The plant formin AtFH4 interacts with both actin and microtubules, and contains a newly identified microtubule-binding domain

Michael J. Deeks1,*, Matyáš Fendrych2,3,*, Andrei Smertenko1, Kenneth S. Bell4, Karl Oparka4, Fatima Čvrčková2, Viktor Žársky2,3 and Patrick J. Hussey1,‡

1School of Biological and Biomedical Sciences, University of Durham, South Road, Durham DH1 3LE, UK
2Department of Plant Physiology, Faculty of Sciences, Charles University, Prague 12844, Czech Republic
3Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague 16502, Czech Republic
4Institute of Molecular Plant Sciences, University of Edinburgh, Mayfield Road, Edinburgh EH9 3JR, UK

*These authors contributed equally to this work
‡Author for correspondence (p.j.hussey@durham.ac.uk)

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Summary
The dynamic behaviour of the actin cytoskeleton in plants relies on the coordinated action of several classes of actin-binding proteins (ABPs). These ABPs include the plant-specific subfamilies of actin-nucleating formin proteins. The model plant species Arabidopsis thaliana has over 20 formin proteins, all of which contain plant-specific regions in place of the GTPase-binding domain, formin homology (FH)3 domain, and DAD and DID motifs found in many fungal and animal formins. We have identified for the first time a plant-specific domain within the group I formin AtFH4 that is capable of coupling aspects of the biophysical behaviour observed in vitro to membrane processes in vivo. Another group I formin, AtFH6, localises to the plasma membrane in expanding giant cells at the plant cell cortex (Fu et al., 2005; Crowell et al., 2009). Preliminary analysis shows that this region (named the GOE domain) binds directly to microtubules. Overexpressed AtFH4 accumulates at the endoplasmic reticulum membrane and co-aligns the endoplasmic reticulum with microtubules. The FH1 and FH2 domains of formins are conserved in plants, and we show that these domains of AtFH4 nucleate F-actin. Together, these data suggest that the combination of plant-specific and conserved domains enables AtFH4 to function as an interface between membranes and both major cytoskeletal networks.

Key words: Actin, Actin regulating proteins, Membrane, Microtubule

Introduction
The activities of the cytoskeleton impact multiple aspects of plant biology (for a review, see Hussey et al., 2006). In addition to supporting mitosis and subsequent cell division, actin filaments and microtubules also guide cell-wall synthesis, endomembrane trafficking and organelle motility. The organisation of the filaments of the eukaryote cytoskeleton requires many accessory proteins, including the formin family of actin-nucleating proteins. The model plant species Arabidopsis thaliana contains more than 20 formin isoforms. These can be divided according to sequence similarity and domain structure into two distinct groups (groups I and II). The divergence of these two groups is likely to be ancient, as representatives of both can be found in mosses (Grunt et al., 2008).

Like their animal and fungal homologues, the formin homology 1 and 2 (FH1 and FH2) domains of group I plant formins modify actin dynamics. The investigation of several isoforms in vitro has identified F-actin nucleation, bundling, capping and severing activities (Ingouff et al., 2005; Michelot et al., 2005; Deeks et al., 2005; Yi et al., 2005). Extensive biophysical studies exploiting total internal reflection fluorescence (TIRF) microscopy monitored the behaviour of the FH1-FH2 domains from group I isoform AtFH1 (Michelot et al., 2005; Michelot et al., 2006). The FH1-FH2 unit establishes filament polymerisation, but does not maintain a long-term association with the barbed end; this non-processive behaviour is accompanied by an ability to enhance the bundling of F-actin through a process of ‘zippering’ (Michelot et al., 2006). The FH1-FH2 unit can simultaneously nucleate and bundle filaments in vitro, creating actin cables that thicken by incorporating filaments that have originated at the cable through de novo nucleation.

