INTRODUCTION

The plant actin cytoskeleton structures undergo dynamic rearrangement during the process of plant–pathogen interactions (Porter & Day, 2016; Takemoto & Hardham, 2004). On one hand, growing evidence demonstrates that an increase in the abundance of actin filaments and bundles occurs in host plant cells in response to pathogen attack. The density of filamentous actin (F-actin) arrays is augmented around the infection sites of various plants (Hardham et al., 2007; Takemoto & Hardham, 2004; Underwood & Somerville, 2008). Tobacco BY-2 cells could be infected by the nonpathogenic Erysiphe pisi when the actin structure was disordered (Kobayashi & Hakuno, 2003). A rapid increase in actin filament abundance could be elicited by Pseudomonas syringae pv. tomato DC3000 during the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) response in Arabidopsis thaliana (Henty-Ridilla et al., 2013). On the other hand, the disruption of actin architecture was demonstrated to be crucial for

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the defence response against the pathogen (Leontovyčová et al., 2019). These cellular changes, including rapid increase or disruption of F-actin, have been found to be associated with various processes in PTI, such as salicylic acid (SA)-mediated defence signalling (Henty-Ridilla et al., 2013; Leontovyčová et al., 2019). Thus, the actin cytoskeleton along with altered actin abundance in the defence response is regarded as a novel but conserved component of PTI or a new sensor of pathogen attack (Henty-Ridilla et al., 2013; Leontovyčová et al., 2020). Due to its vital role in the defence process, the host actin cytoskeleton appears to be a target of invading microbes. The *Verticillium dahliae* toxin (VD toxin) has been demonstrated to cause the destruction of actin filaments in a dose-dependent manner in *Arabidopsis* suspension cells (Yuan et al., 2006). Moreover, effectors released from *P. syringae* cause a reduction in the content of F-actin, which leads to the inhibition of endocytosis in host plants (Kang et al., 2014).

The dynamic restructuring of plant actin organization relies on the participation of different types of actin-binding proteins (ABPs). Several ABPs have been demonstrated to play roles in plant defence. For instance, the expression of several profilin genes is induced on pathogen infection in various plants; overexpression of a cotton *profilin* gene, GhPFN2, enhances protection against *V. dahliae*, and the *Arabidopsis* mutant *prf3* is more sensitive to bacterial infection than the wild type (Sun et al., 2018; Wang et al., 2015, 2017). A capping protein in *Arabidopsis* and the actin-related protein 2/3 complex in wheat and tomato have been demonstrated to regulate the actin cytoskeleton (Li et al., 2015; Qi et al., 2017; Sun et al., 2019). The change in the expression level of the actin-binding protein gene GhADF6 was recently reported to be correlated with disease tolerance against *V. dahliae* in cotton and *Arabidopsis* (Ge et al., 2021).

In addition to the above-mentioned ABPs, several studies have shown that the actin depolymerizing factor (ADF), an important regulator in dynamic actin organization, is involved in plant-pathogen interactions. Alteration in ADF expression levels changes disease tolerance in barley, *Arabidopsis*, and wheat (Fu et al., 2014; Henty-Ridilla et al., 2014; Inada et al., 2016; Miklis et al., 2007; Tang et al., 2015; Tian et al., 2009; Wang et al., 2013; Zhang et al., 2017a), and ADF-mediated dynamic remodelling of the actin cytoskeleton is important for defence-related signal perception (Henty-Ridilla et al., 2014). In addition to modulating actin organization, ADFs play other regulatory roles in plant defence responses. The expression of the resistance (R) gene *RPS5* (*Resistance to P. syringae protein 5*) is down-regulated in the *Arabidopsis adf4* mutant, resulting in a defect in effector-triggered immunity (Porter et al., 2012; Tian et al., 2009). Such findings provide solid evidence of the important roles of ADF in plant immune responses. Thus, understanding the functional mechanisms of these proteins is important.

