The Type II Arabidopsis Formin14 Interacts with Microtubules and Microfilaments to Regulate Cell Division

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Formins have long been known to regulate microfilaments but have also recently been shown to associate with microtubules. In this study, Arabidopsis thaliana FORMIN14 (AFH14), a type II formin, was found to regulate both microtubule and microfilament arrays. AFH14 expressed in BY-2 cells was shown to decorate preprophase bands, spindles, and phragmoplasts and to induce coalignment of microtubules with microfilaments. These effects perturbed the process of cell division. Localization of AFH14 to microtubule-based structures was confirmed in Arabidopsis suspension cells. Knockdown of AFH14 in mitotic cells altered interactions between microtubules and microfilaments, resulting in the formation of an abnormal mitotic apparatus. In Arabidopsis afh14 T-DNA insertion mutants, microtubule arrays displayed abnormalities during the meiosis-associated process of microspore formation, which corresponded to altered phenotypes during tetrad formation. In vitro biochemical experiments showed that AFH14 bound directly to either microtubules or microfilaments and that the FH2 domain was essential for cytoskeleton binding and bundling. However, in the presence of both microtubules and microfilaments, AFH14 promoted interactions between microtubules and microfilaments. These results demonstrate that AFH14 is a unique plant formin that functions as a linking protein between microtubules and microfilaments and thus plays important roles in the process of plant cell division.

INTRODUCTION

Microtubules and microfilaments represent two major plant cell cytoskeletal networks, both of which play important roles in many aspects of the fundamental processes of plant cell growth and development, including cell division, cell expansion, intracellular organization, and cell motility. There is no question that microtubules and microfilaments constitute separate cytoskeletal systems and fulfill distinct functions. However, a growing body of evidence also suggests that functional interactions between microtubules and microfilaments are important for specific cellular processes. In animal cells, microtubules have been proposed to contribute to these processes in part by mediating proper spatial distribution of microfilament structures. For example, during cytokinesis, the microtubules of the mitotic spindle have been shown to play an important role in the positioning of the contractile microfilament ring assembly at the cell cortex (Maddoxa and Oegema, 2003). During cell migration, microtubules have been implicated in steering the microfilament cytoskeleton in the proper direction, suggested by characterization of nerve growth cone guidance (Tanaka et al., 1995; Dent and Gertler, 2003).

In plant cells, microtubules and microfilaments are often codistributed in the cortical area in interphase cells (Blancaflor, 2000) and colocalize in structures, such as the preprophase band, mitotic spindle, and phragmoplast, in mitotic cells (Hoshino et al., 2003) and meiotic cells (Staiger and Cande, 1991). Pharmacological studies using microfilament- and microtubule-specific drugs have demonstrated that microtubules interact with microfilaments. For example, cytochalasin, a microfilament-disrupting drug, prevents the microtubule preprophase band from narrowing in Allium cepa (Eleftheriou and Palevitz, 1992). In addition, long-term treatment with a low level of cytochalasin causes transverse microtubule arrays to reorient into an oblique alignment in growing cotton (Gossypium hirsutum) hairs (Seagull, 1990). These results suggest that microfilaments can influence microtubule organization. This relationship appears to be reciprocal, as microtubules have also been shown to affect microfilament organization (Collings and Allen, 2000). Recently, a few proteins have been shown to interact with both microtubules and microfilaments in plant cells (Petrášek and Schwarzerová, 2009). However, the functional evidence for these interactions is still lacking, and the molecular basis underlying interactions between the microtubule and microfilament cytoskeletons in plants is poorly understood.

Formins are known regulators of the microfilament cytoskeleton (Blanchoin and Staiger, 2008). However, it has been noted recently that formins also behave as prominent regulators of the microtubule cytoskeleton by modulating the dynamics of...
selected microtubules in interphase and mitosis (Bartolini and Gundersen, 2009; DeWard and Alberts, 2009; Chesarone et al., 2010). Some metazoan formins have also been shown to associate with microtubules through interactions with microtubule-associated proteins, including CLIP170, APC, and end binding protein 1 (EB1), via sequences located outside of the formin FH2 domain (Wen et al., 2004; Lewkowicz et al., 2008). Recent evidence has suggested that many formins directly bind microtubules and affect their dynamic properties. For example, experiments using a constitutively active form of mouse formin mDia2 lacking regulatory domains have provided an initial indication that this formin directly interacts with microtubules to alter their dynamics (Bartolini et al., 2008). Subsequent studies have shown that several animal formins, including mDia1, mDia2, Formin1-Lb, INF1, and Capu, interact directly with microtubules (Rosales-Nieves et al., 2006; Zhou et al., 2006; Bartolini et al., 2008; Young et al., 2008). Furthermore, characterization of mDia2 has also suggested the possibility that formins may interact with microfilaments while binding to microtubules (Bartolini et al., 2008).

Recently, significant progress has been made toward understanding the biochemical properties and cellular functions of plant formins. Plant formins can be divided into three classes (Cvrčková et al., 2004; Grunt et al., 2008). However, all of the reported plant formins, including the Class I Arabidopsis thaliana formins AFH1, AFH3, AFH4, AFH5, AFH6, and AFH8 (Deeks et al., 2005; Ingouff et al., 2005; Michelot et al., 2005; Yi et al., 2005; Ye et al., 2009) and a Class II formin from Physcomitrella, For2 (Vidali et al., 2009), have been characterized as microfilament regulators (Blanchon and Staiger, 2008), with the lone exception of AFH4. Recently, AFH4 was reported to bind microtubules through a newly discovered GOE domain, when overexpressed in the leaf cells of Nicotiana benthamiana (Deeks et al., 2010).

In this study, we identified and characterized a previously undiscovered Arabidopsis formin, FORMIN14 (AFH14). We demonstrated that AFH14 plays an important role in regulating both microtubule and microfilament arrays through association with these networks in mitotic BY-2 and Arabidopsis suspension-cultured cells. Furthermore, we found that AFH14 is involved in meiosis through regulation of microtubule structures required for the generation of microspores. To further support these observations, AFH14 was also shown to interact with microtubules and microfilaments and to affect the structures of these polymers in vitro.

**RESULTS**

Identification of AFH14 and Preparation of AFH14 FH1FH2 Domain Recombinant Protein and AFH14-Specific Antibodies

AFH14 was identified based on sequence analysis of the Arabidopsis genome. The gene was found to contain 18 exons and 18 introns, spanning 6653 bp, and to encode a 3102-nucleotide mRNA with a single open reading frame estimated to produce a 113.6-kDa protein of 1033–amino acid residues. The AFH14 protein consisted of three functionally distinct subdomains: an N-terminal PTEN (phosphatase tensin)–related domain, a Pro-rich FH1 domain, and a highly conserved C-terminal FH2 domain (see Supplemental Figure 1 online). However, a 591-bp region within the FH1 domain was absent in the amplified sequence, differing from The Arabidopsis Information Resource prediction (see Supplemental Figure 2 online).

