genes in Arabidopsis (Ruzicka *et al.* 2007). For perspective, the Subclass I ADF gene from Arabidopsis, ADF1, is expressed at moderate levels in 10-day-old seedlings relative to the other Subclass I members, but ADF5 is expressed at 43% the level of ADF1 and ADF9 is only expressed at 1.3% the level of ADF1 (**Figure 1.4**). The same general pattern holds true across most of the tissues and developmental stages Ruzicka et al. assayed: ADF5 is expressed between 73%-42% ADF1 levels (except in callus, root tissue, and immature flower, where ADF5 is expressed at slightly higher levels), and ADF9 is expressed between 13%-0.7% ADF1 levels (except callus, where ADF9 is expressed the highest of all the ADFs).

Despite such a low level of expression, however, plants with a mutant ADF9 allele (*adf9-1*) have several very clear phenotypes. For instance, *adf9-1* seedlings are smaller than WT, *adf9-1* plants flower earlier than WT, and they are down-regulated in the central repressor of flowering, Flowering Locus C (*FLC*) (Burgos-Rivera *et al.* 2008). The down-regulation of FLC is particularly interesting because FLC is up-stream of floral promoters Flowering Locus T (FT) and Suppressor of Overexpression of CONSTANS 1 (SOC1), so down-regulation of *FLC* is sufficient to explain the early flowering phenotype of *adf9-1* (Michaels and Amasino 2001). Because the Subclass III ADFs have such low expression levels compared to all other ADFs in Arabidopsis yet mutants have such clear phenotypes (as I will show in **Chapter 2** for a mutant allele of ADF5, *adf5-1*) and because *adf9-1* has a change in gene expression, I found the Subclass III ADFs particularly interesting.

Purpose of the study

The question that most intrigued me about ADF9 was how an actin-binding protein like ADF could affect gene expression. Ultimately, gene expression is how an organism's genotype is translated (pun intended) into its phenotype. I had yet to learn about the ways that actin polymerization could affect gene transcription in animals, but still, to me, the most intriguing aspect of a link between the cytoskeleton and gene expression is that the system is an elegant way to integrate environmental stimuli into growth and development.

My initial strategy to address the question was to determine the protein-protein interactions and the subcellular localization of ADF9. If, for instance, ADF9 bound a known component of the transcription initiation complex or a chromatin-remodeling complex and localized to the nucleus, these data would suggest ADF9 affected gene transcription through a

direct mechanism. If, however, ADF9 bound only actin and/or other actin-binding proteins and localized to the cytoplasm, these data would suggest ADF9 changed gene expression by an indirect mechanism.

To study protein-protein interactions and subcellular localization, I needed some way of tracking the protein, such as an ADF9 or Subclass III specific antibody or a way of tagging the protein. The Meagher lab group is particularly talented at making antibodies; many of the antibodies generated in the lab are used in labs throughout the world. However, several attempts to make a Subclass III specific antibody failed. So, I began my part in the project by attempting to epitope-tag ADF9. I tried two different epitope tags (FLAG and T7), but unfortunately, though I called on the considerable protein expertise of my advisor, I was unsuccessful in detecting tagged ADF9 on a western blot from plant extracts. Next, I tried tagging ADF9 with a biotin ligase recognition peptide (BLRP) and expressing the fusion protein in a plant line expressing biotin ligase from *E. coli*. The biotin-streptavidin interaction is one of the strongest in biology, so I expected this technique would give me the best chance at success. Once again, however, I was unable to detect tagged ADF9 on a streptavidin blot. Because of these failures with my initial strategy, I decided to try a different approach and chose, therefore, to take a functional genomic and genetic approach to the question.

Functional genomics

The change in *FLC* expression in *adf9-1* (and the complementation of *FLC* expression in *adf9-1*/A2pt:ADF9) shows that ADF9 affects gene expression. I also show in **Chapter 2** that *FLC* expression is changed in *adf5-1* too, where, in contrast to *adf9-1*, *FLC* is up-regulated. Both Subclass III ADFs, therefore, affect gene expression, but I then wondered if the scope of gene expression changes was far-reaching or restricted to one pathway. And if the scope is far-

reaching, could these changes give me insight into ADF's function in gene expression? For instance, if I found that the expression of a number of genes was changed and these genes were enriched in the stress response pathway, these data would suggest ADF functions in stress response signaling. To address these questions, I performed a microarray transcriptome analysis on *adf5-1* and *adf9-1* and I report the results in **Chapter 2**.

Genetic analysis

Regardless of the results of the microarray, I know that both Subclass III ADFs affect transcription of *FLC*, and I next asked if they affected gene expression in an actin-dependent way, as we know they do in animal systems. Based on the literature I have reviewed in this introductory chapter, I have formed three hypotheses for how ADF could function in gene expression. **Figure 1.6** shows an illustration of these three hypotheses. The first, the "actin to the nucleus" hypothesis, is based upon the knowledge that ADFs from animals have a confirmed NLS and plant ADFs are known to localize to the nucleus. The second model, the "cytoplasmic dynamics" hypothesis, is based upon the body of literature that shows that cytoskeletal dynamics in the cytoplasm affects the subcellular localization and activity of several transcription factors in animal systems. Both of these models depend upon cytoskeletal dynamics, but the mechanism is different between the two. The third model, the "direct action" hypothesis, is an alternative hypothesis to the other two, in which ADF's affect on gene expression is independent of actin. I report the results of several genetic experiments designed to test these hypotheses in **Chapter 3**. Finally, in **Chapter 4**, I give my interpretation and perspective on my work to date and where I would like to see the project go in the future.

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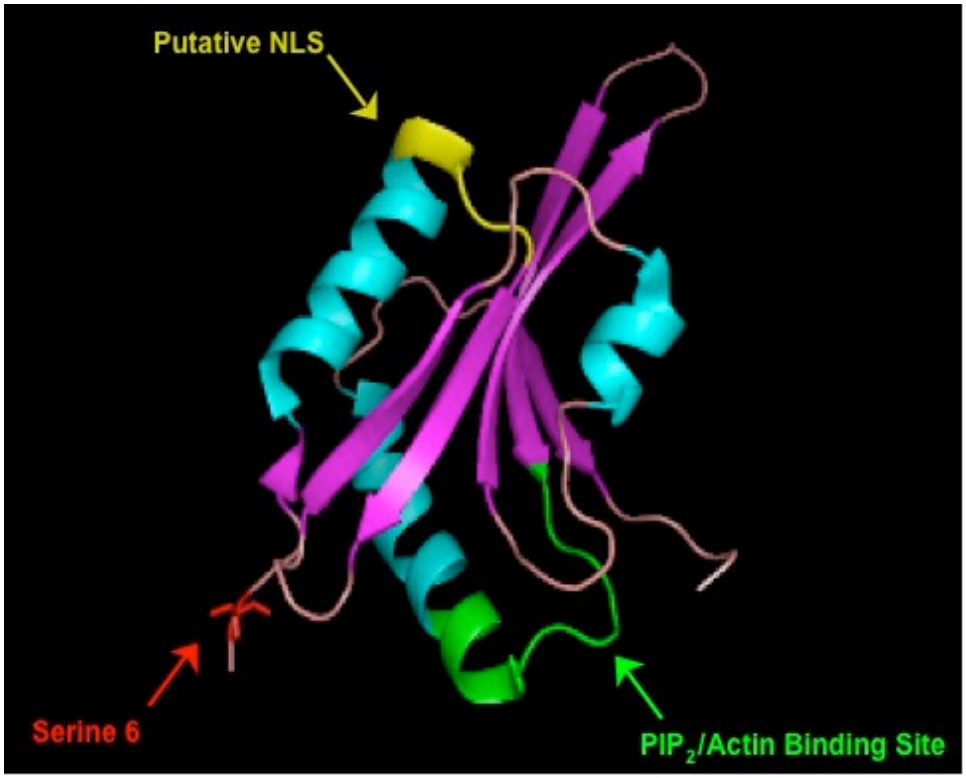

Figure 1.1: Ribbon diagram of ADF1 from Arabidopsis. This image was made in MacPyMol from PDB file 1F7S. The phosporylatable serine (S6) is colored red, the putative NLS yellow, and the PIP_2/Actin binding site in green (Bowman *et al* 2000).

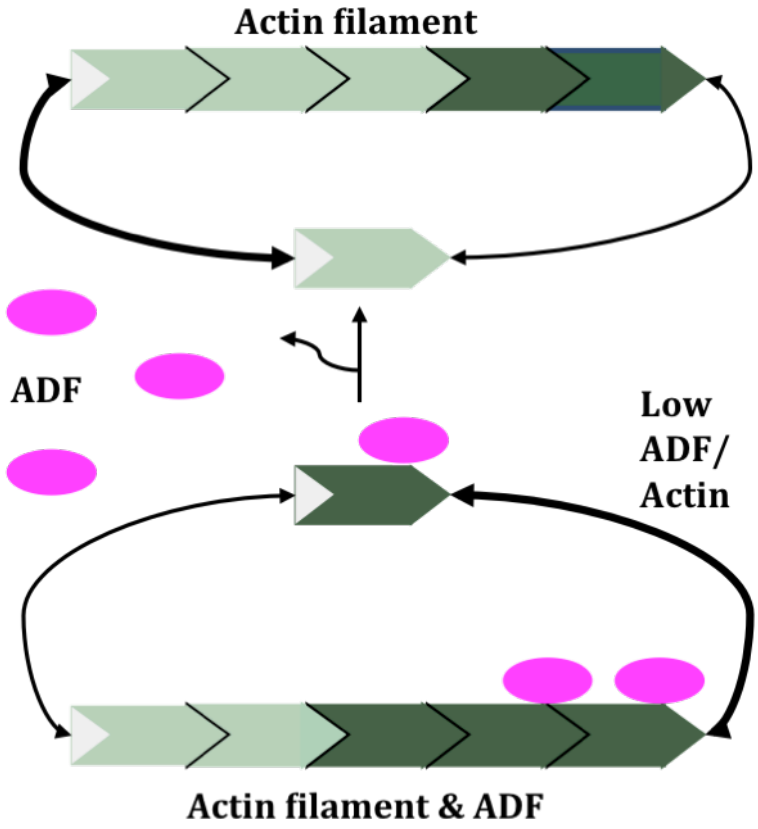

Figure 1.2: Basic fuction of AC proteins. Actin monomers are represented by green polygons. The darker green represents monomers that have hydrolyzed their bound ATP to ADP. The lighter green polygons have yet to hydrolyze their bound ATP. The pink ovals represent ACs. ACs bind at the pointed or minus end of the filaments because they have greater affinity for actin:ADP monomers. In low AC/actin ratios, AC binding induces a conformational change that twists monomers off the end of the filament. Thus, ACs increase the monomeric or G-actin pool for growth at the barbed or plus end.

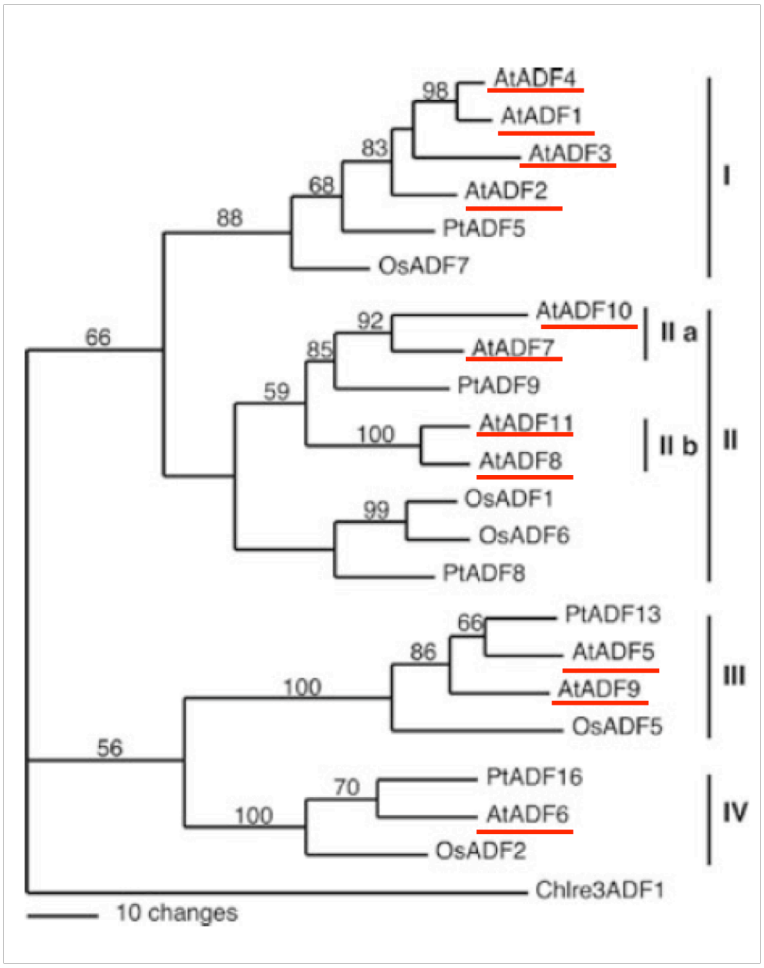

Figure 1.3: The ADF family of proteins is large and has diverse expression patterns in *Arabidopsis thaliana* (Ruzicka *et al* 2007). This figure shows a neighbor-joining protein sequence phylogeny, including rice (Os) and poplar (Pt) sequences and rooted using *Chlamydia reinhardtii* (Chlre) sequence. Bootstrap support is indicated at the branch points. The Arabidopsis ADF proteins are underlined in red. The proteins are grouped into four subclasses, each with a different expression pattern.

Figure 1.4: Subclass III ADF proteins in Arabidopsis are only 80% identical. AtADF5 and AtADF9 protein sequences were downloaded from the TAIR database and aligned using CLUSTAL 2.0.12. This figure is based upon a similar figure in Bowman *et al.* 2000. Amino acid residues that are identical between AtADF5 and AtADF9 are shown in black, residues that are different in AtADF5 and AtADF9 but have similar biochemical properties are shown in blue, and residues that have dissimilar biochemical properties are shown in red. Residues that are at least 75% conserved across the entire AC family of proteins and are identical to residues in AtADF1 are in bold (Bowman *et al* 2000). Shaded residues are those residues that have been mutated in previous studies and shown to participate in actin binding (Bowman *et al* 2000). The tertiary structure of the protein is indicated above the sequence: pink arrows denote alpha-helices and aqua boxes denote beta-sheets.

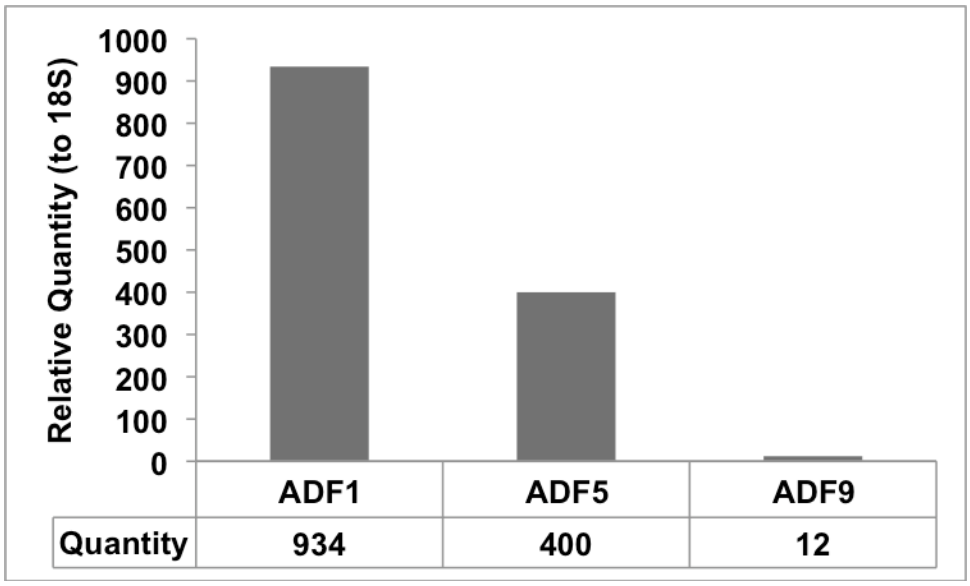

Figure 1.5: Subclass III ADF genes are expressed at the lowest levels. This data was extracted from Ruzicka *et al* 2007. qRT-PCR data for 10-day-old seedlings shows that the Subclass III ADF genes are expressed at much lower levels than ADF1.

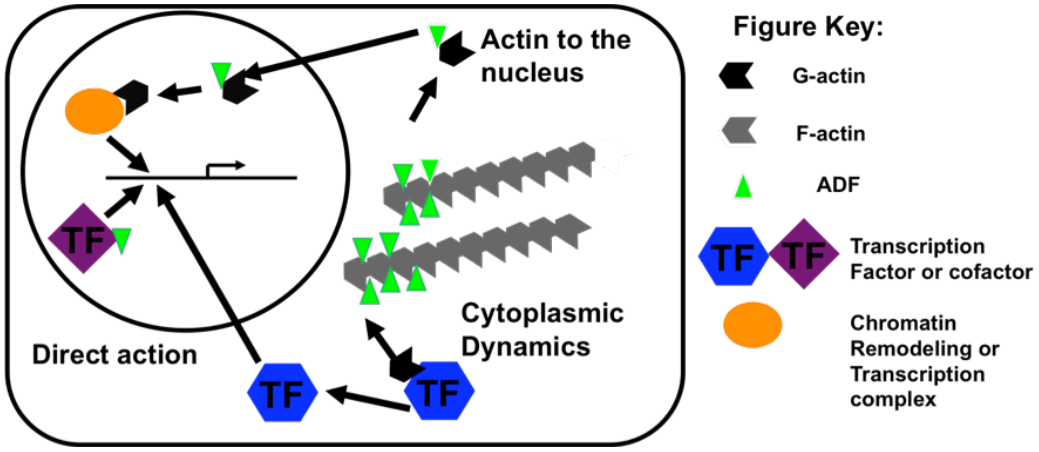

Figure 1.6: Illustrated hypotheses for the function of ADF proteins in gene expression. The "actin to the nucleus" hypothesis, is based upon the knowledge that ADFs from animals have a confirmed NLS and plant ADFs are known to localize to the nucleus. The "cytoplasmic dynamics" hypothesis, is based upon the body of literature that shows that cytoskeletal dynamics in the cytoplasm affects the subcellular localization and activity of several transcription factors in animal systems. Both of these models depend upon cytoskeletal dynamics. The "direct action" hypothesis, is an alternative hypothesis to the other two, in which ADF's affect on gene expression is independent of actin.

