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Summary of the Ph.D. Thesis

ROLE OF FORMINS IN THE ORGANIZATION AND DYNAMICS OF INTRACELLULAR STRUCTURES IN *Arabidopsis thaliana*

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ABSTRACT

On the basis of detailed phenotypic examination of *fh1* and *fh2* mutants we observed that the main housekeeping *Arabidopsis thaliana* formin AtFH1 (At3g25500) and its closest relative, *AtFH2* (At2g43800) are involved in both actin filaments and microtubule dynamics. *fh1* mutants showed increased sensitivity to the actin polymerization inhibitor Latrunculin B (LatB). Formin mutants had cotyledon pavement cells which exhibited more pronounced lobes compared to the wild type, and alterations in vascular tissue patterning were found. The double *fh1 fh2* homozygote was not obtained, suggesting that at least one functional formin gene is required for proper gametophyte development. Methods used to observe and quantify both architecture and dynamics of the cortical cytoskeleton from confocal laser scanning microscopy (CLSM) and variable angle epifluorescence microscopy (VAEM) were standarized and allowed to find that mutants exhibited more abundant but less dynamic F-actin bundles and more dynamic microtubules than wild type seedlings, *fh1* mutant phenotype observed in roots was further aggravated by a (heterozygous) *fh2* mutation. The formin inhibitor SMIFH2 mimicked the alterations observed in *fh1* mutants in plants, it has been the first report of this inhibitor in plants. Defects in membrane trafficking were observed in formin mutants and confirmed by SMIFH2 inhibitor which slowed down the movement of CLC-GFP dots.

ABSTRAKT

Na základě podrobné fenotypové charakterizace *fh1* a *fh2* mutantů bylo prokázáno, že AtFH1 (At3g25500), nejvíce exprimovaný formin *Arabidopsis thaliana*, a jeho nejbližší homolog *AtFH2* (At2g43800) ovlivňuje dynamiku aktinových mikrofilament a mikrotubulů. Mutanti postrádající *fh1* vykazovali zvýšenou citlivost k inhibitoru polymerace aktinu Latrunculinu B (LatB). Epidermální buňky děložních lístků forminových mutantů byly výrazněji laločnaté než u divokého typu, a byly také nalezeny rozdíly v uspořádání vodivých pletiv. Nepodařilo se získat dvojité homozygoty *fh1 fh2*, protože nejméně jeden z těchto dvou forminů je zřejmě nezbytný pro řádný vývoj gametofytu. Byly standardizovány metody pro pozorování a kvantitativní charakterizaci architektury a dynamiky kortikálního cytoskeletu s využitím konfokální laserové skenovací mikroskopie (*confocal laser scanning microscopy*, CLSM) a epifluorescenční mikroskopie s variabilním úhlem osvitu (*variable angle epifluorescence microscopy*, VAEM). Pomocí těchto metod se podařilo zjistot, že mutanti mají více F-aktinových provazců, které jsou méně dynamické než v rostlinách divokého typu, avšak dynamika mikrotubulů je zvýšená. Kořenový fenotyp, *fh1* mutantů byl nadále zesílen v přítomnosti heterozygotní *fh2* mutace. SMIFH2, inhibitor aktivity forminů, vyvolával podobné fenotypové změny jako mutace *fh;* tato pozorování jsou prvním dokladem o citlivosti rostlin k tomuto inhibitoru. Ve forminových mutantech byly nalezeny též poruchy v koloběhu membránových váčků, a úlohu forminů v tomto procesu potvrzuje i pozorování, že inhibitor SMIFH2 zpomaluje pohyb kompartmentů značených lehkým řetězcem klathrinu (CLC-GFP).

