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Plant Actin-Depolymerizing Factors Possess Opposing Biochemical Properties Arising from Key Amino Acid Changes throughout Evolution[OPEN]

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Functional divergence in paralogs is an important genetic source of evolutionary innovation. Actin-depolymerizing factors (ADFs) are among the most important actin binding proteins and are involved in generating and remodeling actin cytoskeletal architecture via their conserved F-actin severing or depolymerizing activity. In plants, ADFs coevolved with actin, but their biochemical properties are diverse. Unfortunately, the biochemical function of most plant ADFs and the potential mechanisms of their functional divergence remain unclear. Here, in vitro biochemical analyses demonstrated that all 11 ADF genes in *Arabidopsis thaliana* exhibit opposing biochemical properties. Subclass III ADFs evolved F-actin bundling (B-type) function from conserved F-actin depolymerizing (D-type) function, and subclass I ADFs have enhanced D-type function. By tracking historical mutation sites on ancestral proteins, several fundamental amino acid residues affecting the biochemical functions of these proteins were identified in Arabidopsis and various plants, suggesting that the biochemical divergence of ADFs has been conserved during the evolution of angiosperm plants. Importantly, N-terminal extensions on subclass III ADFs that arose from intron-sliding events are indispensable for the alteration of D-type to B-type function. We conclude that the evolution of these N-terminal extensions and several conserved mutations produced the diverse biochemical functions of plant ADFs from a putative ancestor.

INTRODUCTION

The functional divergence of paralogs produced by gene duplication is an important genetic source of evolutionary innovation. In general, functional divergence has been proposed to occur via changes in gene expression patterns at the transcriptional level (Force et al., 1999; Hittinger and Carroll, 2007; Gagnon-Arsenault et al., 2013) as well as changes in biochemical function. The primary mechanisms responsible for divergence in biochemical function among paralogs include site-specific regulatory modification of proteins (Marques et al., 2008; Freschi et al., 2011), variation of splicing sites among isoforms (Marshall et al., 2013; Nguyen Ba et al., 2014), and changes in enzymatic activity and protein specificity (Force et al., 1999; Voordeckers et al., 2012). Thus, analyzing key amino acids or functional motifs related to functional divergence could help reconstruct the evolutionary process of paralogs to a certain extent.

The actin cytoskeleton, which exists in all eukaryotic cells, is important for fundamental cellular processes such as vesicle trafficking, organelle movement and rearrangement, cytoplasmic streaming, tip zone organization, and tip growth (Staiger, 2000; Staiger and Blanchoin, 2006; Pollard and Cooper, 2009; Blanchoin et al., 2014). Eukaryotic cells have a highly ordered and dynamic actin architecture that is accurately and directly regulated by numerous actin binding proteins (ABPs) with different functions (Staiger, 2000; Staiger and Blanchoin, 2006; Fu, 2015). Thus, it has been proposed that the evolution of actin was accompanied by the formation of particular ABPs (Kandasamy et al., 2007; Gunning et al., 2015).

The actin-depolymerizing factor (ADF) family is an important class of ABPs that exists in all eukaryotes (Andrianantoandro and Pollard, 2006). The classic members of the ADF family can bind to both monomeric actin (G-actin) and filamentous actin (F-actin), with a notable preference for ADP-G-actin. ADFs can depolymerize or sever F-actin into short fragments, thereby providing new actin filament initiation sites and increasing the dissociation rate of actin monomers at the pointed ends of actin filaments, which supplies more monomers for polymerization at the barbed ends of F-actin and promotes dynamic changes in actin polymerization (Carlier et al., 1997; Galkin et al., 2011; Suarez et al., 2011). In basal eukaryotes such as yeast (*Saccharomyces cerevisiae*), roundworm (*Caenorhabditis elegans*), and alga (*Chlamydomonas reinhardtii*), ADF is encoded by a single gene (Gunning et al., 2015), but multiple ADF genes are found in the genomes of plants, including up to 27 ADF homologs in banana (*Musa acuminata*). In *Arabidopsis thaliana*, a total of 11 ADF genes have been categorized into four subclasses (I to IV) (Ruzicka et al., 2007; Roy-Zokan et al., 2015). The members of subclass I, which includes ADF1 through ADF4, are expressed at a relatively high level in all plant tissues except pollen.

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Subclass II is specifically expressed in polar cells; for example, ADF7 and ADF10 are specifically expressed in pollen grains, whereas ADF8 and ADF11 are localized to root epidermal cells. Subclass III (ADF5 and ADF9) and subclass IV, which contains only ADF6, are universally distributed in plants, although the expression levels of subclass III and IV ADFs are lower than those of subclass I ADFs (Ruzicka et al., 2007). The diversity in the tissue expression patterns of ADFs suggests that ADFs have evolved different physiological activities. For example, Kandasamy et al. (2007) demonstrated that simultaneous overexpression of ADF8 and ADF7, which are specifically expressed in Arabidopsis root hair and pollen grains, but not overexpression of vegetative organ-specific ADF9, rescued the deficiency of dwarfing and aberrancies caused by ectopically pollen-specific ACTIN1 expression.

Plant ADFs display conserved amino acid homologies and key functional sites that are unique to the ADF protein family, and most reported ADFs can sever or depolymerize actin filaments. For example, ADF1, ADF2, ADF4, and ADF7 of Arabidopsis, ADF3 of maize (*Zea mays*), and ADF1 of lily (*Lilium longiflorum*) possess these conserved biochemical activities in vitro (Allwood et al., 2002; Maciver and Hussey, 2002; Ren and Xiang, 2007; Zheng et al., 2013), and their activities are typically increased at elevated pH. Interestingly, ADF9 from Arabidopsis showed actin-bundling and actin-stabilizing activities in vitro, especially at lower pH, that were absolutely distinct from the activities of other conserved ADF family proteins (Tholl et al., 2011). In addition, it has been noted that the activities of ADFs from various organs or plant species may differ slightly. For instance, the capacity of pollen-specific ADF7 from Arabidopsis to sever and depolymerize actin filaments is weaker than that of ADF1, which is expressed in vegetative organs (Zheng et al., 2013). Moreover, compared with vegetative organ-specific ADF3 from maize, pollen-specific ADF1 from lily has higher affinity for, but weaker depolymerization activity against, F-actin (Smertenko et al., 2001). In summary, plant ADFs show broad diversity not only in their tissue expression patterns but also in their biochemical properties, suggesting that ADF genes in plants were duplicated from a common ancestral gene and that they have evolved in a divergent manner over the course of plant diversification. However, the manner in which the multiple functions of ADFs evolved and the key amino acid mutations that caused their divergence in biochemical function are still unclear.

To better understand the mechanisms of functional divergence among ADF proteins and identify key sites associated with their diverse biochemical functions, we performed phylogenetic analysis to describe the evolution of ADF family members across plant lineages. Furthermore, we constructed the most relevant putative ancestral genes to elucidate the ancient origin of Arabidopsis ADFs and to identify the mechanisms by which amino acid substitutions have occurred in recently evolved ADF proteins to change their biochemical functions. The findings of this study show how the opposing and diverse biochemical properties of plant ADFs were acquired via key amino acid changes throughout evolution.

RESULTS

The Biochemical Properties of Arabidopsis ADFs Show Significant Variation

At present, the biochemical properties of most plant ADFs remain unknown. To determine whether the biochemical functions of ADFs have varied throughout evolution, the biochemical properties of all 11 members of the ADF family in Arabidopsis were characterized by high-/low-speed cosedimentation assays and fluorescence microscopy. In general, actin filaments are sedimented after high-speed centrifugation (100,000*g*), although some can remain in the supernatant (Supplemental Figure 1). Typically, ADFs sever or depolymerize F-actin into either shorter fragments or G-actin; thus, when ADFs are added to a high-speed cosedimentation assay, the actin content in the supernatant will increase. To evaluate the capacities of various ADFs to sever or depolymerize F-actin, high-speed cosedimentation assays were performed, and the actin content within the supernatant was quantified (Supplemental Figures 1 and 2). From this, the relative F-actin-severing/depolymerizing activity levels were calculated. As shown in Figure 1, the majority of ADF family members (except ADF5 and ADF9) had the conserved ability to sever or depolymerize actin filaments, especially at pH 7.4. Arabidopsis subclass I ADFs (ADF1–4) exhibited the highest F-actin-severing/depolymerizing activity, whereas subclass III ADFs (ADF5 and ADF9) appeared to be completely lacking in activity. These results are consistent with previous reports on the activity of ADF1, ADF4, ADF7, and ADF9 (Henty et al., 2011; Tholl et al., 2011; Zheng et al., 2013).