CHAPTER 2

Subclass III ACTIN DEPOLYMERIZING FACTORs affect global gene transcription in *Arabidopsis thaliana*[1]

[1] Lori M. King, Daniel R. Ruzicka, Elizabeth C. McKinney, Muthagapatti K. Kandasamy, Kristofer Mussar, Eileen Roy-Zokan, Xiaoyu Zhang, and Richard B. Meagher. To be submitted to G3: Genes, Genomes, Genetics

Abstract

Arabidopsis has 11 ADF protein variants, which group into 4 ancient subclasses. One member of Subclass III, ADF9, affects plant development and gene expression of the central repressor of flowering, FLOWERING LOCUS C (FLC). ADF9's closest homolog in Arabidopsis, ADF5, has yet to be characterized. Here we report that ADF5 affects plant development and expression of *FLC*. To guide understanding of Subclass III ADF's effect on gene expression, we report results from a microarray analysis of *adf5-1* and *ad9-1*. We found that approximately 7% (FDR=0.1) of genes in our WT gene set were differentially expressed in *adf5-1* and approximately 12% in *adf9-1*. Unexpectedly, only 152 of the genes differentially expressed were shared between *adf5-1* and *adf9-1*. We found no Gene Ontology (GO)-term enrichment in differentially expressed genes in *adf5-1* or those shared between *adf5-1* and *adf9-1;* therefore, Subclass III ADFs broadly affect gene expression. Genes shared between adf5-1 and adf9-1 are, however, enriched in 6-mer binding elements found in AGAMOUS (AG) and AGAMOUS-like (AGL) responsive promoters. Subclass III ADFs both localize to the cytoplasm and the nucleus, which opens the possibility that Subclass III ADFs affect gene expression by shuttling actin to the nucleus.

Introduction

The Actin Depolymerizing Factor (ADF)/Cofilin family is an essential actin-binding protein family found in all eukaryotes. ADFs are small, monomeric proteins that bind to either G-actin monomers or F-actin polymers, though they bind with the highest affinity to actin:ADP subunits. ADFs, therefore, tend to bind F-actin filaments on or near the pointed end where the actin subunits have hydrolyzed their bound ATP. When they bind, they induce a conformational change that twists monomers off the pointed end of the filament (Bamburg 1999). This severing function, however, is concentration dependent; only with low ADF/actin ratios does the severing function predominate. With high ADF/actin ratios, F-actin becomes saturated with ADF proteins, so pieces of the larger filament twist off but are stabilized in the twisted conformation as bundles or rods that can serve as nucleation sites for actin in plants (Bamburg and Bernstein 2010, Staiger *et al.* 2010). Indeed, Tholl *et al.* have recently reported that one ADF protein in Arabidopsis, ADF9, may not depolymerize actin filaments; rather, ADF9 may stabilize or bundle actin filaments (Tholl *et al.* 2011).

Regulation of ADF activity is by phosphorylation of a conserved N-terminal serine residue (S6 in most plant variants), by binding to phosphoinositides, and by pH (Allwood *et al.* 2002, Gungabissoon *et al.* 1998). Phosphorylation of the conserved serine residue inactivates ADFs for actin binding. In plant systems, the kinase responsible is likely a calmodulin-like domain protein kinase (CDPK) (Allwood *et al.* 2001). ADFs are also able to bind to phosphoinositides (phosphatidylinositol phosphate or PIP and phosphatidlyinositol (4,5)-bisphosphate or PIP_2), which, like phosphorylation, inactivates them for actin binding. Usually, ADFs bind PIP_2 with higher affinity than PIP, so PIP_2 inhibits ADF activity slightly more than PIP (Gungabissoon *et al.* 1998). pH affects what form of actin ADFs bind. For instance, ADF preferentially binds F-

actin at acidic pH (i.e. pH 6.0) but binds to G-actin at alkaline pH (pH 7.4 or above) (Allwood *et al.* 2002, Gungabissoon *et al.* 1998). Therefore, most of the depolymerization activity occurs at higher pH, while very little occurs at lower pH (Yeoh *et al.* 2002). The Arabidposis Subclass III ADF, ADF9, however, is more efficient in its activity at acidic pH (Tholl *et al.* 2011).

Most plants have large gene families of ADFs (Maciver and Hussey 2002). The *Arabidopsis thaliana* genome encodes 11 ADF proteins, which group into 4 ancient subclasses with differential expression patterns (Feng *et al.* 2006, Ruzicka *et al.* 2007). Two subclasses, Subclass I and Subclass II are predominately expressed in vegetative tissues. The Subclass I ADFs (ADF1, ADF2, ADF3, and ADF4) are expressed in all tissues save pollen and at all developmental stages. Like Subclass I, Subclass III ADFs (ADF5 and ADF9) are expressed broadly in all vegetative tissues, yet their expression is much weaker. They are expressed more strongly in fast growing or differentiating tissues, such as callus and meristem (Ruzicka *et al.* 2007). Unlike Subclass I, however, Subclass III has non-overlapping patterns of expression. ADF5 is expressed strongly in cotyledons, emerging leaves, root vascular tissue, and the root tip meristem, but it is expressed weakly in reproductive tissue, adult leaves, and mature roots. ADF9, in contrast, is expressed strongly in young seedlings, apical meristem, the edges of leaves, trichomes, the style, anther/stamen, root vascular tissue, root elongation zone, and root tip meristem (Ruzicka, Kandasamy, McKinney, Burgos-Rivera and Meagher 2007).

However, to say that Subclass III ADFs are strongly expressed is only with respect to their own subclass expression levels. Using qRT-PCR, Ruzicka *et al.* showed that the Subclass III ADF genes are expressed at the lowest levels of all the ADF genes in Arabidopsis (Ruzicka, Kandasamy, McKinney, Burgos-Rivera and Meagher 2007). For perspective, the Subclass I ADF gene from Arabidopsis, ADF1, is expressed at moderate levels in 10-day-old seedlings

relative to the other Subclass I members, but ADF5 is expressed at 43% the level of ADF1 and ADF9 is only expressed at 1.3% the level of ADF1. The same general pattern holds true across most of the tissues and developmental stages Ruzicka *et al.* assayed: ADF5 is expressed between 73%-42% ADF1 levels (except in callus, root tissue, and immature flower, where ADF5 is expressed at slightly higher levels), and ADF9 is expressed between 13%-0.7% ADF1 levels (except callus, where ADF9 is expressed the highest of all the ADFs).

Functions of several Subclass I proteins in Arabidopsis have been characterized. Dong *et al.* expressed ADF1 cDNA in the antisense orientation to knock down expression, and showed that knockdown caused delays in flowering, increases in cell expansion and organ growth, and increases in actin cabling (Dong *et al.* 2001). Clément *et al*., likewise, used an antisense strategy with ADF2 to show defective embryogenesis, enlargement of some cells, reduced development of infesting nematodes, and, again, increased actin cabling (Clement *et al.* 2009). Tian *et al.* isolated a T-DNA insertion mutant to show that ADF4 is involved in pathogen resistance to *Pseudomanas syringae* (Tian *et al.* 2009).

The function for one of the members of Subclass III, ADF9, has also been characterized. Plants with a mutant ADF9 allele (*adf9-1*) have several clear phenotypes: *adf9-1* seedlings are smaller than WT, flower earlier than WT, and have down-regulation of the central repressor of flowering, Flowering Locus C (*FLC*) (Burgos-Rivera *et al.* 2008). The down-regulation of FLC is particularly interesting because FLC is up-stream of floral promoters Flowering Locus T (FT) and Suppressor of Overexpression of CONSTANS 1 (SOC1), so down-regulation of *FLC* is sufficient to explain the early flowering phenotype of *adf9-1* (Michaels and Amasino 2001). These data suggest, therefore, that ADF9 affects gene expression through an unknown mechanism.

The function of the second member of Subclass III, ADF5, has yet to be described. Given the described function of ADF5's closest homolog, we might expect a similar function for ADF5. However, the members of Subclass III, ADF5 (At2g16700) and ADF9 (At4g34970), are considerably different from one another, with 28 amino acid differences in ADF5's 143 amino acid residues (ADF9 is only 141 amino acid residues). Of these 28 residue differences between ADF5 and ADF9, exactly 50% are amino acids with similar biochemical properties. As mentioned previously, a phosphorylatable serine residue is conserved at the N-terminus across 75% of the ADF/Cofilin family of proteins. In ADF1 this serine is S6 but in ADF9 it is S8. In ADF5, this residue is T10. Two of the amino acid residues at the N-terminus that participate in actin binding are different between ADF5 (M7 and T10) and ADF9 (T5 and S8); however, the remaining residues thought important for actin binding are identical. The remaining changes seen between the ADF5 and ADF9 amino acid sequences are of unknown significance.

Here we describe the phenotype of an ADF5 T-DNA insertion mutant, *adf5-1*, and compare it to the ADF9 T-DNA insertion mutant, *adf9-1*. We show that ADF5 and ADF9 affect many of the same developmental pathways but have opposite effects on them. Additionally, *adf5-1* is up-regulated in *FLC*, suggesting that ADF5 also affects gene expression. To explore Subclass III's effects on gene expression, we present data on microarrays performed on *adf5-1* and *adf9-1* and comparisons between the two.

Materials and Methods

All experiments were performed on *Arabidopsis thaliana*, Columbia (Col) ecotype.

Isolation and confirmation of adf5-1 mutants

A T-DNA insertion mutant (SALK_056064) in the ADF5 gene (At2g16700) was

obtained from TAIR (www.arabidopsis.org) and the allele was named *adf5-1*. To confirm the *adf5-1* mutant allele, primers were designed to amplify identifying fragments from wild-type (WT) ADF5 and mutant *adf5-1* alleles. DNA used as a template for genotype and sequence determination was isolated using the Sigma Plant DNA extraction kit. An 820-bp fragment from the ADF5 allele was amplified using the sense primer ADF5-5´utrS (5´-ATGGCGATGGCTTTCAAGATGGTA) and antisense primer ADF5-3´utrA (5´-AAACATTAACCGATGACCTAATTA). A 900-bp fragment of the *adf5-1* mutant allele was amplified with a left border T-DNA primer LBaI (5´-GGTTCACGTAGTGGGCCATCG) and the sense primer ADF5-5´utrS, and a 400-bp fragment of the *adf5-1* mutant allele was amplified with a left border T-DNA primer LBaI (5´-GGTTCACGTAGTGGGCCATCG) and the antisense primer ADF5-3´utrA. PCR products of the *adf5-1* allele were sequenced to confirm the insertion site.

Once the *adf5-1* allele was confirmed, the mutant was backcrossed twice to the lab strain of WT Columbia (Col), confirmed to have a single T-DNA insertion, and propagated by selfing. Down-regulation of ADF5 was determined by qRT-PCR. For all phenotype analyses, seeds from WT and *adf5-1* plants were sown directly on soil (Fafard 3B), stratified in the dark at 4°C for 48 hours, then placed in a growth chamber maintained at 22°C on a long-day (16 hour light, 8 hour dark) light cycle. Leaf numbers were quantified at bolting (n=15). Epidermal, trichome and stomata cell numbers were assayed on fully mature leaves (n=4) with 6 areas assayed per leaf.

Mutant Complementation and ADF5 overexpression

For complementation, *adf5-1* mutant plants were transformed with *Agrobacterium tumefaciens* C58 via the floral dip method with modifications (Clough and Bent 1998). *A.*

tumefaciens was transformed with a pCAMBIA binary vector containing ADF5 cDNA under the control of an ACTIN2 promoter and terminator. Positive T1 generation transformants were selected by sowing seeds on soil and spraying seedlings with a BASTA solution. Positive transformants were then moved to individual pots and confirmed by a second spraying with BASTA. All complementation analyses were performed on drug-selected T2 generation plants.

Real-time PCR analysis

RNA was isolated from 20-day-old plants (for the complementation analysis) or 10-day-old seedlings using the Qiagen RNeasy Plant Mini Kit. 1.5 μg of total RNA from each sample was treated with RQ1 RNase-free DNase (Promega) and the treated RNA was used for cDNA synthesis using the Super Script III kit (Invitrogen), following the manufacturer's instructions except that incubations were performed for only 30 min. at 55°C using oligo (dT) primer. Aliquots of the cDNA were used as template for qRT-PCR analysis of triplicate reactions for each of the biological replicates on an Applied Biosystems 7500 Real Time PCR Instrument, using SYBR Green detection chemistry. Real time PCR reactions consisted of 2X SYBR GREEN PCR Master Mix (Applied Biosystem), 0.8 μM of each primer, and 1:25 diluted cDNA in a 25 μl reaction volume. The reaction conditions were as follows: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1:00. Data was collected during the last stage of each cycle. The following primers were used to detect transcript levels: *ACT2* (ACT2-RTS: 5'-CAAATCCAGCCTTCACCATA-3', ACT2-RTA: 5'- GAGGCTGATGATATTCAACCA-3'); *ACT7* (ACT7-RTS: 5'-AGAGAAAATACAGTGTCTGGAT-3', ACT7-RTA: 5'-TTTGAAATCCACATCTGTTGGAA-3'); *ADF5* (ADF5-RTS: 5'-CGACTTTGTCACCGTCGATAAC-3', ADF5-RTA: 5'-CTCCGGTGACCATGCAATG-3'); *ADF9* (ADF9-RTS: 5'-ATATAACGAAAGAACAAGAAGACA-3', ADF9-RTA:

5'CACTCGTCGCCGTCTTCAA-3'); *AP1* (AP1-RTS: 5'-CGCAGCAGCACCAAATCC-3', AP1-RTA: 5'-TGAGAAAAGGAGATGGCTGATG-3'); *CO* (CO-RTS: 5'-CCCTATATGCATAAAACCGTGGTAA-3', CO-RTA: 5'-TGGCAAAACTAGACTGCATGCT-3'); *FLC* (FLC-RTS: 5'-TCTTCCGGTGACTCTGTTA-3', FLC-RTA: 5'-ATATGTTTTGGATTTTGATTTCAA-3'); *FT* (FT-RTS: 5'-GGCGCCAGAACTTCAACACT-3', FT-RTA: 5'-CGGGAAGGCCGAGATTG-3'); *LFY* (LFY-RTS: 5'-TTGTCGTCATGGCTGGGATATA-3', LFY-RTA: 5'-GAACATACCAAATAGAGAGACGAGG-3'); *SOC1* (SOC1-RTS: 5'-AAAGCTCTAGCTGCAGAAAACGA-3', SOC1-RTA: 5'-GACCAAACTTCGCTTTCATGAGAT-3'); *UBQ10* (UBQ10-RTS: 5'-AGAAGTTCAATGTTTCGTTTCATGTA-3', UBQ10-RTA: 5'-GAACGGAAACATAGTAGAACACTTAT-3'). Control reactions were performed using UBIQUITIN10 (UBQ10). The $2^{-(ddCT)}$ method of relative quantification was used in all experiments.

GFP reporter for F-actin

We transformed wild-type and *adf9-1* mutant plants with the F-actin reporter GFP:fABD2 (Wang *et al.* 2004). Multiple lines of positive transformants were selected on hygromycin and vertically grown on ½ MS, 1% sucrose, 0.8% agar plates. Microfilaments were visualized in trichomes as described previously (Wang, Motes, Mohamalawari and Blancaflor 2004) using a Leica confocal laser scanning microscope (TCS-SP2, Heidelberg, Germany).

RNA isolation, target synthesis and hybridization to Affymetrix GeneChips for WT, adf5-1, and adf9-1 seedlings

Three replicates of WT, *adf5-1,* and *adf9-1* seeds were surface-sterilized and sown on ½MS, 1% sucrose, and 0.8% agar media. Seeds were then stratified in the dark at 4°C for 48 hours and transferred to a growth chamber maintained at 22°C on a long-day photoperiod. 100 mg (approximately 40 individual seedlings of each genotype for each replicate) of shoot tissue was harvested at 10 days post-germination and frozen in liquid nitrogen. Tissue was collected and processed in parallel for the three biological replicates each of WT, *adf5-1,* and *adf9-1*.

Total RNA was extracted from the shoot tissue using the Spectrum Plant Total RNA kit (Sigma), and RNA quality was assessed using the Experion system (BioRad), according to the manufacturer's protocol. WT, *adf5-1,* and *adf9-1* total RNA was then used for reverse transcription. After second-strand synthesis, double-stranded cDNA was used in in vitro transcription to amplify homologous biotin-modified aRNA, according to the GeneChip® 3' IVT Express Kit User Manual provided by Affymetrix. Size of the aRNA and fragmented aRNA was assayed using the Experion system (BioRad). The fragmented aRNA was hybrized to the Affymetrix ATH1 GeneChips according to the manufacturer's protocol. The nine GeneChips were scanned into the Affymetrix GeneChip scanner 3000.

Affymetrix ATH1 GeneChip transcriptome analysis

Images from the scanned GeneChips were loaded into R and background corrected and normalized using the GeneChip Robust Multiarray Averaging (GCRMA) package (Team 2011, Wu *et al.*). The signal data were returned with Log_2 transformation to adjust for normalcy. The Log_2 transformations were then averaged over the three biological replicates for each sample using the formula: $\bar{A}$ (Adjusted Average) = $\sqrt{(A_1\ A_2\ A_3)}$ (Quackenbush 2002). A two-sample (equal variance) T-test between the adjusted average of the WT and *adf5-1* or WT and *adf9-1* data was performed and the P values were then used to determine a false discovery rate (FDR)

using the QVALUE package in R (Storey and Tibshirani 2003). The P value cut-off was determined for an FDR of 0.1, and genes were considered differentially expressed at $P \leq 0.004$ for *adf5-1* compared to WT and $P \leq 0.01$ for *adf9-1* compared to WT. qRT-PCR analysis was used to confirm approximately differential expression for approximately 50 genes in each mutant.

Venn diagrams were generated with the Venn Diagram Generator at http://www.pangloss.com/seidel/Protocols/venn.cgi and then re-drawn in PowerPoint. Gene Ontology (GO) biological process terms were downloaded from the TAIR database. The cluster analysis was performed in R, using the Cluster package with Ward's method for agglomerative hierarchical clustering (Maechler *et al.* 2005).