To characterize the chemical properties of AFH14 in vitro, a 6-His–tagged truncated recombinant protein (referred to as FH1FH2) containing the FH1 and FH2 domains of AFH14 was expressed and purified from bacterial cells. Polyclonal antibodies directed against the FH1FH2 recombinant protein were then raised in mice. The molecular mass of the purified FH1FH2 proteins was estimated to be ~80 kD by SDS-PAGE. Immunoblot analysis revealed that the polyclonal antibodies effectively recognized the recombinant protein (see Supplemental Figure 3A online). Because the FH1FH2 region is highly conserved across formin family members, we expected that the polyclonal antibodies were likely also to recognize other formins. Therefore, these anti-AFH14 antibodies were exclusively used to examine the biochemical characteristics of AFH14 in vitro. To explore endogenous AFH14 localization in Arabidopsis cells, we raised a monoclonal antibody directed against AFH14 in mice using a fragment of the protein consisting of amino acids 340 to 502 (see Supplemental Figure 1B online). The specificity of the monoclonal antibody was confirmed using protein extracts from 10-d-old Arabidopsis seedlings (see Supplemental Figure 3B online).

AFH14 Localizes to the Preprophase Band, Spindle, and Phragmoplast

To assess the localization of AFH14, a chimeric expression construct harboring AFH14 fused to green fluorescent protein (GFP) under the control of an inducible promoter was stably transformed into wild-type tobacco (Nicotiana tabacum) BY-2 cells. Approximately 30 h after induction, cells were fixed and microtubules were visualized using an anti-α-tubulin antibody. The subcellular localizations of AFH14-GFP and microtubules were then examined by confocal microscopy. In preprophase cells, AFH14-GFP localized primarily to the preprophase bands (Figure 1A). In metaphase and anaphase cells, AFH14-GFP was concentrated along the entire spindle (Figures 1B and 1C). In cells undergoing cytokinesis, AFH14 completely colocalized with the two mirror halves of the phragmoplast (Figure 1D).

To confirm further the localization of endogenous AFH14, antibodies directed against AFH14 and α-tubulin were used to stain Arabidopsis suspension cells. In dividing cells, AFH14 colocalized with α-tubulin at the preprophase bands (Figure 1E), spindles (Figure 1F), and phragmoplasts (Figure 1G) in agreement with the observations in BY-2 cells.

Overexpression of AFH14 Inhibits Turnover of the Mitotic Apparatus and Increases the Resistance of Microtubules to Oryzalin

To explore the contribution of AFH14 to the function of the mitotic apparatus, live cell imaging studies of mitotic progress were performed. At 30 h postinduction with estrogen, there was a pronounced increase in GFP signal in induced BY-2 cells.
expressing AFH14 in comparison to control cells without estrogen treatment. Interestingly, turnover of the spindles and phragmoplasts was severely inhibited in the BY-2 cells overexpressing AFH14-GFP (Figures 2A and 2B). Failure of the turnover of spindles and phragmoplasts resulted in a lack of progression through mitosis in AFH14-expressing cells, which was reflected by a high mitotic index score. Overexpression of AFH14 resulted in an increased mitotic index of 8.8%, compared with 3.9% for control uninduced cells (Figure 2C). The mitotic index of cells transformed with an empty vector carrying only GFP in the presence or absence of estrogen treatment was 4.2 and 4.0%, respectively, similar to uninduced cells from the AFH14-expressing line.

Based on the observations described above, we hypothesized that overexpression of AFH14 may alter the resistance of microtubules to the microtubule-depolymerizing drug oryzalin. Treatment with 10 μM oryzalin had a strong effect on microtubules in uninduced control cells, as 75.0% of spindles and 82.0% of phragmoplasts were disrupted after 15 min of treatment (see Supplemental Figures 4A and 4B online). Overexpression of AFH14 partially suppressed the effect of oryzalin (Figures 2D and 2E), resulting in a decrease in the percentage of depolymerized spindles and phragmoplasts to 52.0 and 58.2%, respectively (Figure 2F). In addition to the effects of AFH14 on microtubule stability, we also found that oryzalin-mediated microtubule depolymerization resulted in changes in the localization of AFH14-GFP. GFP fluorescence was either associated with irregular microtubule arrays or dispersed in the cytoplasm after oryzalin treatment (see Supplemental Figures 5A and 5B online), further confirming that AFH14 binds microtubules.

AFH14 Recruits Microfilaments to the Spindle and Phragmoplast

Significant evidence indicates that the classical formins play important roles in microfilament organization. Therefore, fluorescently labeled phalloidin was used to visualize microfilaments in BY-2 cells. In control uninduced cells, microfilaments formed a cage around the spindle, and very few filaments remained in the spindle (Figure 3A). In addition, the microfilament band in control cells was thinner than the microtubule band in phragmoplasts (Figure 3B). Interestingly, in cells overexpressing AFH14-GFP, microfilaments codistributed with AFH14-GFP in spindles and phragmoplasts (Figures 3C and 3D). Furthermore, similar to results indicating resistance to microtubule depolymerizing drugs, overexpression of AFH14 partially suppressed the effects of treatment with the microfilament-depolymerizing drug latrunculin B (LatB). In BY-2 cells treated with 200 nM LatB for 50 min, 65.4% of AFH14-overexpressing cells exhibited intact mitotic microfilament structures, compared with only 31.6% of control cells (see Supplemental Figure 6 online). In addition, depolymerization of microfilaments with LatB did not appreciably affect the spindle and phragmoplast pattern in cells expressing AFH14-GFP (Figures 3E and 3F). Together, these results indicate that AFH14 likely interacts with both microtubules and microfilaments in vivo, although it binds preferentially to microtubules.

AFH14 Affects the Interaction between Microtubules and Microfilaments

To determine whether connections between microtubules and microfilaments were necessary for cell division in Arabidopsis cells, codistribution of microtubules and microfilaments was analyzed in wild-type suspension-cultured cells. Similar to observations in BY-2 cells, microtubules were arranged to form the mitotic spindle concurrent with formation of a microfilament cage.
surrounding the spindle (Figure 4A). In cytokinesis, the microfilament band appeared thinner than the microtubule band in phragmoplasts (Figure 4B).

To assess the effects of reduced AFH14 expression on microtubule and microfilament arrays, an artificial microRNA construct was prepared and placed under the control of an inducible promoter. Uninduced cells without estrogen treatment were used as a negative control, and the distribution of microfilaments and microtubules in these cells was similar to that of wild-type cells (see Supplemental Figure 7 online). For transformed cells induced with 2\(\mu\)M estrogen for 3 d, changes in cellular phenotypes were classified as normal, mild, strong, or severe, depending upon the severity of the resulting abnormalities. Approximately 25.5% of mitotic arrays in the treated cells exhibited no visible phenotypic changes, and the mitotic apparatus had normal microtubule and microfilament arrangements similar to control cells. Mild abnormalities, in which the spindle and phragmoplast microtubules seemed intact, but the microfilament cages around the spindles and microfilaments in phragmoplasts were not present, were observed in 27.7% of the cells (Figures 4C and 4D). An additional 14.9% of cells exhibited strong phenotypic changes, in which only a remnant of mitotic microtubules were observed, accompanied by severely dispersed microfilaments (Figure 4E). In the remaining 31.9% of cells that showed severe phenotypic abnormalities, severely depolymerized microtubules and microfilaments were observed (Figure 4F). None of these phenotypes were observed in control uninduced cells or in cells transformed with an empty vector cultured in the presence or absence of estrogen.

AFH14 Loss-of-Function Plants Exhibit Defective Microspore Generation

The AFH14 tissue expression pattern was analyzed by transforming Arabidopsis with an AFH14 promoter:β-glucuronidase (GUS) reporter gene transcrip- tional fusion construct. Three independent GUS reporter lines were examined, and all showed the same pattern of expression. In seedlings, high levels of GUS

Figure 2. Overexpression of AFH14 Inhibits Turnover of the Mitotic Apparatus and Increases the Resistance of Microtubules to Oryzalin in BY-2 Cells.