Ten of the eleven group I isoforms of Arabidopsis have an N-terminal secretory signal sequence followed by a transmembrane domain. This domain architecture of formins has not been recognised beyond the Plantae kingdom [with the possible exception of a few invertebrate metazoans and protists (Grunt et al., 2008)]. The overexpression of full-length AtFH1 within pollen tubes causes excessive bundling and membrane-associated accumulation of F-actin in tip-growing cells (Cheung and Wu, 2004). Localisation of this activity at the plasma membrane was dependent on the signalling and transmembrane motifs of the AtFH1 N terminus, confirming that the unique domain structure of group I formins is capable of coupling aspects of the biophysical behaviour observed in vitro to membrane processes in vivo. Another group I formin, AtFH6, localises to the plasma membrane in expanding giant cells of nematode-induced galls (Favery et al., 2004), again consistent with an in vivo role in membrane-associated processes.

Many of the biological processes involving the cytoskeleton depend on the coordination of both actin filaments and microtubules at the plant cell cortex (Fu et al., 2005; Crowell et al., 2009). Preprophase band formation, epidermal cell interdigititation and cytoskeletal polarisation in response to pathogen attack all require local antagonism or cooperation between F-actin and microtubules. Signalling networks orchestrating this coordination are beginning to be characterised (Fu et al., 2005). We have identified a new plant-specific domain within the group I formin AtFH4 that is capable...
of associating with the microtubule cytoskeleton in vivo. In vitro, this domain binds directly to microtubules. Full-length AtFH4-GFP accumulates at the endoplasmic reticulum (ER) and causes microtubule co-alignment. Plant group I formin AtFH4 therefore has the potential to associate microtubule and actin arrays with lipid membranes.

Results and Discussion
AtFH4 associates with microtubules in vivo
To identify cytoplasmic interaction partners of formin AtFH4, the fusion construct AtFH4Δ1 was assembled, containing GFP coupled to the N terminus of the cytoplasmic section of AtFH4 (Fig. 1A). AtFH4 and its closest homologues, AtFH7 and AtFH8, constitute the group Ie subfamily of Arabidopsis formins (Cvrčková et al., 2004). Members of this clade share close sequence homology within the FH2 domain, and significant sequence similarity in a region of unknown function located between the transmembrane domain and the FH1 domain (Fig. 1A). We have named this region the group Ie (GOE) domain. The GOE domain corresponds to the automatically generated ProDom (Bru et al., 2005) domains PD038281 and PD224441, which are mutually related. The GFP-AtFH4Δ1 construct retains the FH1-FH2 region of AtFH4 and encompasses the 138-residue GOE domain.

GFP-AtFH4Δ1 was transiently transformed into leaves of Nicotiana benthamiana. Observation of transformed leaves three days after infiltration revealed the organisation of GFP-AtFH4Δ1 into a filamentous system resembling the cortical microtubule network (Fig. 1B). To label microtubules in vivo, we used the kinesin motor domain (KMD) of plant kinesin-7 fused to RFP (KMD-RFP). Leaves co-infiltrated with GFP-AtFH4Δ1 and KMD-RFP showed colocalisation of the GFP-AtFH4Δ1 and KMD-RFP networks (Fig.1B). The identity of the GFP-AtFH4Δ1 filament system was probed using drugs that target the cytoskeleton. Tissue transformed with GFP-AtFH4Δ1 and incubated with 10 μM of the actin-depolymerising drug latrunculin B retained an intact network resembling control treatments, despite developing a disorganised ER and cytoplasm (Fig. 1C). Equivalent treatments with the plant microtubule-disrupting agent amiprophosmethyl (APM) caused ER and cytoplasm (Fig. 1C). Equivalent treatments with the plant microtubule-disrupting agent amiprophosmethyl (APM) caused severe reduction in the number and length of filaments, progressing to the complete loss of GFP-AtFH4Δ1 structures. These data, together with the observed colocalisation of KMD-RFP, suggest that GFP-AtFH4Δ1 associates with microtubules. To confirm that the localisation of AtFH4Δ1 was not an artefact of the N. benthamiana-Agrobacterium tumeficiens transformation system, mature A. thaliana leaves were transformed with GFP-AtFH4Δ1 using a particle bombardment method, also revealing a microtubule network (supplementary material Fig. S1). The identity of the cytoskeletal network labelled by AtFH4Δ1 was further confirmed by imaging dynamic AtFH4Δ1-decorated filaments (Fig. 1D; supplementary material Movie 1). The elongation rate of GFP-AtFH4Δ1 is approximately 0.05 μm per second, similar to rates of microtubule elongation measured in Arabidopsis of approximately 0.1 μm per second (e.g. Kawamura and Wasteneys, 2008; Yao et al., 2008).