Subcellular localization of ADF5

We had previous success generating antibodies to Subclass I ADFs (Ruzicka *et al.* 2007). However, we were unsuccessful in making either a mouse monoclonal or a rabbit polyclonal antibody using either purified protein or synthetic peptide immunogens that were ADF5- and/or ADF9-specific yet still sensitive enough to detect the weakly expressed native protein in plant tissues. C-terminally FLAG and T7 epitope tagged ADF5 and internally tagged ADF5-GFP constructs following the design for yeast cofilin (Okreglak and Drubin 2007) produced reasonable transcript levels, but very poor protein expression *in planta*. Constructs tagging ADF5 with a C-terminally located yellow fluorescent protein (YFP) or biotin ligase recognition peptide (BLRP) were each expressed at sufficient levels to detect the fusion proteins in tissue using fluorescent microscopy (490 ex/520 em) or on Streptavidin-horse radish peroxidase (GE Healthcare) probed blots, respectively. The YFP fusion is an original design using a YFP sequence modified from pEarlyGate 104 (http://sites.bio.indiana.edu/~pikaardlab/) (Earley *et al.*

2006). The design of the BLRP fusion is modified from vectors previously reported by the Henikoff laboratory (Furuyama, 2006). We used an *Arabidopsis thaliana* (Col) plant line expressing biotin ligase (BirA) from *E. coli* supplied by the Henikoff laboratory in the biotin tagging experiments.

Results

adf5-1 mutant identification and description

We obtained an *ADF5* T-DNA insertion allele, *adf5-1*, in the *Arabidopsis thaliana* Columbia (Col) ecotype from ABRC (Salk_018325). The insertion was located 69 base pairs (bp) into the third exon of the ADF5 gene, and it deleted a total of 36 bases of coding sequence, as shown in **Figure 2.1A**. We assayed *ADF5* mRNA levels using quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) in wild-type (WT) Col, the *adf5-1* mutant, and a complementation line (*adf5-1*/A2pt:ADF5) relative to UBIQUITIN10 (UBQ10) mRNA levels. Levels of *ADF5* mRNA in both *adf5-1* and *adf5-1*/A2pt:ADF5 were normalized to WT. *ADF5* mRNA levels in *adf5-1* were reduced to 0.3% of WT levels (**Figure 2.1B**). We classified the *adf5-1* allele as a null allele because of the loss of exonic sequence, and the qRT-PCR data shows negligible transcript levels.

The *adf5-1* plants showed several morphological and developmental phenotypes compared to WT, including an increase in the numbers of rosette and cauline leaves, a decrease in leaf size, a change in leaf morphology, and a delay in flowering, as shown in **Figure 2.1 (C - E and M)**. WT plants bolted 24 days after germination with cotyledons and 13 true leaves when grown under long day conditions (16 h light, 8 h dark; see **Materials and Methods**). In contrast, at 24 days post-germination, *adf5-1* plants had cotyledons and 22 true leaves. They bolted with

approximately 25 leaves 5 days after WT. The *adf5-1* mutant also had an increased number of cauline leaves at the nodes of the primary inflorescence stem (**Figure 2.1F and G**). WT Arabidopsis primary inflorescence stems have a single cauline leaf at secondary branch junctions, while 67% of the nodes of the primary inflorescences of *adf5-1* plants had two or three cauline leaves at secondary branch junctions. Thus, the *adf5-1* mutant showed alterations in organ-number and organ initiation.

In addition to increased leaf number, trichome cells on the adaxial surface and stomatal cells on the abaxial surface showed an increased density on *adf5-1* leaves relative to WT ratios. To correct for potential differences in leaf-surface cell density caused by differences in leaf growth rates, we performed trichome and stomatal cell counts on mature leaves from 20 day-old plants. Epidermal pavement cells showed some variations in cell architecture compared to WT, though they were not different in average size or number (**Figure 2.1H**). Therefore, the density differences for trichomes and stomata is not because of differences in surrounding cell size.

Fully mature WT leaves had an average of 30.3 +/- 0.9 trichomes per $1cm^2$. In comparison, *adf5-1* leaves had an average of 67.0 +/- 5.0 trichomes per $1cm^2$ (**Figure 2.1N**). We took scanning electron-microscope images of WT and *adf5-1* leaf surfaces to determine if the trichome phenotypes also signaled changes in leaf epidermal and stomatal cell size, shape, or number. Mature WT leaves had an average of 17.8 +/- 0.7 stomata per 0.22 mm^2, but mature *adf5-1* leaves had an average of 26.5 +/- 1.3 stomata per 0.22 mm^2 (**Figure 2.1O**).

Genetic complementation and overexpression

We transformed a construct expressing *ADF5* cDNA from the strong, constitutive *ACTIN2* promoter-terminator cassette (A2pt:ADF5) into *adf5-1* and WT plants to genetically

complement the *adf5-1* allele and to test the effect of ADF5 overexpression. The *A2pt:ADF5* transgene produced high levels of *ADF5* transcripts, generally ranging from 35- to 90-fold above WT levels, as shown for one complemented *adf5-1* mutant line (**Figure 2.1B**). The *A2pt:ADF5* transgene quantitatively complemented all *adf5-1* morphological phenotypes, as shown in **Figures 2.1B, C, M, N, and O**. Rosette and cauline leaf size and number were indistinguishable from WT in the *adf5-1/A2pt:ADF5* complemented lines, and trichome and stomata numbers were restored to WT levels. Of note, the overexpression of *ADF5* did not produce any detectable dominant morphological phenotypes in relation to leaf number or any other obvious morphological phenotype in either the mutant (**Figure 2.1M, N, and O**) or the WT genetic backgrounds (not shown). Interestingly, over-expression of the other Subclass III member *ADF9*, the closest sequence homolog to *ADF5* in the Arabidopsis ADF family, in the *adf5-1* mutant background did not complement any of the above-described mutant phenotypes (data not shown).

adf5-1 mutants have slight changes to the morphology of the F-actin cytoskeleton

Since ADF5 represents such a small part of the ADF protein pool (approximately 2 to 5% of the total ADF transcript pool in various vegetative organs), we expected minimal changes in F-actin organization in *adf5-1*. To assay the organization of the actin cytoskeleton in the *adf5-1* mutant, we transformed WT and *adf5-1* plants with an F-actin reporter known to have minimal effects on the actin cytoskeleton (GFP:fABD2, see **Materials and Methods**) (Wang *et al.* 2008). We observed actin cytoskeletal morphology in both root cells and leaf trichomes, where ADF5 is normally expressed and GFP-labeled actin filaments are easily visualized, for several independent transformants of WT/*GFP:fABD2* and *adf5-1/GFP:fABD2* lines using confocal microscopy. In WT leaf trichome cells, the actin cytoskeleton is organized into a network of fine

filaments and bundles primarily arrayed longitudinally along the axis of cell growth (**Figure 2.1K**). In *adf5-1* leaf trichome cells, the general organization of filaments was indistinguishable from WT; however, we observed slightly thicker F-actin bundles and fewer fine filaments in several lines compared to WT (**Figure 2.1L**). We failed to detect changes in F-actin cytoskeletal organization in the root cells in *adf5-1* (data not shown). The slightly altered F-actin cytoskeleton in *adf5-1* leaf trichomes suggests that ADF5 has a minimal effect on F-actin cytoskeletal remodeling and organization, so we conclude that the loss of ADF5 results in only a minor increase in F-actin stability. These effects on the filamentous actin cytoskeleton were similar to effects we saw in *adf9-1* mutants (Burgos-Rivera *et al.* 2008).

Comparison of adf5-1 and adf9-1 mutant phenotypes

ADF5 and ADF9 are the sole members of Subclass III ADFs in Arabidopsis, so we were surprised to find that the *adf5-1* and *adf9-1* mutants were phenotypic opposites for a number of morphological traits. For instance, in mass of seedlings, rosette leaf morphology, and flowering time, *adf5-1* mutant seedlings between 10 and 12 days post-germination are slightly larger than WT, but *adf9-1* seedlings at the same age are smaller than WT (**Table 2.1**). As mentioned above, the *adf5-1* mutant flowers later than WT when using rosette leaf number as a proxy for flowering time (25 vs. 13 leaves, respectively). When considered as days post-germination to flowering, *adf5-1* flowers slightly later than WT (29 days for *adf5-1* vs. 25 days for WT). In contrast, *adf9-1* mutants flower significantly earlier than WT when using either rosette leaf number (7 for *adf9-1* vs. 12 for WT) or days post-germination to flowering (22.5 days for *adf9-1* vs. 27.5 for WT). Additionally, *adf5-1* mutants produce epinastic rosette leaves with short petioles, so the rosette is dense and lays flat on the soil (**Figure 2.1C**). But, *adf9-1* mutants

produce hyponastic rosette leaves with long petioles, so the rosette is sparse and bends away from the soil.

Because *adf5-1* and *adf9-1* are morphological opposites in flowering time, we determined the relative quantities of selected genes in the flowering pathway in the *adf5-1* mutant, some of which we had previously published data for *adf9-1* (**Figure 2.2B**) (Burgos-Rivera *et al.* 2008). **Table 2.1** summarizes the gene expression phenotypes for *adf5-1* and *adf9-1* mutants. They have opposite phenotypes for FLOWERING LOCUS C (FLC) (up in *ad5-1* vs. down in *adf9-1*). So, their molecular phenotypes for the known central regulator of flowering time is also opposite. Since FLC is up-stream of the floral integrator gene, FT, the change in expression of FLC is sufficient to explain the change in flowering time we observed in *adf5-1* and *adf9-1* (Michaels and Amasino 2001). These data suggest that ADF5 and ADF9 affect gene transcription by an unknown mechanism. Also, ADF9 expression was up-regulated in an *adf5-1* mutant (**Figure 2.2A**), while ADF5 expression was down-regulated in an *adf9-1* mutant, which suggests a feedback loop between ADF5 and ADF9.

Transcriptome analysis of adf5-1 and adf9-1

ADF5 and *ADF9* alter gene expression of floral pathway genes and they do so in an antagonistic way. To determine if the gene expression changes in *adf5-1* and *adf9-1* are specific to flowering time and if the changes are usually antagonistic, we performed a microarray analysis on *adf5-1* and *adf9-1* and compared their transcriptomes to WT. Three biological replicates each for WT, *adf5-1*, and *adf9-1* seedling shoots were taken at 10 days post-germination because at this stage the seedlings are indistinguishable from one another (**Figure 2.3A)**, and because the plants have yet to begin bolting. Genes were determined as differentially expressed in the mutants by comparison with WT (see **Materials and Methods**). For

visualization, we determined that our appropriate comparison is genes expressed in the same tissue and at the same developmental stage as our mutant samples: 10-day-old seedlings are approximately between the 2 to 4 leaf visible stages (LP.02-LP.04) (developmental stage), and we sampled only seedling shoots (tissue). Therefore, our comparison is between genes differentially expressed in the mutants and genes expressed in the shoot system at LP.02-LP.04 (WT gene set).

Of the 11,667 genes expressed in the WT gene set, 786 are differentially expressed in *adf5-1* (6.7%) (**Figure 2.3B**) and 1350 are differentially expressed in *adf9-1* (11.6%) (**Figure 2.3E**). 268 of those genes differentially expressed in *adf5-1* are expressed in a range of tissues that we normally associate with the shoot system but were not specifically annotated in the Plant Ontology (PO) database at TAIR (www.arabidopsis.org) as shoot system genes (**Figure 2.3C**). The same holds true for 278 of those genes differentially expressed in *adf9-1* (**Figure 2.3F**). However, approximately 8% of the 268 ectopically expressed genes in *adf5-1* are annotated to the root system, 1% to cultured plant cells, and 5% to seeds (**Figure 2.3C**). Approximately 8% of the 278 ectopically expressed genes in *adf9-1* are also annotated to the root system, yet 4% are annotated to cultured plant cells, 0.4% to callus, and 4% to seeds (**Figure 2.3E**).

Most of the genes ectopically expressed in *adf5-1* and *adf9-1* are annotated to developmentally relevant stages (**Figures 2.3D** and **G)**, but 6% and 8% are specific to pollen development in *adf5-1* and *adf9-1*, respectively. Finding genes specific to pollen development is unexpected, especially in the case of *adf9-1*, because in promoter:GUS fusions of Subclass III ADF proteins, expression was absent in pollen (Ruzicka *et al.* 2007).

Because ADF5 and ADF9 are the closest paralogs in the ADF gene family in Arabidopsis, we expected wide overlap in the number and types of genes changed in *adf5-1* and *adf9-1* mutants; however, we found that only 152 genes were shared between those differentially expressed in *adf5-1* and *adf9-1* (**Figure 2.3H**).

Gene ontology of differentially expressed genes

Next, we addressed if the gene expression changes we saw in *adf5-1* and *adf9-1*were in specific biological processes. Because the morphological changes we observe are in developmental processes, for instance, leaf shape and size and flowering time, we expected to see that most of the genes changed were developmental process genes. To do this, we classified the WT gene set and the genes differentially expressed in *adf5-1* and *adf9-1* according to the biological process category of the Gene Ontology (GO) terms in the TAIR database. We summarize the GO data for *adf5-1* and *adf9-1* and shared genes in **Table 2.2**, **Table 2.3**, and **Table 2.4**, respectively.

Genes can be assigned to more than one GO category, so we determined the proportion of genes annotated to each GO biological process category per genotype by dividing the genes annotated to each category by the total number of GO biological process annotations. **Figure 2.4** shows these proportions for the WT gene set, *adf5-1*, *adf9-1*, and shared genes. Enrichment for genes in a particular process category is not apparent based on **Figure 2.4**, but using the GO::TermFinder tool (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) (Boyle *et al.* 2004) with default settings to analyze our data (using the WT gene set as our background) and performing a Bonferroni correction on the p-values, we found that genes associated with the GO biological process categories of stress response (GO:0006950, p = 2.37E-04), S-glycoside

biosynthetic process (GO:001614, p = 1.03E-03), and indole-containing compound biosynthetic process (GO:0042435, p = 4.84E-03) are significantly enriched in *adf9-1*. No GO biological process categories are enriched in *adf5-1* or those genes shared between *adf5-1* and *adf9-1*.

GO::TermFinder found, however, that *adf5-1* is significantly enriched in the GO molecular function terms hydrogen ion transmembrane transporter activity (GO:0015078, p = 2.54E-02) and oxygen binding (GO:0019825, p = 3.52E-02), but *adf9-1* is significantly enriched in the molecular function terms anthranilate synthase activity (GO:0004049,p = 4.11E-03), and oxo-acid lyase activity (GO:0016833, p = 2.10E-02). Once again, no GO molecular function terms are enriched in genes shared between *adf5-1* and *adf9-1*.

The differences in GO term enrichment we observe between *adf5-1* and *adf9-1* could account for some of the differences we observe in morphological phenotypes between the mutants; however, it does not account for the antagonistic developmental phenotypes we see, such as flowering time and leaf morphology. No GO terms in biological process, molecular function, or cellular component are enriched in the 152 differentially expressed genes shared between *adf5-1* and *adf9-1*, which suggests that Subclass III ADFs change gene expression across a broad range of processes or functions.

Cluster analysis of differentially expressed genes shared between adf5-1 and adf9-1

Since the differentially expressed genes shared between *adf5-1* and *adf9-1* have no GO term enrichments, we performed a cluster analysis in R on the 152 genes in this set to gain some insight into how ADF5 and ADF9 affect gene transcription. Genes that share a similar function cluster together in genome-wide studies (Eisen *et al.* 1998). The analysis was performed with reference to the p-value of the differentially expressed genes and the ratios of the log

transformed florescence values in the mutants to WT in both *adf5-1* and *adf9-1*. **Figure 2.5** shows that with an arbitrary cut-off of 10 (chosen to reduce the number of clusters to analyze but still enough clusters to give some meaningful information), the 152 differentially expressed genes shared between *adf5-1* and *adf9-1* fall into 8 clusters, as indicated by the colored bars to the left of the dendrogram. The number of genes in each cluster is shown in parentheses beside the colored bars.

We used Promomer (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi) (Toufighi *et al.* 2005) to analyze the promoters (defined as 1000-bp upstream of the start of transcription) of the genes in each cluster to identify over-represented promoter motifs. We looked for 6-mer "words" represented in at least 90% of the genes in a cluster. We summarize these data in **Table 2.5**. Clusters 4 and 6 have no over-represented 6-mers that meet the criteria. Cluster 3 has no 6-mer element that matches a characterized Arabidopsis promoter motif, Clusters 1 and 8 have only one. Clusters 2 (which contains FLC), 5, and 7 share many AT-rich 6-mer elements that match the characterized promoter motifs AGAMOUSATCONSENSUS (AGAMOUS (AG) MADS domain site), AGATCONSENSUS (AG binding site that also contains a CArG box), AGL1ATCONSENSUS (AGAMOUS-like 1 (AGL1) binding site), AGL2ATCONSENSUS (AGL2 binding site), AGL3ATCONSENSUS (AGL3 binding site), CARGATCONSENSUS (CArG box), CARGCW8GAT (canonical CArG box, AGL15 binding site), and CARGNCAT (noncanonical CArG box, AGL15 binding site).

Subclass III ADF proteins are distributed to both the cytoplasm and the nucleus

The subcellular localization of ADF5 and ADF9 has a bearing on the models and mechanisms that we could propose to test their role in regulating gene expression. Considering

the spectrum of activities described for plant and animal ADF/cofilin (Bernstein and Bamburg 2010), Subclass III ADF proteins may control gene expression from the cytoplasm and/or from the nucleus and/or via nuclear transport. Using ADF subclass-specific monoclonal antibodies we previously demonstrated that Arabidopsis subclass I ADFs are distributed in the cytoplasm and the nucleus, yet subclass II ADFs are restricted to the cytoplasm (Ruzicka, Kandasamy, McKinney, Burgos-Rivera and Meagher 2007). Tholl *et al.* show that ADF9-GFP biolistically transformed into tobacco cells localizes to both actin filaments and the nucleus (Tholl *et al.* 2011). However, the subcellular distribution of the remaining Subclass III member, ADF5, has yet to be reported.

We examined two C-terminally tagged versions of ADF5 from several independently transformed plants (**see Materials and Methods**). Of the 13 lines expressing ADF5 tagged with yellow fluorescent protein (ADF5-YFP) we examined by fluorescent microscopy, 12 plant lines showed fluorescent fusion protein levels considerably above background fluorescence detected in WT. The root hairs and trichomes from typical plants expressing moderate levels of ADF5-YFP are shown in **Figure 2.6.** In these images and in all 12 lines fluorescent lines, ADF5-YFP fluorescence was distributed to both the cytoplasm and nucleus, but fluorescence was more concentrated in the nucleus than in the cytoplasm. GFP and its derivatives like YFP distribute on their own to the nucleus to some extent, possibly by passive diffusion, but the nuclear/cytoplasmic ratio for GFP-derivatives varies among constructs and target cell types. Nuclear levels are higher when fluorescent protein derivatives are highly overexpressed. That we observed nuclear staining in different plant lines and tissues with both low and high expression levels suggests that ADF5 rather than YFP is the driving factor for nuclear staining.