Wild-type BY-2 cells were transformed with inducible AFH14-GFP vectors. After induction of expression, the cells were observed at the indicated time points ([A] to [C]) or were treated with 10 \(\mu\)M oryzalin for 15 min ([D] to [F]). Mock-induced cells treated with DMSO served as negative controls. ([A] and [B]) Serial images show representative cells arrested at metaphase ([A]) and cytokinesis ([B]). For each stage, at least 15 cells were observed and exhibited similar results. ([C]) The mitotic index was analyzed in AFH14-overexpressing cells (OX) and control cells. The mitotic index of OX cells was found to be significantly different than that of control cells (t-test, P < 0.05). Error bars indicate SE (n > 1500). Results represent data from three independent experiments. ([D] and [E]) The metaphase spindle ([D]) and cytokinetic phragmoplast ([E]) remained partially intact after treatment with oryzalin in OX cells. Bar = 5 \(\mu\)m. ([F]) The number of mitotic cells with depolymerized microtubules (MT) was analyzed in OX and control BY-2 cells. Significant differences in levels of spindle and phragmoplast microtubule depolymerization between OX and control cells were observed, respectively (t-test, P < 0.05). Error bars indicate SE (n > 300). Results represent data from three independent experiments.
activity were detected in the shoot apex, leaf primordia, guard cells, trichomes, and roots, with the exception of the root tip (see Supplemental Figures 8A to 8D online). In the inflorescence, GUS activity was strong in all floral buds (see Supplemental Figures 8E and 8F online). However, GUS activity could not be detected in the wild-type floral buds (see Supplemental Figure 8G online). This pattern is consistent with the expression of AFH14 reported in publicly available expression array data (http://www.genevestigator.ethz.ch/).

To characterize the function of AFH14 in vivo, two distinct Arabidopsis mutant alleles harboring T-DNA insertions in AFH14 were identified. The afh14-1 allele harbored a T-DNA insertion in the second exon, and the afh14-2 allele harbored an insertion in the 3′ untranslated region (see Supplemental Figure 9 online). Further characterization revealed that both mutants exhibited a similar phenotype with respect to decreased pollen counts (Figure 5A). To explore further the underlying reasons for this phenotype, meiosis was compared between the wild-type and mutant plants. In wild-type plants, all tetrads observed had four spores, and the tetrahedral microspore arrangement appeared normal (Figure 5B). By contrast, the afh14-1 and afh14-2 lines produced dyads, triads, polyads, and abnormal tetrads with an irregular microspore distribution patterns (Figures 5C to 5F; see Supplemental Figures 10B to 10E online). These phenotypes accounted for 40.0% of all meiotic tetrads. In addition, no tetrads from afh14-1 and afh14-2 had the swollen domain present in all wild-type tetrads (Figure 5B; see Supplemental Figure 10A online).

AFH14 Affects the Arrangement of Microtubule Arrays in Microspore Formation

We first analyzed the subcellular localization of AFH14 during meiosis in pollen mother cells (PMCs) by immunofluorescence microscopy. As shown in Figure 6, the AFH14 protein was bound to spindle microtubules at metaphase I and II and to phragmoplast microtubules at anaphase I and advanced cytokinesis (Figures 6B, 6C, 6E, and 6G). Although the radial microtubule system (RMS) was present surrounding the nuclei during prophase I, telophase I, early cytokinesis, and in tetrads, AFH14 was dispersed throughout the cytoplasm in these phases (Figures 6A, 6D, 6F, and 6H).

Next, the microtubule arrays were compared in wild-type and mutant plants during the process of microspore generation. These results showed that microtubule arrays behaved irregularly in afh14-1 and afh14-2 PMCs. For example, metaphase I spindles exhibited a typical fusiform configuration in wild-type cells (Figure 7A) but became visibly smaller in afh14-1 cells (Figure 7D). At telophase I, two RMSs formed properly around the nucleus in wild-type cells (Figure 7B) but displayed a skewed configuration in afh14-1 cells (Figure 7E). In addition, the two adjacent spindles present during metaphase II were perpendicular to one another in wild-type cells (Figure 7C) but were parallel to one another in 58.4% of afh14-1 cells (n = 24) (Figure 7F). Furthermore, in wild-type cells, four RMSs surrounded four nuclei symmetrically during early cytokinesis (Figure 7G), whereas in afh14-1 cells, the microtubules became slightly waved (Figure 7J). Also, during advanced cytokinesis, the phragmoplast microtubules were concentrated between two adjacent nuclei (Figure 7H), but this was not the case in 100% of afh14-1 cells observed (n = 36) (Figure 7K). Finally, in wild-type cells, the tetrad developed into four microspores with microtubules radiating from each nucleus (Figure 7I). However, the afh14-1 mutants produced some dyads that included two nuclei in each spore, although microtubules still radiated from each nucleus in some afh14-1 cells (Figure 7L). These observations indicate that mutations in AFH14 have a deleterious effect on microtubule formation.
arrangement. The afh14-2 mutant displayed similar microtubule array defects to those observed for afh14-1 (see Supplemental Figure 11 online).

The FH1FH2 Domain of AFH14 Directly Binds Both Microtubules and Microfilaments in Vitro

Because AFH14 colocalized with microtubules and microfilaments in vivo, the direct binding activity of FH1FH2 to the two cytoskeletal polymers was analyzed in vitro. To assess microtubule binding activity, taxol-stabilized microtubules were incubated with varying concentrations of the recombinant protein, and the amount of tubulin and FH1FH2 in the resulting pellets was quantified by SDS-PAGE and densitometry (Figures 8A and 8C). In the absence of microtubules, FH1FH2 was found predominantly (>90%) in the supernatant (Figure 8A, lane 17), indicating that the recombinant protein alone did not sediment. However, with the addition of microtubules, FH1FH2 was largely found in the pellet with the microtubules. Therefore, we conclude that FH1FH2 can bind and cosediment with taxol-stabilized microtubules in vitro, behavior typical of a microtubule binding protein.

We next analyzed the binding activity of FH1FH2 for microfilaments using a high-speed cosedimentation assay. As shown in Figures 8B and 8D, addition of increasing concentrations of FH1FH2 to microfilaments resulted in concentration-dependent increases in the amount of FH1FH2 present in the pellets. These results indicate that, in addition to microtubules, FH1FH2 also binds microfilaments in vitro.

Additional experiments assessing the effects of FH1FH2 on actin polymerization were also performed using the high-speed cosedimentation assays. These results showed that, in samples containing FH1FH2, more microfilaments were present in the pellets (with a concomitant decrease in the supernatants) than when FH1FH2 was not added during the first 5 min of the assay (see Supplemental Figure 12 online), indicating that FH1FH2 can bind and nucleate microfilaments.

