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Hledání a charakterizace interakčních partnerů rostlinných forminů

Identification and characterization of proteins interacting with plant formins

Diplomová práce

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze,

Podpis

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Abstract:

Formins are evolutionarily conserved proteins participating in actin and microtubule organisation, affecting thus also intracellular transport, cell growth, morphogenesis and cell polarity. All formins contain formin homology-2 domain (FH2), well known to dimerize and act as a nucleator of actin. Angiosperms have two formin clades, Class I and Class II, which are distinguished by domain organisation. Based on knowledge from animal models and protein sequence homology, two groups of candidate membrane-associated formin interactors have been proposed in Arabidopsis (Cvrčková, 2013).

First group of candidates consists of FYVE domain-containing proteins FAB1A (At4g33240) and FAB1B (At3g14270), the other group contains proteins with BAR and SH3 domains AtSH3P1 (At1g31440), AtSH3P2 (At4g346600) and AtSH3P3 (At4g18060).

Yeast two hybrid assay was used to examine protein interactions of selected proteins from both candidate interactor groups (FAB1A, SH3P2 and SH3P3) with FH2 domains representing both plant formin clades. The same experimental setup was also used to test dimerization among FH2 domains of plant formins. Translational fusions of FH2 domains from Class I formins AtFH1 (At3g25500), AtFH5 (At5g54650) and Class II representatives AtFH13 (At5g58160) and AtFH14 (At1g31810) with the GAL4 activation domain have been co-expressed in yeast with GAL4 DNA binding domain fusions of the candidate interactors or other plant formin FH2 domain.

Strong interaction between the FH2 domains of AtFH5 with AtSH3P3 protein has been confirmed, while the other candidates did not interact in this experimental setup.

Homodimerization of FH2 domain of AtFH13 has been confirmed as well as heterodimerization of FH2 domains of AtFH13 with AtFH14, showing that heterodimerization between FH2 domains of closely related non-identical formins may take place. However, no other dimerization was observed, albeit this does not rule out the possibility that other interactions may take place under different experimental conditions.

Key words: Formins, FH2 domain, dimerization, interactions, FAB, BAR, FYVE, SH3, yeast two hybrid assay

Abstrakt:

Fominy jsou evolučně konzervované protein, účastnící se organizace aktinového a mikrotubulárního cytoskeletu, ovlivňují tedy intracelulární transport, buněčný růst, morfogenezi a buněčnou polaritu. Všechny forminy obsahují doménu formin homology-2 (FH2), která je známá pro svou vlastnost tvořit dimery a nukleovat aktin.

Cévnaté rostliny mají dvě forminové evoluční větve, třídu I a třídu II, které jsou mezi sebou rozdílné doménovou organizací. Na základě znalostí živočišných modelů a homologie proteinových sekvencí byly navrženy dvě skupiny membránově-asociovaných proteinů jako interaktoři forminů v huseníčku (Cvrčková, 2013).

První skupina kandidátů zahrnuje proteiny s FYVE doménou: FAB1A (At4g33240) a FAB1B (At3g14270), druhá skupina zahrnuje tři proteiny s doménami BAR a SH3: AtSH3P1 (At1g31440), AtSH3P2 (At4g346600) a AtSH3P3 (At4g18060). Kvasinkový dvouhybridní systém byl použit na testování proteinových interakcí vybraných proteinů z obou skupin kandidátních interakčních partnerů (FAB1A, SH3P2 a SH3P3) spolu s FH2 doménami z obou tříd rostlinných forminů. Stejný experimentální systém byl použit na testování dimerizace mezi FH2 doménami rostlinných forminů.

Translační fúze FH2 domén z forminové třídy I reprezentované AtFH1 (At3g25500), AtFH5 (At5g54650), AtFH8 (At1g70140) a z třídy II reprezentované AtFH13 (At5g58160) a AtFH14 (At1g31810) s fúzované s GAL4 aktivační doménou byli ko-exprimované v kvasinkách s vybranými interaktory nebo dalšími FH2 doménami rostlinných forminů fúzovanými s GAL4 DNA vazebnou doménou.

Potvrdila se silná interakce mezi FH2 doménami AtFH5 s AtSH3P3 ale další kandidátní proteiny nevykazovaly žádnou interakci v použitém experimentálním systému.

Dále se potvrdila homodimerizace FH2 domén proteinu AtFH13 a heterodimerizace FH2 domén forminů AtFH13 a AtFH14, prokazující že heterodierizace mezi blízce příbuznými, neidentickými forminy je možná. Avšak žádné další dimerizace nebyly potvrzeny i když to nevylučuje možnost interakce v jiných experimentálních podmínkách.