A low-speed cosedimentation assay at a centrifugal force of 13,500*g* can be used to separate single actin filaments and actin filament bundles. To evaluate the capacity of the various ADFs to bundle F-actin, low-speed cosedimentation assays were performed, and the actin content of the resultant sediment was quantified (Supplemental Figures 3 and 4). From this, the relative F-actin-bundling activity was calculated. As shown in Figure 1, only ADF5 and ADF9 bundled F-actin, especially at pH 6.6; this result is in agreement with previous reports concerning ADF9 (Tholl et al., 2011).

To validate the results of the above experiments, the activities of ABP29 and WLIM1, two other well-documented actin-severing and -bundling proteins, were also evaluated in the same assays (Supplemental Figures 1 to 4). The activities of ABP29 and WLIM1 were shown to be in accordance with previous reports (Xiang et al., 2007; Papuga et al., 2010). In addition, to further confirm the results, fluorescence microscopy was used to directly observe actin filaments after the addition of ADFs, ABP29, or WLIM1. As shown in Supplemental Figure 5, both the length of the actin filaments and the number of actin bundles present were consistent with the results obtained by the cosedimentation assays. Taken together, these findings indicate that the biochemical properties of various Arabidopsis ADFs differ greatly. The biochemical functions of these ADFs can be divided into opposing categories: (1) D-type (depolymerizing F-actin) activity, defined as the capacity to sever or depolymerize F-actin, which is possessed by all ADF members of subclasses I, II, and IV; and (2) B-type (bundling F-actin) activity, defined as the capacity to bind to and bundle F-actin, which is

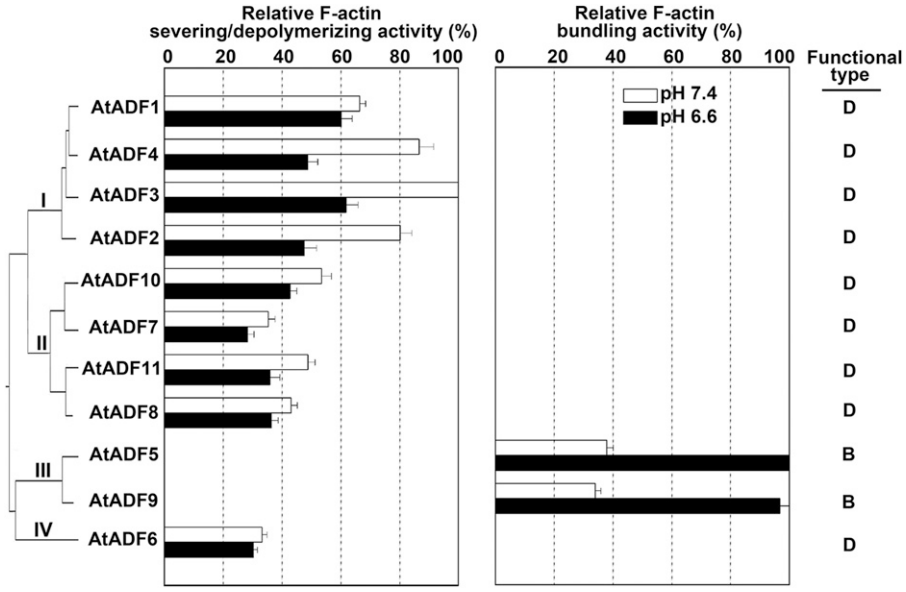

Figure 1. Relative F-Actin-Severing/Depolymerization and -Bundling Activities of 11 AtADFs at pH 6.6 and 7.4.

The F-actin-severing/depolymerizing and -bundling activities of AtADFs were determined using high-/low-speed cosedimentation assays at pH 6.6 and pH 7.4. The severing/depolymerization activity of 6 μM ADF3 at pH 7.4 was used to normalize the activity of all other AtADFs at pH 6.6 and 7.4; the resulting activities ranged from 0 to 100%. The bundling activity of AtADF5 at pH 6.6 was used to normalize the activity of all other AtADFs at pH 6.6 and 7.4; the resulting activities ranged from 0 to 100%. Functional type D indicates a protein having only severing/depolymerizing activity, whereas functional type B indicates a protein having only bundling activity. The data are presented as the mean ± SD from at least three replicate assays.

possessed by subclass III ADFs (ADF5 and ADF9). Interestingly, the F-actin-severing/depolymerizing activity of subclass I ADFs is stronger than that of other D-type ADFs. Moreover, the F-actin-severing/depolymerizing activity of D-type ADFs is enhanced at high pH, whereas the capacity of B-type ADFs to bundle F-actin is increased at low pH.

Phylogenetic Analysis of the ADF Gene Family in Plant Lineages

To evaluate the evolutionary history of the ADF gene family in plants, phylogenetic analysis of all ADF variants from nine plants was performed (Supplemental Table 1). The ADF variants clustered into four subclasses (I–IV) (Figure 2) in angiosperms. The moss *Physcomitrella patens* had a single ADF variant grouped outside the four subclasses, and two variants of ADF from *Selaginella moellendorffii* were basal to subclass I/II and to subclass III/IV (Figure 2), respectively. In each subclass, the ADF genes from eudicot and monocot plants were clustered together.

In Arabidopsis, ADFs have two introns. The first intron of all ADFs, except ADFs 5, 6, and 9, displays an unusual pattern beginning after the ATG codon. Importantly, the first intron in Arabidopsis ADFs affects their transcriptional expression because it contains putative enhancer motifs (Ruzicka et al., 2007; Rose et al., 2008). We analyzed the gene structures of all ADFs from nine plants. As shown in Figure 2, PpADF has no introns; SmADF2 has one intron, whereas SmADF1 has two introns, the first of which starts after the ATG codon. The intron patterns of ADFs from other plant species are similar to those of AtADFs. For instance, most subclass I and II ADFs have two introns, and the first intron begins after the ATG codon (35/40). In contrast, most subclass III and IV ADFs have two introns; in these ADFs, the first exons are longer and the lengths of the first exons are unequal (20/21). By comparing the coding sequences of the ADF genes with the genomic sequences, we deduced that the altered N-terminal amino acid residues in subclass III and IV ADFs may have arisen from an intron-sliding event (Rogozin et al., 2000; Lehmann et al., 2010). In the above subclass of ADFs, the conserved splicing sites (GT) after the ATG codon were changed, which led to spicing events that occurred at the next splicing sites (GT). The results also suggest that such intron-sliding events were conserved during the evolution of angiosperm plants.