The AFH14 FH1FH2 Domain Bundles Microtubules and Microfilaments

Because the AFH14 FH1FH2 recombinant proteins were found to bind directly to microtubules and microfilaments in vitro, we further analyzed the effects of the FH1FH2 protein on microtubule and microfilament organization. First, confocal microscopy was used to examine the effects of 1 μM FH1FH2 on rhodamine-conjugated microtubules. In the absence of FH1FH2 and in the presence of heat-denatured FH1FH2, microtubules were scattered individually throughout the solution (see Supplemental Figure 13A online). However, in the presence of active FH1FH2, the microtubules organized almost exclusively into densely packed bundles, with few microtubules found outside of the bundles (see Supplemental Figure 13B online). Consistent with a role for FH1FH2 in this process, FH1FH2 staining was observed in dot-like structures along the lengths of the microtubules (see Supplemental Figure 13C online). To investigate further this microtubule bundling activity, 200 mM NaCl was added to the reaction to detach the fusion protein from the microtubule bundles. Two hours after NaCl treatment, the large aggregated bundles disassembled completely into single microtubules (see Supplemental Figure 13D online). In the absence of FH1FH2, the microtubules remained dispersed in cells with the severe phenotype (F). Bars = 5 μm.
FH1FH2, appeared to become bundled (see Supplemental Figure 13H online). Similar to observations in microtubules, dot-like structures corresponding to active FH1FH2 staining were visualized alongside the filaments (see Supplemental Figure 13I online). Electron microscopy confirmed that individual microfilaments were bundled together in the presence of active FH1FH2 (see Supplemental Figure 13K online). By contrast, in the absence of the recombinant protein or in the presence of denatured recombinant protein, only individual microfilaments were observed (see Supplemental Figures 13G and 13J online). Also similar to microtubules, treatment with 200 mM NaCl promoted disassembly of the microfilament bundles (see Supplemental Figure 13L online). The observed bundle formation and association of FH1FH2 were not due to nonspecific antibody binding, as microtubules and microfilaments stained with the fluorescently labeled secondary antibodies alone failed to exhibit dot-like structures along the length of the polymers (data not shown).

The AFH14 FH1FH2 Domain Bundles and Cross-Links Microtubules with Microfilaments

To determine whether the AFH14 FH1FH2 domain could interact with microtubules and microfilaments concomitantly, binding experiments were performed using 0.5 μM preformed microtubules, microfilaments, and 100 nM active FH1FH2. As shown in Figure 9A, microfilaments assembled into bundles in the presence of FH1FH2. When preformed taxol-stabilized microtubules were added to reactions containing microfilaments and FH1FH2, microtubule bundles began to form after 5 min and, at the same time, some microfilament bundles became looser (Figure 9B). After 40 min and 4 h incubations, increased numbers of microtubule bundles were observed, whereas microfilament bundles completely disassembled into individual filaments (Figures 9C and 9D). These observations suggest that the microtubules induce detachment of FH1FH2 from the microfilament bundles.

In contrast with microfilament disassembly, when preformed microfilaments were added to reactions containing bundled microtubules and FH1FH2 (Figure 9E), the microtubule bundles remained intact, and microfilament bundling was not observed after 5 min, 40 min, or 4 h incubations (Figures 9F to 9H). This result suggests that microfilaments cannot strip FH1FH2 from microtubule bundles. In addition, when preformed microtubules and microfilaments were mixed together in the absence of FH1FH2, both cytoskeletal polymers remained as individual filaments scattered randomly throughout the solution (Figure 9I). However, addition of FH1FH2 promoted microtubule bundling, but not microfilament bundling (Figure 9J). These results indicate that the FH1FH2 protein preferentially bundles microtubules over microfilaments.

Interestingly, when higher concentrations of FH1FH2 were added to reaction mixtures (up to 1 μM), both microtubules and microfilaments formed bundles within 30 min (Figure 9K), and in some cases, the two types of bundles colocalized (Figure 9L). When an excess of anti-AFH14 antibodies was added to these reactions, nearly all of the bundles disassembled into individual filaments (Figure 9M), indicating that a dynamic exchange may exist between free FH1FH2 in the solution and bound FH1FH2 on microtubule and microfilament bundles. Negative staining electron microscopy was used to confirm the microtubule and microfilament bundling and cross-linking activities of FH1FH2 (Figures 9O to 9R). The statistic analysis of the thickness of the filaments and filament bundles corresponding to the result of negative staining electron microscopy are shown in Supplemental Figure 15 online.

Similar experiments were also performed to test the activity of the FH2 domain alone in binding or bundling microtubules and microfilaments. Similar to the FH1FH2 recombinant protein, treatment with 1 μM active FH2 resulted in binding to microtubules and microfilaments, decoration of filament bundles with FH2 immunoreactivity, and microtubule and microfilament bundling (see Supplemental Figure 16 online).

Figure 5. Microspore Formation Is Defective in afh14 Arabidopsis Plants.

(A) Pollen counts from afh14-1, afh14-2, and wild-type anthers. Both mutant Arabidopsis plants produced significantly less pollen than did wild-type plants (t test, P < 0.05). Error bars indicate ± SEM (n > 100). Results include data from three independent experiments. (B) A representative tetrad from a wild-type anther. The arrow indicates the swollen domain. (C) to (E) Representative dyad (C), triad (D), and polyad (E) from the afh14-1 line. (F) Representative irregular tetrahedral arrangement of afh14-1 microspores. Bar = 5 μm.
AFH14 Serves as a Linker Protein Coordinating Microtubule and Microfilament Assembly in Plant Cells

Formins have been primarily shown to associate with actin and regulate actin dynamics by nucleating, capping, severing, elongating, and bundling microfilaments (Ingouff et al., 2005; Michelot et al., 2005; Yi et al., 2005; Vidali et al., 2009; Ye et al., 2009). Recently, various studies have suggested that formins can also directly regulate microtubule organization and stability in animal (Wen et al., 2004; Yasuda et al., 2004) and yeast cells (Delgehyr et al., 2008). However, all formins identified to date in plant cells have been characterized as microfilament regulators (Blanchoin and Staiger, 2008), with the exception of the most recently reported formin, AFH4, which can bind microtubules in addition to microfilaments (Deeks et al., 2010). In this study, we found that a type II formin from Arabidopsis, AFH14, appears to play essential roles in regulation of both microtubules and microfilaments. First, in vitro experiments revealed that the FH1FH2 fragment of AFH14 binds to purified microtubules and microfilaments directly and induces the formation of microtubule or microfilament bundles. Additional in vitro experiments conducted in the presence of both microtubules and microfilaments indicate that FH1FH2 binds preferentially to microtubules. This result differs from the function of the well-characterized animal formin, mDia2, which has a much higher in vitro binding affinity for microfilaments than for microtubules (Bartolini et al., 2008). mDia2-mediated promotion of microfilament polymerization and stabilization of microtubules are functionally separate events, and stable dimerization of mDia2 is not necessary to generate stable Glu microtubules (Bartolini et al., 2008). With respect to AFH4, it has been shown that the GOE domain located between the transmembrane and the FH1 domain binds directly to microtubules (Deeks et al., 2010). By contrast, our results show that the FH2 domain of AFH14 is critical for microtubule and microfilament binding. Although we have not directly analyzed the requirement for dimerization of AFH14 in bundling of microtubules or microfilaments, our results showed that microtubules compete with microfilaments to bind FH1FH2 and that an excess of FH1FH2 promotes bundling of microtubules with microfilaments. Combining these observations with the fact that FH1FH2 has a highly conserved domain structure and has been suggested to function as a tethered dimer (Kovar, 2006; Michelot et al., 2006), we propose that FH1FH2 or FH2 dimers of AFH14

Localization of AFH14 was assessed by confocal microscopy in wild-type PMCs during meiosis (A), metaphase I (B), anaphase I (C), telophase I (D), metaphase II (E), early cytokinesis (F), advanced cytokinesis (G), and the tetrad (H). AFH14 was dispersed throughout the cytoplasm in prophase I (A), telophase I (D), early cytokinesis (F), and in the tetrad (H). The RMS was present around the nuclei in these cells. AFH14 colocalized with spindle microtubules in metaphase I (B) and II (E). AFH14 colocalized with the phragmoplast microtubules in anaphase I (C) and in advanced cytokinesis (G). Images were derived from stacks of three or four sections containing the appropriate information. Bar = 5 μm.
are probably involved in the bundling of the filaments. In addition, for FH1FH2 and FH2, dots distributing along the bundled cytoskeletons seem uneven, which implies that AFH14 might form different sizes of multimers like the mouse formin mDia1 does (Li and Higgs, 2003) Furthermore, the microtubule bundling domain may overlap with the domain required for microfilament bundling. Bundling of microtubules with microfilaments has previously been observed for Cappuccino, a related formin in Drosophila melanogaster (Rosales-Nieves et al., 2006). These results seem to indicate that formins are evolutionarily conserved proteins that mediate interactions between microtubules and microfilaments in eukaryotic cells.