Klíčová slova: Forminy, FH2 doména, dimerizace, interakce, FAB, BAR, FYVE SH3, kvasinkový dvouhybridní test

List of abbreviations:

ATG8	Autophagy-related protein 8
BAR	Bin–Amphiphysin–Rvs domain
Cdc42	Cell division control protein 42 homolog
CD21	Complement receptor type 2 (CR2)=C3d receptor=Epstein-Barr virus receptor
DAAM	Disheveled-associated activator of morphogenesis 1
DAD	Diaphanous autoregulatory domain
DID	Diaphanous inhibitory domain
DMSO	Dimethyl sulfoxide
DRF	Diaphanous related formins
eEF1A	Eukaryotic elongation factor 1-alpha 1
EDTA	Ethylenediaminetetraacetic acid
FBP17	Formin-binding protein 17
FH	Formin homology
FH1	Formin Homology 1 domain
FH2	Formin homology 2 domain
FHOS	Formin homology 2 domain containing ortholog
FMNL3	Formin-Like 3
FRET	Förster or Fluorescence Resonance Energy Transfer
FYVE	FYVE zinc finger domain, named after Fab 1, YOTB, Vac 1, EEA1
GAL4	Regulatory protein, positive regulator for the gene expression of the galactose-induced genes
GBD	GTPase-binding domain
GTPase	Large family of enzymes that can bind and hydrolyze GTP
GUS	Beta-glucuronidase (in context of GUS reporter system)
LatB	Latrunculin B
LD	Limb deformity
mDia1	Diaphanous-related formin-1
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PCR	Polymerase chain reaction

PDZ	Structural domain of 80-90 amino acids, named after proteins where discovered: Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), Zonula occludens-1 protein (zo-1)
PTEN	Phosphatase and tensin homolog domain
RBD	Rho binding domain
Rho	Family of small signaling G proteins, a subfamily of the Ras superfamily
RhoGAP	Evolutionary conserved protein domain of GTPase activating proteins
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium dodecyl sulfate
SH3	SRC Homology 3 Domain
srGAP2	SLIT-ROBO Rho GTPase-activating protein 2
TAIR	The Arabidopsis Information Resource
TMR	Tetramethyl-rhodamine-labeled
Tris-HCl	Tris(hydroxymethyl)aminomethane-HCl

Table of content:

1. Introduction

1.1. Formins

Formins are proteins interacting with cytoskeleton, which is an indispensable part of each eukaryotic cell; in plant cells virtually all processes are connected to the cytoskeleton. Not only it has a stabilizing function, working as a cell "scaffold", but it also has irreplaceable role in all dynamic processes such as targeting and moving organelles, cytoplasmic streaming, formation and transport of vesicles, establishing cell polarity, cell division including creation of phragmoplast followed by deposition of components of cell wall etc. Components of the cytoskeleton are being rebuilt continuously, and its central two components – actin and tubulin – can occur as monomers, polymeric filaments (microfilaments and microtubules), and other more complex formations as complicated networks of filaments.

Obviously, such networks need to be controlled very precisely. One of the essential groups of proteins responsible for dynamic interaction with (mainly actin) cytoskeleton are formins. Formins or formin homology (FH) proteins are presents in eukaryotes but have not been confirmed in any prokaryotes (Grunt et al., 2008). They all contain evolutionarily conserved domain formin homology 2 (FH2, see chapter 1.4.), which has been confirmed in all eukaryotic model organisms, for example *Arabidopsis thaliana* (Rivero et al., 2005, Cvrčková et al., 2014,), *Saccharomyces cerevisiae* (Liu et al., 2010), *Caenorhabditis elegans* (Heil-Chapdelaine et al., 1999*)*, *Drosophila melanogaster* (Castrillon and Wasserman, 1994), *Mus musculus* (reviewed in Dietrich et al., 2013, and see below) and human (Schoenichen and Geyer, 2010).

First discovered formin was Limb Deformity (LD) of mice. Name is due to phenotype in mice where mutants had malformed limbs, in other words, "wrongly formed" thus the later name formin (Kleinebrecht et al., 1982). However, these results were rejected in the light of discovery of neighboring gene Gremlin coding a transcription factor, which is responsible for the phenotype instead of formin (Zuniga et al., 2004).

Main function of formins lies within their capability to nucleate actin (see chapter Formin Homology domain, reviewed for example by Campelone and Welsch, 2010, Skau and Watterman, 2015). Interestingly, plant formins are able to interact with microtubules as well (recently reviewed by Wang et al., 2012, Hamada, 2014, Cvrckova et al., 2015, Liu et al., 2015).