The Potential Duplication History of ADF Genes in Arabidopsis

Chromosomal location analysis showed that the 11 ADF genes in Arabidopsis are distributed among five chromosomes. The gene pairs with synteny are located in a pair of paralogous blocks distributed on different chromosomes (Supplemental Figure 6) and could be defined as direct results of whole-genome or random duplication events (Raes et al., 2003; Zeng et al., 2014). ADF1/2 and ADF3/4 are arranged in tandem, as shown by the observation that the two gene pairs are located <200 kb apart on the same chromosome. For ADF6, located on chromosome 2, no similar duplicated block with other ADF gene segments was found. Summarizing the results of phylogenic analysis of ADF genes (Figure 2), all ADF genes potentially descended from a single

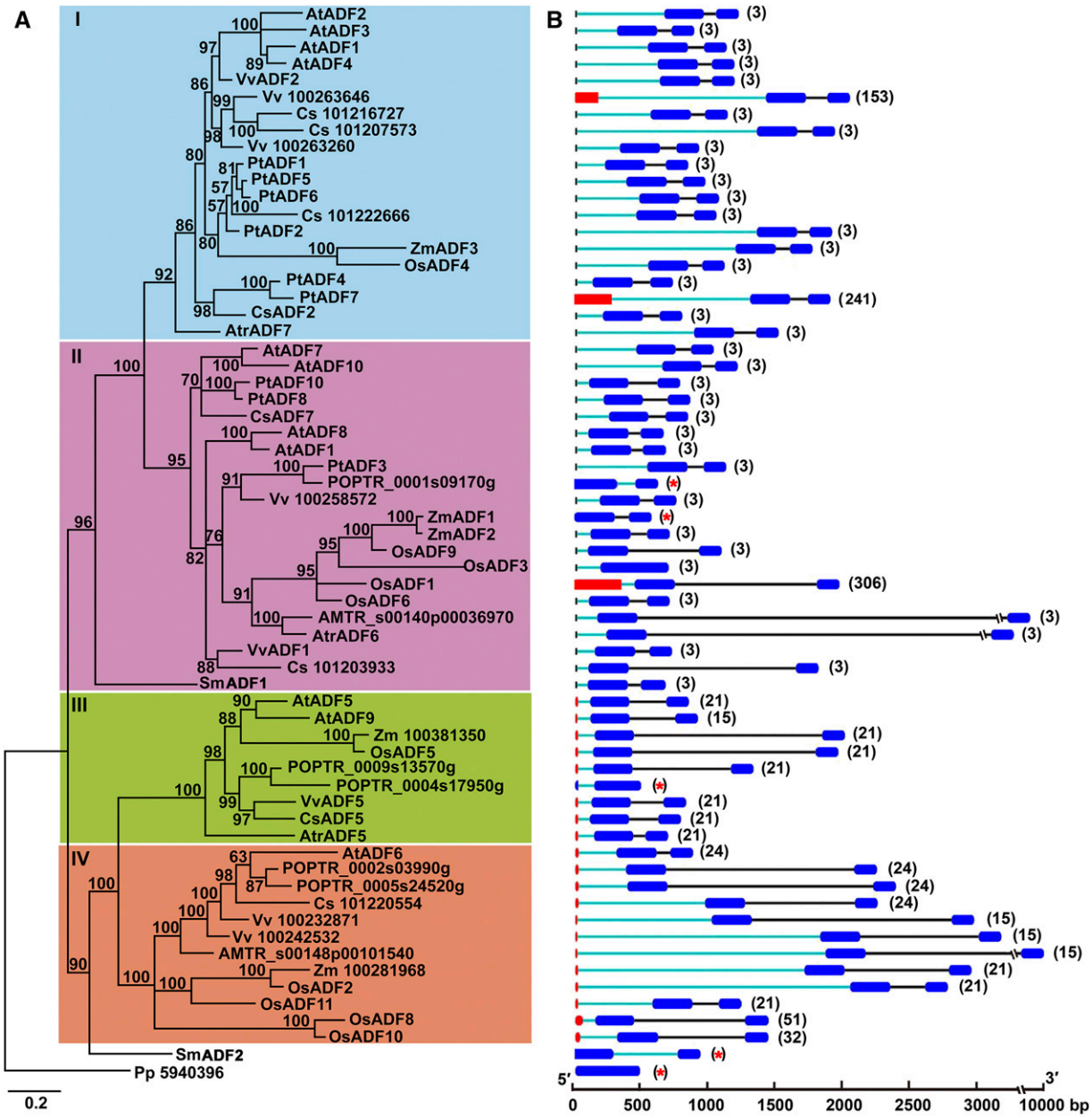

Figure 2. Phylogenetic Analysis of ADF Gene Subclasses in Plants under the Bayesian Information Criterion.

(A) Phylogenetic analysis of ADF gene subclasses in plants under the Bayesian information criterion. The four subclasses of ADFs are highlighted in differently colored boxes. Species abbreviations are as follows: At, *Arabidopsis thaliana*; Atr/AMTR, *Amborella trichopoda*; Pt/POPTR, *Populus trichocarpa*; Zm, *Zea mays*; Os, *Oryza sativa*; Vv, *Vitis vinifera*; Cs, *Cucumis sativus*; Sm, *Selaginella moellendorffii*; and Pp, *Physcomitrella patens*. *PpADF* was used as an outgroup, and the numbers on the branch nodes of the phylogenetic tree refer to posterior probabilities.

(B) Schematic diagram of ADF gene structures. In Arabidopsis, all ADF variants contain three exons. The first intron is marked by a light blue line, and the second intron is marked by a black line. The first exon, which only contains the ATG codon, is marked by a black box, the first exon displaying an N-terminal extension due to intron sliding is marked by a red box, and the second and third exons are marked by blue boxes. Lines and boxes are proportional in length and represent the full-length genomic sequences of ADFs. The numbers in the bracket represent the length of the first exon in the ADF gene; the red asterisks show that the conserved exon pattern of ADFs are lacking. The scale bar represents 500 nucleotides.

common ancestor, and whole-genome duplication, random duplication, and tandem duplication could have been responsible for the expansion of the ADF gene family. Furthermore, we estimated the evolutionary frequencies of these homologous genes using Ks (synonymous [silent] substitution rates) as the proxy (Supplemental Table 2). The results showed that the ADF genes

can be grouped into four ancient subclasses that have been conserved in angiosperms for ~250 million years; within each subclass, the gene pairs began to differentiate between 271.3 (ADF7/8) and 35.79 (ADF3/4) million years ago. Most of the gene pairs diverged after the predicted origin of angiosperms (~150 million years ago) (Lawton-Rauh et al., 2000) and subsequent gene duplication.

Potential Selection among Subclasses of ADF Genes

To evaluate the potential effects of natural selection on ADF genes, we used the branch and sites models provided in PAML package version 4.4 (Yang, 2007). The results showed that selective pressure on the four subclasses of ADFs differed significantly ($P < 0.001$). Under the two-ratio model, the ω values for ADF gene subclasses I, II, III, and IV ranged from 0.00193 to 0.09357, indicating that all ADFs in Arabidopsis were under purifying selection (Supplemental Table 3). To detect whether directional selection acted on the branches and sites leading to the F-actin-bundling function, random effects likelihood and Bayes Empirical Bayes were employed to detect periodic diversifying selection, MEDS (model of episodic directional selection) was employed to detect directional selection acting on specific branches and sites, and DEPS (directional evolution of protein sequence) and FADE (FUBAR approach to directional evolution) (Kosakovsky Pond and Frost, 2005; Kosakovsky Pond et al., 2008; Murrell et al., 2012) were used to detect directional selection acting on sites in a phylogeny. However, these results showed that no amino residues with significant selection signals were detected by at least two tests (Supplemental Table 4).

The Ancestor of Plant ADFs Evolved D-Type Biochemical Function

To explore the evolution of the biochemical functional divergence of ADFs in Arabidopsis, a maximum likelihood model for phylogenetic analysis was used, particularly for the sites with a high posterior probability (PP) (Supplemental Figures 7 and 8). The 10 putative ancestral proteins at critical nodes on the tree were reconstructed (Figure 3A), and the biochemical properties of these proteins were identified via high- and low-speed cosedimentation assays. As shown in Figure 3B and Supplemental Figures 9 to 11, the putative ancestors ancADF-A-I were classified as D-type. ancADF-J was classified as B-type because it could bundle but not sever/depolymerize F-actin. Importantly, in *P. patens*, only one PpADF was identified; it was classified as D-type, and its biochemical properties and activity were similar to those of ancADF-A. Moreover, two ADFs in *S. moellendorffii* were also characterized as D-type. These results suggest that the prediction of the most putative ancestral protein is reliable. Compared with their putative ancestral proteins, ADFs in flowering plants have diversified widely in terms of biochemical function. Most ADFs completely retain the typical D-type function of their ancestors, whereas the remaining ADFs, characterized as B-type, have completely lost the conserved function and have undergone neofunctionalization. Compared with subclass I and II ADFs, the N termini of the reconstructed ancestral ADFs contain seven additional amino acids that are mainly based on ADF5, ADF6, and ADF9. To eliminate the influence of N-terminal extension in ancADF-A-H, we constructed the mutations ancADF-A'-H', in which the 7 N-terminal amino acids were deleted (Supplemental Figure 8). We found that the biochemical activities of ancADF-A'-H' are the same as those of ancADF-A-H. Thus, the N-terminal extension of seven amino acids does not influence the F-actin-severing/depolymerizing activity of ancADFs (Supplemental Figures 12 and 13).