1. INTRODUCTION

Plant cytoskeleton plays important roles in cell shape determination (see Szymanski and Cosgrove, 2009; Smith and Oppenheimer, 2005; Mathur, 2004); microtubules are associated with the cellulose microfibrils arrangement as a key determinant of cell expansion pattern (Bibikova et al., 1999) while microfilaments promote and regulate growth by controlling intracellular trafficking affecting cell wall composition and membrane homeostasis (see Smith and Oppenheimer, 2005; Hussey et al., 2006).

Actin cytoskeleton is a highly dynamic network essential for a wide variety of basic cellular processes (see Schmidt and Hall, 1998; Dobrak et al., 2004; Hussey et al., 2006). The actin filaments turnover depends of accessory proteins activity which maintain the balance of actin monomers, initiate polymerization, restrict the length of actin filaments, regulate the assembly and turnover of actin filaments, and cross-link filaments into networks or bundles (see Pollard and Cooper, 2009; Chen et al., 2000). These events conform the known actin stochastic dynamic model which generate flexibility in the actin filament network (Smertenko et al., 2010; Staiger et al., 2009).

Actin filaments arrangement and dynamics modulate the membrane trafficking (see Pollard and Cooper, 2009; Mooren et al., 2012). In mammalian and yeast cells, F-actin dynamics are required for multiple distinct stages of clathrin-coated vesicle formation, including coated pit formation, constriction, and internalization (Yarar et al., 2005). Actin cables are involved in the transport of secretory vesicles (see Pruyne and Bretscher, 2000); localization of components of exocytic machinery and secretory vesicles are affected by disruption of

cytoskeleton (Zhang et al., 2001); however, vesicle tethering at the plasma membrane by the exocyst seems to be cytoskeleton- independent mechanism (Bendezu and Martin, 2011).

Actin filaments and their constant remodelling (Staiger et al., 2009) affect the microtubule dynamic due their strong crosstalk (Collings et al., 2006; Smertenko et al., 2010; Sampathkumar et al., 2011) may be mediated by bifunctional proteins or protein complexes (see Petrasek and Schwarzerova, 2009), from this side, formins seems to play important role in both actin filaments and microtubules organization and dynamics (Rosero et al., 2013).

The formin family is conformed by 21 *Arabidopsis* genes that are predicted to contain FH2 domain (Deeks et al., 2002). A subset of plant formins, referred to as group I, is distinct from formins from other species in having evolved a unique N-terminal structure with a signal peptide, a Pro-rich domain, potentially glycosylated extracellular domain, and a transmembrane domain (Banno and Chua, 2000, Cheung and Wu, 2004). Group-II plant formins have FH1 and FH2 domains, no known protein motif has yet been identified in group-II N-termini (Deeks et al., 2002).

AtFH1 formin protein, the main housekeeping of formin family, contains a putative signal peptide and a transmembrane domain suggesting its association with membrane Banno and Chua, 2000. Its overexpression of AtFH1 resulted in an abundance of membrane-associated actin cables, induced tube broadening, growth depolarization, and growth arrest in transformed pollen tubes (Cheung and Wu, 2004). Recently, the extracellular domain of AtFH1 formin was studied, AtFH1 forms a bridge that provides stable anchor points for the actin cytoskeleton, across the plasma membrane and is anchored within the cell wall. Proline-rich extracellular domain of AtFH1 has homology with cell-wall extensins (Martiniere et al., 2011).

2. AIMS OF THE THESIS

The main aim of this thesis was:

To characterize the effect of formin mutations on the organization and dynamics of intracellular structures, in particular the cortical cytoskeleton

This aim was addressed through the following particular aims:

1. To evaluate phenotypes of knockout AtFH1 and AtFH2 lines (Paper 1 and 2).
2. To investigate the possibility of using cytoskeletal inhibitors in order to uncover the effect of formin mutations.
3. To observe the effect of formin mutation in *in vivo* fluorescent protein-tagged cytoskeletal markers (GFP-FABD for actin and GFP-MAP4 for microtubules) using CSLM and VAEM microscopy.
4. To identify different intracellular processes affected by alterations in cytoskeleton organization and dynamics.
5. To optimize methods for quantifying actin filament and microtubule organization and dynamics.