1.2. Structure and composition of formins

Formins are rather large proteins, often exceeding length of 1000 amino acids, and contain well-conserved domains. However, their domain structure is not uniform, since different kingdoms and organisms often don't share the same domain architecture and their domain occur in different combinations, depending on organism (see figure 1, Chalkia et al., 2008, Grunt et al., 2008). They all share conserved core domain FH2, located mostly at the C-terminal end (Chalkia et al., 2008, Grunt et al., 2008). FH2 domain is crucial for reaction with actin (see chapter 1.4.). Other most commonly shared domain is prolin-rich Formin Homology 1 domain (FH1), which mediates certain interaction, such as with profilin or SH3 proteins (Castrillon and Wasserman, 1994, Uetz et al., 1996).

Other characteristic domains are regions binding small GTPases. They are well described in animals and fungi, but missing in plants. The domain FH3 has been described in yeasts and animals (Rivero et al., 2005) and can interact with small GTPases (Dames et al., 2011) and also is responsible for localization of the formin within the cell (Evangelista et al., 2003, Petersen et al., 2008). The FH3 domain is loosely conserved and is partially overlapping with the GTPase-bidning domain (GBD) (Deeks et al., 2002). Given such evidence, it is speculative if above mentioned functions are really a responsibility of FH3 domain or they are more likely caused by different structures (Higgs, 2005). GBD-GTPase binding domain, in other words RBD-Rho binding, is typical for Diaphanous-related formins and is characteristic for subfamily of Diaphanous-related formins (DRF) (Ridley 2006). Active (GTP-bound) forms of small GTPases from Rho subfamily bind to this domain, and interaction with GDP-bound GTPases is also known (Tian et al., 2002). Rho GTPases are notorious for their participation in cytoskeletal dynamics control and, among other, participate in cell division as well as formins (Wu and Leu, 2013, Munoz et al., 2014). The binding of GTPase onto GBD can be regulated by Diaphanous autoregulatory domain (DAD), which is located on the C-terminal end of the DRFs, while Diaphanous inhibitory domain (DID) is located at the N-terminal end so that they can interact within the same molecule (Copeland et al., 2007). The linkage DID-DAD blocks the binding site for GTPase, however, activated GTPase can replace attached DAD, and at the same time, the DAD is blocked. In such case, formins function is inhibited (Higgs, 2005). Behind GBD domain, N-terminal to the FH1, is sometimes located Coiled-coil domain (Wallar and Alberts, 2002), allowing oligomerization of proteins. Confirmed interactions include binding of catenins, proteins involved in signaling related to adhesion between neighboring cells (Chesarone et al., 2010).

In the yeast formin Bni1p, a region responsible for interaction with elongation factor eEF1A was discovered. eEFA1 belongs in the group of small GTPases. This region is located between domain FH1 and FH2 and the interaction is suggesting a possibility of participation in regulation of protein expression or targeting mRNA with necessary proteins along the cytoskeleton to the translation apparatus (Gross and Kinzy, 2007).

Other domain worth mentioning is PDZ, which was found in formin called delfilin, presented in the neural cells. Thanks to this domain, delfilin is able to interact with glutamate receptor. The binding of the receptor then results in the rebuilding of cytoskeleton, which can influence the direction of the growth of axon and possibly also stimulate endocytosis of glutamate receptors as a part of negative feedback (reviewed by Wallar and Alberts, 2003). Summary of formins domain organization is in figure 1.

Formins may contain some additional specific domains; those specific to plant formins are discussed in following chapter.

Recently, the nomenclature and classification has been reviwed based on the domain organisation from avaliable dates of various eucariotic organisms (Grunt et al., 2008). Based on the domain analysis, cruicial differences between opisthokonts and plants were discovered (Cvrčková et al., 2004, Higgs and Peterson 2004, Rivero et al., 2005).

1.3. Plant formins

Since formins are in general well conserved, the characteristics mentioned above apply also for plants, albeit some differences between plants and opisthokonts exist. Plant formins are more numerous (21 isoforms in *Arabidopsis thaliana*); they can be divided into three classes based on FH2 domain phylogeny (Deeks et al 2002, Grunt et al., 2008, reviewed Blanchoin et al., 2010). The quantity of genes probably coincides with expansion of the plants to dry land. Family II and I are present in angiosperms (including *Arabidopsis thaliana*) but class III is present only in some algae, mosses and lycophytes making it a specific branch. Class III is characterized by possession of RhoGAP-related domain (Grunt et al. 2008). Such domain is probably replacement of GBD/FH3 (mentioned in previous chapter). Formin from classes I and II also include unique, alternative membrane attachment mechanisms. Class I formins (in Arabidopsis including AtFH1-AtFH11) typically contain unique N-terminal structure with signal peptide together with serin/prolin rich region and a transmembrane domain Banno and Chua 2000, Cvrčková 2000, (Cheung and Wu, 2004). Class II formins (in Arabidopsis including AtFH12-AtFH21) on the other hand contain Phosphatase and tensin homolog domain (PTEN) domain, which was predicted and most probably target formin to the plasmatic membrane