We further examined the nuclear localization of ADF5 with an alternative tagging technique, fusing the biotin ligase recognition peptide sequence (BLRP) to ADF5 and expressing this construct in a transgenic plant line producing *E. coli* biotin ligase (BirA). We then prepared cytoplasmic and nuclear protein fractions from these lines using formalin fixed tissues to prevent the redistribution of cytoplasmic ADF to the nucleus during preparation. We resolved the protein preparations by SDS-PAGE and examined membrane imprints with a Streptavidin reporter. ADF5 is found in both the nuclear and cytoplasmic fractions, as shown in **Figure 2.6B**.

Discussion

Like ADF9, ADF5 affects developmental pathways

ADF5 and ADF9 are the sole members of Subclass III ADF in Arabidopsis. We previously described the function for ADF9 (Burgos-Rivera *et al.* 2008). *adf9-1* mutants are smaller and flower earlier than WT, and they are down-regulated in the master regulator of flowering, Flowering Locus C (*FLC*) (Burgos-Rivera *et al.* 2008). The function of the second member of Subclass III, ADF5, has yet to be described. Because ADF5 and ADF9 are the closest homologs in Arabidopsis, we would expect a very similar phenotype, yet are only 80% identical in amino acid sequence (28 changes/143 amino acid residues).

Here we describe the phenotype of an ADF5 T-DNA insertion mutant, *adf5-1*, and compare it to the ADF9 T-DNA insertion mutant, *adf9-1*. The *adf5-1* mutant showed alterations in organ-number and organ initiation: *adf5-1* plants had several morphological and developmental phenotypes, including an increase in the numbers of rosette (25 at bolting vs. 13 for WT) and cauline leaves, a decrease in leaf size, and a change in leaf morphology. Because *adf5-1* produces more rosette leaves at bolting (and bolts slightly later than WT), one could argue

that *adf5-1* flowers late; whereas, *adf9-1* flowers early, with reduced expression of *FLC*. We show that *adf5-1* has increased expression of *FLC,* suggesting that ADF5, like ADF9, affects gene expression. To explore Subclass III's effects on gene expression we analyzed the transcriptomes of WT, *adf5-1,* and *adf9-1* in 10-day-old shoot tissue, and we made comparisons between the two.

Transcriptome analysis of adf5-1 and adf9-1

We found that 6.7% of genes in the control data-set (genes expressed in WT shoot tissue at a developmental stage when 2 and 4 leaves have been produced) were differentially expressed in *adf5-1* and 11.6% of genes were differentially expressed in *adf9-1*. So, approximately 7%-12% of the WT genes are differentially expressed in *adf5-1* and *adf9-1*, which is a substantial proportion of the WT gene set in this tissue and at this developmental stage. Because ADF5 and ADF9 are the closest paralogs in the ADF gene family in Arabidopsis and because the mutants show changes in what we presume are similar developmental pathways (flowering time and leaf morphology, for instance), we expected wide overlap in the number and types of genes changed in *adf5-1* and *adf9-1* mutants; however, we found that only 152 genes were shared between those differentially expressed in *adf5-1* and *adf9-1*.

Gene ontology of differentially expressed genes

To help make sense of the data on differentially expressed genes, we began looking for patterns in the types and numbers of differentially expressed genes in *adf5-1, adf9-1*, and those genes shared between them. We first classified the WT gene set and the genes differentially expressed in *adf5-1*, *adf9-1,* and shared genes according to the biological process category of the Gene Ontology (GO) terms in the TAIR database. Using the GO::TermFinder tool, we found

that the genes differentially expressed in *adf5-1* and the genes shared between *adf5-1* and *adf9-1* were not enriched in a particular GO biological process. However, the genes differentially expressed in *adf9-1* were enriched in the stress response, S-glycoside biosynthetic process, and the indole-containing compound biosynthetic process. 15% of the genes differentially expressed in *adf9-1* are involved in the stress response, including two MADS-box transcription factors, FLC and AGAMOUS-like 24 (AGL24).

1% of the genes differentially expressed in *adf9-1* are involved in the S-glycoside biosynthetic process. Glycosides are molecules with a sugar group bound through its anomeric carbon to some other non-carbon group via a glycosidic bond. Glycosides perform important functions in many organisms, but in plants, glycosides are involved in, for instance, host-pathogen interactions and pollen development (Hrmova and Fincher 2007). Similarly, 1% of the genes differentially expressed in *adf9-1* are involved in indole-containing compound biosynthesis impinges upon indole-3-acetic acid *(*IAA*)* or auxin synthesis. IAA is the most potent endogenous auxin, an important plant hormone with many effects, most notably plant growth and development in response to its environment (Zhao 2010). The common theme of these biological processes is the plant's developmental response to its environment, which is intriguing given the *adf9-1* phenotype. But we are left wondering why the genes differentially expressed in *adf5-1* and the genes shared between *adf5-1* and *adf9-1* are not also enriched in these processes.

The differences in GO term enrichment we observe between *adf5-1* and *adf9-1* could account for some of the differences we observe in morphological phenotypes between the mutants; however, it does not account for the antagonistic developmental phenotypes we see, such as flowering time and leaf morphology. No GO terms in biological process, molecular

function, or cellular component are enriched in the 152 differentially expressed genes shared between *adf5-1* and *adf9-1*, which suggests that Subclass III ADFs change gene expression in different pathways across a broad range of processes or functions. The most reasonable mechanism for Subclass III ADF effects on gene expression is through its effects on actin cytoskeletal dynamics, so how the effects are modulated to specific pathways is unclear.

Cluster analysis of differentially expressed genes shared between adf5-1 and adf9-1

To attempt to determine possible targets for future genetic studies on genetic pathways affected in adf5-1 and adf9-1, we performed a cluster analysis to determine if genes with similar function would cluster together. We used an agglomerative hierarchical clustering method to group the genes and then chose an arbitrary threshold of 10. These criteria gave eight clusters, and we performed promoter motif analyses on the genes in each cluster. We found that three clusters: Clusters 2, 5, and 7 share many AT-rich 6-mer elements that match the characterized promoter motifs AGAMOUSATCONSENSUS (AGAMOUS (AG) MADS domain site), AGATCONSENSUS (AG binding site that also contains a CArG box), AGL1ATCONSENSUS (AGAMOUS-like 1 (AGL1) binding site), AGL2ATCONSENSUS (AGL2 binding site), AGL3ATCONSENSUS (AGL3 binding site), CARGATCONSENSUS (CArG box), CARGCW8GAT (canonical CArG box, AGL15 binding site), and CARGNCAT (noncanonical CArG box, AGL15 binding site). These data suggest that the genes affected in *adf5-1* and *adf9-1* are regulated by several AG and AGL transcription factors, which make good targets for further genetic studies on the effects of ADF proteins on gene expression.

Subclass III ADF proteins are distributed to both the cytoplasm and the nucleus

Several groups have shown in animal systems that ADF/Cofilin proteins control gene expression by several mechanisms (Zheng *et al.* 2009). One of the possible mechanisms is by ADF/Cofilin binding to actin and carrying it into the nucleus. Therefore, the subcellular localization of Subclass III is important to know; if Subclass III were restricted to the cytoplasm, then carrying actin to the nucleus would be an unlikely mechanism for Subclass III ADF's function in the nucleus. Using two different methods, an ADF5-YFP reporter to visualize under the microscope and an ADF5-BLRP tagged version to show on a streptavidin blot, we show that ADF5 localizes in both the cytoplasm and the nucleus, just as ADF9 has been reported to do (Tholl, Moreau, Hoffmann, Arumugam, Dieterle, Moes, Neumann, Steinmetz and Thomas 2011). This subcellular localization pattern fails to exclude one hypothesis for the mechanism of Subclass III ADF's control of gene expression.

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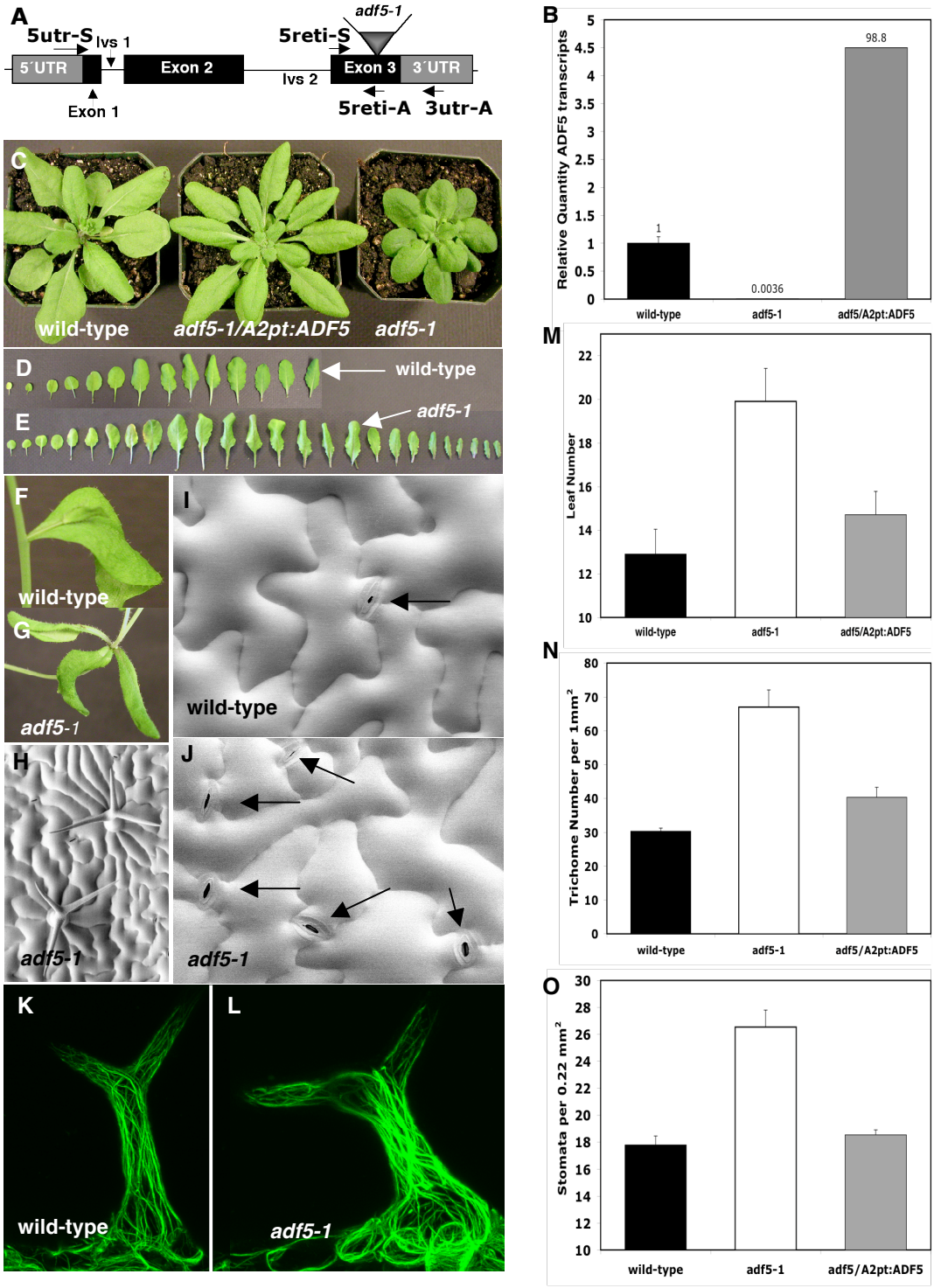
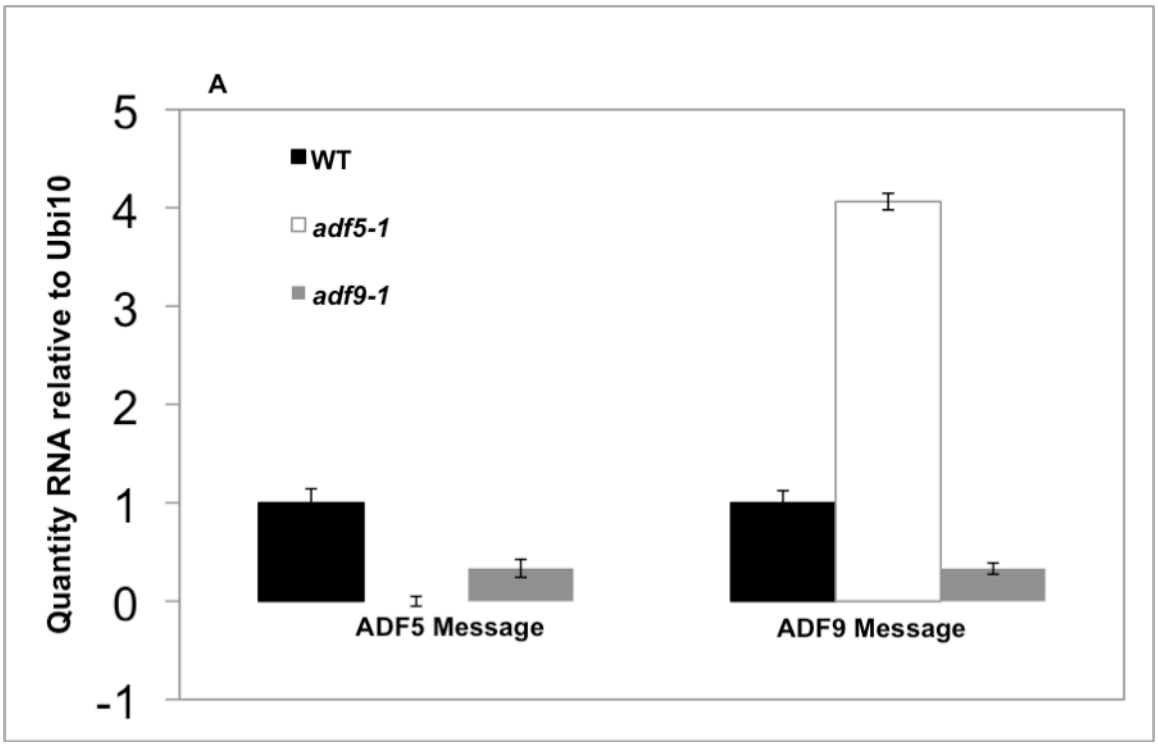

A
5utr-S
Ivs 1
5´UTR
Exon 2
Exon 1
adf5-1
5reti-S
Ivs 2
Exon 3
3´UTR
5reti-A
3utr-A
B
Relative Quantity ADF5 transcripts
98.8
1
0.0036
wild-type
adf5-1
adf5/A2pt:ADF5
C
wild-type
adf5-1/A2pt:ADF5
adf5-1
D
wild-type
E
adf5-1
F
wild-type
G
adf5-1
I
wild-type
H
adf5-1
J
adf5-1
K
wild-type
L
adf5-1
M
Leaf Number
N
Trichome Number per 1mm²
O
Stomata per 0.22 mm²

Figure 2.1: The *adf5-1* mutant allele exhibits multiple developmental phenotypes. The *adf5-1* mutant allele has an insertion in the 3rd exon (A). *ADF5* expression levels in wild-type, *adf5-1* mutant, and *A2pt:ADF5* over-expression lines were quantified by qRT-PCR (B). Rosette leaf number and morphology are significantly altered in the *adf5-1* mutant (C to E). The *adf5-1* mutant also exhibits an increase in cauline leaf number (F, G). SEM images of the leaf surface show an increase in the number of stomata and trichomes (as indicated by the arrows), but not a change in the number or size of epidermal pavement cells (H to J). The GFP/fABD2 F-actin reporter decorates actin filaments and bundles. Confocal microscopy images of mutant and wild-type trichome cells show the *adf5-1* cytoskeleton exhibits thicker bundles and fewer fine filaments indicating a more stabilized, less dynamically remodeled actin cytoskeleton phenotype (K, L). Leaf and cell phenotypes are quantified in M to O.


A
Quantity RNA relative to Ubi10
5
4
3
2
1
0
-1
WT
adf5-1
adf9-1
ADF5 Message
ADF9 Message

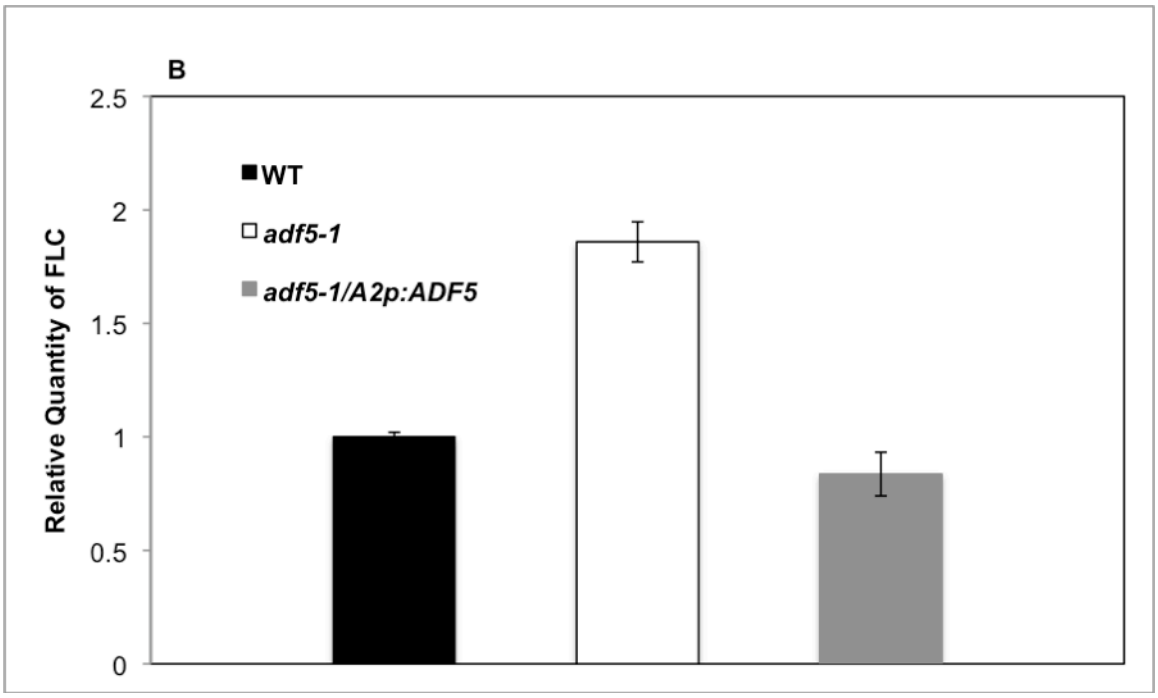

B
Relative Quantity of FLC
2.5
2
1.5
1
0.5
0
WT
adf5-1
adf5-1/A2p:ADF5

Figure 2.2: ADF5 changes gene expression of genes in the flowering pathway and its closest homolog in Arabidopsis, ADF9. WT, *adf5-1* and *adf5-1*/A2pt:ADF5 seeds were plated on ½ MS media supplemented with 1% sucrose and allowed to grow for 10 days. Samples were taken 3 hours after the beginning of the photoperiod. qRT-PCR analysis was performed with reference to UBQ10 and normalized to WT. **A)** ADF9 expression is up in an *adf5-1* mutant and ADF5 expression is down in an *adf9-1* mutant. **B)** FLC expression is up-regulated in the *adf5-1* mutant but restored to WT levels in the complementation line.