Cotton is an important cash crop that produces natural fibres. *Verticillium* wilt caused by the fungus *V. dahliae* is a severe vascular disease in upland cotton (Fradin & Thomma, 2006). In recent years, many efforts have been made to generate cotton cultivars with enhanced resistance to the pathogen by molecular breeding. Several genes have been identified to participate in the defence against *V. dahliae* invasion in cotton (Cheng et al., 2016; Gaspar et al., 2014; Han et al., 2019; Hu et al., 2018, 2020; Li et al., 2014, 2016; Liu et al., 2018; Qin et al., 2018; Wang et al., 2020a, 2020b; Xiong et al., 2021; Yang et al., 2015; Zhang et al., 2017b; Zhou et al., 2019); however, the pool of candidate genes for cotton breeding is limited.

In this study, we identified a *V. dahliae*-responsive ADF, GhADF6, from upland cotton. Based on biochemical and cellular analyses, GhADF6 is an active actin depolymerizing and severing protein. The defence-related down-regulation of GhADF6 was found to augment the abundance of actin filaments and bundles, which was accompanied by enhanced disease tolerance against *V. dahliae* in cotton. Our findings demonstrate that the actin cytoskeleton was reorganized and F-actin abundance was increased on *V. dahliae* attack. Furthermore, regulating the expression of GhADF6 was identified to be crucial for this cellular process. Thus, we discuss the functional mechanism for F-actin reorganization involving ADF in plant innate immune responses.

## RESULTS

### 2.1 Identification of *V. dahliae*-responsive ADF genes from upland cotton

In our previous study, proteomic analysis revealed that some cytoskeleton-associated proteins, including ADF, responded to *V. dahliae* infection in the Verticillium wilt-resistant sea-island cotton, *Gossypium barbadense* (Wang et al., 2011). Accordingly, we sought to identify the pathogen-responsive ADF genes from the upland cotton *G. hirsutum*, the major variety of cotton in cultivation. Briefly, the genome sequence of *G. hirsutum* was searched and 37 ADF genes were identified. Phylogenetic analysis indicated that these ADFs can be grouped into four subclasses (Figure S1). Thus, we proceeded to examine the expression profiles of these ADF genes in the cotton root where the invasion of *V. dahliae* is initiated. Quantitative reverse transcription PCR (RT-qPCR) showed that two genes in subclass I, seven genes in subclass II, four genes in subclass III, and seven genes in subclass IV were expressed relatively higher than the other genes (Figure S2). To determine which ADFs are *V. dahliae*-responsive, the transcript levels of the 20 ADFs with high constitutive expression in the root were quantified at different time points following *V. dahliae* inoculation. As shown in Figures 1a and S3, the expression patterns of these genes were diverse. Among them, *GH_A09G0773/GH_D09G0714* exhibited a unique characteristic. Its gene expression was decreased significantly at 6 h postinoculation (hpi), and the down-regulated expression continued over the timeframe tested (Figure 1a). The gradual decline in the transcript expression suggests that *GH_A09G0773/GH_D09G0714* may be continuously responsible for cotton’s defence response. Based on phylogenetic analysis, *GH_A09G0773/GH_D09G0714* had the closest relationship with *Arabidopsis* ADF6 (Figure 1b). Thus, *GH_A09G0773/GH_D09G0714*
was named GhADF6. In addition to the root, GhADF6 was found to be expressed in other cotton organs, including leaf, stem, and flower (Figure S4), as reported for other ADFs. Accession numbers: ADF1 (AT3G46010), ADF2 (AT3G46000), ADF3 (AT5G59880), ADF4 (AT5G59890), ADF5 (AT2G16700), ADF6 (AT2G31200), ADF7 (AT4G25590), ADF8 (AT4G00680), ADF9 (AT4G34970), ADF10 (AT5G52360), ADF11 (AT1G01750). Scale bar stands for evolutionary distance.