Increasing evidence has shown that interactions between the microtubule and microfilament cytoskeletons are common phenomena in plant cells. Coordination of microtubules and microfilaments in the cortical cytoplasm plays a key role during directional expansion of cells as well as during positioning of the plane of cell division. Microtubules and microfilaments cooperate during cell division to coordinate the mitotic and cytokinetic apparatus (Panteris and Galatis, 2005; Yasuda et al., 2005; Collings, 2008; Petrášek and Schwarzerová, 2009). However, little information is available at the protein level to understand the mechanisms by which microtubules and microfilaments interact. The cotton calponin homology domain (KCHs)–containing kinesins, Gh-KCH1 and Gh-KCH2, which have been shown to bind both microtubules and microfilaments in vitro, colocalize with microtubules and microfilaments in the cortical cytoplasm and in the midzone of the phragmoplast in dividing root tip cells (Xu et al., 2009), and the cytoskeletal dual binding activity of Gh-KCH2 has been demonstrated in Arabidopsis protoplasts. Os-KCH1, recently identified in rice (Oryza sativa), is associated with cortical microtubules and microfilaments in vivo and in vitro. These kinesins may play a role in both cell elongation and cell plate formation. Microtubule-associated 190-kD polypeptide (MAP190) from BY-2 cells, a protein that cosediments with both microtubules and microfilaments in vitro, has been shown to associate with the spindle and the phragmoplast during cell division (Igarashi et al., 2000; Hussey et al., 2002), but the in vitro cross-linking ability of MAP190 is not clear. Microtubule-associated protein SB401, which has been shown to bind both microtubules and microfilaments in vitro, colocalizes with cortical microtubules in pollen tubes (Huang et al., 2007).

**Figure 7.** Arrangement of Microtubule Arrays in Meiosis Is Affected in afh14 Male Meiocytes.

Immunostaining was used to assess tubulin localization in wild-type ([A] to [C]) and [G] to [I]) and afh14-1 ([D] to [F]) and [J] to [L]) male meiocytes in metaphase I ([A] and [D]), telophase I ([B] and [E]), metaphase II ([C] and [F]), early cytokinesis ([G] and [J]), midcytokinesis ([H] and [K]), and in tetrads ([I] and [L]). In metaphase I, the spindle in afh14-1 plants ([D]) was smaller than that of wild-type plants ([A]). In telophase I, two RMSs were present surrounding the nuclei in wild-type male meiocytes ([B]), but the RMS configuration was skewed in afh14-1 male meiocytes ([E]). In metaphase II, the spindles were perpendicular in wild-type plants ([C]) and parallel in afh14-1 plants ([F]). In early cytokinesis, microtubules radiate from the four nuclei in both wild-type ([G]) and afh14-1 plants ([J]). In advanced cytokinesis, phragmoplast microtubules were concentrated between the two nuclei in wild-type male meiocytes but were absent in the afh14-1 mutant ([K]). Four normal microspores were present in a tetrad from wild-type plants ([I]). Two 2n microspores were present in afh14-1 dyad ([L]). Bar = 5 μm.

**Figure 8.** FH1FH2 Recombinant Protein Binds Taxol-Stabilized Microtubules and Polymerized Microfilaments in Vitro.

(A) and (B) Taxol-stabilized microtubules (A) or polymerized microfilaments (B) were incubated with varying concentrations of recombinant FH1FH2 protein. After centrifugation, supernatants (S) and pellets (P) were analyzed by SDS-PAGE and Coomassie blue staining. (C) Quantification of scanned SDS-PAGE gel from (A). (D) Quantification of scanned SDS-PAGE gel from (B).
Figure 9. FH1FH2 Recombinant Protein Preferentially Binds Microtubules and Links Microfilaments and Microtubules in Vitro.

(A) to (M) To compare the binding of FH1FH2 to microtubules and microfilaments, 0.5-μM preformed microtubules (red) and/or 0.5-μM microfilaments (green) were incubated with 100 nM [A] to [J] or 1 μM [K] to [M] FH1FH2, and polymerization was assessed by confocal microscopy.

(A) Microfilaments exhibited considerable bundling 60 min after addition of FH1FH2.

(B) to (D) Addition of preformed taxol-stabilized rhodamine-conjugated microtubules to the reaction in (A) resulted in the formation of microtubule bundles within 5 min (B). Microtubule bundling increased and microfilament bundling decreased at 40 min (C) and 4 h (D) after the addition of polymerized microtubules.

(E) Microtubules exhibited significant bundling 60 min after the addition of FH1FH2.

(F) to (H) Microtubules remained bundled and microfilaments were randomly scattered throughout the solution 5 min (F), 40 min (G), and 4 h (H) after the addition of preformed microfilaments to the reaction in (E).

(I) In the absence of FH1FH2, microfilaments and microtubules were randomly scattered throughout the solution.

(J) Microtubule bundles were present at 5 min, 30 min, and 4 h after the addition of FH1FH2 to the reaction in (I), but microfilaments were not bundled.

(K) and (L) Excess FH1FH2 protein (1 μM) induced bundling of both microtubules and microfilaments at 30 min (K) and 4 h (L) after addition.

(M) Addition of excess antibodies directed against FH1FH2 to the reaction described in (K) caused the majority of cytoskeletal bundles to dissociate into single filaments.

(N) to (R) After incubation of 0.5 μM globular actin (G-actin) with preformed taxol-stabilized rhodamine-conjugated microtubules or microtubule bundles (0.5 μM) for 20 min, microfilaments and microtubules were examined by electron microscopy. White arrows indicate microtubules, and black arrows indicate microfilaments.
Consistent with this observation, SB401 binds preferentially to microtubules in the presence of both microtubules and microfilaments and has been shown to target specific organelles to microtubules (Huang et al., 2007). However, it is not clear whether this protein also binds microfilaments in vivo. Two microtubule plus end-interacting proteins, EB1 and CLASP, may be involved in root hair tip growth through interactions with microfilaments. Despite the identification of these proteins as in vitro cross-linkers of the microtubule and microfilament networks, significant evidence is still needed to bridge the gap between in vitro biochemical interactions and in vivo functional cellular interactions. Intriguingly, AFH14 decorates almost all mitotic and cytokinetic cytoskeletal apparatuses but does not localize to cortical cytoskeletal networks in interphase cells, which is different from the localization of PTEN-containing formins in moss (Vidali et al., 2009). In combination with our genetic, cellular, and biochemical studies, these results strongly suggest that AFH14 may have a function distinct from cytoskeletal linkers involved in interactions between microtubules and microfilaments in the cell cortex (Collings et al., 1998; Schwab et al., 2003; Preuss et al., 2004; Frey et al., 2009).