A domain for microtubule association is located within the GOE region
To identify the domain responsible for microtubule association, a further three deletion constructs of AtFH4 fused to GFP were transformed into leaves (Fig. 2A). AtFH4Δ2 contains the FH1 and FH2 domains, AtFH4Δ3 encompasses the FH2 domain only, and AtFH4Δ4 contains the GOE and FH1 domains. Besides AtFH4Δ1, only AtFH4Δ4 associates with microtubules (as summarised in Fig. 2A). AtFH4Δ1 and AtFH4Δ4 contain both the GOE domain and...
the FH1 domain, but the isolated FH1 domain of AtFH4A2 was not sufficient for microtubule association. A further deletion construct containing only the GOE domain, AtFH4A5, was transformed into plants to test the hypothesis that the GOE region encompasses the microtubule-association domain. AtFH4A5 localised to microtubules, as shown by a co-transformation with KMD-RFP (Fig. 2B); however, the ratio of cytoplasmic to microtubule association was noticeably higher for AtFH4A5 than AtFH4A4, suggesting that, although the GOE domain is sufficient for microtubule association, the neighbouring FH1 region has a minor influence on microtubule association in vivo.

The GOE domain binds to microtubules directly in vitro

The microtubule association of AtFH4 in vivo leads to the hypothesis that AtFH4 can interact directly with microtubules. Deletion fragments of AtFH4 were expressed and purified from Escherichia coli. The microtubule-binding capability of AtFH4A4 was compared with that of AtFH4A5 (the GOE domain) and with a new deletion, AtFH4A6, which contains only the FH1 domain. The protein fragments were mixed with taxol-stabilised microtubules and centrifuged to pellet the microtubules and associated proteins. Fig. 2C shows that both AtFH4A4 and AtFH4A5 sedimented with microtubules, whereas AtFH4A6 remained in the supernatant. Fragment AtFH4A4 noticeably decreased the proportion of tubulin remaining in the supernatant (Fig. 2C; supplementary material Fig. S2). To test its bundling potential, AtFH4A4 was mixed with taxol-stabilised and Oregon-Green-labelled microtubules. Increasing concentrations of AtFH4A4 were correlated with the co-alignment of microtubules (supplementary material Fig. S3), suggesting that the presence of AtFH4A4 promotes microtubule bundling. Together, these data show that the GOE domain exhibits microtubule-binding activity in vivo and in vitro, and that the neighbouring FH1 region also influences AtFH4-microtubule interactions.

In animals, three formins have been found to associate directly with microtubules: mDia1-2, Capu and INF1. The FH2 domains of mDia2 and Capu are responsible for mediating microtubule interactions in these proteins (Bartolini et al., 2008; Rosales-Nieves et al., 2006), with mDia1 also containing an N-terminal region in an analogous position to the AtFH4 GOE domain that is essential for mitotic spindle association (Kato et al., 2001). INF1 is a formin with an unusual domain structure, with two novel microtubule-binding motifs in an extended C terminus (Young et al., 2008). Neither the mDia1 N terminus nor the INF1 C terminus have primary sequence similarity to the GOE domain. When considering the evolutionary distance between the formin families of animals and angiosperm plants (Grunt et al., 2008; Chalkia et al., 2008), as well as the absence of GOE-related non-plant sequences detectable in GenBank by BLAST or PSI-BLAST, it seems likely that the microtubule affinity of the AtFH4 GOE domain originates from independent convergence towards similar functions rather than the ancient conservation of a shared function.