2.2 | GhADF6 possesses actin depolymerizing and severing activities

As one of the key regulators of actin organization, ADFs play different roles in the remodelling of the cytoskeletal structure, most commonly depolymerizing and severing F-actin. However, Arabidopsis ADF9 has been reported to possess antagonist activities because it can stabilize and bundle actin filaments (Tholl et al., 2011). To determine the biochemical function of GhADF6 in modulating the actin structure, the actin-binding activity of GhADF6 was first tested. Polyhistidine (His)-tagged GhADF6 proteins were expressed in Escherichia coli, and a high-speed co-sedimentation assay was conducted with the purified proteins and polymerized F-actin. Figure 2a shows that GhADF6 was detected in the pellets in the presence of self-polymerized F-actin, whereas the band was only observed in the supernatant when polymerized F-actin was absent, indicating that GhADF6 possesses F-actin-binding activity. We proceeded to assess the actin depolymerizing and severing activities of GhADF6. The stained actin filaments were combined with a series of concentrations of GhADF6 (0, 1, and 2 μM) and viewed under a fluorescence microscope. As shown in Figure 2b, the lengths of F-actin decreased with increasing concentrations of GhADF6. F-actin depolymerization kinetics was measured via the pyrene fluorescence-monitored actin filament disassembly assay. When a series of concentrations of GhADF6 proteins (0, 10, 50, and 200 nM) was added to pyrene-labelled actin filaments for 10 min, a gradient reduction in fluorescence absorption was induced (Figure 2c). Thus, the inverse correlation demonstrated that GhADF6 can destabilize F-actin.

To determine whether the shortening of F-actin involved a filament severing activity of GhADF6, we visualized single-filament dynamics using a time-lapse total internal reflection fluorescence (TIRF) microscope. Without the GhADF6 protein, cleavage of the actin filaments was hardly detectable (Figure 3a,1 and Video S1); however, increased filament breaks were observed with the accumulated concentrations of GhADF6 (10, 30, and 100 nM) (Figure 3b,ii,iii and Videos S2–S4). Statistical calculations of the breakage revealed that the severing activity of GhADF6 depended on its concentration (Figure 3b).

To test the actin depolymerizing and severing activities of GhADF6 in vivo, fission yeast cells, the division of which was precisely regulated by the F-actin structures, was used as an experimental system. The cells were transformed with a yeast plasmid harbouring GhADF6 whose expression was driven by a thiamine-repressible promoter. The yeast transformants were then cultured in media without or with thiamine, and the cell morphology and F-actin structures were examined. As shown in Figure S5a–c, when GhADF6 expression was induced by removing thiamine from the medium, many yeast cells appeared longer (middle row) than those growing in repressive medium with thiamine (right row) or transformed with the empty plasmid (left row); however, the width of cells did not significantly change. 6-diamidino-2-phenylindole (DAPI) and calcofluor staining (Figure S5a, middle panel) revealed that approximately 45% of yeast cells were binucleate or multinucleate in GhADF6-induced cells, yet only approximately 13% of cells appeared binucleate in the two types of control cells (Figure S5d), indicating that GhADF6 expression resulted in a defect in yeast cell division. The F-actin structures in yeast cells were also visualized by staining with Alexa Fluor 488-phalloidin (Figure S5a, lower panel). In the control and GhADF6-repressed cells, normal F-actin patches, longitudinal cables, and the contractile F-actin ring which are essential for cytokinesis can be clearly observed. In contrast, in the cells with induced expression of GhADF6, the actin cables were reduced and contractile F-actin rings were hardly detected. These findings indicate that ectopic expression of GhADF6 can disturb yeast cell division by reducing F-actin cables and rings.
2.3 | GhADF6 proteins are colocalized with F-actin in plant cells

A functional ADF should colocalize with actin filaments in plant cells, thus we assessed the subcellular distribution of GhADF6 proteins in vivo. An enhanced green fluorescent protein (eGFP)-tagged GhADF6 (GhADF6-eGFP) was co-expressed with a well-characterized actin reporter, fABD2-mCherry, in the epidermal cells of *Nicotiana benthamiana* through agroinfiltration. The co-expression of free eGFP and fABD2-mCherry was used as a control, and the transient expression of the fusion proteins was visualized using confocal microscopy. As shown in Figure 4, GhADF6-eGFP fusion proteins, but not free eGFP, exhibited a distribution pattern that matched the fluorescent pattern of fABD2-mCherry, confirming that GhADF6 localized at F-actin. Interestingly, in addition to targeting periplasmic actin microfilaments, GhADF6-eGFP was observed in the nucleus.