3. MATERIAL AND METHODS

Plants

Two T-DNA insertional mutants (*fh1-1:* SALK-032981 and *fh1-2:* SALK-009693) in the *FH1* gene (At3g25500) and one (*fh2:*GK-066D02) in the *FH2* gene (At2g43800) were obtained through NASC (Alonso et al., 2003; Kleinboelting et al., 2012). Crossing between *fh1-1* and *fh2* mutants, and with reporter lines GFP-MAP4 (for microtubules) and GFP-FABD (for microfilaments) (Marc et al., 1998; Ketelaar et al., 2004) were performed. Formin wild type seedlings expressing both FABD-GFP and mCherry-TUA5 (Sampathkumar et al., 2011) or tagged clathrin light chain CLC-GFP (Ito et al., 2012) were used in some experiments.

Growth conditions and inhibitor treatments

Seed germination was synchronized by several days at 4°C, followed by growth on vertical MS or inhibitor-containing plates for 5, 10 or 15 days at 22°C with a 16h-light/8h-dark cycle. Inhibitor stock solutions were prepared in dimethylsulfoxide (DMSO), stored at −20°C (Lat B, Oryz, Tax, Jas, BFA and TyrA23) or 4°C (SMIFH2) and added to liquid agar to desired concentrations.

Pollen and silique analysis

Histochemical analysis of pollen was done according to Howden et al., 1998. Fertilized and aborted seeds were counted from mature siliques.

Organ and cell morphometric analyses

Root diameter, root growth and pavement cell shape parameters were determined (described in (Rosero et al., 2013; Rosero et al., submitted) using light microscope (BX-51, Olympus) or confocal laser-scanning microscope (LCS 510; Leica). Measurements were performed using the ImageJ software (http://rsbweb.nih.gov; Abramoff et al., 2004) using its built-in functions for calculating circularity.

Analysis of vascular tissue development

Cotyledons from 15 DAG seedlings were dissected and cleared, and venation complexity and connectivity were characterized according to Cnops et al., 2006.

Confocal microscopy and image analysis

GFP-tagged cytoskeleton was observed in roots or cotyledons using a confocal laser-scanning microscope (LCS 510; Leica) with a 63x/1.2 water-immersion objective and 488-nm argon laser (25 mW) excitation as described previously (Rosero et al., 2013). Microfilament bundling and density was quantified according to Higaki et al., 2010. Microtubule density was determined as the number of microtubules in an area of 400 μm^2 from confocal images in 5 cells from several plants. For measuring clathrin compartment dynamics, time-lapse images spanning two minutes were analyzed with the aid of the MTrackJ plugin incorporated in the Fiji ImageJ distribution (Meijering et al., 2012).

VAEM Microscopy

To evaluate cytoskeletal dynamics, we used the Leica AF6000 LX fluorescence platform with integrated TIRF module, the HCX PL APO 100x/1.46 oil inmersion objective. Time sequences were analyzed by ImageJ software as described before by Rosero et al., 2013. Kymographs were generated using Multiple Kymograph ImageJ plug-in (Sampathkumar *et al.*, 2011). The distribution of microtubule growth and shrinkage rates was estimated from at least 150 microtubule ends.

Electron microscopy

For transmission electron microscopy (TEM), 5-8 anthers or 9-10 cotyledons per sample were fixed and observed.

4. RESULTS AND DISCUSSION

The phenotypic characterization of T-DNA mutants with insertions in the main housekeeping Arabidopsis Class I membrane-targeted formin *AtFH1* (At3g25500) and its closest relative, *AtFH2* (At2g43800) grown on standard or cytoskeletal inhibitors (LatB or Oryz) medium allowed us to uncover the role of formins in cytoskeleton organization and dynamics, and in the cellular-tissue development.