Formins of animals associate with the plus ends of microtubules using intermediates such as CLIP170, APC and EB1 (Lewkowicz et al., 2008; Wen et al., 2004). Our data do not exclude such additional interactions in plants; however, we have not isolated any known plus-end-binding proteins from AtFH4 interactor screens. In animals, the plus-end-associated interactions show functional
specificity. For example, mDia1 interactions with CLIP170 are only essential for phagocytosis mediated by CR3 and not for phagocytosis mediated by FcR (Lewkowicz et al., 2008). For plants, it is possible that formin isoforms other than AtFH4 use alternative methods to interact with microtubules, such as plus-end-binding proteins.

The actin cable network is distinct from AtFH4-decorated microtubules in vivo

The potential for the AtFH4Δ1 fragment to associate with actin filaments was assessed by coating Ni-NTA sepharose beads with 6×His-tagged AtFH4Δ1. Coated and uncoated beads were exposed to a polymerisation solution containing 2 μM of rhodamine-labelled actin. Rhodamine fluorescence accumulated in a growing corona around the coated beads before reaching a steady state approximately 10 minutes after initial exposure (Fig. 3A; supplementary material Movie 2). A growing corona of fluorescence was not observed to develop around either beads coated with AtFH4Δ4, which lacks the FH2 domain (supplementary material Fig. S4A), or uncoated beads (supplementary material Fig. S4B). The inclusion of 10 μM latrunculin B in the polymerisation medium prevented the formation of an AtFH4Δ1 corona (Fig. 3A; supplementary material Movie 3), confirming that the fluorescence was generated by rhodamine-actin polymerisation. Leaves were co-transformed with CFP-AtFH4Δ1 and GFP fused to the N terminus of Lifeact, a 17-residue peptide with affinity for G-actin and F-actin (Riedl et al., 2008). GFP-Lifeact revealed a network of F-actin cables distinct from the network of CFP-AtFH4Δ1 (Figs 2D.3B), showing that, under these in vivo conditions, the actin-interaction domains of AtFH4Δ1 do not serve to decorate F-actin arrays. Similar behaviour has been reported for Arabidopsis formin AtFH1. This formin in vitro nucleates actin filaments and has side-binding activity (Michelot et al., 2005; Michelot et al., 2006), but an equivalent AtFH1 fragment expressed transiently in pollen tubes does not decorate actin filaments (Cheung and Wu, 2004). Taken together, these data would suggest a dual role for AtFH4, whereby microtubules act as a scaffold to which the N-terminal GOE domain of AtFH4 is attached, allowing the C-terminal FH2 domain to freely regulate the nucleation of actin filaments from a relatively fixed position.

Full-length AtFH4 associates with microtubules in vivo

AtFH4 contains a secretory signal sequence and a single transmembrane domain within the N terminus. These domains are absent from the AtFH4Δ1 deletion, but are adjacent to the GOE microtubule-binding domain in the full-length protein (Fig. 1A). The influence of these domains on microtubule interaction was assessed by expressing full-length AtFH4-GFP in leaf epidermal cells. AtFH4-GFP was found to align with microtubules, but was simultaneously associated with an unidentified compartment (Fig. 4A; supplementary material Fig. S5). The coexpression of HDEL-CFP (a fusion protein retained by the ER) showed that AtFH4-GFP was localised to the ER (Fig. 4B). The organisation of the HDEL-CFP-labelled ER is aberrant in the presence of overexpressed AtFH4-GFP (Fig. 4B). Epidermal cells expressing GFP-AtFH4Δ1, which lacks the transmembrane domain, do not show ER-microtubule co-alignment (Fig. 4B, panel ii; supplementary material Fig. S6A). This contrasts with cells expressing AtFH4-GFP, in which ER networks adopt the configuration of the microtubule cytoskeleton (Fig. 4B panel vii; supplementary material Fig. S6B). ER tubules can be observed bridging co-aligned sections of the ER (supplementary material Fig. S7). The strong ER-microtubule co-alignment induced by AtFH4-GFP suggests that full-length AtFH4 can associate membranes and microtubules.