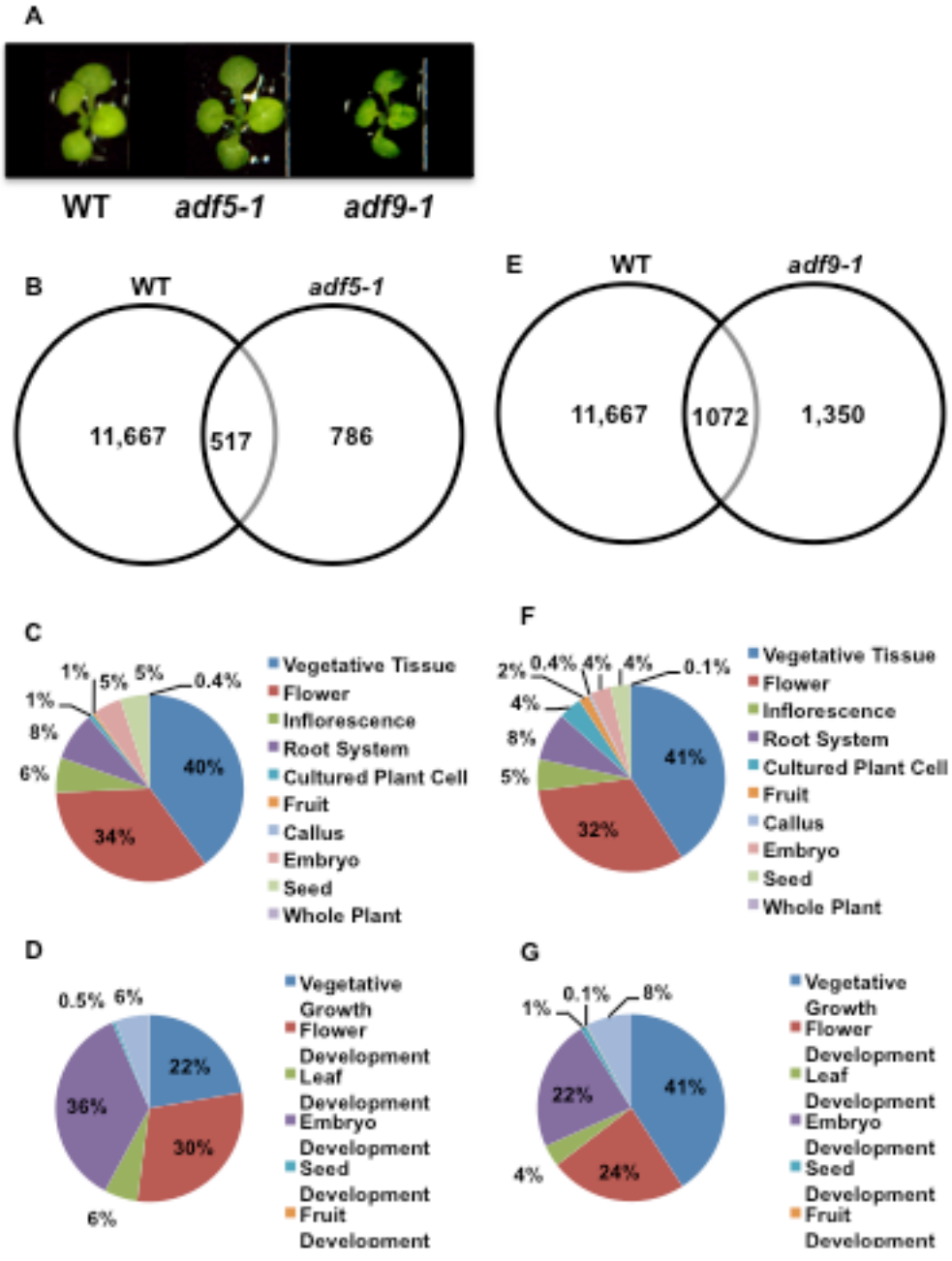

A
WT
adf5-1
adf9-1
B
WT
adf5-1
11,667
517
786
E
WT
adf9-1
11,667
1072
1,350
C
1%
5%
5%
0.4%
1%
8%
6%
40%
34%
Vegetative Tissue
Flower
Inflorescence
Root System
Cultured Plant Cell
Fruit
Callus
Embryo
Seed
Whole Plant
F
2%
0.4%
4%
4%
0.1%
4%
8%
5%
41%
32%
Vegetative Tissue
Flower
Inflorescence
Root System
Cultured Plant Cell
Fruit
Callus
Embryo
Seed
Whole Plant
D
0.5%
6%
22%
36%
30%
6%
Vegetative Growth
Flower Development
Leaf Development
Embryo Development
Seed Development
Fruit Development
G
1%
0.1%
8%
41%
22%
4%
24%
Vegetative Growth
Flower Development
Leaf Development
Embryo Development
Seed Development
Fruit Development

Figure 2.3: Approximately 7% or 12% of the genes expressed in the WT gene set are changed in *adf5-1* and *adf9-1* respectively*;* however, only 20%-12% of these genes are shared between them. A) Three biological replicates of approximately 40 10-day-old (between the 2 and 4 leaf stages according to TAIR classification) seedling shoots for WT, *adf5-1*, and *adf9-1* were collected for the microarray analysis. The photograph shows examples of 10-day old seedling shoots for each genotype. **B)** Approximately 24,000 loci are represented on the Affymetrix ATH1 Genechip; however, we base our comparisons on genes expressed in the WT shoot system between the 2 and 4 leaf visible stages (LP.02-LP.04). Of the 786 loci mis-regulated in *adf5-1*, 518 genes are expressed in WT. **C)** The 268 genes changed in *adf5-1* that are not classified as shoot system genes or LP.02-LP.04 genes are ectopically expressed in the tissues and **D)** developmental stages shown. **E)** Of the 1,350 loci mis-regulated in *adf9-1*, 1,072 are expressed in WT. **F)** The 278 genes changed in *adf9-1* that are not classified as shoot system genes or LP.02-LP.04 genes are ectopically expressed in the tissues and **G)** developmental stages shown. **H)** Only 152 genes of those changed in *adf5-1* and *adf9-1* are shared between them.

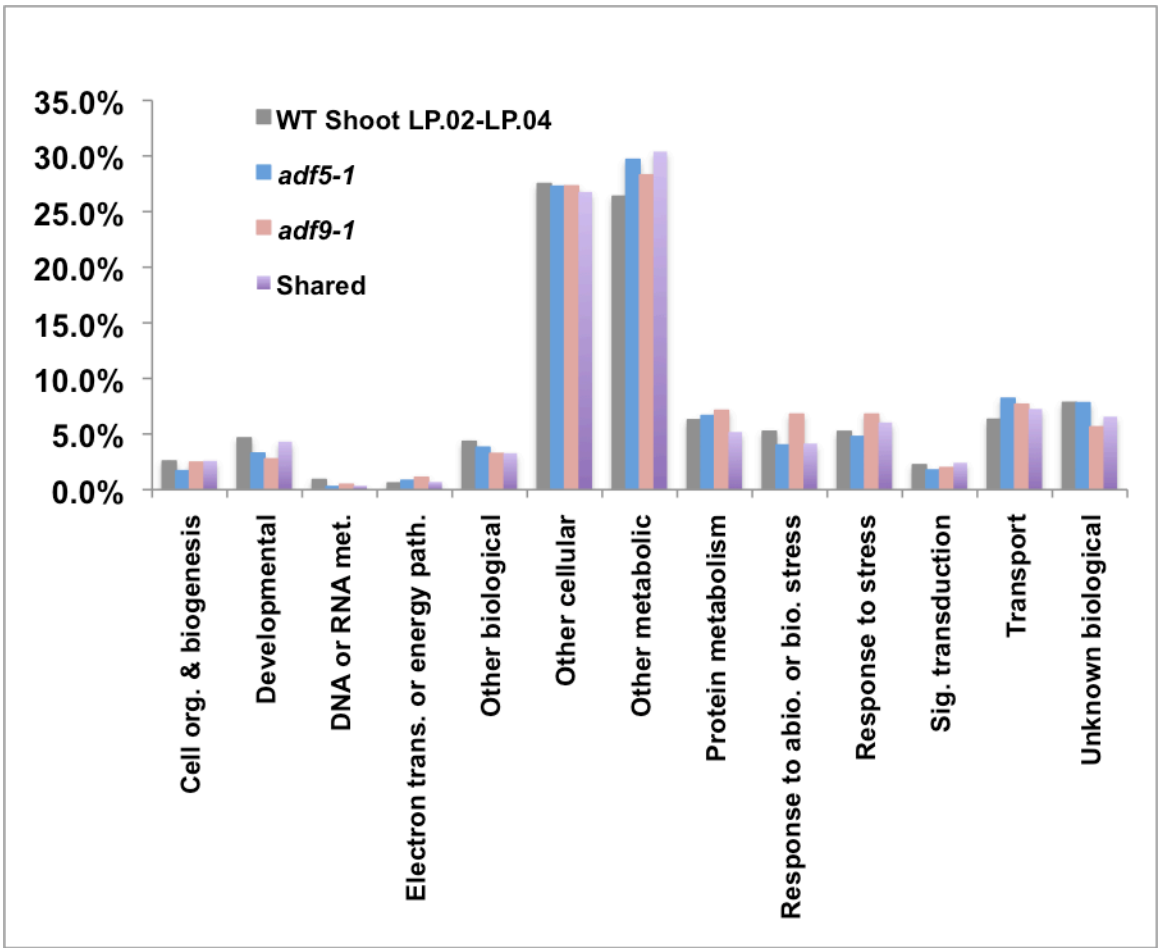


Figure 2.4: Comparison of the proportion of genes in each GO biological process category for genes expressed in WT and differentially expressed in *adf5-1*, *adf9-1*, and genes shared between *adf5-1* and *adf9-1*. GO biological process annotations were downloaded for genes expressed in WT shoot system at LP.02-LP.04 and the genes differentially expressed in *adf5-1* and *adf9-1*. Genes can be assigned to more than one category, so the proportion of genes in each category is determined separately for each genotype by the following formula: genes in a sample annotated to each GO category/total number of GO annotations for that sample.

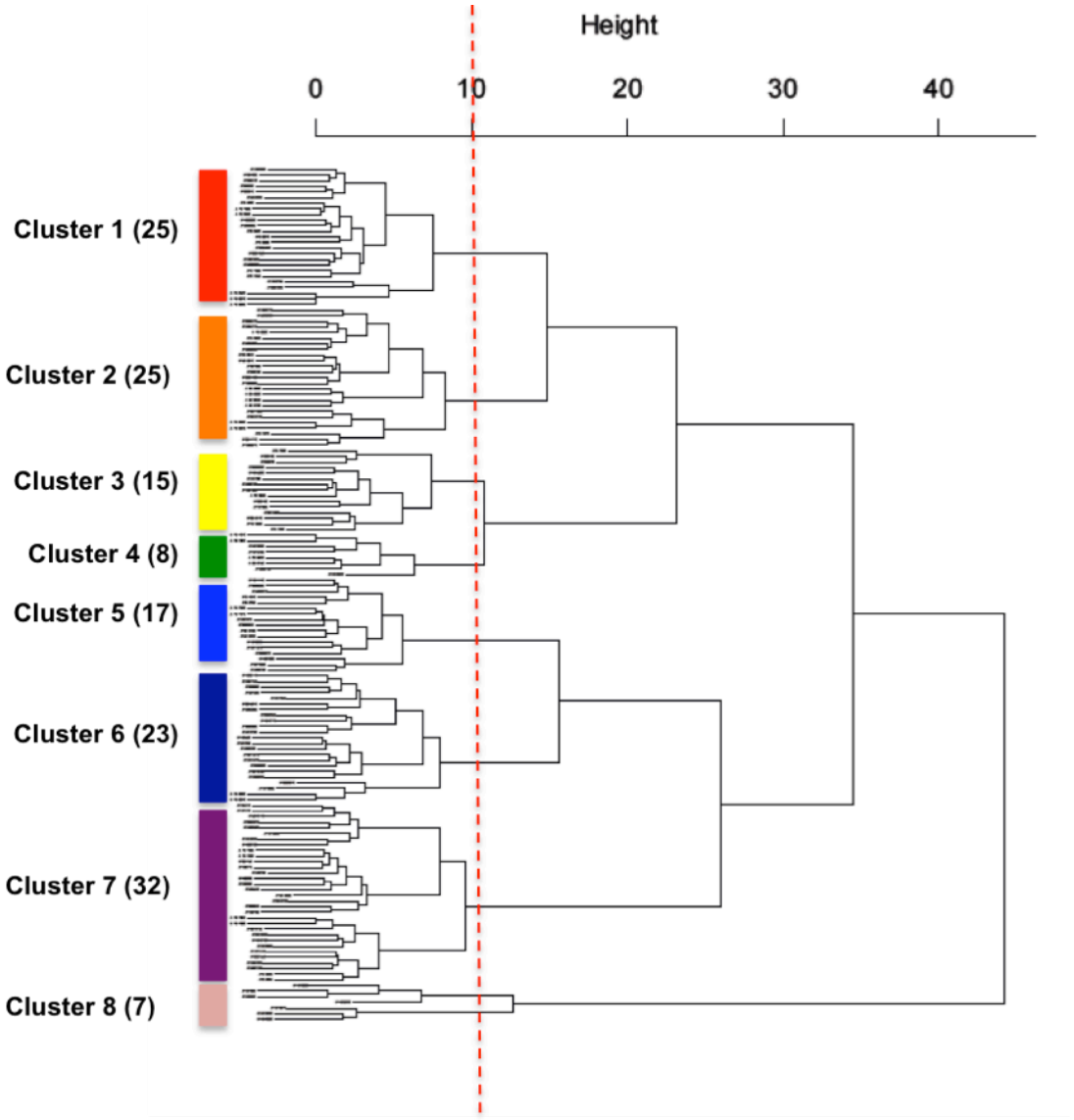

Height
0
10
20
30
40
Cluster 1 (25)
Cluster 2 (25)
Cluster 3 (15)
Cluster 4 (8)
Cluster 5 (17)
Cluster 6 (23)
Cluster 7 (32)
Cluster 8 (7)

Figure 2.5: Cluster analysis of differentially expressed genes shared between *adf5-1* and *adf9-1*. The analysis was performed in R using agnes in the Cluster package (agglomerative clustering). The agglomerative coefficient was 0.97. Clusters were determined by P-value and the ratio of the log transformed values of the mutant to WT. With an arbitrary cut-off of 10, the genes fall into 8 different clusters, indicated by the colored bars to the left. The number of genes in each cluster is shown in parentheses.

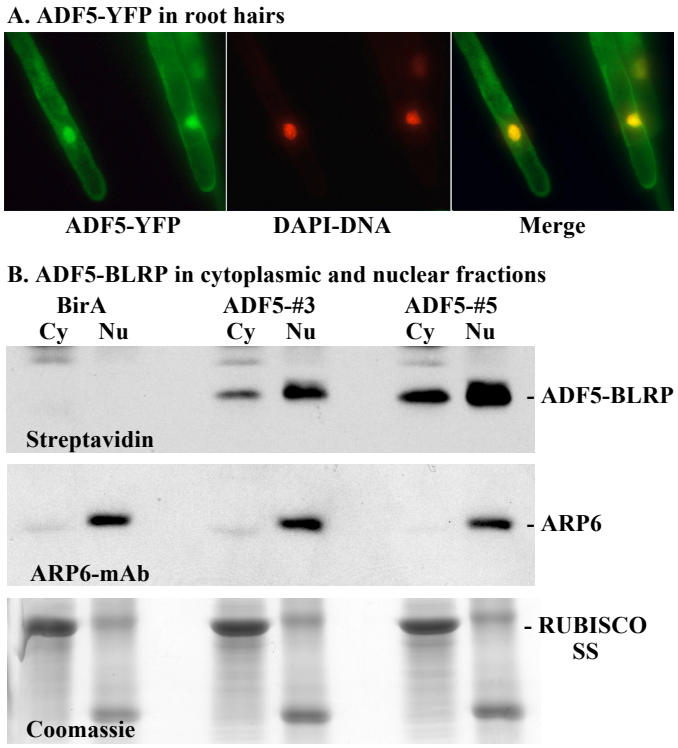


Figure 2.6: ADF5 localizes to both the cytoplasm and the nucleus. A) C-terminally tagged ADF5-YFP was transformed into WT(Col) visualized using fluorescent microscopy. 13 independent insertion lines were examined, and we show a representative picture here. ADF5-YFP fluorescence was distributed to both the cytoplasm and nucleus, but fluorescence was more concentrated in the nucleus than in the cytoplasm. **B)** ADF5-BLRP was transformed into a transgenic plant line expressing *E. coli* biotin ligase (BirA). We prepared cytoplasmic and nuclear protein fractions from these lines using formalin fixation and resolved the protein preparations by SDS-PAGE. We examined membrane imprints with a Streptavidin reporter. ADF5 is found in both the nuclear and cytoplasmic fractions.