4.1. Role of AtFH1and AtFH2 in actin and microtubule organization and dynamics

Altered balance between fine actin filaments and bundles was observed in formin mutants; thicker, more compact and less dynamic actin bundles were observed in roots of *fh1* mutant plants (Rosero et al., 2013). AtFH1 was previously proposed to participate in regular actin nucleation and elongation (Michelot et al., 2005; Michelot et al., 2006) and in actin filaments anchoring across the plasmalemma into the cell wall (Martiniere et al., 2011), which may effectively constrain bundling. Decreased availability of formins might disrupt the association between actin and cell wall, by reduction of new filaments polymerization from cell membrane and rendering some fine cortical actin filaments free to move and bundle, with the net effect being a shift in the balance between fine, presumably cortically anchored filaments and massive, to more stable bundles (Rosero et al., 2013 submitted). The less dense and more dynamic microtubules found in the formin mutants are the subsequent response to the absence or reduction of the restrictions on their distribution or movement imposed by cell wall-anchored formin-actin complexes (Martiniere et al., 2011).

4.2. Formin inhibition mimics the mutant phenotype

The small molecule SMIFH2, a 2-Thiooxodihydropyrimidine-4,6-dione derivative, is an inhibitor of FH2 domain-mediated actin assembly, active *in vitro* against several formins, and eliciting actin-related phenotypes in yeast and mammalian cells (Rizvi *et al.*, 2009).

In plants, SMIFH2 reduced root growth, increased microfilament bundling and decreased cortical microtubule density (Rosero et al., 2013), increased cell lobing and slowed down the movement of CLC-GFP (GFP-tagged clathrin light chain) dots (Rosero et al., 2013 submitted).

4.3. Role of AtFH1 in root isotropic growth

Consistent with primarily actin-related formin function, *fh1* mutants showed increased sensitivity to the actin polymerization inhibitor Latrunculin B (LatB). Disruption of microtubules affected *fh1* mutants and wt similarly. However, mutants exhibit increased sensitivity to Oryz in the presence of LatB and showed altered microtubule organization and dynamics, suggesting that AtFH1 may participate in a cross-talk between microfilaments and microtubules. Mutants had more dynamic microtubules and were partially resistant towards the root growth inhibition, radial root swelling and root twisting induced by the GFP-MAP4 marker and Taxol (Rosero et al., 2013), which can stabilize and bundle microtubules (Granger and Cyr, 2001; Hashimoto, 2002).

4.4. Role of AtFH1and AtFH2 in interdigitating pavement cell growth

Mutation AtFH1 and AtFH2 or formin inhibition by SMIFH2 reduced the leaf polarity affecting the planar pavement cell expansion by increasing cell size and lobing (Rosero et al., 2013 submitted). The currently accepted model of interdigitating growth highlights the role of microtubule bands in neck formation, while lobe expansion depends on patches of

microfilaments (Fu et al., 2005; Fu et al., 2009; Xu et al., 2010). The Rho of plant (ROP) GTPases and their RIC family interactors play an important role and recently, the important role of microtubule turnover induced by ROP6 GTPase for organizing paralleled cortical microtubule arrays was reported (Lin et al., 2013). Formins modulate cytoskeleton arrangement and dynamics, changes in actin filaments density and reduction in their dynamics probably affect the microtubules stability in *fh1* mutant which also showed alterations in pavement cell size and lobing (Rosero et al., 2013 submitted).

Absence of *AtFH1* and *AtFH2* showed to decreased membrane turnover by analogy with other systems via modulating actin filament arrangement and turnover, probably due the role of microfilament assembly anchorage to plasmalemma (Martiniere et al., 2011), both vesicle formation and transport could be affected. The inhibition of PIN1 endocytosis results in PIN1 polarization that enhances the lobe growth (Nagawa et al., 2012), thus both accumulation of cortical actin filaments and reduction of single actin filaments affect endocytosis.