Several Crucial Sites Are Responsible for the Divergent Biochemical Functions among Arabidopsis ADFs

To explore the mechanisms underlying the differences in biochemical function among Arabidopsis ADFs, we focused on variations in key functional sites in ancient ADFs that directly resulted in the conversion of the original D-type function to B-type function. For this purpose, forward mutation was performed on ancient ADFs, and reverse mutation was performed on modern ADFs.

First, ADF1 and ADF5, which share high sequence identity but have opposing biochemical characteristics, were analyzed to identify domains or sites crucial for differences in function. To accomplish this, we generated different ADF1 and ADF5 variants and compared their biochemical characteristics in vitro (Supplemental Figure 14A). As shown in Supplemental Figures 14B and 14C, unlike ADF5, ADF5$\Delta N_{1\text{-}32}$, and ADF1$_{1\text{-}28}$-ADF5$_{33\text{-}143}$ did not efficiently bundle actin filaments, which suggests that the opposing biochemical characteristics of ADF1 and ADF5 may result from differences in their N-terminal domains. The results also suggest that the N-terminal domain of ADF5 is essential for its ability to bundle F-actin. The biochemical data indicated that ancADF-I could be the key node for functional differentiation. Thus, ancADF-I was selected to identify crucial amino residues driving functional alteration. According to the above results, we mainly focused on the N terminus of ancADF-I and created four mutations, ancADF-I2-5, within its N terminus. The mutated sites were similar among D-type ADFs and ancADFs and differed among B-type ADFs and ancADFs. In ancADF-I2, hydrophobic Ala (PP = 0.455) and Val (PP = 0.996) at positions 16 and 17 were replaced by hydrophilic Trp (PP = 0.187) and Met (PP = 0.051), respectively, based on the same sites in ADF5 and ADF9 (Supplemental Figure 8 and Supplemental Table 5). In ancADF-I3, hydrophilic Asn (PP = 0.985) at position 10 was replaced by hydrophobic Leu (PP = 0.189). ancADF-I4 and I5 involve the replacement of noncharged Asn (PP = 0.985) and Thr (PP = 0.490) at positions 10 and 23 with positively charged Lys (PP = 0.002) and Lys (PP = 0.111), respectively, based on the same sites in ADF5 and ADF9 (Supplemental Figure 8 and Supplemental Table 5). As shown in Figure 4A and Supplemental Figure 15, ancADF-I1-5 display the same biochemical activities.

In terms of sequence alignment, the N-terminal sequences of ADF5, ADF9, and ancADF-J, which all possess B-type function, contain two additional amino acids compared with other D-type ADFs and ancADFs; the additional amino acids are F5K6, L3K4, and L8K9, respectively (Supplemental Figure 8). Moreover, the 13th, 19th, and 16th residues of ADF5, ADF9, and ancADF-J, respectively, were changed to K/R, adding a positive charge (Figure 4A; Supplemental Figure 8). The mutant ancADF-I1 m

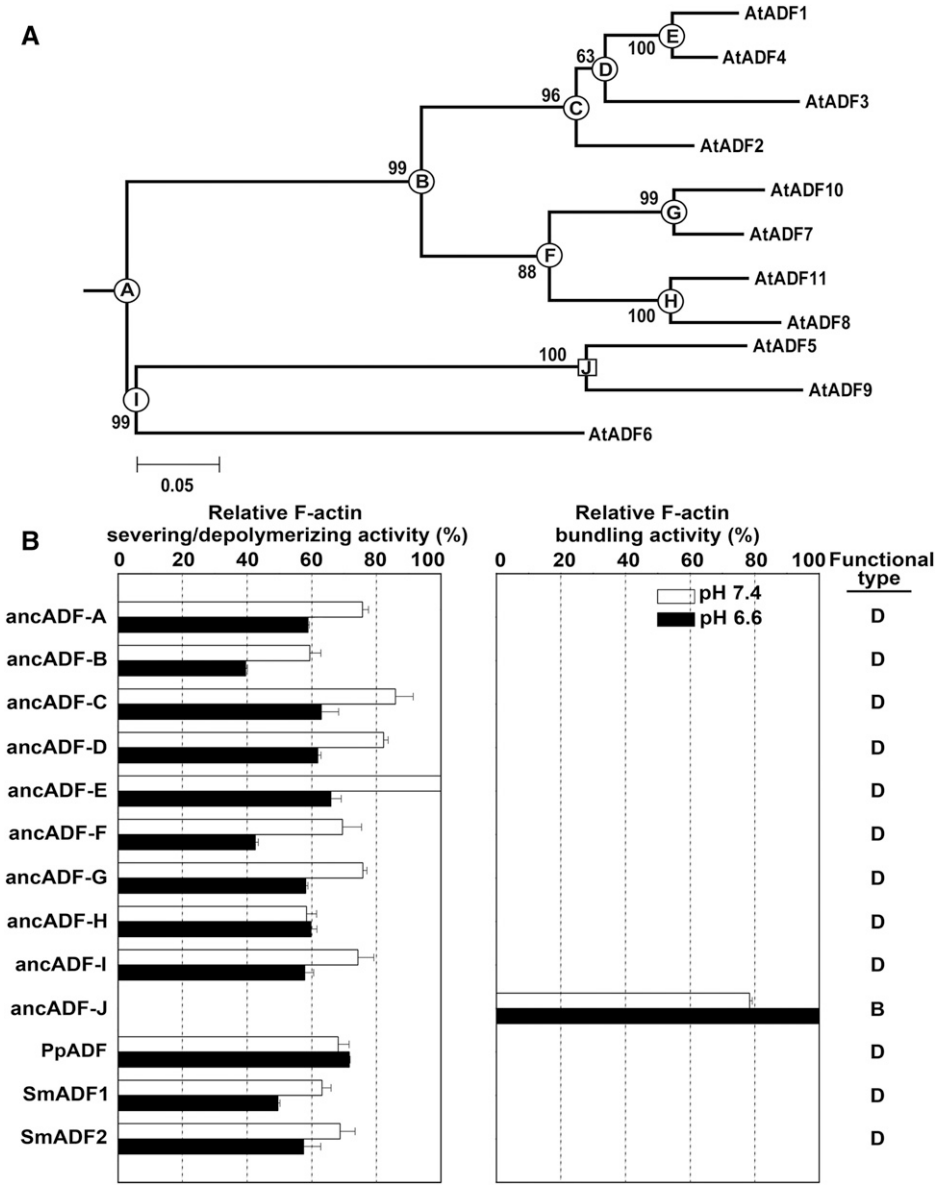

Figure 3. Relative F-Actin-Severing/Depolymerization and -Bundling Activities of 10 Putative ancADFs Corresponding to AtADFs at pH 6.6 and 7.4.

(A) Reconstruction of the most recent common ancestral protein of each subclass of ADFs in Arabidopsis. Bootstrap percentages >50 are shown next to the corresponding nodes. Nodes A to J refer to the recent common putative ancestral proteins.

(B) The actin-severing/depolymerization and -bundling activities of ancADFs at pH 6.6 and pH 7.4 were determined using high-/low-speed cosedimentation assays. The severing/depolymerization activity of 6 μM ancADF-E at pH 7.4 was used to normalize the activity of all other ancADFs at pH 6.6 and 7.4; the resulting normalized activities ranged from 0 to 100%. The bundling activity of ancADF-J at pH 6.6 was used to normalize the activity of all other ancADFs at pH 6.6 and 7.4; the normalized activities ranged from 0 to 100%. PpADF and SmADFs were used as a positive control. Functional type B indicates a protein having only bundling activity, whereas functional type D indicates a protein having only severing/depolymerizing activity. Data are presented as the mean ± SD from at least three replicate assays.