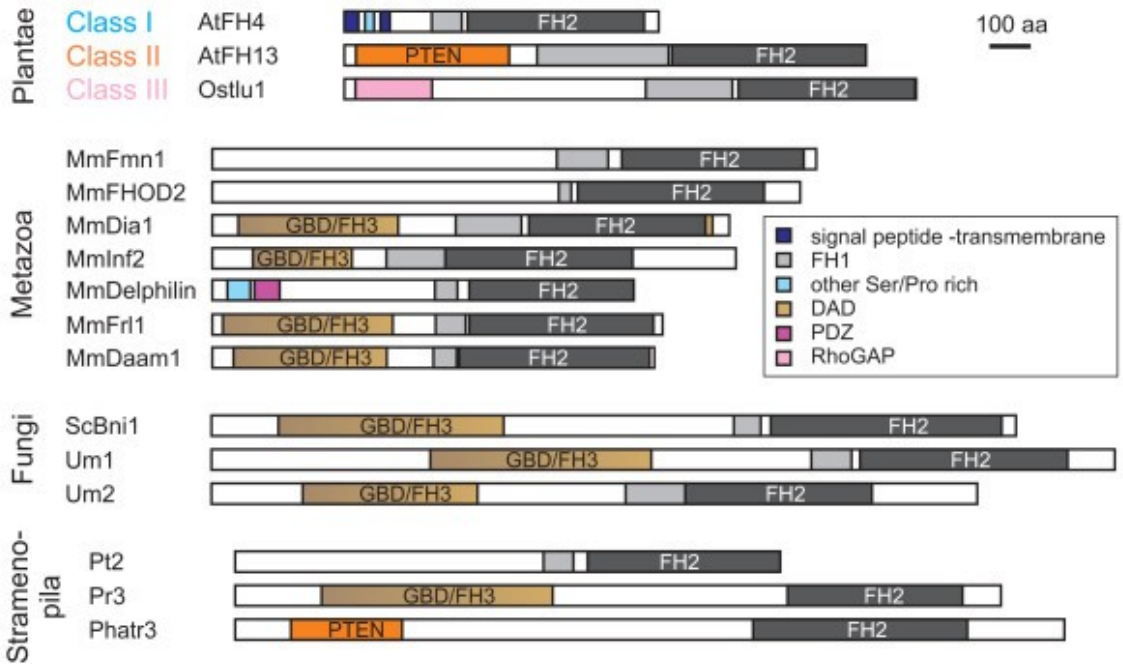


Figure 1 Summary of formin domains organization

Abbreviations of names of organisms: At – *Arabidopsis thaliana*; Mm – *Mus musculus*; Sc – *Saccharomyces cerevisiae*; Um – *Ustilago maydis*; Pt – *Paramecium tetraurelia*; Pr – *Phytophtora ramorum*; Phatr – *Phaeodactylum tricornutum*. Names of proteins: FH – Formin homology; Fmn – Formin; Fhod – FH domain-containing protein; Dia – Diaphanous; Inf – Inverted formin; FRL – Formin- related gene in leukocytes; Daam – Dishevelled-associated activator of morphogenesis; Bni – Bud neck involved. Names of domains: FH – Formin homology; DAD – Diaphanous autoregulatory domain; PDZ – Postsynaptic-density protein; RhoGAP – GTPase-activator protein for Rho-like GTPases; GBD – GTPase-binding domain; PTEN – Phosphatase and tensin-related. From Grunt et al., 2008.

(Cvrckova et al., 2004, Favery et al., 2004). Experimentally, such hypothesis has been confirmed in *Physcomitrella patens* (Vidali et al., 2009, van Gisbergen et al., 2012). The comparison with other formins can be seen at figure 1.

Regarding biochemical properties of plant formins, all so far examined formins can nucleate actin Their physiological functions are various, most often related to polarized growth, cell division, reorganization and spatial organization of cytoskeleton etc. (reviewed Blanchoin and Staiger, 2010, Wang et al., 2012).

Formin AtFH1, a typical Class I member, is involved in creating bridges from the actin cytoskeleton, across the plasma membrane and is anchored within the cell wall (Martiniere et al., 2011). Its mutation leads to increased sensitivity to the actin polymerization inhibitor latrunculin B (LatB). Mutant fh1 had thicker and shorter roots compared to wild-type as well as reduced cell elongation of trichoblasts on low LatB doses (Rosero et al., 2013). Its closest relative AtFH2 lacks published studies, even though it has been investigated (Rosero, 2013). AtFH3 is expressed in pollen and responsible for the formation of longitudinal actin cables in pollen tubes, which are important for cytoplasmic streaming and polarized growth. Its overexpression in tobacco (*Nicotiana tabacum*) pollen tubes induced excessive actin cables, which extended into the tubes' apices (Ye et al., 2009).