In animal cells, interactions with the microtubule cytoskeleton drive rearrangements of the ER. Kinesin motor proteins and plus-end-binding proteins both contribute to this process (Bola and Allan, 2009). Complementing these interactions are proteins such as CLIMP-63 and VAP-B, which provide static links between microtubules and the ER. Both CLIMP-63 and VAP-B are type II membrane proteins integrated into the ER membrane; the cytosolic domain of CLIMP-63 binds microtubules directly, but the mechanism for VAP-B remains unclear (Amarilio et al., 2005). Overexpression of both these classes of proteins causes ER rearrangement and ER-microtubule co-alignment (Amarilio et al., 2005; Vedrenne et al., 2005). To date, formins have not been identified as contributing to these ER-microtubule interactions, but mammalian formin INF2 was recently found to associate with the ER periphery of Swiss 3T3 cells (Chhabra et al., 2009). Constitutively active INF2 mutants caused actin rearrangements that were detrimental to the ER organisation, but the contribution of wild-type INF2 to ER function remains unknown. In plants, it has been thought that actin plays the major role in ER organisation and motility (Boevink et al., 1998), although during cell division the ER uses microtubules of the spindle and phragmoplast as an organisational template (Hepler and Jackson, 1968; Gupton et al., 2006). In characean algae, the alignment of the ER at the cell cortex during interphase is reliant on the cortical microtubule array during periods of cell elongation (Foisanner et al., 2009). Although AtFH4-GFP accumulates at the ER, AtFH4 does not contain a characterised ER membrane retention motif. Type I membrane proteins in plants are thought to be delivered by default to the plasma membrane in the absence of other targeting signals presented to the cytoplasm or encoded within transmembrane domains (Brandizzi et al., 2002). Membrane-integrated plant formins AtFH1 and AtFH6 have been shown to be trafficked to the
Although AtFH4 accumulates within the ER of transiently transformed *N. benthamiana* epidermal cells, it is plausible that AtFH4 can also be trafficked to the plasma membrane, because, for example, the passage of some transiently expressed integral membrane proteins is dependent on the coexpression of specific cofactors (Ribeiro et al., 2009). The destination for AtFH4 along the default secretory pathway could also be regulated by developmental or environmental factors. Immunofluorescence imaging of sections of Arabidopsis mesophyll labelled with anti-AtFH4 showed that endogenous AtFH4 in this tissue is localised to the cell cortex in proximity to the plasma membrane (Deeks et al., 2005); however, the transverse mesophyll tissue sections did not permit the observation of filamentous or reticulate structures. The arrangement of the cytoskeleton immediately adjacent to the plasma membrane is essential for the development of the cell wall. Membrane-integrated cellulose synthase complexes use microtubules to guide the deposition of cellulose microfibrils (Paredez et al., 2006). Moreover, coordination with the actin cytoskeleton is thought to be necessary for the correct delivery of cellulose synthase particles to sites of cell-wall assembly (Wightman and Turner, 2008; Crowell et al., 2009). A recent study using high-resolution scanning electron microscopy has confirmed the existence of ordered microtubule arrays in contact with the plasma membrane, and resolved physical linkages between microtubules and the membrane (Barton et al., 2008). The identity of these linkages remains unknown. Few protein candidates have been proposed, apart from plant phospholipase D (PLD), a peripheral membrane protein (Gardiner et al., 2001). Our data show that AtFH4 can co-align microtubules with membranes, demonstrating the potential for an additional microtubule-plasma membrane coupling mechanism (Fig. 4C), whereby the AtFH4 integral membrane protein acts as a scaffold for cytoskeletal organisation.