**AFH14 Plays an Important Role in Cell Division**

During mitosis and cytokinesis, microtubule arrays form the basis for mitotic and cytokinetic apparatuses, including the preprophase band, the mitotic spindle, and the phragmoplast. It is well accepted that the microfilament cytoskeleton is strongly linked to these microtubule structures during cell division, although subtle differences in microfilament distribution have been identified (Panteris, 2008). While some studies have shown that microfilaments codistribute with microtubules in the prophase band (Kakimoto and Shibaoka, 1987; Palevitz, 1987; Traas et al., 1987; Zachariadis et al., 2001, 2003), others have shown that cortical actin filaments do not always follow the pattern of the prophase microtubule band (Cho and Wick, 1990, 1991; Panteris et al., 1992, 2007; Collings and Wasteneys, 2005). In the mitotic spindle, microfilaments are usually present (Schmit and Lambert, 1987; Seagull et al., 1987; Traas et al., 1987; Loyd and Traas, 1988; Panteris et al., 1992; Cleary, 2001; Collings et al., 2001), but their absence from the spindle has also been reported (Liu and Palevitz, 1992; Baluska and Hasenstein, 1997; Vitha et al., 2000; Voigt et al., 2005). It is generally accepted that, in the phragmoplast, actin filaments lie parallel to microtubules (Panteris, 2008). This paradigm is consistent with a previous finding showing that microfilaments localize to the central region of the phragmoplast in BY-2 cells (Yoneda et al., 2004).

In agreement with previous results, our experiments in both *Arabidopsis* and BY-2 cells showed that microfilaments form a cage around spindle and that the length of phragmoplast microfilaments is shorter that than of microtubules. However, in AFH14-overexpressing cells, the microfilaments and microtubules appeared to be similar in length and aligned evenly with one another. By contrast, in cells with decreased AFH14 expression, the connections between microtubules and microfilaments were disturbed at several different levels. These results indicate that proper microfilament structure is important for stabilizing the spindle apparatus. We hypothesize that downregulation of AFH14 disturbs the interaction between microtubules and microfilaments, which initially causes microfilament instability and is shortly followed by alterations in microtubule structures important for mitosis and cytokinesis. Consistent with this idea, in animal cells, mDia2-mediated regulation of the microfilament cytoskeleton has been suggested to be required for proper distribution of stable microtubules (Bartolini and Gundersen, 2006).

The precise nature of microfilament distribution relative to microtubule distribution has remained somewhat unclear due to discrepancies in the observed distribution of microfilaments. These discrepancies could be explained by differences in techniques used for visualization (Maupin and Pollard, 1986; STAiger and Schliwa, 1987; Liu and Palevitz, 1992) and differences in cell types (Derkson et al., 1986; Schmit and Lambert, 1987; Haraguchi et al., 1997; Hasezawa et al., 1998; Silverman-Gavril and Forer, 2000). In addition, we propose that some of these discrepancies may also be due to differences in the timing of the images, as it is very difficult to obtain images at the exact same time point due to the extreme dynamics of the two cytoskeletal structures, particularly microfilaments. In this regard, functional studies characterizing cross-linking proteins that coordinate the two cytoskeletal networks may help us to understand the precise relationship between cytoskeletal microtubules and microfilaments.

Through analysis of two mutant *Arabidopsis* lines harboring T-DNA insertions within *AFH14, afh14-1*, and *afh14-2*, we found that AFH14 is involved in microspore generation. These mutant plants exhibited a variety of meiosis-related phenotypes, including dyads, triads, polyads, and irregular tetrahedral tetrads with abnormal microspore distributions. Dyads and triads may contribute primarily to the reduction of the total amount of pollen. These phenotypes imply that AFH14 is involved in the regulation of male meiotic division. Meiotic microtubule arrays have been described in a number of different species. During premeiotic interphase, microtubules form random networks throughout the...
cytoplasm. In telophase I, two RMSs are formed around the nuclei. In metaphase II, the spindles lie perpendicular to one another, and in early cytokinesis, RMSs radiate from the four nuclei. During advanced cytokinesis, microtubules are not only radially distributed around the entire nucleus but also form phragmoplast microtubules located between the two nuclei (Hogan, 1987; Brown and Lemmon, 1988; Traas et al., 1989; Staiger and Cande, 1991). We observed similar structures during tetrad formation in wild-type plants. However, in afh14-1 plants, abnormal microtubule structures were observed that included skewed RMSs in telophase I, parallel spindles in metaphase II, and the absence of phragmoplast microtubules in advanced cytokinesis. Compared with the strong disturbance of mitosis in suspension-cultured cells after downregulation of AFH14, T-DNA insertion mutants show relatively weak deviations in meiosis. The difference is probably due to gene redundancy or off-target silencing in RNA interference experiments.

The best documented and described meiotic abnormalities indicate that the RMS, the orientation of the spindle, and the cytokinetic microtubule systems affect the distribution of microspore nuclei in tetrads (Brown and Lemmon, 1988; Bretagnolle and Thompson, 1995; Magnard et al., 2001). For example, the RMS determines spindle positioning, and the orientation and position of the spindles reflect the positions of the microspore nuclei in the meiotic coenocyte (Heslop-Harrison, 1971; Dover, 1972; Brown and Lemmon, 1988). A multianipar second division spindle in Lonicera japonica causes a tetrahedral arrangement of nuclei, whereas a uniplanar division in Impatiens sultani results in a tetragonal nuclear arrangement (Brown and Lemmon, 1988).

During advanced cytokinesis, the structure of the phragmoplast microtubules maintains the microspore nuclear distance and positioning (Hogan, 1987). Extensive studies have characterized the mechanism of dyad formation and have found that parallel spindle orientation is the most common cause of dyads (Bretagnolle and Thompson, 1995; Genualdo et al., 1998; Consiglio et al., 2004; Jiang et al., 2009).

Microtubule–microfilament interactions have been proposed to be indispensable for male meiotic division. In support of this idea, in metaphase I and II, microfilaments codistribute with the spindle microtubules. During the second division, microfilaments intimately connect with the radiating microtubules and guide outgrowth of the phragmoplast (Schmit and Lambert, 1987; Traas et al., 1989; Staiger and Cande, 1991; Preuss et al., 2004; Yasuda et al., 2005). Drug-based experiments have shown that fragmentation of microfilaments leads to the disappearance of spindles and phragmoplasts. In addition to the disappearance of these microtubule-based structures, microfilaments normally concentrated around the chromosomes are absent (Traas et al., 1989). Microtubules also affect microfilament distribution, for example, the maize (Zea mays) dv and ms17 mutants display defects in microtubule distribution and a dramatic reorganization of microfilaments (Staiger and Cande, 1991). In animals, the mitotic microfilament cortex has been shown to guide spindle orientation in response to extracellular stimuli (Kunda and Baum, 2009). Consistent with this observation, dyad production appears to be related to deviations in the spatial configurations of microtubules and microfilaments in plants (Genualdo et al., 1998). Furthermore, treatment with cytochalasin D, a potent inhibitor of actin polymerization, often results in tetrads with disoriented cell walls (Magnard et al., 2001).

In summary, coordinate regulation of microtubules and microfilaments is thought to be important for the formation of regular microtubule configurations necessary for meiosis and mitosis. Based on direct genetic and biochemical evidence, we have demonstrated that AFH14 plays a key role in spatial regulation of cell division by mediating interaction between microtubules and microfilaments.