AtFH4 has been proved to interact with both actin and microtubule cytoskeleton (Deeks et al., 2010). Unique combination of conserved and plant specific domain makes of AtFH4 a mediator of network between membranes and both major cytoskeletal networks. Its closest relativeAtFH8 is also important for polarized growth since overexpression of the protein in Arabidopsis caused changes in root hair cell development and its actin organization (Yi et al., 2005). According to fluorescent protein fusion and immunolocalization assays, the AtFH8 protein has been localized to cross-walls of root cortex cells (together with AtFH4, Deeks et al., 2005), as well as to the nuclear envelope in interphase and to the new cell wall after cytokinesis and GUS histochemical staining revealed AtFH8 to be predominantly in Arabidopsis root meristem, vasculature, and outgrowth points of lateral roots (Xue et al., 2011).

AtFH5 was experimentally proven to be involved in cytokinesis; loss of its function compromises cytokinesis in the seed endosperm (Ingouff et al., 2005). Furthermore the AtFH5 gene is target of Polycomb group (PcG). PcG activity silences the paternal allele and thus restricts its expression to the maternal allele. AtFH5 is thus also responsible for morphological defects caused by the loss of PcG activity in the seed (Gerald et al., 2009).

Favery et al. (2004) studied AtFH6, which is highly upregulated in giant cells, and concluded that AtFH6 regulates polarized growth by controlling the assembly of actin cables.

They also showed that AtFH6 is anchored to the plasma membrane and uniformly distributed, and suggested that this protein might be involved in the isotropic growth of hypertrophied feeding cells via the reorganization of the actin cytoskeleton.

There are no available data on other plant *Arabidopsis* formins from class I. Partially it could be caused by the abundance of formin genes and their similar and overlying physiological functions making this research more challenging. To a smaller extent, plant formins are being studied also in model crop *Oryza sativa.* Formin homology 1 (OsFH1) regulates root-hair elongation in rice. The mutant *Osfh1* exhibited root-hair defects when roots were grown submerged in solution, and mutant roots produced normal root hairs in the air (Huang et al., 2013). *BENT UPPERMOST INTERNODE1* (BUI1) in rice encodes a formin (OsFH5), which affects cell expansion and plant morphogenesis in rice. The mutant was dwarfish, had wavy panicle rachis and showed enhanced gravitropic response. Such growth defects were caused mainly by inhibition of cell expansion confirming role of OsFH5 in the regulation of de novo actin nucleation and spatial organization of the actin filaments (Yang et al. 2011).

Members of plant formins class II are even less extensively studied than members of class I. AtFH12 does not have an obvious phenotype but can produce minor phenotypic alterations depending on environment; however abnormal actin structures were observed in atfh12 mutants expressing GFP-mTalin as compared to wild type. GFP-mTalin is an actin marker known to interfere with actin dynamics (Cvrčková et al., 2012).

AtFH14 was found to regulate both microtubules and microfilaments and is localized at microtubule-based structures. Knockdown of this gene in mitotic cells results in the formation of an abnormal mitotic apparatus. AtFH14 is thought to be another “linker” between microtubules and microfilaments (Li et al., 2010).

AtFH16 has an exceptional domain structure with a long C-terminal extension. Its FH1FH2 domains cannot nucleate actin polymerization efficiently but can bind and bundle microfilaments. In vitro, AtFH16 FH1FH2 preferentially binds microtubules over microfilaments and similarly as AtFH14 colocalizes with microtubules (in onion epidermal cells) thus AtFH16 is also rather bifunctional protein (Wang et al., 2013).

Last formin from *Arabidopsis* with published data is AtFH19, which was characterized biochemically. Results confirmed general properties of the formin family such as nucleation and barbed end capping however the activity is lower that at main housekeeping formin AtFH1 and moreover those two proteins are competing in binding actin filaments (Zheng et al., 2012). Figure 2 reviews known functions of plant formins.