Table 2.1: Comparison between *adf5-1* and *adf9-1* phenotypes.

	adf5-1	***adf9-1***
Morphology (cf WT)		
Fresh weight at 10-days post-germination (mg)		4±1.5[1]
Size of 12-day-old seedlings		0.5[1]
Number of rosette leaves at flowering	20±2	7±1[1]
Number of lateral branches off primary inflorescence		Decreased[1]
Length of lateral branches off primary inflorescence		Increased[1]
Leaf morphology	Short petioles	Long petioles[1]
Number of stomata	Increased	
Number of trichomes	Increased	
Gene Expression (cf WT)		
ADF5	0.1	0.25[1]
ADF9	4	0.25[1]
Flowering Time Genes		
CO	1	1.5[1]
FLC	2	0.25[1]
FT	0.4	1.75[1]
SOC1	1	2.25[1]
LFY		

The morphological and gene expression phenotypes of the adf5-1 and adf9-1 mutants known before the transcript analysis is summarized here. [1]The *adf9-1* phenotypes are taken from a previous publication (Burgos-Rivera *et al.* 2008).

Table 2.2: Summary of GO biological process terms for differentially expressed genes in *adf5-1* (786 genes).

Cell organization and biogenesis	45
Developmental processes	62
DNA or RNA metabolism	7
Electron transport	15
Others (biological, cellular, metabolic)	1353
Protein metabolism	155
Response to abiotic or biotic stress	92
Response to stress	109
Signal transduction	46
Transport	180
Unknown biological processes	133
Total annotations	2197

Each gene differentially expressed in *adf5-1* was categorized according to GO biological process, as determined from information in the TAIR database. Most genes are annotated to multiple process groups, so each category contains the number of differentially expressed genes annotated to that category.

Table 2.3: Summary of GO biological process terms for differentially expressed genes in *adf9-1* (1350 genes).

Cell organization and biogenesis	116
Developmental processes	130
DNA or RNA metabolism	22
Electron transport	52
Others (biological, cellular, metabolic)	2778
Protein metabolism	336
Response to abiotic or biotic stress	247
Response to stress	320
Signal transduction	93
Transport	362
Unknown biological processes	266
Total annotations	4722

Each gene differentially expressed in *adf9-1* was categorized according to GO biological process, as determined from information in the TAIR database. Most genes are annotated to multiple process groups, so each category contains the number of differentially expressed genes annotated to that category.

Table 2.4: Summary of GO biological process terms for differentially expressed genes shared between *adf5-1* and *adf9-1* (152 genes).

Cell organization and biogenesis	15
Developmental processes	25
DNA or RNA metabolism	2
Electron transport	4
Others (biological, cellular, metabolic)	350
Protein metabolism	30
Response to abiotic or biotic stress	24
Response to stress	35
Signal transduction	14
Transport	42
Unknown biological processes	38
Total annotations	579

Each differentially expressed gene shared between *adf5-1* and *adf9-1* was categorized according to GO biological process, as determined from information in the TAIR database. Most genes are annotated to multiple process groups, so each category contains the number of differentially expressed genes annotated to that category.

Table 2.5: Over-represented 6-mers in the 8 clusters identified in differentially expressed genes shared between *adf5-1* and *adf9-1*

Cluster	# of Genes	Genes in the Cluster	Over-represented 6-mers (Z-score, P value)	Specific Arabidopsis consensus matches to 6-mer
1	25	AT1G02260, AT2G16380, HHP3, AT2G20360, CYP96A1, AT5G10620, AT1G52100, ROP3, EMB2076, AT4G29890, AT5G06265, AT4G31530, AT2G20270, VHA-A3, AT2G05830, LAX2, MSRB2, AT5G02800, SMAP1, AT5G16250, EXPA10, AT5G02630, RUS4, AT4G25710, AT4G27390	AAAAGAA (2.3, 0.05) AACAAAA (2.2, 0.05)	N/A CGF1ATCAB2
2	25	MRP10, CBB3, AT5G08000, AT2G16940, ACT7, AHA1, AT3G01860, AT2G24420, AT4G08280, RPS4A, FAD2, AT5G38930, AT5G38940, AT2G17670, AT2G44770, AT4G38810, AT1G42705, AT3G04000, AT3G61520, AT5G28370, AT5G28460, AT1G54120, AT1G74710, FLC, AT2G26970	AAATTT (2.4, 0.01) AAAAAT (2.0, 0.05)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS, AGL3ATCONSENSUS, CARGATCONSENSUS AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS, AGL3ATCONSENSUS, CARGATCONSENSUS,

Table 2.5: Over-represented 6-mers in the 8 clusters identified in differentially expressed genes shared between *adf5-1* and *adf9-1*

Cluster	# of Genes	Genes in the Cluster	Over-represented 6-mers (Z-score, P value)	Specific Arabidopsis consensus matches to 6-mer
				CARGCW8GAT, CARGNCAT CCA1ATLHCB1
			AGAAAA (1.8, 0.05)	N/A
3	15	AT4G33520, AT4G12260, AT4G19200, PDE327, FUT13, PAM68, AT3G47540, AT4G24175, AT4G20020, AT4G39860, AT5G55450, AT5G23020, MAM3, ADF5, GST21	AGAAAA (1.7, 0.05)	N/A
4	8	AT4G21650, AT1G74280, AT1G74290, AT3G16750, AT3G19030, AT4G12790, AT4G19830, AT1G74440	None	None
5	17	AT2G27550, RLP46, AT5G27430, AT3G03100, AT5G08535, HB31, AT2G20230, MAM1,	AAAATAT (2.4, 0.01)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS AGL3ATCONSENSUS CARGATCONSENSUS, CARGCW8GAT, CARGNCAT, EVENINGAT
			AAAAAA (3.1, 0.001)	AGAMOUSATCONSENSUS,

Table 2.5: Over-represented 6-mers in the 8 clusters identified in differentially expressed genes shared between *adf5-1* and *adf9-1*

Cluster	# of Genes	Genes in the Cluster	Over-represented 6-mers (Z-score, P value)	Specific Arabidopsis consensus matches to 6-mer
5	17	AT1G49740, AT1G61790, GGH2, LUP1, PEPCK, XBAT31, PIN4, AT5G38220, HAC1	AAAAAA (3.1, 0.001)	AGATCONSENSUS, AGL1ATCONSENSUS, CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
			TTTTTT (2.8, 0.005)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
			TATTTT (2.3, 0.05)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
			ACAAAA (2.6, 0.005)	N/A
			AACAAA (2.1, 0.05)	CGF1ATCAB2
			TAAAAT (1.8,	AGAMOUSATCONSE

Table 2.5: Over-represented 6-mers in the 8 clusters identified in differentially expressed genes shared between *adf5-1* and *adf9-1*

Cluster	# of Genes	Genes in the Cluster	Over-represented 6-mers (Z-score, P value)	Specific Arabidopsis consensus matches to 6-mer
			0.05)	NSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS AGL3ATCONSENSUS CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
			AATTTT (1.8, 0.05)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS AGL3ATCONSENSUS CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
6	23	AT4G11270, IAA8, AT1G67860, AT3G16080, AT4G38100, AT1G63110, ML1, AT2G02970, GASA4, ,AT4G17350, SCPL48, AT5G65685, DDE1, AT4G16770, CAF1, AT4G12760, GAD2, SEC10, AT4G29490, AT2G14910, AARE, ATL5, AT3G18420	None	None
7	32	AT4G29260, AT3G20370, AT4G16265, AT4G21830,	TAAAAA (2.5,	AGAMOUSATCONSENSUS

Table 2.5: Over-represented 6-mers in the 8 clusters identified in differentially expressed genes shared between *adf5-1* and *adf9-1*

Cluster	# of Genes	Genes in the Cluster	Over-represented 6-mers (Z-score, P value)	Specific Arabidopsis consensus matches to 6-mer
7	32	AT4G21840, AT1G72920, AT4G19500, AT4G20480, AT4G03060, AT4G16870, AT4G16880, AT4G16890, AT1G73330, AT2G01090, AT2G03710, AT2G03980, AT4G15260, AT4G17340, AT4G28100, AT4G37330, AT4G18390, AT2G21830, AT4G13560, AT2G14660, AT2G18170, AT4G09680, AT4G11830, AT2G16990, AT2G18280, AT2G18290, AT4G39180, AT4G11310	0.01)	NSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS AGL3ATCONSENSUS CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
			AAAAAT (2.2, 0.05)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS, AGL3ATCONSENSUS, CARGATCONSENSUS, CARGCW8GAT, CARGNCAT, CCA1ATLHCB1
			ATTTTT (1.8, 0.05)	AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS, AGL3ATCONSENSUS,

Table 2.5: Over-represented 6-mers in the 8 clusters identified in differentially expressed genes shared between *adf5-1* and *adf9-1*

Cluster	# of Genes	Genes in the Cluster	Over-represented 6-mers (Z-score, P value)	Specific Arabidopsis consensus matches to 6-mer
			AATTTT (1.7, 0.05)	CARGATCONSENSUS, CARGCW8GAT, CARGNCAT AGAMOUSATCONSENSUS, AGATCONSENSUS, AGL1ATCONSENSUS, AGL2ATCONSENSUS, AGL3ATCONSENSUS, CARGATCONSENSUS, CARGCW8GAT, CARGNCAT
8	7	AT4G11320, AT4G13630, AT4G15420, AT4G37320, AT5G04750, AT5G03190, AT4G16860	AGGAAA (3.0, 0.0005) AAAGAA (1.8, 0.05)	AGL1ATCONSENSUS N/A

The promoter motif analysis was done using Promomer (http://bar.utoronto.ca/ntools/cgi-bin/BAR_Promomer.cgi)

CHAPTER 3

Arabidopsis thaliana ACTIN DEPOLYMERIZING FACTOR9 (ADF9) affects gene transcription in an actin-dependent manner[1]

[1] Lori M. King, Daniel R. Ruzicka, and Richard B. Meagher. To be submitted to Genetics

Abstract

The Actin Depolymerizing Factor (ADF)/cofilin family is an essential modulator of actin dynamics in eukaryotes. In animal systems, actin filament dynamics perform important functions in gene expression. For instance, a cofactor for the MADS-box transcription factor, Serum Response Factor (SRF) binds to G-actin. Depletion of the G-actin pool activates this cofactor, which binds SRF and turns on transcription of downstream targets. We previously reported that a Subclass III ADF in Arabidopsis, ADF9, affected transcription of a MADS-box transcription factor, *FLC*, a master repressor of flowering. Treatment of WT Arabidopsis seedlings with an actin filament disruptor, Cytochalasin D, up-regulates expression of *FLC*, suggesting that *FLC* transription responds to an increase in the G-actin pool. Overexpression of an actin variant in *adf9-1* suppresses the down-regulation of *FLC* and early flowering of the mutant, suggesting that ADF9 affects gene transcription in an actin-dependent manner, likely by modulating the G-actin pool.

Introduction

The Actin Depolymerizing Factor (ADF)/Cofilin family is an essential actin-binding protein family found throughout eukaryotes. ADFs are small, monomeric proteins that bind to F-actin filaments on or near the pointed end and, in low ADF/actin ratios and high pH, induce a conformational change that twists monomers off the end (Bamburg 1999). ADFs are negatively regulated by phosphorylation of a conserved N-terminal serine residue and by binding to phosphoinositide (Allwood *et al.* 2002, Gungabissoon *et al.* 1998).

Most plants have large gene families of ADFs (Maciver and Hussey 2002). The *Arabidopsis thaliana* genome encodes 11 ADF proteins, which group into 4 ancient subclasses (Feng *et al.* 2006, Ruzicka *et al.* 2007). We previously reported that the Arabidopsis Subclass III protein, ADF9 affects gene expression of the central repressor of flowering, FLOWERING LOCUS C (FLC) through an unknown mechanism (Burgos-Rivera *et al.* 2008).
The most parsimonious hypothesis for ADF9's effect on FLC is through its effect on actin cytoskeletal dynamics because modulating actin filament dynamics is ADF's well-characterized function; however, we propose three mechanisms based upon ADF's effect on cytoskeletal dynamics.

Serum response factor (SRF) is a MADS-box transcription factor upstream of many of the immediate-early genes responsible for cell cycle control, apoptosis, cell growth, and cell differentiation in animal cells. SRF binds to so-called CArG box promoter elements (consensus sequence $CC[A/T]_2A[A/T]_3GG$) in the promoters of target genes and activates transcription, ultimately in response to serum stimulation (Sun *et al.* 2006). Evidence now shows that a cofactor of SRF, MAL, binds G-actin in the cytoplasm through RPEL motifs (Guettler *et al.* 2008). Activation of signaling cascades by serum leads to actin polymerization, which depletes the G-actin pool. Polymerization releases MAL, which translocates to the nucleus, binds to and

activates SRF poised on CArG boxes, thereby leading to transcription of target genes (Posern and Treisman 2006, Zheng *et al.* 2009). A similar mechanism could be responsible for transcription of *FLC*, which is also a MADS-box transcription factor.

A second mechanism based upon ADF9's effect on cytoskeletal dynamics upon FLC transcription is also dependent upon actin cytoskeletal dynamics. Cofilin is a known inhibitor of glucorticoid receptor (GR) target genes (Ruegg *et al.* 2004). GR is held in the cytoplasm by interaction with hsp90, which anchors it to the actin cytoskeleton, where it is activated by glucocorticoid. Activation by glucocorticoid causes GR to release from the cytoplasm and translocate to the nucleus. Overexpression of cofilin inhibits transcription of GR target genes, possibly because the increased G-actin pool has two inhibitory effects. First, the increased G-actin pool induces the known GR inhibitor *c*-Jun, and second, the increase in actin treadmilling may release GR before it has been activated by glucocorticoid, thereby saturating nuclear GR binding sites with inactive GR (Ruegg, Holsboer, Turck and Rein 2004).

Nuclear localization of ADF proteins has been observed in metazoans (Abe *et al.* 1993, Nebl *et al.* 1996, Ohta *et al.* 1989, Samstag *et al.* 1994, Sanger *et al.* 1980). In vertebrates, nuclear localization of ACs usually accompanies stress. Human cofilin has a Nuclear Localizaiton Signal (NLS) with a sequence similar to that of the SV40 large T antigen (Bamburg 1999). The first two lysine residues (K30 and K31) of the critical KKRKK motif, however, are formed from a vertebrate-specific nine residue insertion in mammals (Bowman *et al.* 2000).

No NLS has been confirmed in the plant specific variants of ADFs, but ZmADF3 localizes to the nucleus (taking actin with it) during cellular stress, just as the vertebrate variant does (Jiang *et al.* 1997). Ruzicka *et al*. show, using a monoclonal antibody raised against recombinant ADF4 (mADF4a) and which reacts with Subclass I and Subclass II ADFs, that

Subclass I ADFs from Arabidopsis localize to both the cytoplasm and nucleus (Ruzicka, Kandasamy, McKinney, Burgos-Rivera and Meagher 2007). Tholl *et al.* show that a Subclass III ADF9-GFP fusion protein biolistically transformed into tobacco cells localizes to both actin filaments and the nucleus (Tholl *et al.* 2011).

That ADFs often bring actin with them when they redistribute to the nucleus suggests that the function of the NLS in ADFs is to help localize actin to the nucleus, where it has important functions in gene transcription, chromatin remodeling, and the formation of heterogeneous nuclear ribonucleoprotein complexes (Zheng, Han, Bernier and Wen 2009). Actin serves these important functions in the nucleus, but does not have an NLS. Although the 42 kDa actin monomer is technically small enough to passively diffuse into the nucleus through the nuclear pore complex, actin is rarely seen in the nucleus, possibly because it has two Nuclear Export Signals (NES) that ensure its cytoplasmic localization under ordinary circumstances (Wada *et al.* 1998). Some debate exists about the form actin takes in the nucleus, but since phalloidin cannot stain nuclear actin (phalloidin only stains actin filaments of at least seven subunits), researchers have suggested that nuclear actin is mostly monomeric, forms short polymers, or is in some nuclear-specific conformation (Vartiainen 2008). Whatever form it takes once there, actin monomers must get into the nucleus, and therefore cytoskeletal dynamics and the G-actin pool in the cytoplasm likely has a bearing on the nuclear actin pool.

Here we present data to address if ADF9 affects transcription of FLC through its effect on cytoskeletal dynamics, and if so, by what mechanism it does so. We show by treatment with Cytochalasin D (CytD) and overexpression of ACTIN2 that an increased G-actin pool is sufficient for the expression of *FLC*, which suggests that ADF9 affects transcription in an actin-dependent manner.

Materials and Methods

GFP reporter for F-actin

We transformed wild-type and *adf9-1* mutant plants with the F-actin reporter GFP:fABD2 (Wang *et al.* 2004). Multiple lines of positive transformants were selected on hygromycin and vertically grown on ½ MS, 1% sucrose, 0.8% agar plates. Microfilaments were visualized in trichomes as described previously (Wang, Motes, Mohamalawari and Blancaflor 2004) using a Leica confocal laser scanning microscope (TCS-SP2, Heidelberg, Germany).

Drug Treatment

WT (Col) seeds were surface sterilized and sown into sterile ½ strength Murashige and Skoog (MS) media supplemented with 1% sucrose in 16 well culture dishes. Approximately 10 seeds were sown into each well to allow ample room for growth. Seeds were stratified in the dark at 4°C for 48 hours and then were placed in an incubator on a long-day photoperiod (16 hour light, 8 hour dark). Seedlings were allowed to grow for 9 days and then were either left untreated or were treated with sterile deionized water (water control), filter sterilized DMSO (DMSO control), 0.1 μM filter sterilized Cytochalasin D in DMSO (CytD; Boehringer Mannheim) or 0.1μM filter sterilized Latrunculin B (Lat B; Calbiochem) in DMSO. Drug treatment was continued approximately 24 hours, and seedlings were harvested and frozen in liquid nitrogen 2.5 hours after the beginning of the photoperiod.

Suppression studies

For actin overexpression, *adf9-1* mutant plants were transformed with *Agrobacterium tumefaciens* C58 via the floral dip method with modifications (Clough and Bent 1998). A. tumefaciens was transformed with a pCAMBIA binary vector containing the unmutated ACTIN2 (ACT2) or a mutated, nonpolymerizable form of ACT2 ($ACT2_{G13R}$) cDNA

under an ACT2 promoter and terminator. Positive T1 generation transformants were selected by plating seeds on ½ MS, 1% sucrose, and 0.8% agar under Hygromicin (Hyg) selection. T1 plants were rescued and grown on soil. T2 seeds were collected and 3 lines with individual insertions (as determined by a 3:1 segregation ratio on Hyg selection) were selected for further analysis. All suppression analyses were performed on T2 generation plants.