2.4 | GhADF6-silenced cotton plants are more tolerant to *V. dahliae* infection

To investigate the physiological significance of GhADF6 down-regulation in response to *V. dahliae* infection, we first examined the upland cotton plants with a decreased GhADF6 expression by employing the tobacco rattle virus (TRV)-induced gene silencing (VIGS) technique (Liu et al., 2002). As a trial step, the *GhPDS* gene encoding a 15-cis-phytoene desaturase was used as a visual marker to evaluate the gene silencing effect. Two weeks after *Agrobacterium*-mediated VIGS infiltration, the albino phenotype appeared on the cotton leaves (Figure S6), thereby confirming the VIGS efficiency under our experimental conditions. To silence GhADF6, a sequence corresponding to a 243-bp fragment at the 3’ untranslated region (UTR) of GhADF6 mRNA was cloned into the VIGS vector, and then the *Agrobacterium* cells harbouring this construct were infiltrated into cotton cotyledons. Two weeks later, total RNA was extracted from top leaves to verify the silencing competence by RT-qPCR. As shown in Figure 5b, the GhADF6 mRNA level declined by approximately 80% in GhADF6-silenced seedlings compared with that in the control.

At the next step, the GhADF6-silenced and control cotton plants were inoculated with *V. dahliae*, and the disease-related symptoms were analysed and compared. Although the leaves of the control plants appeared yellow and were severely withered at 20 days post-inoculation (dpi), the symptoms of GhADF6-silenced plants appeared to be markedly weaker (Figure 5a). Statistical calculations proved that the disease index (DI) for the GhADF6-silenced plants was
significantly lower than that for the control (Figure 5c). These results indicate that the down-regulation of GhADF6 can positively affect the disease tolerance of cotton plants.

As knockdown of ADF1-4 can activate the SA pathway in Arabidopsis (Inada et al., 2016), we performed RT-qPCR to analyse the transcript levels of several defence-related genes in the GhADF6-silenced plant and compared these levels with those of the control plant. The relative expression of SA- and jasmonic acid (JA)-responsive genes, PR5 and PDF1.2, was significantly activated in GhADF6-silenced plants, whereas no significant change was detected for other genes (Figure 5d).

### 2.5 Down-regulation of GhADF6 increases the percentage occupancy and skewness of actin filaments in cotton root epidermal cells

In our previous study, we observed an increase in both actin filaments and actin bundles in cotton roots in response to *V. dahliae* infection (Wang et al., 2017). To observe the actin structural changes in GhADF6-silenced cotton plants compared with those in wild-type plants, actin filament arrays in the root epidermal cells were examined. The roots of cotton plants grown under hydroponic conditions were subjected to microscopy observation. As expected, both the abundance of actin filaments and the degree of actin bundling significantly increased in the GhADF6-silenced plant roots compared to the control (Figure 6a). The values of percentage occupancy and skewness of actin filaments were higher in GhADF6-silenced plants than in the control plant (Figure 6b,c).