(ancADF-I1 N10LKT. . .T23K) was generated by inserting Leu and Lys into the original site of the 10th amino acid of ancADF-I; the 23rd residue of this protein was also changed from Thr to Lys. As a result of these mutations, the F-actin-severing/depolymerizing capacity of ancADF-I was lost, and its activity appeared similar to that of ADF9. In contrast, the mutants ADF9 m1 (ADF9 K4A) and ADF9 m2 (ADF9 K18A) showed weaker F-actin-bundling activity than ADF9, and the mutant ADF9 m3 (ADF9 K4A. . .K18A) exhibited significantly decreased F-actin-bundling function (Figure 4A; Supplemental Figure 15). These results show that alterations in these three key amino acid residues resulted in conversion to B-type function over the process of

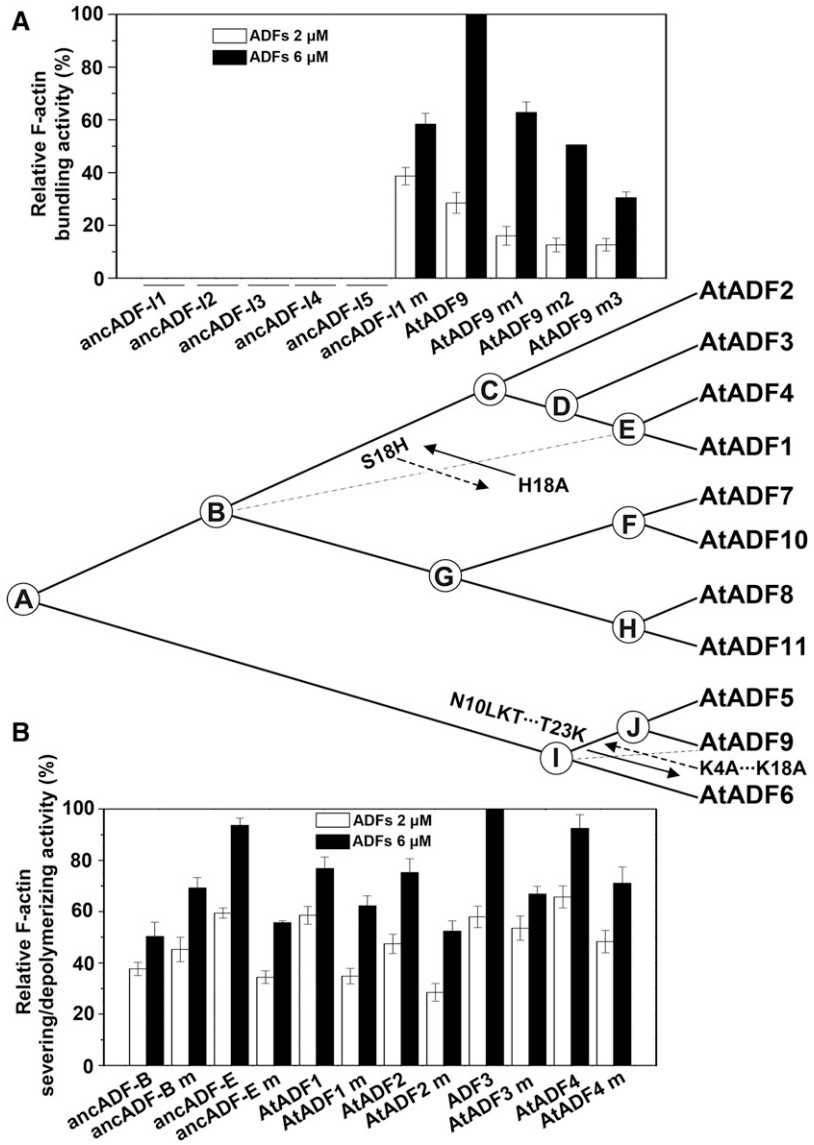

Figure 4. The Putative Fate of ancADF Activity after Functional Mutational Shifts.

Relationships among Arabidopsis ADFs and putative ancADFs, simplified from the illustration shown in Figure 3A.
(A) and **(B)** F-actin-severing/depolymerization or -bundling activities of forward mutants of ancADFs or reverse mutants of current ADFs were determined.
(A) The ancADF at node I had no actin-bundling activity but subsequently evolved into ADF9, which possesses F-actin-bundling activity at pH 7.4. This change was concomitant with the replacement of Asn at position 10 and Thr at position 23 by Leu, Lys, and Thr at positions 2 to 4 and Lys at position 18, respectively. ancADF-I2 involved the replacement of Ala and Val at positions 16 and 17 by Trp and Met, respectively; ancADF-I3 involved the replacement of Asn at position 10 by Leu; ancADF-I4 and I5 arose from the replacement of Asn and Thr at positions 10 and 23 by Lys, respectively; ancADF-I1 m involved the replacement of Asn by Leu, Lys, and Thr at position 10 and Thr at position 23 by Lys, respectively; ADF9 m1 involved the replacement of Lys by Ala at position 4; ADF9 m2 involved the replacement of Lys by Ala at position 18; and ADF9 m3 involved the replacement of Lys by Ala at positions 4 and 18.
(B) ancADF at node B appears to have low F-actin-severing/depolymerization activity but subsequently evolved to possess increased activity, as shown for node E. This change was partly concomitant with the replacement of Ser at position 18 by His. ancADF-B m involved the replacement of Ser at position 18 by His, ancADF-E m arose from the replacement of His at position 18 by Asn, and ancADF-1-4 m arose from the replacement of His at position 11 by Asn. Data are presented as the mean ± SD from at least three replicate assays.

evolution from ancient ADFs. Because ancADF-B and ancADF-I are D-type ADFs and the biochemical functions of AtADF6 and ancADF-J have been differentiated, we speculate that ancADF-I was the key node for functional differentiation.

The F-actin-severing/depolymerizing activity of ancADF-C/D/E was higher than that of other ancient ADFs. Similarly, the F-actin-severing/depolymerizing capacities of subclass I Arabidopsis ADFs were higher than those of other D-type ADFs. In addition, we

found that the mutant ancADF-E m (ancADF-E H18A) had reduced F-actin-severing/depolymerizing activity and, based on sequence alignment, that this His site was conserved only in ancADF-C-E and ADF1-4 (Figure 4B; Supplemental Figure 16). Interestingly, the F-actin-severing/depolymerizing activities of mutants ADF1-4 m (ADF1-4 H11A) were dramatically reduced, whereas the mutant ancADF-B m (ancADF-B S18H) did not show enhanced severing activity (Figure 4B; Supplemental Figure 16). These results demonstrate that mutation of the His site occurred when the ancient gene ancADF-B evolved to form ancADF-C. Due to this mutation, the F-actin-severing/depolymerizing activity of subclass I Arabidopsis ADFs was enhanced.

The basic residues found in β-sheet 4, β-sheet 5, α-helix 3, and α-helix 4 are conserved in several ADF/cofilin genes and are important for F-actin binding activity (Jiang et al., 1997; Lappalainen and Drubin, 1997; Pope et al., 2000; Dong et al., 2013). According to published reports, β-sheets 4 and 5 and α-helix 4 of ADF are in spatial proximity to one another and likely form an essential F-actin binding surface (F-surface 1) with a relatively high level of conservation of key basic residues (Lys-86, Lys-88, and Arg-141) (Figure 5A) (Lappalainen and Drubin, 1997; Bowman et al., 2000; Pope et al., 2000). In addition, the N terminus and the long α-helix 3 found on ADFs may form another F-actin binding surface (F-surface 2) (Figure 5A) (Ressad et al., 1998; Mannherz et al., 2007). As shown in Figure 5B, the N-terminal amino acids that regulate the actin bundling activity of ADF5, ADF9, and ancADF-J are located in F-surface 2. Furthermore, amino acid His, which enhances the actin-severing activity of subclass I ADFs and ancADF-C/D/E in Arabidopsis, is also present in F-surface 2. Therefore, changes in these amino acids might affect the capacity of an F-surface to bind to actin, thereby altering the biochemical properties of the ADFs.