Quantitative Real-time PCR (qRT-PCR) analysis

RNA was isolated from 10-day-old whole seedlings (for the drug treatment experiments) or 10-day-old shoot tissue (for the mutant and suppression experiments) using the Qiagen RNeasy Plant Mini Kit. 1.5 µg of total RNA from each sample was treated with RQ1 RNase-free DNase (Promega) and the treated RNA was used for cDNA synthesis using the Super Script III kit (Invitrogen), following the manufacturer's instructions except that incubations were performed for only 30 min. at 55°C using oligo (dT) primer. Aliquots of the cDNA were used as template for qRT-PCR analysis of triplicate reactions for each of the biological replicates on an Applied Biosystems 7500 Real Time PCR Instrument, using SYBR Green detection chemistry. Real time PCR reactions consisted of 2X SYBR GREEN PCR Master Mix (Applied Biosystem), 0.8 µM of each primer, and 1:25 diluted cDNA in a 25 µl reaction volume. The reaction conditions were as follows: 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 1:00. Data was collected during the last stage of each cycle. The following primers were used to detect transcript levels: *ACT2* (ACT2-RTS: 5'-CAAATCCAGCCTTCACCATA-3', ACT2-RTA: 5'- GAGGCTGATGATATTCAACCA-3'); *ACT7* (ACT7-RTS: 5'-AGAGAAAATACAGTGTCTGGAT-3', ACT7-RTA: 5'-TTTGAAATCCACATCTGTTGGAA-3'); *ADF5* (ADF5-RTS: 5'-CGACTTTGTCACCGTCGATAAC-3', ADF5-RTA: 5'-CTCCGGTGACCATGCAATG-3');

ADF9 (ADF9-RTS: 5'-ATATAACGAAAGAACAAGAAGACA-3', ADF9-RTA: 5'CACTCGTCGCCGTCTTCAA-3'); *FLC* (FLC-RTS: 5'-TCTTCCGGTGACTCTGTTA-3', FLC-RTA: 5'-ATATGTTTTGGATTTTGATTTCAA-3'); *UBQ10* (UBQ10-RTS: 5'-AGAAGTTCAATGTTTCGTTTCATGTA-3', UBQ10-RTA: 5'-GAACGGAAACATAGTAGAACACTTAT-3'). Control reactions were performed using UBIQUITIN10 (UBQ10). The $2^{-(ddCT)}$ method of relative quantification was used in all experiments.

Results

ADF9 has a minimal effect on the actin cytoskeleton

Tholl *et al*. recently reported that ADF9 acts as an actin-filament bundler, suggesting it stabilizes actin filaments (Tholl, Moreau, Hoffmann, Arumugam, Dieterle, Moes, Neumann, Steinmetz and Thomas 2011). However, they performed their in vivo experiments in biolistically transfected tobacco cells. To confirm their data in Arabidopsis, we stably transformed WT and *adf9-1* with a GFP:fABD2 F-actin reporter construct known to have minimal effects on the cytoskeleton (Wang, Motes, Mohamalawari and Blancaflor 2004). We observed F-actin morphology using confocal microscopy in multiple independently transformed WT/*GFP:fABD2* and *ad95-1/GFP:fABD2* lines. F-actin morphology was examined in root cells and leaf trichomes, where ADF59 is normally expressed and GFP-labeled actin filaments are easily visualized.

In WT trichome cells, the actin cytoskeleton is organized into a network of fine filaments and bundles primarily arrayed longitudinally along the axis of cell growth (**Figure 3.1A and B**). In *adf9-1* trichomes, the general organization of filaments is the same as WT, but the thickness of bundles is increased and the number of fine filaments is decreased (**Figure 3.1C and D**). The

minimal effects on the F-actin cytoskeleton in *adf9-1* trichomes suggests that ADF9 has a minimal effect on F-actin cytoskeletal remodeling and organization, and, contrary to what Tholl *et al.* saw in tobacco cells, the loss of ADF9 gives a minor increase in F-actin stability. If ADF9 acts as an actin-filament bundler, we would expect that *adf9-1* would have fewer actin bundles and more fine filaments. The difference in what we observe compared to what Tholl *et al*. report may be because of differences in expression or stability of proteins in transiently transfected compared to stably transformed cells. Also, our *adf9-1* mutant is not a null; rather, it is a hypomorphic allele, and ADF9 expression is comparatively weak in leaf cells (Burgos-Rivera *et al*. 2008, Ruzicka *et al*. 2007). Nevertheless, our data suggest that ADF9 acts to destabilize actin filaments.

Treatment with Cytochalasin D (CytD) up-regulates FLC in WT seedlings

We have previously reported that *FLC* is down-regulated in a mutant allele of ADF9, *adf9-1* (Burgos-Rivera *et al.* 2008). These data suggest that ADF9 affects gene expression by an unknown mechanism. The most parsimonious hypothesis for the function of an ADF protein in gene expression is through its effect on actin cytoskeletal dynamics; modulating actin filament dynamics is their well-characterized function. All of the mechanisms we mention in our introduction depend upon cytoskeletal dynamics. To test if cytoskeletal dynamics plays a part in *FLC* expression, we treated WT(Col) seedlings with actin filament disrupting drugs. When both a disruptor and stabilizer of actin polymerization inhibit a biological process, the assumption is that actin dynamics are required for that process (Staiger 2000).

Cytochalasin D (CytD) is a well-characterized actin filament disruptor that works through a complex mechanism; CytD binds to actin filaments at both the pointed and the barbed end and prevents depolymerization (pointed end), yet it allows some polymerization (barbed end) (Sampath and Pollard 1991). Latrunculin B (LatB) is another actin filament disruptor, but it

works through a different and simpler mechanism than CytD; LatB binds to actin monomers, so it disrupts actin filaments by sequestering G-actin (Morton *et al.* 2000).

Our observation is that ADF9 acts as an actin filament disruptor (**Figure 3.1**), and in an *adf9-1* mutant, FLC expression is down-regulated. If actin dynamics affect transcription of *FLC*, then we expect an actin filament disruptor to have the opposite phenotype as *adf9-1*. In **Figure 3. 2**, we show that the actin filament disruptor CytD, but not LatB (at least at the concentration we tested), up-regulates *FLC*. With only one concentration and without a stabilizing drug treatment to compare, interpretation is difficult. For that reason, we will be titrating out concentrations of CytD and LatB to determine if the effect on *FLC* is concentration dependent and to confirm that both CytD and LatB give the same effect. As we mentioned above, since CytD and LatB have different modes of action (CytD binds actin filaments and LatB binds actin monomers), an observable difference in their effect on *FLC* may suggest that ADF9 binds actin monomers and carries them into the nucleus. In addition, we will be adding an actin filament stabilizing drug, Jasplakinolide, to our experiment, titrated out as for CytD and LatB.

All our proposed mechanisms depend on actin cytoskeletal dynamics, so our data thus far suggest one of these three mechanistic models is the correct one.

Overexpression of ACTIN2 suppresses the down-regulation of FLC in adf9-1, the altered rosette morphology, and the early flowering phenotype.

Drug treatment can cause pleiotropic effects, therefore to further test the hypothesis that ADF9 affects expression of FLC through its ability to modulate actin cytoskeletal dynamics, we over-expressed ACTIN2 (ACT2) cDNA under its endogenous promoter in *adf9-1*. We chose ACT2 because it is normally expressed in vegetative tissues and is an unregulated actin variant (unlike another vegetative actin, ACTIN7, which is responsive to hormones and stress). A

similar approach was used previously in an experiment on Serum Response Factor (SRF). Kuwahara *et al.* hypothesized that a muscle-specific actin-binding protein, STARS, activated SRF's cofactor MAL by depleting the G-actin pool. They show that overexpressing WT actin (which increases the G-actin pool but does not alter the F-actin/G-actin ratio) reduced the ability of a muscle-specific actin-binding protein, STARS, to activate MAL-dependent transcription of SRF target genes (Kuwahara *et al.* 2005). Adding more actin reestablishes the pool and inhibits MAL.

Based on our drug treatment data, if actin filaments are destabilized with CytD (which binds filaments not monomers), presumably the G-actin pool is increased and *FLC* expression goes up. If it is true that ADF9 helps maintain the G-actin pool through depolymerization, then an *adf9-1* mutant would have a decreased G-actin pool and *FLC* expression would drop. This is, indeed, the effect we see. If we increase the G-actin pool by overexpressing actin, then we should see *FLC* expression go up. **Figure 3.3** shows this is the case for one of our three independent insertions of *adf9-1*/A2:ACT2 (#16). We will expand this analysis to include all three insertion lines; we are particularly interested to see if the suppression of the down-regulation of FLC in these *adf9-1*/A2:ACT2 lines is concentration-dependent or if we can discern a saturation point. In **Figure 3.4** we show that the sparse rosette morphology seen in adf9-1 is likewise suppressed by ACT2 overexpression, and in **Figure 3.5** we show that the early flowering phenotype of *adf9-1* is also suppressed, and, indeed, these plants bolt, on average, with even more rosette leaves than WT, though we see more variability.

These data suggest that some level of G-actin is required for the appropriate FLC expression, but this experiment alone, once again, fails to distinguish between the three mechanisms. Therefore, we will also be analyzing overexpression of a nonpolmerizable form of

ACT2, $ACT2_{G13R}$, in the *adf9-1* background. Kuwahara *et al.* found in their system that the nonpolymerizable actin decreased the F-actin/G-actin ratio, which inhibits MAL further. In our system, based upon the data we have already shown, we expect to see a further increase in *FLC* expression and possibly even later flowering than that already seen for *adf9-1*/A2:ACT2.

Discussion

Known FLC regulation pathways

FLOWERING LOCUS C (FLC) is a MADS-box transcription factor that represses expression of the floral integrators FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESION OF CONSTANS 1 (SOC1), and FD, which are required for the transition to flowering (He 2009). FRIGIDA (FRI) up-regulates *FLC* expression to inhibit flowering. Rapid flowering accessions of Arabidopsis, like Columbia (Col), have non-functional or loss-of-function FRI alleles. WT (Col), specifically, has a loss-of-function FRI allele with a 16-base pair deletion that results in a truncated protein; however, *FRI* transcript is detectable in Col and so is *FLC* transcript from its strong FLC allele (Gazzani *et al.* 2003, Johanson *et al.* 2000). Therefore, even though Col is a rapid flowering accession, some level of *FLC* is expressed and *FLC* levels do correlate with flowering time.

FLC is also up-regulated by the PAF1 complex, H2A.Z deposition by the SWR1 complex, and H2B ubiquitination (He 2009). *FLC* is repressed by proteins in the autonomous pathway (FCA, FPA, FVE, LUMINIDEPENDENS, *and* FLOWERING LOCUS D (FLD)) in response to internal signals. Of these autonomous pathway proteins, only LUMINIDEPENDENS is a likely transcription factor. FCA and FPA are involved in RNA-mediated chromatin silencing, FVE is part of the CUL4-RING ubiquitin ligase complex, and FLD is the homolog of a SWIRM domain containing protein found in histone deactylase

complexes in mammals.

We previously reported that the Arabidopsis Subclass III protein, ADF9 acts as a positive regulator of *FLC* through an unknown mechanism (Burgos-Rivera *et al.* 2008). ADF9 most likely does so through its effect on the actin cytoskeleton, but cytoskeletal dynamics has never, to our knowledge, been previously shown to affect *FLC* expression.

Treatment with Cytochalasin D (CytD) up regulates FLC in WT seedlings

To test if cytoskeletal dynamics plays a part in *FLC* expression, we treated WT(Col) seedlings with actin filament disrupting drugs. When both a disruptor and stabilizer of actin polymerization inhibit a biological process, the assumption is that actin dynamics are required for that process (Staiger 2000). We show that the actin filament disruptor Cytochalasin D (CytD) up regulates *FLC*, which suggests that actin filament dynamics (or an increased G-actin pool) are sufficient for the expression of *FLC*.

Overexpression of ACTIN2 suppresses the down-regulation of FLC in adf9-1 and the early flowering phenotype

If actin filaments are destabilized with CytD, presumably the G-actin pool is increased and *FLC* expression goes up. ADF9 helps maintain the G-actin pool through depolymerization, so an *adf9-1* mutant has a decreased G-actin pool and decreased *FLC* expression. We show that increasing the G-actin pool by overexpressing actin increases *FLC* expression (**Figure 3)**. These data confirm what we observed for CytD treatment; an increased G-actin pool is sufficient for the expression of *FLC*.

In the Serum Response Factor (SRF) literature, an increased G-actin pool (or decreased polymerization) has an inhibitory effect on MAL activation and SRF-dependent transcription. In this model, FLC would be one of the down stream targets of some unknown transcription factor

and/or cofactor, and here we see the opposite effect: an increased G-actin pool leads to increased expression of the downstream target (*FLC*, in this case). If this mechanism is correct, then the target we are looking for is a repressor of *FLC* that may bind G-actin and allow *FLC* expression. As mentioned above, some of the known repressors of *FLC* are proteins in the autonomous pathway. Among these, LUMINIDEPENDENS, is the only likely transcription factor, and perhaps the best target for genetic experiments with double mutants to test this mechanism.

If ADF9 affects *FLC* expression by shuttling actin into the nucleus, then we would expect that an increased G-actin pool would lead to increased shuttling of actin into the nucleus (in the absence of increased poylmerization), which may increase the activity of actin-dependent chromatin-modifying complexes (that FCA, FPA, and FLD are components of). Experiments with the nonpolymerizable actin $ACT2_{G13R}$ may help show if this is the case; we expect overexpression of nonpolymerizable actin to give the same or an even more dramatic *FLC* expression and flowering time suppression as overexpression of polymerizable actin.

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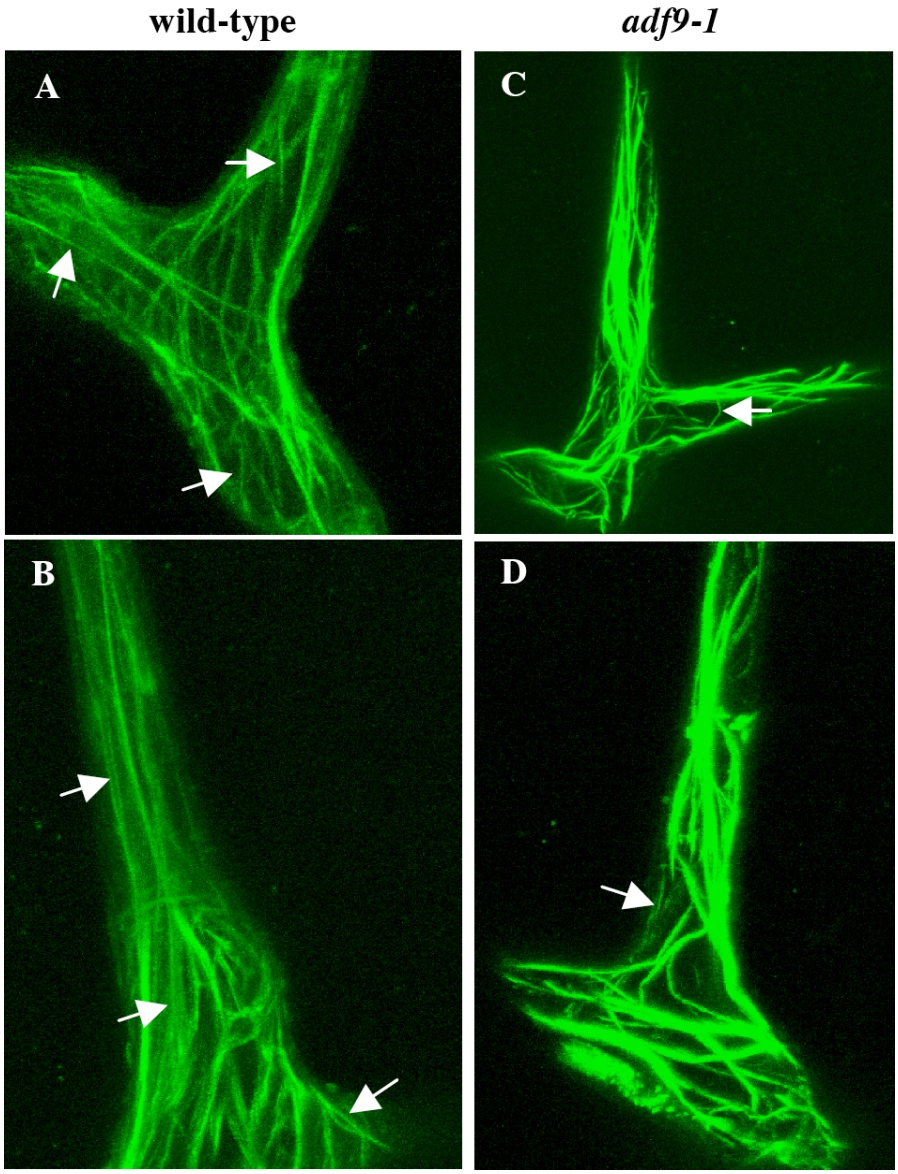

Figure 3.1: ADF9 has minimal effects on the actin cytoskeleton. Trichomes from T2 generation WT and adf9-1 plants transformed with FimABD-GFP were visualized with a confocal microscope. Representative images are shown. ADF9 has minimal effects on the actin cytoskeleton in trichomes, although adf9-1 trichomes have fewer fine actin filaments and thicker actin cables (as indicated by the arrows).

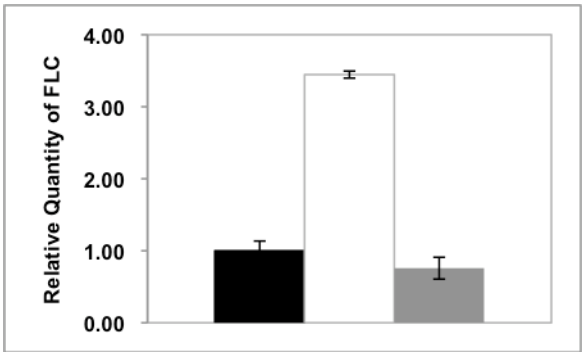

Figure 3.2: CytD treatment up-regulates *FLC* expression in WT. WT (Col) were grown in sterile liquid ½ MS media supplemented with 1% sucrose and treated with Dimethyl Sulfoxide (DMSO) as a control (black bar), 0.1 μM Cytochalasin D (CytD) in DMSO (white bar), or 0.1 μM Latrunculin B (LatB) in DMSO (gray bar). Seedlings were harvested 10 days after germination. Samples were taken 2.5 hours after the beginning of the photoperiod. qRT-PCR analysis was performed with reference to UBQ10 and normalized to WT treated with DMSO. Three technical replicates were performed for each reaction, and the error bars represent standard deviation of the three replicates.