### 2.6 Knockdown of GhADF6 decreases the actin depolymerizing and severing activities in cotton cells

The increase in actin density and bundles in GhADF6-silenced cotton demonstrated that GhADF6 can actively destabilize F-actin structures. Thus, the dynamic parameters of actin filament depolymerizing and severing rates in the GhADF6-silenced and control plants were compared. Live-cell imaging of the cytoskeleton in cotton was achieved using a transgenic line stably expressing the F-actin reporter ABD2-GFP (Yu et al., 2019). As the dynamic changes of actin filaments in leaf cells can be visualized more clearly than those in root cells using a spinning disk confocal microscope and the transcript level of GhADF6 was knocked down significantly in the leaves.
(Figures S4 and 5b), the actin structures in the leaf epidermal cells were observed via microscopy. As shown in Figure 6d–g, the actin depolymerizing rate and severing frequency were reduced in the GhADF6-silenced plants compared to those of the control; however, the difference in the actin severing frequency was not statistically significant. These findings indicate that the down-regulation of GhADF6 can reduce the actin depolymerizing/severing capabilities or stabilize the actin filament in cotton cells, as reported for AtADF4 (Henty-Ridilla et al., 2014).

3 | DISCUSSION

The dynamic remodelling of actin structures has been commonly observed in plant defence against pathogen infection (Day et al., 2011; Lipka & Panstruga, 2005). In a previous study, we observed increases in the density of F-actin and the extent of actin bundling in the root cells of cotton plants on V. dahliae infection (Wang et al., 2017). Several ABPs have been shown to be responsible for the defence-related changes in the actin cytoskeleton (Li et al., 2015; Qi et al., 2017; Schütz et al., 2006; Sun et al., 2018, 2019; Wang et al., 2017). Further evidence has demonstrated that ADF plays critical roles in plant-pathogen interactions by modulating actin rearrangement (Fu et al., 2014; Henty-Ridilla et al., 2014; Inada et al., 2016; Miklis et al., 2007; Zhang et al., 2017a).

As a modulator of actin organization, typical ADFs can balance the F- and G-actin pools by severing F-actin in the middle and depolymerizing F-actin from the pointed filament end into actin monomers (Maciver & Hussey, 2002). In contrast, AtADF9, an atypical ADF, was reported to effectively stabilize F-actin and concomitantly bundle actin filaments (Tholl et al., 2011). Here, we observed that GhADF6, a typical ADF, actively depolymerizes and severs actin filaments. Based on these activities, we believe that the regulated expression of GhADF6 primarily contributed to the remodelling of actin architecture during the host immune responses.

The functional mechanisms of ADFs in plant innate immunity seem intricate. The Arabidopsis adf4 mutant was found to exhibit reduced disease tolerance to the bacterial pathogen P. syringae (Tian et al., 2009). However, a later study reported that knockout of ADF4 enhanced the resistance of Arabidopsis plants to the fungal pathogen Golovinomyces orontii (Inada et al., 2016). In addition, it has been reported that actin cytoskeleton in the Arabidopsis adf4 mutant failed to respond to the bacterial PAMP elf26, but could respond to the fungal PAMP chitin by enriching actin density (Henty-Ridilla et al., 2014).
These findings suggest that the mechanism by which ADF4 participates in the defence against filamentous fungal and bacterial pathogens may vary. Likewise, ADFs may participate in the immune responses via alternative pathways in the same plant. Silencing TaADF7 or TaADF4 in wheat was found to result in plant susceptibility to the wheat stripe rust, Puccinia striiformis, whereas inhibition of TaADF3 expression could confer disease tolerance to the plant (Fu et al., 2014; Tang et al., 2015; Zhang et al., 2017). In this study, we found that GhADF6 expression decreased in defence response. Furthermore, its down-regulation by VIGS led to a higher tolerance of cotton plants to V. dahliae, while some other cotton ADFs exhibited distinct expression patterns in response to V. dahliae infection, suggesting that the functional mechanisms among these ADFs may differ.