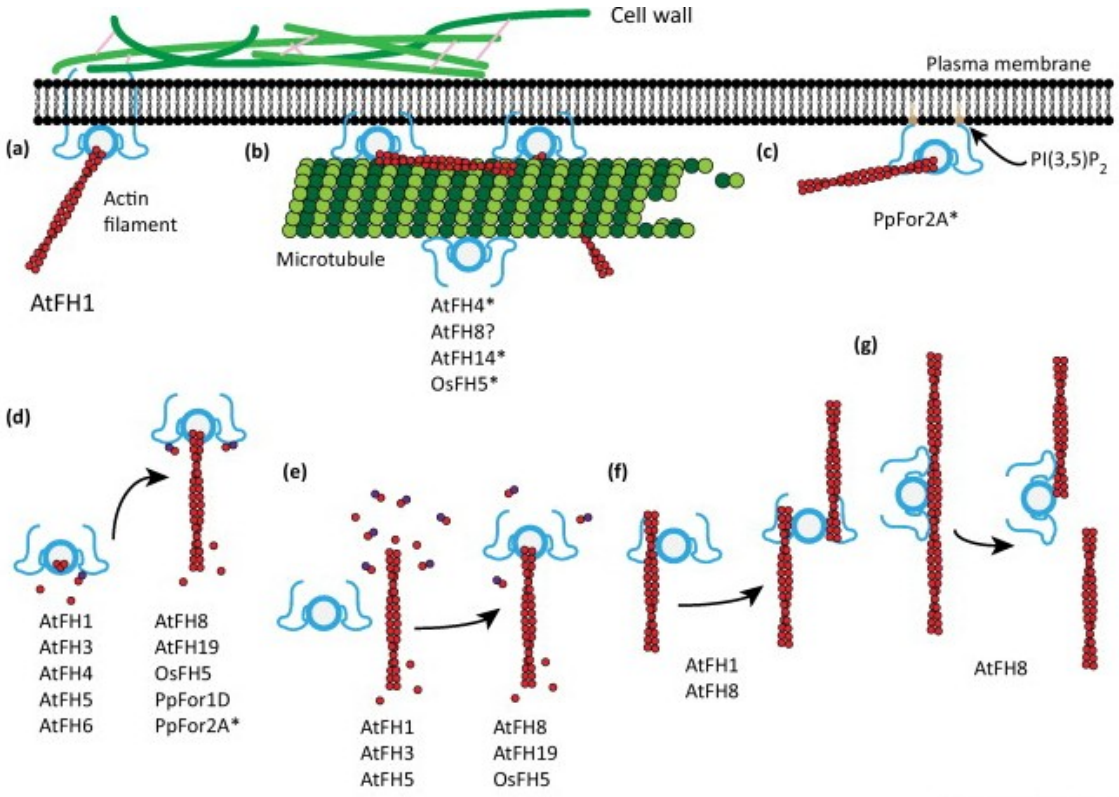


Figure 2 Summary of plant formin functions

(**a**) Cell wall anchoring (**b**) Microtubule binding (**c**) Specific phosphoinositide binding is essential for moss For2A function (**d**) Nucleation and elongation of actin filaments and interaction with profilin (**e**) Capping the barbed end, preventing or slowing polymerization (**f**) Bind at the side and bundling actin filaments (**g**) AtFH8 binds to the side and severs actin filaments. Asterisks indicate that the interaction was studied both *in vitro* and *in vivo*. Abbreviation: $PI(3,5)P_2$, phosphatidylinositol 3,5-biphosphate. From van Gisbergen and Bezanilla, 2013.

1.4. Formin Homology domain

Formin homology domain 2 (FH2), composed of approximately 400 amino acids residues, is hallmark domain whose presence is sufficient to define a protein as a member of the formin family. Its main feature is the ability to form dimmers that can nucleate and progressively elongate actin filaments from their barbed ends. The domain is also very ancient since no homologues have been detected in prokaryote species but proteins containing conserved FH2 are to be found in all examined species across all eukaryotic kingdoms (Rivero and Cvrčková, 2007). The FH2 domain is also the minimal unit of formins necessary for nucleation and elongation of actin filaments (Pruyne et al., 2002, Pring et al., 2003)