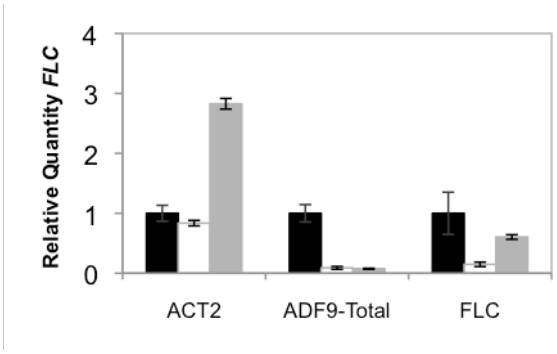

Figure 3.3: Overexpression of ACTIN2 suppresses the down-regulation of FLC in *adf9-1*. WT (black bars), *adf9-1* (white bars), and T2 generation seeds of *adf9-1*/A2:ACT2 #16 (gray bars) were germinated on ½ MS, 1% sucrose, agar plates and were harvested 10 days after germination. Samples were taken 3 hours after the beginning of the photoperiod. qRT-PCR analysis was performed for the primer pairs shown, with reference to UBIQUITIN10 (UBQ10) and normalized to WT. Three technical replicates were performed for each reaction, and the error bars represent standard deviation of the three replicates.

Figure 3.4: Overexpression of ACT2 suppresses the rosette morphology of adf9-1. WT, *adf9-1*, and T2 generation seeds for three independent insertion lines of *adf9-1*/A2:ACT2 were germinated on soil, and seedlings were transferred to individual pots 10 days after germination. Pictures were taken at 24 days post-germination (n≥5 for each genotype).

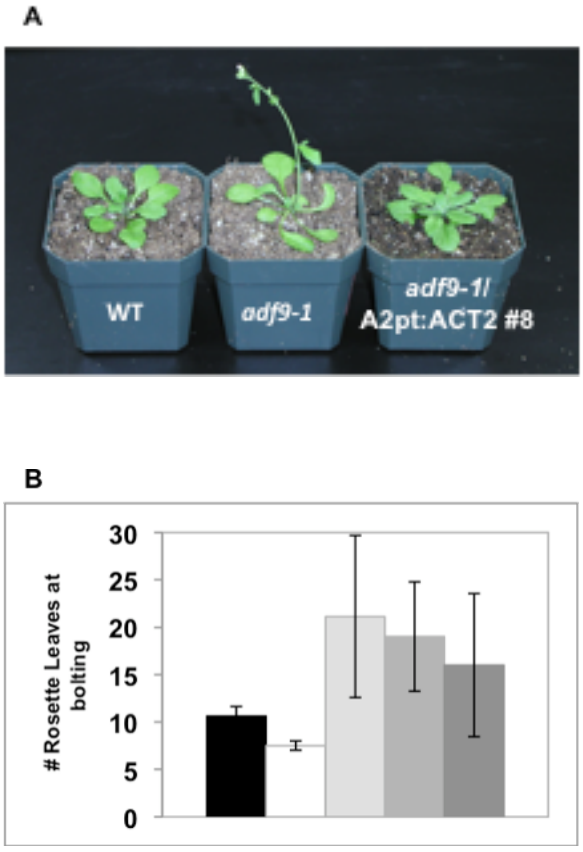

Figure 3.5: Overexpression of ACT2 suppresses the early flowering time of *adf9-1*. WT, *adf9-1*, and T2 generation seeds for three independent insertion lines of *adf9-1*/A2:ACT2 were germinated on soil, and seedlings were transferred to individual pots 10 days after germination. The number of rosette leaves at bolting was scored for plants of each genotype (n≥5). Pictures were taken at 24 days post-germination (pg) **A)** One representative picture shows that by 24 days pg, *adf9-1* has bolted, but WT and the suppression line has not. **B)** The number of rosette leaves at bolting is quantified for WT (black bars), *adf9-1* (white bars), #8 (lightest gray bars), #16 (medium gray bars), and #20 (darkest gray bars).

CHAPTER 4

Conclusion

Current knowledge of Subclass III Actin Depolymerizing Factor (ADF)s

When I began my project, I was focused on ADF9. I was interested in ADF9 because plants with a mutant ADF9 allele (*adf9-1)* flowered earlier than WT and had reduced expression of the master regulator of flowering, Flowering Locus C (*FLC*) (Burgos-Rivera *et al.* 2008). ADF9 flowers early and is down-regulated at FLC, despite an almost negligible expression level compared to co-expressed ADFs (**Figure 1.6**). In my example, ADF9 is only expressed at 1.3% the level of ADF1 in 10-day-old seedlings, and the same general pattern holds true across most tissues and developmental stages: except in callus where ADF9 is expressed the highest of all the ADFs, ADF9 is expressed between 13%-0.7% ADF1 levels (Ruzicka *et al.* 2007). The question that most intrigued me about ADF9 was how such a weakly expressed actin-binding protein like ADF9 could change the expression of such an important, high-level regulator of development as *FLC*.

Even though its expression is low, ADF9 is expressed in important tissues. It is expressed in fast growing and differentiating tissues such as young seedlings, shoot apical meristem, the edges of leaves, root elongation zone, and root tip meristem (Ruzicka, Kandasamy, McKinney, Burgos-Rivera and Meagher 2007). The shoot apical meristem (SAM), for instance, is the home and organizing center for a population of undifferentiated stem cells from which above ground organs are derived (Ha *et al.* 2010). During vegetative growth, the SAM produces leaves, then, after the switch to reproductive growth, the SAM produces floral meristems that make flowers. FLC expressed in the SAM binds to the promoters and represses expression of

the floral integrators SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and FLOWERING LOCUS D (FD) (Searle *et al.* 2009). To my knowledge, no one has specifically looked at actin cytoskeletal organization in the SAM, but it would be interesting to see if Subclass III mutants had alterations in the organization of the actin cytoskeleton in the SAM.

When I began my project, little was known about the remaining member of Subclass III, ADF5, except the expression levels and expression patterns. The amino acid sequence of ADF5 is only 80% identical to ADF9 (115 out of 143 residues) (Ruzicka *et al.* 2007). Perhaps most significantly, ADF5 does not have a phosphorylatable serine (phosphorylation at this site negatively regulates ADF for actin binding) at the conserved N-terminal position; rather, ADF5 has a threonine at this position (T10). ADF5 also differs from ADF9 in one other residue thought important for actin binding, M7 (ADF9 has a threonine at this position) (Bowman *et al.* 2000). ADF5's expression overlaps with that of ADF9 but is also different in that it is expressed strongly in cotyledons, emerging leaves, root vascular tissue, and the root tip meristem, but is expressed weakly in reproductive tissue, adult leaves, and mature roots. ADF5 is expressed at a higher level than ADF9, too; ADF5 is expressed at 43% the level of ADF1 in 10-day-old seedlings (Ruzicka *et al.* 2007).

One of the side projects I have been working on was in collaboration with the Staiger lab group; I was attempting to isolate and purify ADF5 and ADF9 protein for use in *in vitro* depolymerization assays. ADF5 has yet to be biochemically characterized, but a paper biochemically characterizing ADF9 has been recently published. In their paper, Tholl *et al.* suggest that ADF9 has a different function and pH dependence than the already well-characterized ADF1 (Tholl *et al.* 2011). ADF1 induces depolymerization of actin filaments *in vitro* in a concentration-dependent way with greater efficiency at alkaline pH (8.0) than acidic

pH (6.0). In their study, Tholl *et al.* report that ADF9 did not depolymerize actin filaments; rather, ADF9 promotes actin bundling, and it does so with more efficiency at acidic pH than alkaline pH. An ADF9-GFP construct biolistically transformed into tobacco cells shows that ADF9 can bundle actin filaments *in vivo* as well as *in vitro*, and localizes to both the cytoplasm and the nucleus. We show the same subcellular localization pattern for a stably transformed ADF5-YFP reporter in **Chapter 2**.

Hypotheses to test ADF9's function in *FLC* expression

My initial strategy to address how ADF9 functioned in the expression of *FLC* was to determine the protein-protein interactions and the subcellular localization of ADF9, but protein work in Subclass III has always been difficult, so I changed my approach to a genetic analysis, focusing on suppression studies. Both Subclass III ADFs affect transcription of *FLC*. The most likely hypothesis is that ADF5 and ADF9 affect expression of FLC indirectly through their effects on the actin cytoskeleton.

One of my hypotheses I call the "cytoplasmic dynamics" hypothesis, and I have based it upon the body of literature showing that cytoskeletal dynamics in the cytoplasm affects the subcellular localization and activity of several transcription factors in animal systems. For instance, Serum response factor (SRF) is a MADS-box transcription factor upstream of many of the immediate-early genes responsible for cell growth and differentiation in animal cells. SRF binds to CArG box elements in the promoters of target genes (Sun *et al.* 2006). Actin cytoskeletal dynamics is one of the factors that controls expression of SRF target genes.

A cofactor of SRF, MAL, binds G-actin in the cytoplasm, and activation of signaling cascades by serum stimulation causes actin polymerization, which removes G-actin from the

monomeric actin pool. As actin monomers are polymerized, MAL is released and translocates to the nucleus where it binds to and activates SRF-dependent transcription of target genes (Posern and Treisman 2006, Zheng *et al.* 2009).

Actin-binding proteins and drugs that can affect actin treadmilling also affect MAL:SRF-dependent gene transcription. For instance, treatment with Cytochalasin D (CytD) activates MAL:SRF-dependent gene transcription (Posern and Treisman 2006). To test if cytoskeletal dynamics plays a part in *FLC* expression, I treated WT(Col) seedlings with CytD and Latrunculin B (LatB). CytD treatment up-regulates *FLC*, which suggests that decreased actin filament dynamics (or an increased G-actin pool) are sufficient for the expression of *FLC*. If actin filaments are destabilized with CytD, presumably the G-actin pool is increased and *FLC* expression goes up.

ADF9 helps maintain the G-actin pool through depolymerization, so an *adf9-1* mutant has a decreased G-actin pool and decreased *FLC* expression. I showed that increasing the G-actin pool by overexpressing actin increases *FLC* expression. In the SRF literature, an increased G-actin pool (or decreased polymerization) has an inhibitory effect on MAL activation and SRF-dependent transcription. If this model were true in my system, FLC would be one of the down-stream targets of some unknown transcription factor and/or cofactor – yet, unlike in the SRF literature, in my system I see that an increased G-actin pool (or decreased polymerization) leads to increased expression of the downstream target (*FLC*, in this case). If this mechanism is correct, then a candidate mediator of this process (the MAL or SRF to our downstream target FLC) is a repressor of *FLC* for which binding to G-actin inhibits activity and thus allows *FLC* expression. An interesting experiment to pursue would be to make crosses between *adf9-1* and mutants in known repressors of FLC, such as those in the autonomous pathway (FCA, FPA,

FVE*,* LUMINIDEPENDENS*, and* FLOWERING LOCUS D (FLD)). If an interaction exists between ADF9 and one of these proteins, then I would expect a suppression of the FLC down-regulation in *adf9-1*.

Another of my hypotheses I call my "actin to the nucleus" hypothesis. As mentioned previously, mammalian ADF/Cofilins have an NLS. Nuclear localization of ADF proteins has been observed in metazoans (Abe *et al.* 1993, Nebl *et al.* 1996, Ohta *et al.* 1989, Samstag *et al.* 1994, Sanger *et al.* 1980). No NLS has been confirmed in the plant specific variants of ACs, but ZmADF3 localizes to the nucleus (taking actin with it) during cellular stress, just as the vertebrate variant does (Jiang *et al.* 1997). Ruzicka *et al*. show, using a monoclonal antibody raised against recombinant ADF4 (mADF4a) and which reacts with Subclass I and Subclass II ADFs, that Subclass I ADFs from Arabidopsis localize to both the cytoplasm and nucleus (Ruzicka *et al.* 2007). As I mentioned previously, I show in **Chapter 2** that ADF5 from Subclass III similarly localizes to both the cytoplasm and nucleus, and Tholl *et al.* show that ADF9-GFP biolistically transformed into tobacco cells localizes to both actin filaments and the nucleus (Tholl *et al*. 2011).

If ADF9 affects *FLC* expression by shuttling actin into the nucleus, then we would expect an increased G-actin pool would lead to increased shuttling of actin into the nucleus (in the absence of increased poylmerization), which may increase the activity of actin-dependent chromatin-modifying complexes (of which FLC repressors in the autonomous pathway, FCA, FPA, and FLD, are components). Experiments with the nonpolymerizable actin $ACT2_{G13R}$ may help show if this is the case; we expect overexpression of nonpolymerizable actin to give the same or an even more dramatic *FLC* expression and flowering time suppression as overexpression of polymerizable actin.

That ADFs often bring actin with them when they redistribute to the nucleus may mean that the function of the NLS in ADFs is to help localize actin to the nucleus, where it has important functions in gene transcription, chromatin remodeling, and the formation of heterogeneous nuclear ribonucleoprotein complexes (Zheng *et al.* 2009). Actin serves these important functions in the nucleus, but does not have an NLS. Although the 42 kDa actin monomer is technically small enough to passively diffuse into the nucleus through the nuclear pore complex, actin is rarely seen in the nucleus, possibly because it has two Nuclear Export Sequences (NES) that ensure its cytoplasmic localization under ordinary circumstances (Wada *et al.* 1998).

Functional Genomics: Exploring the effects of Subclass III ADF proteins on global gene expression using microarrays

As I mentioned before, both Subclass III ADFs affect transcription of *FLC*. Using a microarray, we found that a substantial proportion of the genes in our control data set (genes expressed in WT shoot tissue at a developmental stage when 2 and 4 leaves have been produced) were differentially expressed in *adf5-1* (~7%) and in *adf9-1* (~12%). Only152 genes were shared between those differentially expressed in *adf5-1* and *adf9-1*, however. Using the GO::TermFinder tool to determine if these differentially expressed genes were enriched in any particular Gene Ontology (GO) biological process, we found that the genes differentially expressed in *adf5-1* and the genes shared between *adf5-1* and *adf9-1* were not enriched. However, the genes differentially expressed in *adf9-1* were enriched in the stress response, S-glycoside biosynthetic process, and the indole-containing compound biosynthetic process. 15%

of the genes differentially expressed in *adf9-1* are involved in the stress response, including two MADS-box transcription factors, FLC and AGAMOUS-like 24 (AGL24).

1% of the genes differentially expressed in *adf9-1* are involved in the S-glycoside biosynthetic process. Glycosides are molecules with a sugar group bound through its anomeric carbon to some other non-carbon group via a glycosidic bond. Glycosides perform important functions in many organisms, but in plants, glycosides are involved in, for instance, host-pathogen interactions and pollen development (Hrmova and Fincher 2007). Similarly, 1% of the genes differentially expressed in *adf9-1* are involved in indole-containing compound biosynthesis, which impinges upon indole-3-acetic acid *(*IAA*)* or auxin synthesis. IAA is the most potent endogenous auxin, an important plant hormone with many effects, most notably plant growth and development in response to its environment (Zhao 2010). The common theme of these biological processes is the plant's developmental response to its environment, which is intriguing, given the *adf9-1* phenotype.

The differences in GO term enrichment we observe between *adf5-1* and *adf9-1* could account for some of the differences we observe in morphological phenotypes between the mutants; however, it does not account for the antagonistic developmental phenotypes we see, such as flowering time and leaf morphology. A cluster analysis revealed enrichment for elements several AGAMOUS (AG) and AGAMOUS-like (AGL) transcription factors are known to bind.

That I could find no clear enrichment in a biological process, molecular function, or cellular component in the 152 differentially expressed genes shared between *adf5-1* and *adf9-1* suggests that Subclass III ADFs change gene expression in different pathways across a broad

range of processes or functions. The most reasonable mechanism for Subclass III ADF's effect on gene expression remains an actin cytoskeletal-dependent mechanism.

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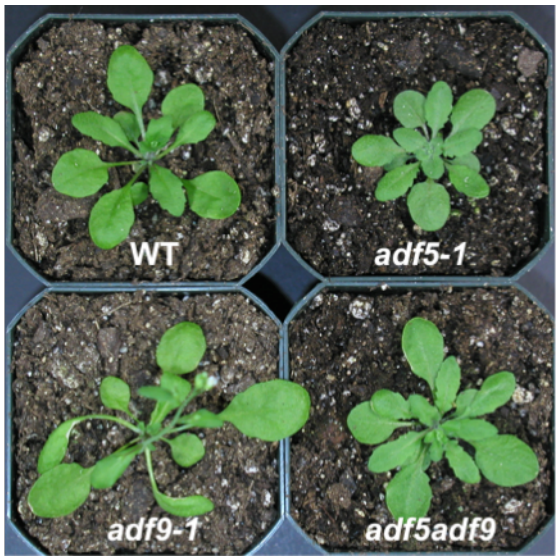
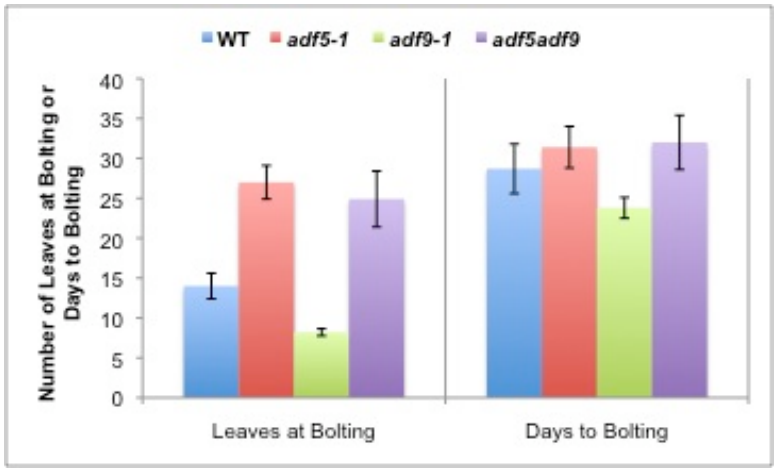
APPENDIX A

Subclass III double mutant analysis

A



B



A.1: *adf5adf9* double mutants morphologically more like *adf5-1* single mutants than like *adf9-1*. A) WT, *adf5-1*, *adf9-1*, and *adf5adf9* seeds were sown on soil and transferred to individual pots 10 days post germination. Pictures were taken 24 days post germination ($n \geq 15$). **B)** Rosette leaves at bolting and days post germination to bolting were scored for all four genotypes.