The actin cytoskeleton has been suggested to act as a sensor to monitor the invasion of pathogens and trigger downstream defence responses (Henty-Ridilla et al., 2013). The PAMPs flg22 or chitin are sufficient to activate the reorganization of the actin cytoskeleton (Henty-Ridilla et al., 2013). Thus, the enhanced F-actin density and actin bundles in GhADF6-silenced cotton may behave as the pathogen-induced signal that leads to an increase in the basal defence level. Besides, actin cytoskeleton-mediated intracellular trafficking may also be critical (Hardham et al., 2007; Takemoto & Hardham, 2004; Underwood & Somerville, 2008). The reorganization of the actin cytoskeleton in defence against pathogen attack may be required for alternative material transportation. Several studies have presented data to support this notion. For instance, the actin cytoskeleton was found to mediate the accumulation of the resistance proteins RPW8.2 and PEN3 to the pathogen penetration sites (Wang et al., 2009). Furthermore, the deposition of callose on the cell wall was found to require the actin cytoskeleton (Day et al., 2011). Accordingly, the enhanced F-actin density and actin bundles
in GhADF6-silenced cotton may be beneficial for the transport of defence-related components in cotton root cells, consequently leading to higher tolerance to *V. dahliae* invasion.

When all members of subclass I ADFs genes are suppressed (ADF1-4 RNAi), the *PR1* mRNA accumulates in uninfected leaf cells of *Arabidopsis* (Inada et al., 2016). In our study, we observed the induced expression of *PR5* and *PDF1.2* genes in GhADF6-silenced cotton plants. Thus, we speculate that the defence-related actin rearrangement that involves the down-regulation of GhADF6 may associate with SA- or JA-mediated defence signalling. Finally, we cannot exclude the possibility that the nuclear

**FIGURE 6** Knockdown of GhADF6 results in increased density of actin filaments due to the decreased actin depolymerizing and severing activities in cotton cells. (a) Confocal microscopy visualization of actin filament arrays in root cells. Cotton plants were grown in a climate simulator for 14 days after agroinfiltration. Representative images of root epidermal cells are displayed for each sample. Bar = 10 μm. (b, c) Determination of actin percentage occupancy (density) and bundling (skewness) in (a). Values represent mean ± SD (*n* = 100). Asterisks denote statistically significant differences as determined by Student’s *t*-test (*p* < 0.05). A similar tendency was obtained from three biological replicates. (d) Dynamic observation of actin depolymerization in the leaf epidermal cells of cotton plants. The white circle marks the depolymerizing end of the filamentous actin. The red circle marks the stable end. (e) Observation of actin severing event in the leaf epidermal cells of cotton plants. The red arrows indicate the actin severing event. (f, g) Statistical calculations of actin depolymerizing rate and severing frequency. Values represent mean ± SD (*n* = 100). *p* < 0.05, as determined by Student’s *t*-test. A similar tendency was obtained from three biological replicates.
localization of GhADF6 also contributes to the defence response, as some ADFs have been found to localize in the nucleus to regulate gene expression or to act as a chaperone of actin monomers (Bernstein & Bamburg, 2010; Clément et al., 2009; Inada et al., 2016; Jiang et al., 1997). For instance, a recent study showed that ADF1 can directly interact with transcription factors to regulate downstream genes in Arabidopsis (Yu et al., 2020). Further study is needed to identify the underlying mechanisms.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials

The upland cotton (G. hirsutum) cv. Xinluzao 7 used in this study was kindly provided by Professor Jie Sun (Shihezi University). The 35S::ABD2-GFP transgenic cotton lines were provided by Professor Zhaosheng Kong (Institute of Microbiology, CAS). Seedlings were cultured in the soil in a greenhouse or grown under hydroponic conditions in an artificial climate simulator at 28°C under 16 h light/8 h dark conditions. Plants were irrigated with Murashige and Skoog (MS) nutrient solution weekly.

4.2 | Pathogen cultivation and inoculation

The highly destructive defoliating isolate of V. dahliae, strain V991, was used. Fungi mycelium was cultured in Czapek medium (1 g KH₂PO₄, 2 g NaNO₃, 1 g MgSO₄·7H₂O, 1 g KCl, 2 mg FeSO₄·7H₂O, and 30 g sucrose per litre) at 28°C for 5 days. The spores were harvested at 0, 6, 12, 24, and 48 hpi, respectively.