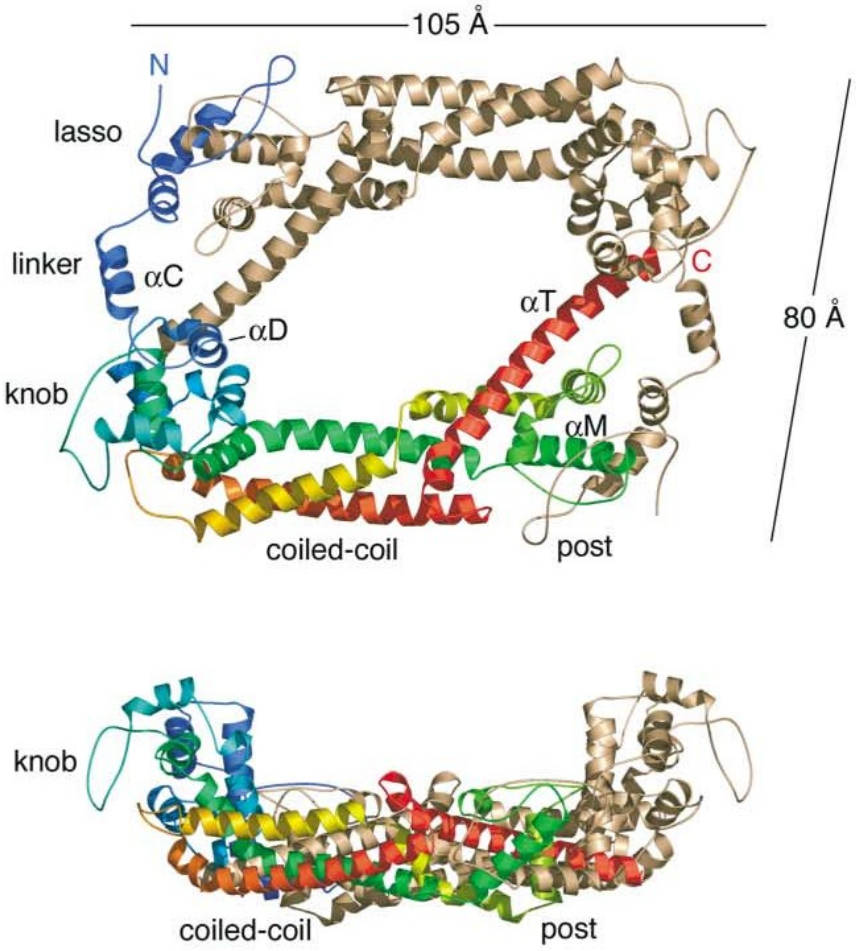


Figure 3 Three-dimensional structure of the FH2 domain dimer (*S. cerevesiae* Bni1p)

Ribbon diagram showing the overall architecture of the FH2 dimer. One molecule is colored using the spectrum (from blue-N terminus to red-C terminus). All characteristic features are marked. From Xu et al., 2004.

The mostly α-helical FH2 domain forms a unique "tethered dimer" in which two elongated actin binding heads are tied together at either end by an unusual lasso/linker/post tructure. Such structure exhibits very stable yet flexible properties. The flexibility is thanks to linker segments (see figure 3) and although each half of dimer can interact with microfilament ends, whole intact dimer is required for its function (Xu et al., 2004). Crystallography study of Bni1p FH2 domain interacting with tetramethyl-rhodamine-labeled actin (TMR actin) revealed place inside the hole of the parallelogram-shaped FH2 dimer where the actin filament sits. FH2 associates in total with 3 actin monomers: upper-binding the knob of one subunit, middle-binding post+lasso of the same subunit and the knob of the half of the dimer. Last actin monomer binds to the post of the second FH2 subunit (Otomo et al., 2005). The configuration of actin monomers prevents additional monomers from binding, thus effectively capping the

filament. For incorporation of additional actin monomer, the FH2 has to get rid of the obstruction and provide extra binding spot for actin monomer.

Dissociation of one actin-binding region of FH2 dimer is necessary for additional incorporation of actin monomer. Such configuration makes it possible for the dimer to move along the barbed end during elongation. The system is in rapid equilibrium between blocked and accessible conformations. In blocked conformation, the FH2 dimer binds 3 actin monomers with all 4 binding sites. During conformational change, one FH2 domain frees its two binding sites and moves toward the barbed end. Here, the knob site reattaches to terminal actin monomer and FH2 dimer binds 2 actins, leaving one post/lasso site unbound. The complex is then in accessible conformation and additional actin subunit can enter and bind near the barbed end. By binding onto the post/lasso site, the complex once again becomes blocked for another addition until FH2 bridge moves and so on (Otomo et al., 2005). Such model of actin nucleation and filament elongation derived from structure of Bni1p FH2+TMR-actin data is called nucleating ratchet model. In general the higher the concentration of actin is, than less favorable the dissociation of actin monomer is and vice versa since model is depending on concentration of free actin subunits in cytoplasm (Otomo et al., 2005; see figure 4 and 5). There is a possibility, that the movement of formin dimer is causing a tension. The tension then allows only addition of actin monomer in certain position and the helical filament is created. The question of rotation and formation of helix remains however remains unclear (Shemesh et al., 2005).

However, the FH2 domain is often not acting alone in process of nucleation but the prolin-rich FH1 domain (Pring et al., 2003) plays very important role. The Formin Homology 1 (FH1) domain is usually adjacent directly on the N-terminal of the FH2 domain and contains one or more stretches of polyproline that bind the actin-binding protein profilin (Courtemanche and Pollard, 2012), allowing thus for local increase of actin subunit concentration in the vicinity of the nucleation site.