**METHODS**

**Plant Materials, Growth Conditions, and Transformations**

Arabidopsis thaliana afh14-1 (SALK_058886) and afh14-2 (SALK_038277) lines were obtained from the Salk Institute for Biological Studies. Wild-type and mutant plants were of the Columbia-0 ecotype. Plants were grown under similar conditions as described previously (Yi et al., 2005).

Arabidopsis thaliana suspension cultures and tobacco BY-2 (Nicotiana tabacum Bright Yellow-2) cells were grown as described by Smertenko et al. (2004). Agrobacterium tumefaciens (strain GV3101) was used for transformation of Arabidopsis and BY-2 cells. Cells were transformed as previously described (Yu et al., 2006). Exogenous gene expression in transgenic cell lines was induced by the addition of 2 μM or 100 nM 1β-D estradiol (Sigma-Aldrich) to Arabidopsis or BY-2 culture medium, respectively. Mock induction treatments consisting of DMSO alone were used as negative controls for all experiments.

**Screening of T-DNA Insertion Mutants and RT-PCR Analysis**

Genomic DNA extract was performed using a plant genomic DNA kit according to the manufacturer’s recommended protocol (Tiangen). T-DNA insertions in afh14-1 and afh14-2 were detected by PCR using the following primer sets: RP1, 5′-TGGTTGTTGTTGCTGTCAGG-3′; and LP1, 5′-GGCTCCCAAAGAATATCCAG-3′; and RP2, 5′-CTCGAGTGTCCTTTTGGCAAG-3′; and LP2, 5′-AACAGATGTCTTATCGAG-3′. LP1, LP2, and LBa1 (http://signal.salk.edu/tdnaprimers.2.html) were used to detect the wild-type gene.

For RT-PCR, total RNA was isolated from the 10-d-old wild-type, afh14-1, and afh14-2 plants using a plant RNA extraction kit (AutoLab), and 2 μg RNA was used for reverse transcription using a commercial kit (Invitrogen). Primers for PCR were as follows: afh14-1 (forward, 5′-TGATGCTAGAAGCTTTGAGCA-3′; reverse, 5′-AACAAAACTCCCTTTGTCGCCA-3′), afh14-2 (forward, 5′-AATAACGCTGCTACTAAAGAGGT-3′; reverse, 5′-CTCTGCTGTTCATGTAAGCAG-3′), and ACTIN2 (forward, 5′-AGGGCGTCTTTTCCAGTGTTG-3′; reverse, 5′-GTCGCTGTGATTCTTGGCTAC-3′).

**Cloning, Recombinant Protein Expression, and Construct Design**

PCR products were amplified using the primers shown below and were digested with the indicated restriction enzymes prior to introduction into different expression vectors. The AFH14 CDNA was amplified using the following primers: 5′-GGCGGCGCCATGCTGTTGTTAAGTAGTCTTTTAC-3′ (forward) and 5′-GGTACCTGTCTATGCTTAGGCTCT-3′ (reverse). Ascl and KpnI (underlined) were used for digestions. GFP was amplified from pCAMBIA1300-GFP-Fabd2 using 5′-CTCGAGATGTGTTGTTGTTTAC-3′ (forward) and 5′-GTCGCTGTGATTCTTGGCTAC-3′ (reverse), and KpnI or XhoI and SpeI were used for digestion, respectively. The ligated AFH14:GFP and GFP fragments were individually inserted into the pEBI vector.
plant transformation vector, which contains the G10-90 promoter (Zuo et al., 2000). AFH14 promoter was amplified from Arabidopsis genomic DNA using the following primers: 5′-GAGGTCCGTTGAAATCCTGAATTTGAGGTTT-3′ (forward) and 5′-CCATGGGAAAGCCTTGTTAATATGAAGAGCGG-3′ (reverse). SacI and Ncol were used for digestion. The AFH14 promoter fragment was then inserted into the pCAMBIA1305.1 vector (CAMBIA), which contains the GUS gene. The FH1FH2 and FH2 fragments were amplified from the AFH14 cDNA with the following primers 5′-GATACCCATGAGTCCAACACCTTCTCT-3′ and 5′-GGATCCATGTAGAGAAAGATGACCGGG-3′ (forward; respectively) and 5′-GGCGGGTCTTGCTCTTGAT GCTGTC-3′ (reverse). KpnI and Ncol were used to digest the fragments, which were then inserted individually into pet30-a expression vectors (Novagen) for protein expression in the Escherichia coli BL21 (DE3) strain. Recombinant protein was affinity purified as previously described (Sambrook and Russell, 2001).

An artificial microRNA construct was designed according to published protocols (Schwarb et al., 2006). The amiR-AFH14 construct included the miR319a precursor as a backbone (http://www.weigelworld.org), a designed duplex 21-nucleotide sequence (miRNA, 5′-TATTACGTCAT CATTCTGCGTG-3′, and miRNA*, 5′-CAAGCAAGATGTGCTGTAAT-3′) unique to Arabidopsis. FH1FH2 cDNA, and Xhol and SpeI restriction sites at the 5′ and 3′ ends, respectively. After digestion with Xhol and SpeI, the amiR-AFH14 fragment was introduced into the pX7 vector (Guo et al., 2003). All constructed plasmids were confirmed by DNA sequencing.

Antibody Preparation and Immunoblotting

The FH1FH2 fusion protein was used as an antigen to raise polyclonal antibodies in mouse, which was used to assess FH1FH2 chemical activity on microtubules and microfilaments for in vitro experiments. To assess endogenous AFH14 localization in Arabidopsis thaliana, actin was centrifuged at 100,000 g.

Rabbit muscle actin was purified according to published methods (Pardee and Spudich, 1982). Actin was centrifuged at 100,000 g at 25 °C for 10 min. Pellets were washed with 100 mM taxol-stabilized microtubules were mixed with 2, 4, 6, 8, 10, 12, 14, or 18 μM FH1FH2 recombinant protein in 100 μL PEMT buffer for 30 min at room temperature. The samples were centrifuged at 25,000g for 30 min at 25 °C. A volume of 480 or 304 μL 1× loading buffer was added to the resulting pellet or to 80 μL of supernatant, respectively.

To assess microtubule binding activity of FH1FH2, 2 μM preformed taxol-stabilized microtubules were mixed with 2, 4, 6, 8, 10, 12, 14, or 18 μM FH1FH2 recombinant protein in 100 μL PEMT buffer for 30 min at room temperature. The samples were centrifuged at 25,000g for 30 min at 25 °C. A volume of 480 or 304 μL 1× loading buffer was added to the resulting pellet or to 80 μL of supernatant, respectively.

For both assays, all samples were resolved on 12% SDS-PAGE gels. Gels were stained with Coomassie Brilliant Blue R 250 (Dingguo). FH1FH2-bound microtubules and microfilaments were quantified by scanning gels with UMAX PowerLook 2100XL (Umax). Protein concentrations were measured three times using a 318C microplate reader (Sanke).

Assessment of Microtubule and Microfilament Bundling Activity

To assess microtubule and microfilament preparations as described above were incubated without or with 1 μM active or heat-denatured FH1FH2 or FH2 at room temperature in a 10 μL total volume for 1 h. To determine whether FH1FH2 changed the stability of microtubules in vitro, solutions containing microtubules and active or heat-denatured FH1FH2 were incubated at 4°C for 30 min or 2 h. A 1-μL sample was then placed on a slide and visualized by confocal microscopy or fluorescence microscopy (Zeiss). Microtubules or microfilaments were also observed by negative staining under an electron microscope (Hitachi H600).