**Fig. 4. AtFH4-GFP co-aligns microtubules and ER.** (A) Full-length AtFH4 fused to GFP (green) colocalises simultaneously with the microtubule cytoskeleton (labelled with KMD-RFP; red) and a globular compartment. (B) Coexpression of AtFH4-GFP, KMD-RFP and the ER marker HDEL-CFP within cells identified co-alignment between AtFH4-GFP, the microtubule cytoskeleton and the ER. Panels i, ii, iii and iv show cells expressing the control construct GFP-AtFH4Δ1, which contains only the cytosolic domains of AtFH4. Panels v, vi, vii and viii show cells expressing full-length AtFH4-GFP. Panels i and v compare AtFH4-GFP localisation; panels ii and vi compare ER organisation; panels iii and vii compare microtubule organisation, whereas panels iv and viii show merged images of all panels. (C) A model depicting a putative role for AtFH4 at the plant membrane-cytoskeletal interface. Formins are depicted in contact with the barbed end of filaments, but the non-processive nature of AtFH1 and the ability of AtFH1 to bind to the flanks of filaments (Michelot et al., 2006) might suggest an alternative F-actin arrangement for AtFH4. Actin filament anchoring could theoretically occur in parallel to microtubule association, or microtubule and F-actin binding could be mutually exclusive.
Tagged protein expression and live cell imaging

The following fragments of AfTH4 were amplified and recombined into Gateway entry vector pDONR207: AfTH4A1 (codons 102 to 762), AfTH4A2 (codons 221-763), AfTH4A3 (codons 302-763), AfTH4A4 (codons 302-338), AfTH4A5 (codons 102-237) and AfTH4A6 (codons 221-302). For in vivo analysis, LR reaction mix was used to transfer inserts to N terminus fusion GFP vector pMD4C3 (Curts and Grossniklaus, 2003) and to GFP fusion vector pH7WG2 (Kartini et al., 2002). Construct KMD-RFP encodes 400 residues of the N terminus of N. benthamiana A90 (1 mM DTT, 0.2 mM AtF10, 10% glycerol, 50 mM KCl, 1 mM MgCl2, 2 mM DTT). Microtubules were polymerised from purified porcine brain tubulin at 30°C for 30 minutes in the presence of 1 mM GTP. Oregon-Green-labelled tubulin (Molecular Probes) was mixed with unlabelled tubulin for 10 minutes. Pellet and supernatant fractions were mixed immediately for 4 hours and imaged using a Zeiss LSM510 scanning confocal microscope (excitation 543 nm, 40x objective).

Protein expression and microtubule-binding assay

For protein expression, AfTH4 fragments were recombined into vector pGAT4 (Ketelaar et al., 2004), resulting in the fusion of a 6xHis tag to the N terminus of the respective fragments. Constructs were expressed in E. coli strain BL21 DE3 pLYS Rosetta 2 and purified using Ni-NTA resin (Qiagen) according to manufacturer’s guidelines. For the microtubule-binding assay, purified recombinant proteins were dialysed against PEM buffer with approximately 100 mM sodium phosphate, 10% glycerol, 50 mM NaCl, 2 mM DTT. Microtubules were polymerised from purified porcine brain tubulin at 30°C for 30 minutes in the presence of 1 mM GTP and 100 µM taxol. 4 µM of polymerised tubulin was co-incubated with 10 minutes in PEM buffer with approximately 1 µM of recombinant protein before centrifugation at 230,000g for 10 minutes. Pellet and supernatant fractions were mixed immediately after spinning with SDS-PAGE loading buffer. For microtubule-binding assays, Oregon-Green-labelled tubulin (Molecular Probes) was mixed with unlabelled tubulin at a ratio of 1:5. Tubulin was polymerised as described above and microtubules incorporating Oregon Green tubulin were sedimented, then resuspended in polyacrylamide buffer (10 mM imidazole pH 7.0, 50 mM KCl, 1 mM MgCl2, 2 mM EGTA, 1 mM DTT, 0.2 mM ATP) and diluted to a concentration of approximately 10 beads per microlitre. 1 µl of bead suspension was mixed with 2 µM of rhodamine-labelled actin monomer (cytoskeleton) and polymerisation buffer to a total volume of 10 µl. After gentle mixing, the reaction was transferred to a microscope slide. The slide was coated with a paraffin spacer with a cut square (5 mm sides) into which the bead suspension was deposited. A cover slip 10 mm×10 mm was used to seal the chamber. Images were taken every 100 seconds using a Zeiss LSM510 scanning confocal microscope (excitation 543 nm, 40x objective).

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References


Microtubule- and actin-binding plant formin