4.3 | Phylogenetic analyses

The ADF protein sequences were accessed from http://cgp.genomics.org.cn and http://arabidopsis.org. Sequences were aligned using ClustalW, and the neighbour-joining method was used to produce the phylogenetic tree of ADFs using MEGA v. 4.0 (Tamura et al., 2007).

4.4 | RNA extraction and RT-qPCR

The Plant Total RNA Purification Kit (GM Biolab) was used to extract total RNA from different organs. For synthesis of cDNA, 2 µg of total RNA was added into the reaction system for reverse transcription according to the protocol of TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Transgen). SYBR Green Realtime PCR Master Mix (TOYOBO) and a real-time PCR detection system (CFX96 Touch) were used for the qPCR assays. The cotton Histone3 gene was used as the internal control. The primers used for RT-qPCR are listed in Table S1.

4.5 | Protein expression and purification

The cDNA sequence of GhADF6 digested with EcoRI and HindIII was cloned into the bacterial protein expression vector pET28a (Novagen/Merck). The recombinant plasmid was transformed into Escherichia coli BL21 (DE3). His-tagged GhADF6 proteins were extracted and purified through HISTRAP HP using AKTA purifier UPC10 (GE Healthcare) at 4°C. The proteins were stored at −80°C.

4.6 | High-speed co-sedimentation assay

The high-speed co-sedimentation assay was modified according to Han et al. (2013). G-actin extracted from rabbit muscle was incubated in ME buffer (10 mM EGTA, 1 mM MgCl₂) for 2 min to convert Ca-ATP-actin to Mg-ATP-actin. Thereafter, 3 µM G-actin was polymerized to F-actin in 1× KMEI buffer (10× stock: 500 mM KCl, 10 mM MgCl₂, 10 mM EGTA, 100 mM imidazole–HCl, pH 7.0) and incubated with 5 µM GhADF6 proteins for 30 min at 25°C. The samples were centrifuged at 200,000 × g for 60 min at 4°C in an Optima TLX ultracentrifuge (Beckman). The pellet and supernatant fractions were analysed by SDS-PAGE and stained with Coomassie Brilliant blue R250. All proteins and solutions were pre-clarified at 160,000 × g for 30 min at 4°C.

4.7 | Fluorescence microscopy of actin filaments

Actin filaments were polymerized as described above. F-actin (2 µM) was incubated with GhADF6 proteins at the indicated concentrations for 30 min at 25°C and then labelled with 0.5 µM Alexa Fluor 488-phalloidin (Invitrogen) (Han et al., 2013). A sample of 50× diluted actin filament mixture was then placed on a poly-L-lysine-coated glass slide (Sigma). The lengths of actin filaments were observed under a fluorescent microscope (Observer Z1; Zeiss).

4.8 | Actin filament depolymerization kinetics

The pyrene fluorescence-monitored actin filament disassembly assay was performed as described by Moseley et al. (2006). In brief, 5 µM G-actin (including 50% pyrene labelled actin) was polymerized in the dark as described above. Pyrene fluorescence was monitored immediately after adding the indicated concentration.
of the His-GhADF6 proteins. The fluorescence values were recorded for 10 min at 365 nm excitation and 407 nm emission using a QuantaMaster Luminescence QM 3 PH fluorometer (Photon Technology International).

### 4.9 | Visualization of actin filament severing using TIRF microscopy

Direct visualization of GhADF6-mediated actin filament severing was conducted using a TIRF microscope according to the method described by Amann and Pollard (2001). After the flow cell chamber was coated with 10 nM N-ethylmaleimide-myosin for 1 min, HS-bovine serum albumen (BSA) (50 mM Tris-HCl, pH 7.5, 600 mM NaCl, 1% BSA) and LS-BSA (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA) were sequentially injected; thereafter, the chamber was washed with 1× TIRF buffer (1× KME1 buffer, 100 mM dithiothreitol, 0.2 mM ATP, 15 mM glucose, 20 μg/ml catalase, 100 μg/ml glucose oxidase, 0.2% BSA, 0.5% methylcellulose). Preassembled 100 nM F-actin (50% rhodamine-labelled) was perfused into the chamber. After identifying the focal plane, a range of GhADF6 concentrations in 1× TIRF buffer was perfused into the chamber followed by image acquisition. All images were captured as a time-lapse series at 2-s intervals by TIRF illumination with an IX81 microscope (Olympus).