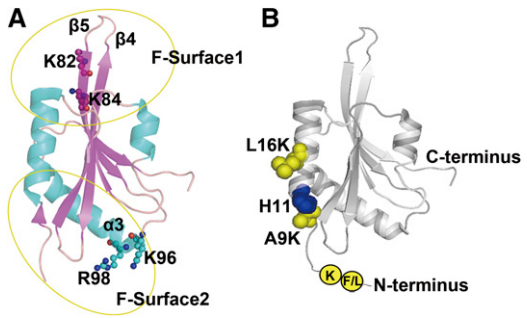

Figure 5. Mapping of Hot Spot Residues Responsible for the Functional Divergence of ADF Proteins onto the Crystal Structure of ADF1 (PDB Code 1F7S).

(A) The two previously proposed F-actin binding surfaces, respectively labeled as surfaces F1 and F2, are circled, and the side chains of the key residues involved in F-actin binding are shown in ball-and-stick format.

(B) The residues responsible for the enhanced actin-bundling activity of certain ADFs are shown in yellow spheres, and the residue responsible for the enhanced actin-severing activity of certain ADFs is shown in a blue sphere. All structural images were prepared using PyMOL (DeLano).

The Key Sites Affecting the Biochemical Functions of ADFs Are Somewhat Conserved among Various Plant Species

To determine whether the crucial sites involved in the above-mentioned functions of ADFs have been conserved throughout plant evolution, ADF subclasses I and III were further compared among plant species (Figure 6A; Supplemental Figure 17A). The His residue at site 11 was extremely well conserved among ADF subclass I members, and this key site enhanced the actin severing/depolymerizing activity of these proteins. Interestingly, almost all members of ADF subclass I in other species possessed a His residue at the same site. Moreover, the Lys residue at site 4 and the Lys/Arg residue at site 18 (refer to ADF9) in the sequences of ADF subclass III members in Arabidopsis are two pivotal sites. These two sites are highly conserved among different species, especially the Lys at site 4 (refer to ADF9) (Figure 6A; Supplemental Figure 17A). Moreover, two ADFs categorized into different subclasses in rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) were randomly selected, and both of these ADFs possessed similar functions to the corresponding ADF subclass members in Arabidopsis (Figure 6B; Supplemental Figure 17B). Thus, the mechanism leading to the differentiation of the biochemical functions of ADFs in plants appears to have been consistent over the course of evolution.

DISCUSSION

Multiple Retention Mechanisms Involved in the Evolution of ADFs

The angiosperm genome has undergone at least two whole-genome duplication (WGD) events throughout evolution (Jiao et al., 2011). Following these WGD events, the genes from a common ancestor formed a gene family in which regions of some duplicated genes became pseudogenes while the remaining genes were retained and either preserved or evolved various functions. These surviving genes have provided an important genetic resource for functional novelty (Flagel and Wendel, 2009). However, the manner in which these duplicated genes have been retained remains a crucial but unsolved problem. The ADF gene family is divided into four conserved subclasses in angiosperms (Ruzicka et al., 2007), and the WGDs described above have likely led to ADF gene duplications. ADF variants were likely retained by dosage selection in early gene duplications. The duplicated ADF variants exhibit multiple expression patterns in response to biotic/abiotic stresses and in different tissues (Supplemental Figure 18), suggesting that they have undergone subfunctionalization (Force et al., 1999). However, in view of the inconsistent mRNA and protein expression levels associated with some genes, further testing and verification is needed. The most recent putative ancestral protein (ancADF-A) had a D-type function similar to that of ADF in *P. patens*, while 9 of the 11 extant ADF genes have maintained D-type function, and two genes (*ADF5* and *ADF9*) in subclass III have evolved to possess B-type function. These findings suggest that ADF variants have undergone neofunctionalization during the evolution of Arabidopsis. Taken together, multiple gene retention mechanisms may have been

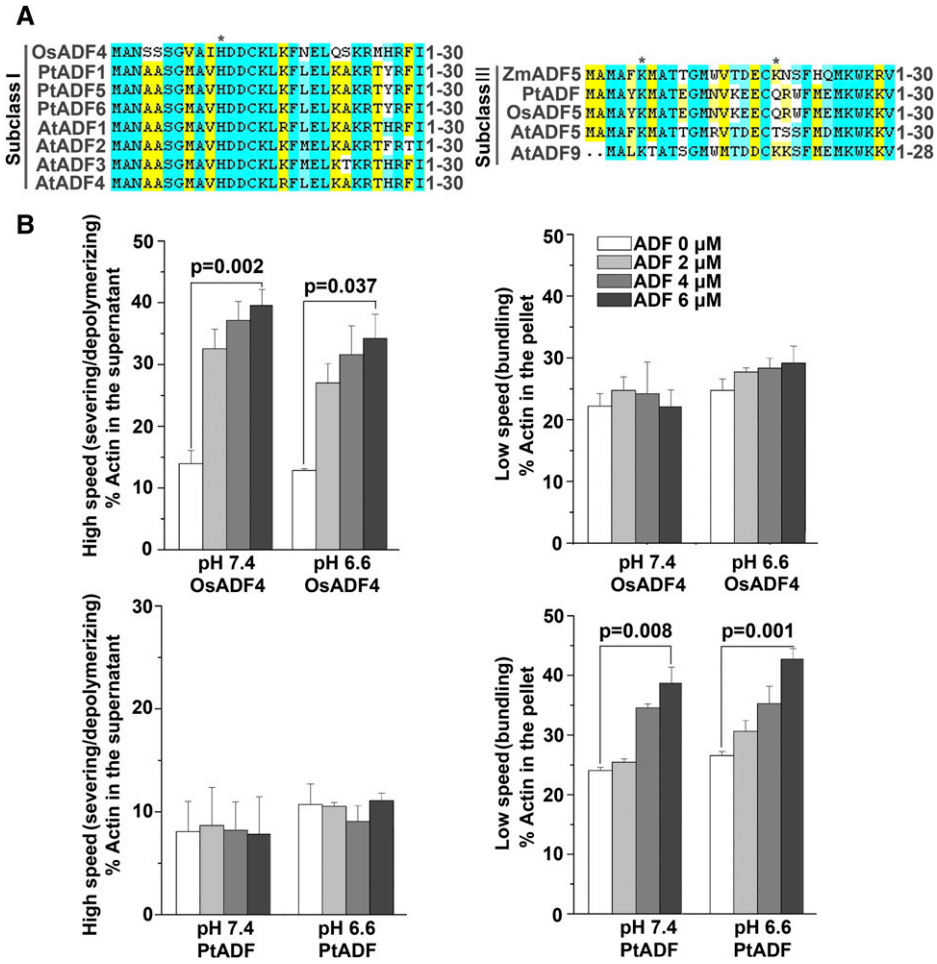

Figure 6. Alignment of Key Basic Residues and Functional Verification of Subclass I and III ADFs from Different Plant Species.

(A) Alignments of ADFs between different species were generated using DNAMAN. The asterisks indicate certain key conserved amino acid residues that are crucial sites for ADF function. At, Arabidopsis; Os, *O. sativa*; Pt, *P. trichocarpa*; and Zm, *Z. mays*. PtADF indicates POPTR_0009S13570g.
(B) Functional verification of predicted key basic residues of subclass I and III ADFs from rice and *P. trichocarpa* based on high-/low-speed cosedimentation assays. Values plotted are means, and the error bars represent SD, $n = 3$. At least three independent experiments were performed.

involved in the evolution of ADFs, which increased the chance of ADF genes being retained.

ADFs Have Divergent Functions in Plant Growth and Development

ADFs are involved in the formation of the actin cytoskeleton in all eukaryotic cells through their interactions with actin, the isoforms of which also show divergent expression patterns (Kijima et al., 2016). Based on the evolution of actin (Gunning et al., 2015), ADF gene duplication and subsequent sub- or neofunctionalization in plants have contributed to the ability of the ADF proteins to interact with actin to perform diverse physiological activities by regulating actin cytoskeletal architecture and the dynamic turnover of actin filaments. Furthermore, the divergence in biochemical function and tissue differentiation among Arabidopsis ADFs has also specifically contributed to the process of plant development and adaption. Subclass I ADFs are the most highly expressed and most broadly distributed in various tissues, and their capacities to sever/depolymerize F-actin are greater than those of other D-type ADFs, as shown both here and by the work of Ruzicka et al. (2007) and Zheng et al. (2013). The lack of or overexpression of AtADF1 induces deficiencies in the growth, development, and morphogenesis of plants (Dong and Hong, 2013). Loss-of-function mutations in *AtADF4* lead to the formation of longer hypocotyls in seedlings and reduced rates of actin bundling and actin turnover (Henty et al., 2011). Recently, AtADF4 has been shown to act as a substrate of casein kinase 1-like protein 2 and to play a role in ABA- and drought-induced stomatal closure (Zhao et al., 2016). Moreover, the defective Arabidopsis mutant *adf4* displays enhancement of activities that confer resistance to multiple pathogenic bacteria and fungi (Tian et al., 2009; Porter et al., 2012; Henty-Ridilla et al., 2014; Inada et al., 2016), while *AtADF2* contributes to innate immunity in roundworm (Clément et al., 2009).