4.5. Gametophytic function of AtFH1 and AtFH2

AtFH1 and/or *AtFH2* formins are required for normal gametophytic development, both defective pollen grains and aborted seeds in siliques of self-pollinated double heterozygotes or single copy *AtFH2* plants. Ultrastructural aberrations in tapetum and endothecial cells of the formin-impaired plants, including detachement of the plasma membrane from the cell wall and presence of amorphous lipid bodies suggest a possible downstream effects on intracellular dynamics including the endomembrane system (Rosero et al., 2013 submitted).

4.6. Formins contribute to membrane trafficking and tissue and organ development

The role of endocytosis during interdigitating cell growth was probed using TyrA23, an endocytosis inhibitor, which increased cell lobing and its effect was stronger in *fh1* plants. The formin inhibitor SMIFH2 slowed down the movement of CLC-GFP dots comparable with the LatB effect on endosomal movements (Voigt et al., 2005), confirming thus the important role of cytoskeleton in cell membrane recycling. The ultrastructure of single copy *AtFH2* pavement cells showed plasma membrane invaginations and thickened cell walls similar to the phenotype of dynamin mutants *drp1(*Carter et al., 2004) and *adl1A-2* (Kang et al., 2003), which may be due of inhibited membrane recycling. *AtFH1* and *AtFH2* participate in endocytosis via modulating actin filament arrangement and turnover, probably due the role of microfilament assembly anchorage to plasmalemma (Martiniere et al., 2011), both vesicle formation and transport could be affected; thus insufficient formin function results in decreased membrane trafficking that also affect the distribution of PIN auxin carriers. Thus, alterations in cytoskeleton turnover affect pavement cell lobe development as reported previously by localized inhibition of PIN1 endocytocis induces PIN1 polarization in the lobing region (Nagawa et al., 2012), affect auxin transport and auxin-carriers recycling (Geldner et al., 2001; Dhonukshe et al., 2008) and auxin-dependent developmental processes as vascular tissue patterning (Rosero et al., 2013 submitted). The role of cytoskeleton in vascular pattern was observed in formin mutants and using SMIFH2, LatB and Oryz treatments. Auxin maxima visualized by DR5-GFP expression were altered in formin mutants (Rosero et al., 2013 submitted), consistent with possible defects in PIN polarity due to alterations in their recycling (Geldner et al., 2001; Dhonukshe et al., 2008).

5. CONCLUSIONS

Formin AtFH1 (At3g25500) and its closest relative, AtFH2 (At2g43800) modulate both actin and microtubule organization and dynamics and their mutations produced root growth sensitivity to LatB, defects in interdigitating cell expansion, abnormal gametophyte development and alterations in the vascular pattern. These defects are associated with the downstream effect of formin mutations in membrane trafficking. This phenotype was mimicked by SMIFH2 Formin inhibitor.

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PUBLICATIONS AND RESEARCH

From PhD

Accepted and submitted papers

Rosero, A.; Grunt, M.; Schiebertová, P.; Žárský, V.; Cvrčková, F. Arabidopsis FH1 and FH2 formins affect pavement cell shape and vascular patterning by modulating cytoskeleton and membrane dynamics. Plant Cell (Submitted).

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Rosero, A.; Žárský, V.; Cvrčková, F. 2013. AtFH1 formin mutation affects actin filament and microtubule dynamics in *Arabidopsis thaliana.* Journal of Experimental Botany 64(2): 585–597.

Selected abstracts

Rosero. A.; Cvrčková, F. and Žárský, V. 2012. AtFH1 formin mutation affects actin filament and microtubule dynamics in Arabidopsis thaliana. Plant and microbial cytoskeleton/Gordon Conference. Andover-USA.

Rosero. A.; Sebesta, O.; Cvrčková, F. and Žárský, V. 2011. Microtubules distribution and cell shape are affected by AtFH1 formin mutation in Arabidopsis. Abstract book Society Experimental Biology Annual Main Meeting 2011. Glasgow Scotland.

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