### 4.10 | Ectopic expression of GhADF6 in fission yeast

The cDNA sequence of GhADF6 was cloned into the yeast vector pREP1 (Maundrell, 1993), and the resulting vector and the empty pREP1 were transformed into the fission yeast, *Schizosaccharomyces pombe* (h*, leu1-32), by electroporation using standard methods (Alfa et al., 1993). GhADF6 expression was repressed by adding thiamine in the medium. For observation of the nucleus, yeast cells were stained with DAPI (Roche) and fluorescent brightener 28 (calcofluor white M2R; Sigma). At least 500 cells were measured for each treatment. For F-actin structure observation, the cells were stained with 0.5 μM Alexa Fluor 488-phalloidin (Invitrogen). The images were captured under a fluorescent microscope (Observer Z1; Zeiss).

### 4.11 | Subcellular localization of GhADF6

The cDNA sequences encoding eGFP, GhADF6-eGFP, or fABD2-mCherry were cloned under the control of the CaMV 35S promoter into the plant expression vector pPZP111 (Hajdukiewicz et al., 1994). The plasmid construct was transformed into *Agrobacterium tumefaciens* GV3101 cells, which were subsequently infiltrated into 5-week-old leaves of *Nicotiana benthamiana*. Cells were visualized via confocal microscopy (TCS SP8; Leica) at 36 h after infiltration.

### 4.12 | Agrobacterium-mediated VIGS

The *Agrobacterium* mediated VIGS using a TRV-based binary vector was described previously by Gao et al. (2011). The recombinant plasmid vector was transformed into *A. tumefaciens* GV3101. A mixture (1:1 ratio, vol/vol) of *Agrobacterium* cultures (OD$_{600}$ = 1) harbouring the pTRV1 or pTRV2/pTRV2-GhADF6 plasmid was injected into 7-day-old cotton seedlings with two fully expanded cotyledons using a needleless syringe.

### 4.13 | Labelling of actin filaments in cotton root epidermal cells and quantification

The hydroponically grown cotton roots were washed twice with phosphate-buffered saline (PBS), and then soaked in Alexa Fluor 488-phalloidin staining buffer (0.1 M PIPES, pH 6.9, 0.05% vol/vol Triton X-100, 1 mM MgCl$_2$, 3 mM dithiothreitol, 0.3 mM PMSF, 5 mM EGTA, and 0.25% glutaraldehyde in PBS) to observe the actin filament arrays, as described previously (Gestel et al., 2001). Confocal microscopy (TCS SP8; Leica) was used to collect the z-series stacked images. The excitation wavelength was 488 nm and the emission filter was 500–540 nm. The density and skewness value of the actin filament arrays were analysed based on the method described previously by Higaki et al. (2010) using ImageJ software (http://imagej.net/Fiji). The subtracting background was set to 50 pixels and the filter of Gaussian blur was set to 1.0.

### 4.14 | Live-cell imaging of actin filaments via spinning-disc confocal microscopy

The leaves of the 35S::ABD2-GFP transgenic cotton lines were subjected to live-cell imaging with a spinning disk confocal microscope (UltraView VoX; Perkin Elmer) equipped with a Yokogawa Nipkow CSU-X1 spinning disk scanner, a Hamamatsu EMCCD 9100-13, and a Nikon TiE inverted microscope. The captured images were analysed by ImageJ. The F-actin depolymerizing rate was defined as the shrinking rate of a filament undergoing depolymerization per μm per second. The F-actin severing frequency was counted using the number of severing events in the observed filaments over the length in unit time.

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### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.
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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.