These data reveal that subclass I ADFs function to maintain normal plant growth and to confer resistance to various abiotic/biotic stresses.

The F-actin-severing/depolymerizing activity of subclass II ADFs is largely similar to those of the products of their ancient genes, but their gene expression is specifically limited to polarized cells such as pollen and root hairs (Supplemental Figure 18) (Ruzicka et al., 2007). Compared with other plant cell types, polarized cells grow very quickly and grow only at the tips. Given that pollen germination and pollen tube growth are two crucial steps in the sexual reproduction of flowering plants and that root hairs in plants play a key role in nutrient absorption, flowering plants have specifically evolved subclass II ADFs to accurately regulate the actin structures in these cell types. Recently, it was shown that in the Arabidopsis loss-of-function mutant *adf7*, pollen tube growth is significantly suppressed, primarily due to the effect of reduced F-actin severing/depolymerizing activity on actin turnover in the pollen tube (Zheng et al., 2013).

F-Actin-Bundling Function Is Conserved and Specific in Plant Lineages

In animals and yeast, the homologous ADF genes possess a simple F-actin-depolymerizing function, while F-actin bundling is performed by other proteins (Stagier and Blanchoin, 2006). However, in plants, ADF genes conferring B-type function were produced from ancient D-type genes, suggesting that the independent retention and diversification of F-actin-bundling function was important for the diversification and development of the plant lineage. In Arabidopsis, ADF5 and ADF9 have B-type function, and their tissue-specific gene expression patterns appear to have diverged (Supplemental Figure 18) (Ruzicka et al., 2007; Tholl et al., 2011). AtADF5 is expressed in whole organs at a level similar to subclass I ADFs; in contrast, AtADF9 is nearly exclusively expressed in callus tissue and flowers (Ruzicka et al., 2007). AtADF9 deficiency has a marked effect on callus growth, and its gene expression can be increased by many plant hormones. Additionally, a loss-of-function mutation in *AtADF9* caused the formation of scrubby seedlings and skimpy lateral branches as well as early flowering (Burgos-Rivera et al., 2008). Although there is no direct evidence for a relationship between this phenotype and turnover of actin structure, it has been speculated that AtADF9 functions as a transcription factor to regulate the expression of *FLOWERING LOCUS C* (Burgos-Rivera et al., 2008). Moreover, it has been demonstrated that the expression of subclass III Arabidopsis ADF genes can be specifically induced by low or high temperatures, whereas other ADFs lack this feature (Fan et al., 2015, 2016). Similarly, the biochemical functions of subclass III Arabidopsis ADF genes are regulated by acidic conditions, while classical D-type ADFs are strongly activated at high pH (Figure 1; Supplemental Figure 1 to 4). In addition, there is evidence that AtADF9 and AtADF1 are mutually antagonistic in vitro (Tholl et al., 2011). One possible explanation for these findings is that oscillations or alterations in pH or other physiological activities in plant cells caused subclass III Arabidopsis ADFs to evolve new protein functions and regulatory mechanisms. Unfortunately, little is known about the physiological and biochemical functions of this ADF subclass in Arabidopsis.

The Gene Structure of Plant ADFs Shows a Conserved Pattern among Plant Species

Intron-sliding events have been a major force in diversifying intron positions among eukaryotic homologs (Fedorov et al., 2002; Rogozin et al., 2003; Carmel et al., 2007; Lehmann et al., 2010) and have led to changes in eukaryotic gene structure. Such changes have further affected encoded protein sequences and/or post-transcriptional regulation and are thus a potentially important source of genetic novelty (Rogozin et al., 2000; Nielsen et al., 2010). In angiosperm plants, ADF gene structures show diversity in intron positions between subclasses I/II and subclasses III/IV across species (Figure 2). The novel intron position and pattern in ADF subclasses III and IV could have resulted from intron sliding that produced N-terminal extensions of conserved ADF protein variants. In Arabidopsis, subclass I, II, and IV ADFs are all D-type, whereas ADF5 and ADF9, which have N-terminal extensions, are a neofunctionalized B-type. Importantly, the Leu/Phe/Lys residues that arose from the N-terminal extension of subclass III ADFs are necessary for their F-actin-bundling activity. ancADF-I can be mutated to resemble AtADF9, but the reverse mutations do not produce the opposite effects, such as severing activity, which may be the result of epistasis (Bridgham et al., 2009; Smith et al., 2013; Bartlett et al., 2016). Unfortunately, we could not identify epistasis sites because numerous possible mutation sites are potentially involved in affecting B-type function. In addition, it is well documented that a conserved serine residue at position 6 in plant ADFs plays a vital role in their biochemical function (Jiang et al., 1997; Chen et al., 2002; Dong and Hong, 2013). However, N-terminal extensions lead to the loss of this conserved serine in subclass III and IV ADFs, e.g., this key site is substituted with a Thr residue in ADF5 (Supplemental Figure 8). Taken together, the conservation of ADF gene structure during the evolution of angiosperm plants provided the genetic basis for their functional divergence.

The Potential Role of Mutated Amino Acids in the Activities of B-Type and D-Type ADFs

Divergence in the biochemical functions of proteins is primarily attributable to mutations in their amino acid sequences. It has been reported that ADF has two potential F-surfaces that bind F-actin, one at the N terminus and the other at the C terminus, and that the crucial amino acids responsible for this biochemical function are located at these sites (Tholl et al., 2011). Therefore, it is implied that mutation of the key sites responsible for binding actin might change the actin binding activity of ADFs or induce their functional differentiation. Compared with ancADF-I, ADF5 and ADF9 contain two mutations in F-surface 2. It was confirmed that these two mutated amino acids, which both carry a positive charge, enhance the binding of F-surface 2 to F-actin. In contrast, F-surface 1 could bind to other forms of actin; consequently, ADF5 and ADF9 retain the capacity to bundle F-actin (Figure 5). The potential mechanism driving ADF5/9 actin-bundling activity could be similar to that of LIM, another actin-bundling protein with a small molecular mass (there are 193 amino acids residues in *Nicotiana tabacum* WLIM1) that possesses two well-conserved LIM domains (Thomas et al., 2007; Hoffmann et al., 2014). We also found that N-terminal His-tagged ADF1, ADF11, and ancADF-A-I exhibit F-actin-bundling

activity only at a low pH. Thus, the addition of six positively charged His residues to F-surface 2 could influence the binding of the recombinant ADFs to F-actin (Supplemental Figures 1 to 4). Similar results were obtained in rice and poplar, indicating that the conserved mutation sites related to B-type function have remained fixed since the diversification of the angiosperm lineage.

METHODS

Phylogenetic Analysis

For phylogenetic analyses, nucleotide coding sequences of ADFs from each species were acquired from NCBI (www.ncbi.nlm.nih.gov) and TAIR (www.arabidopsis.org); the accession numbers are listed in Supplemental Table 1, and the protein sequences are listed in Supplemental File 1. All ADF protein sequences were verified using BLASTp (e-value ≤ 1) (Camacho et al., 2009), aligned using ClustalW within MEGA5.2 (Tamura et al., 2011), and adjusted manually to optimize the alignments. For phylogenetic analysis, the MrBayes 3.1 program (Ronquist and Huelsenbeck, 2003) with the GTR + I +G model was used, based on the Akaike information criterion in ModelTest 3.7 (Posada and Crandall, 1998). The trees were rooted by the moss *Physcomitrella patens* ADF. The computational runs were implemented for 10,000,000 generations with a tree sample frequency of every 100 generations, and the first 25% of sampled trees were discarded to allow for "burn-in" of the process.