Staining and Immunolabeling for Fluorescence Microscopy

For immunolabeling, Arabidopsis and BY-2 cells were fixed in 4% (w/v) paraformaldehyde in PEM buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO4, 0.1 M mannitol, pH 6.9) for 30 min at room temperature. The cells were then rinsed three times with PEM buffer and treated with a solution of 0.5% Pectolyase and 1% cellulase R-10 in PEM buffer for 10 min at room temperature. After digestion with Pectolyase and Spel, the amir-AFH14 fragment was introduced into the pX7 vector (Guo et al., 2003). All constructed plasmids were confirmed by DNA sequencing.

Microtubule and Microfilament Cosedimentation Assays

FH1FH2 protein was centrifuged at 100,000g at 4°C for 1 h. To analyze microfilament binding activity of FH1FH2, 5 μM microfilaments and 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, or 1.0 μM FH1FH2 recombinant protein in a 100-μL total volume were incubated for 30 min at 37 °C. The samples were centrifuged at 100,000g for 30 min. The resulting pellets were then resuspended in 120 μL 1× loading buffer, and 16 μL 5× loading buffer was added to 80 μL of the resulting supernatant.

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To assess microtubule binding activity of FH1FH2, 2 μM preformed taxol-stabilized microtubules were mixed with 2, 4, 6, 8, 10, 12, 14, or 18 μM FH1FH2 recombinant protein in 100 μL PEMT buffer for 30 min at room temperature. The samples were centrifuged at 25,000g for 30 min at 25 °C. A volume of 480 or 304 μL 1× loading buffer was added to the resulting pellet or to 80 μL of supernatant, respectively.

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Staining and Immunolabeling for Fluorescence Microscopy

For immunolabeling, Arabidopsis and BY-2 cells were fixed in 4% (w/v) paraformaldehyde in PEM buffer (50 mM PIPES, 5 mM EGTA, 5 mM MgSO4, 0.1 M mannitol, pH 6.9) for 30 min at room temperature. The cells were then rinsed three times with PEM buffer and treated with a solution of 0.5% Pectolyase and 1% cellulase R-10 in PEM buffer for 10 min at room temperature. Afterward, the cells were incubated for 40 min in 3% (w/v) BSA in PEM buffer, followed by incubation with primary antibodies overnight at 4°C in a dark, moist chamber. Primary antibodies used for immunostaining included mouse AFH14 monoclonal antibodies (1:100 dilution) and rat monoclonal anti-α-tubulin YL1/2 antibodies (AbD Serotec; 1:200 dilution). The specimens were then washed three times for 10 min each in PEM buffer. For double immunolabeling, ant-mouse tetramethyl rhodamine isothiocyanate-conjugated antibodies (cross-adsorbed against rat and other IgGs; catalog number 715-025-151) and anti-rat fluorescent isothiocyanate-conjugated antibodies (cross-adsorbed against mouse and other IgGs; catalog number 112-095-167) were used for secondary antibody labeling. Finally, the cells were washed four times with PEM buffer and stained with DAPI (1 μg/mL) or 110 nM rhodamine-phalloidin (Invitrogen) if needed for 10 min prior to visualization by confocal laser scanning microscopy. As a negative control, each sample was incubated with only one of the primary antibodies, followed by incubation with a mixture of both secondary antibodies used in the experiment. No detectable signal was recorded in fluorescent channels that did not correspond to the primary antibody.

Inflorescences were fixed in Carnoy’s solution (6:3:1, ethanol:chloroform:glacial acetic acid) at room temperature for at least 24 h. To observe the tetrads, the inflorescences were stained in Carbol fuchsin for 48 h. An anther was then placed on a slide and gently rubbed between the cover glass and slide under the light microscope. This process resulted in the release of tetrads from the anther.

Preparation of PMCs for immunofluorescence microscopy was performed by as previously described (Jiang et al., 2009). Briefly, inflorescences were fixed in methanol:acetone (4:1, v/v) for 3 h, washing at 100g, and washed two times with PEM buffer. Anthers were then gently flattened on poly-L-lysine-coated slides, resulting in the release of PMCs from the anthers. Subsequently, slides were dried for 10 min. A thin layer of
agarose/gelatin (0.75% low melting agarose, 0.75% gelatin, and 0.3% sucrose) was spread on the male meiocytes. PMCs were then soaked in PEM buffer containing 2% Drilease (Dingguo) for 1 h at 37°C in the absence of light. Drilease was removed by washing in PEM buffer. Slides were then incubated in 1% Triton X-100/10% DMSO for 1 h at room temperature, followed by three washes. Afterward, the slides were incubated with AFH14 antibody and anti-α-tubulin antibodies (1:100 dilution) overnight, washed, and incubated with the appropriate secondary antibody (1:200 dilution) for 2 h. Finally, nuclei were stained by incubation with DAPI for 40 min as described above.

Drug Treatments
Cytoskeletal inhibitors used at the following concentrations: 10 μM oryzalin (Sigma-Aldrich) and 200 nM LatB (Invitrogen). After 30 h of induction, cells were incubated in fresh medium containing oryzalin or LatB for 15 or 50 min, respectively. Cells were then fixed and labeled with rhodamine-phalloidin or anti-α-tubulin as described above.

Microscopy
Confocal microscopy and image acquisition were performed using a Zeiss LSM 510 META confocal microscope. Zeiss ×63 oil objectives (Plan-Apochromat; numerical aperture of 1.4) were used for visualization. Serial confocal optical sections were taken at a step size of 0.5 to 1.0 μm. Images are presented as either single sections or stacks of neighboring sections. All images were processed using Adobe Photoshop 7.0 software.

Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers At1g31810/HM016081 (AFH14).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Predicted Domain Organization of the AFH14 Protein.
Supplemental Figure 2. Differences between the Amplified and Predicted AFH14 Coding Sequences (CDSs).
Supplemental Figure 3. FH1FH2 and AFH14 Are Specifically Recognized by Polyclonal and Monoclonal AFH14 Antibody, Respectively.
Supplemental Figure 4. Oryzalin Depolymerizes Microtubules in Uninduced Control BY-2.
Supplemental Figure 5. AFH14-GFP Localization Is Irregular or Dispersed in BY-2 Cells Treated with Oryzalin.
Supplemental Figure 6. Overexpression of AFH14-GFP Increases the Stability of Mitotic Microfilaments.
Supplemental Figure 7. Phragmoplast Microfilaments Codistribute with Microtubules in Uninduced Cells.
Supplemental Figure 8. Analysis of the AFH14 Expression Pattern in Arabidopsis Tissues and Organs.
Supplemental Figure 9. Schematic Showing the AFH14 T-DNA Insertion Locus.
Supplemental Figure 10. Microspore Formation Is Defective in afh14-2 Arabidopsis Plants.
Supplemental Figure 11. Microtubule Array Arrangement Is Defective in afh14-2 Plants.
Supplemental Figure 12. The FH1FH2 Recombinant Protein Nucleates Microfilaments in Vitro.
Supplemental Figure 13. Microtubule and Microfilament Organization in the Presence of FH1FH2.
Supplemental Figure 14. FH1FH2 Inhibits Cold-Induced Disassembly of Microtubules in Vitro.
Supplemental Figure 15. The Thickness of Filaments and Filament Bundles.
Supplemental Figure 16. Microtubule and Microfilament Organization in the Presence of the FH2 Recombinant Protein.

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