Tests for selection were conducted among all AtADFs with maximum likelihood codon models in PAML version 4.4 (Yang, 2007). The branch models were used to estimate the ω (dN/dS) ratio differences in selective pressure between the branches, while the site model was used to detect positive selection sites in ADFs. To identify which of the models best fit the data, likelihood ratio tests were performed by comparing twice the difference in log likelihood values between pairs of the models using a χ^2 distribution (Yang et al., 2000). MEDS (model of episodic directional selection), DEPS (directional evolution of protein sequence), and FADE (FUBAR approach to directional evolution), provided by the DataMonkey web server (Kosakovsky Pond and Frost, 2005; Kosakovsky Pond et al., 2008; Murrell et al., 2012), were used to detect potential directional selection acting on specific branches and sites (Bartlett et al., 2016). The chromosomal locations of the 11 AtADF genes were determined using data on gene position, length, chromosome size, and chromosome position obtained from TAIR (http://www.arabidopsis.org/index.jsp). The syntenic regions of Arabidopsis ADFs were downloaded from LegumeIP (Li et al., 2012). To evaluate the timing of ADF gene diversification, the Ks value was calculated for each of the gene pairs and was used to calculate the approximate date of each duplication event ($T = Ks/2\lambda$), assuming a clock-like rate (λ) of synonymous substitution of 6.1×10^{-9} substitutions/synonymous site/year for *Arabidopsis thaliana* (Lynch and Conery, 2000).

To understand how the multiple functions of ADFs evolved, ancestral proteins were reconstructed using the maximum likelihood method (Yang, 2007). The ancestral sequences obtained were similar to those predicted by maximum parsimony methods, particularly for the sites with high posterior probability. To examine the robustness of the predicted sites, those sites along with the codon-based sites estimated from the gene tree were checked.

Plasmid Construction and Recombinant Protein Expression and Purification

To investigate the biochemical basis of ADF function, ADF cDNA was amplified using corresponding forward and reverse primers and then subcloned into the pET30a vector using specific restriction endonuclease sites. To obtain untagged recombinant proteins, His-ADFs were digested with enterokinase overnight at 4°C to cleave the His-tag and further purified using anion exchange chromatography to remove the His-tag and enterokinase. A Multipoints Mutagenesis Kit (Takara) was used to generate ADF mutants. All amplified fragments were confirmed by DNA sequencing. The primers used for cloning are described in Supplemental Data Set 1. All putative ancient genes were synthesized by Genewiz, and their sequences were confirmed by DNA sequencing (Supplemental Data Set 2).

The His-tagged recombinant proteins were purified using previously described methods (Xiang et al., 2007; Jia et al., 2013). The purified proteins were dialyzed overnight against buffer A3 (10 mM Tris-HCl, 0.2 mM $CaCl_2$, 0.2 mM ATP, and 0.5 mM DTT, pH 7.0). Prior to use, the proteins were further purified by centrifugation at 100,000*g* for 1 h. Actin was purified from rabbit skeletal muscle acetone powder using previously published methods (Pardee and Spudich, 1982).

High-/Low-Speed Cosedimentation Assays

A high-speed cosedimentation assay was used to evaluate the F-actin severing/depolymerization activity of the recombinant proteins at various concentrations, as described previously (Xiang et al., 2007). To assess the F-actin-bundling activity of the recombinant proteins, low-speed cosedimentation assays were performed as described by Jia et al. (2013). Depending on the pH used in the cosedimentation assays, the reaction medium was buffered with 7 mM PIPES or 10 mM Tris adjusted to pH 6.6 or 7.4, respectively. The relative amounts of actin in the supernatant and the pellet were quantified by densitometry using ImageJ software.

Direct Observation of Actin Filaments and Bundles via Fluorescence Microscopy

Fluorescence microscopy was used to directly visualize the effects of the recombinant proteins on the organization of actin filaments as described previously (Jia et al., 2013).

Sequence Alignment and Structural Modeling

Alignments of full-length sequences were generated using DNAMAN. A homology model of ADF5 was constructed using SWISS-MODEL (http://swissmodel.expasy.org) based on the crystal structure of ADF1 (PDB ID code 1F7S) (Bowman et al., 2000). The structure was illustrated using PyMOL (version 1.4.1; DeLano Scientific) and VMD software (Humphrey et al., 1996).

Statistical Analysis

Statistical analysis was performed using the Mann-Whitney U-test or Student's *t* test when data were normally distributed. Values plotted are means, and the error bars represent SD ($n = 3$); at least three independent experiments were performed.

Accession Numbers

The accession numbers of the proteins used in this study are shown in Supplemental Table 1.

Supplemental Data

Supplemental Figure 1. F-actin-severing/depolymerization activity of 11 AtADFs and PpADF at pH 6.6 and 7.4.

Supplemental Figure 2. Quantitative analysis of the F-actin-severing/depolymerization activities of AtADFs at pH 6.6 and 7.4.

Supplemental Figure 3. F-actin-bundling activity of 11 AtADFs and PpADF at pH 6.6 and 7.4.

Supplemental Figure 4. Quantitative analysis of the F-actin-bundling activities of AtADFs at pH 6.6 and 7.4.

Supplemental Figure 5. Direct visualization of F-actin-severing/depolymerization and -bundling by AtADFs.

Supplemental Figure 6. Chromosomal distribution of the ADF genes in Arabidopsis.

Supplemental Figure 7. Site-specific posterior probabilities for ancestral amino acid estimates for the ancestral protein.

Supplemental Figure 8. Multiple alignment of AtADFs, PpADF, and putative ancADFs.

Supplemental Figure 9. F-actin-severing/depolymerization activities of the putative ancADFs, PpADF, and SmADFs.

Supplemental Figure 10. F-actin-bundling activities of the putative ancADFs, PpADF, and SmADFs.

Supplemental Figure 11. Quantitative analysis of actin-severing/depolymerization or -bundling activity of ancADFs at pH 6.6 and 7.4.

Supplemental Figure 12. F-actin-severing/depolymerization activity of putative ancADFs and mutants of ancADFs at pH 6.6 and 7.4.

Supplemental Figure 13. F-actin-bundling activity of putative ancADFs and mutants of ancADFs at pH 6.6 and 7.4.

Supplemental Figure 14. N-terminal domain is crucial for ADF5 actin-bundling activity.

Supplemental Figure 15. F-actin-severing/depolymerization and -bundling activity of ADF9, mutants of ADF9, ancADF-I, and mutants of ancADF-I at pH 7.4.

Supplemental Figure 16. F-actin-severing/depolymerization activity of putative ancADF-B, mutants of ancADF-E, ADF1-4, and mutants of ADF1-4 at pH 6.6 and 7.4.

Supplemental Figure 17. Multiple alignments, severing/depolymerization, and bundling activities of subclass I and III ADFs from different plant species.

Supplemental Figure 18. Expression of AtADF genes in different tissues and under various stress treatments.

Supplemental Table 1. Accession numbers for ADF gene sequences used in multiple sequence alignment and phylogenetic analysis.

Supplemental Table 2. Ks-based estimation of the timing of ADF duplication in Arabidopsis.

Supplemental Table 3. CODEML analysis of the selective pattern for Arabidopsis ADFs.

Supplemental Table 4. Amino acid residues under selection, detected using multiple methods.

Supplemental Table 5. Posterior probabilities for original and mutated sites for all mutations.

Supplemental Data Set 1. Primers used in this study.

Supplemental Data Set 2. DNA and amino acid sequences for ancestral ADF genes.

Supplemental File 1. Alignments used to produce the phylogenetic trees in Figure 2.

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AUTHOR CONTRIBUTIONS

Y.X. and D.W. conceived the project and designed the study. Q.N., D.Q., Y.N., Y.Y., and S.T. performed most of the experiments. Y.H. performed the structural analysis and reconstructions. D.W., Z.N., and J.M. performed the phylogenetic analysis and phylogenetic reconstructions. Y.X., D.W., Y.N., Y.H., and Q.N. analyzed the data and wrote the article.

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