As a result of interaction with profilin–actin complexes and thus increased local actin concentration, acceleration of filament elongation and shuttling actin to the barbed end occur (Romero et al., 2004, Vavylonis et al., 2006). So even if FH2 domain can nucleate actin itself, ability to effectively elongate actin polymerization into filaments depends on close co-operation of both FH2 and FH1 domains.

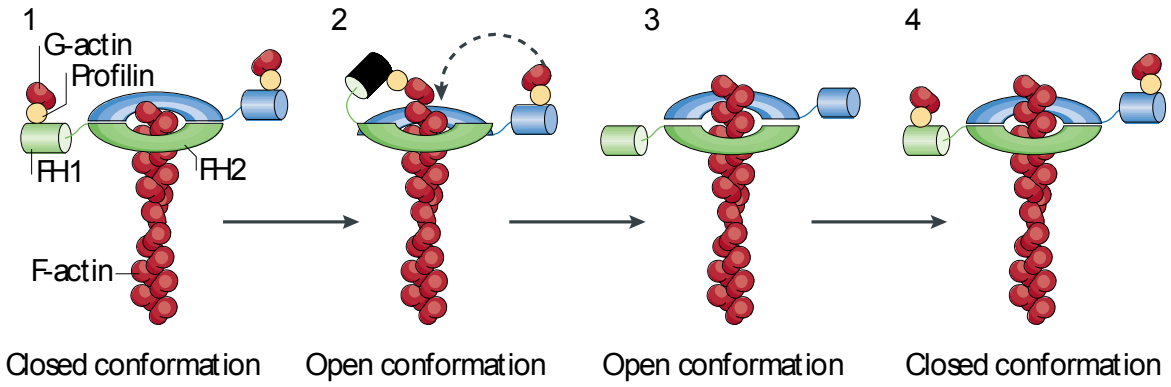


Figure 4 Model of formin-mediated actin polymerization. An FH2 dimer associates with the barbed end of an actin filament, while the FH1 domains recruit profilin–actin (1). The FH1 domain delivers profilin–actin to the barbed end, and this is either preceded by or follows the FH2 domain stepping towards the barbed end (2). The second FH2 repeats this process (3). The formin closed conformation prevents capping by other factors (4). From Campellone and Welsh, 2010.

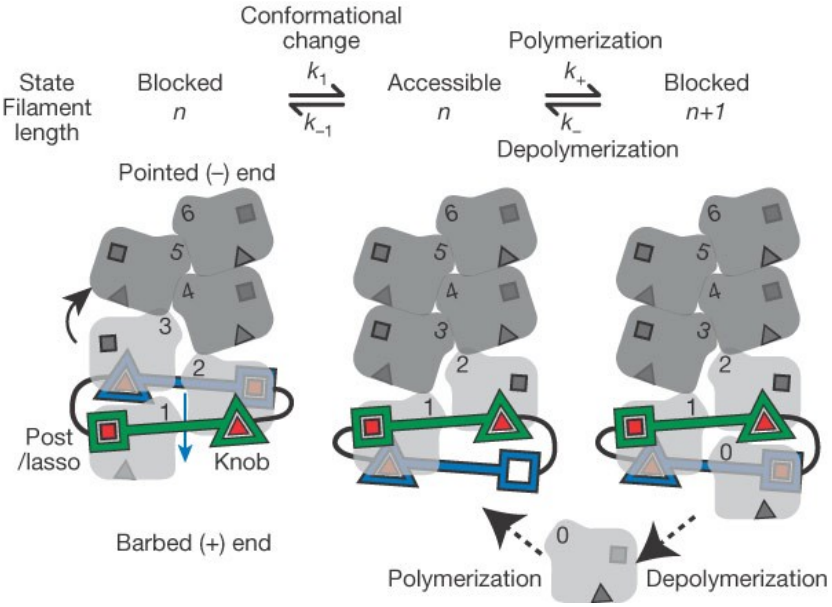


Figure 5 Model of FH2 –mediated barbed end dynamics and conformational change

Actin monomers bind on binding sites of FH2 dimer and their respective partners on actin. FH2-bound site is in red, unbound grey, triangle being for knob and square for post/lasso. In blocked state, actin cannot be added. In accessible state, the blue bridge moves towards the +end and exposes one of its binding sites. From Otomo et al., 2005.

1.4.1. Dimerization of FH2 domain

The fact that FH2 domain of formins makes dimers and its responsibility for nucleation of actin is well known and rigorously described and almost unanimously presented in literally every paper dealing with formins (reviewed e.g. by Goode and Eck, 2007, Paul and Pollard, 2009, Campellone and Welsh, 2010, Cvrčková 2012). However the question, whether FH2 domains are forming exclusively homodimers or whether they can form heterodimers (and if so, do they heterodimerize only within a clade or even between clades) remains unanswered and literature is lacking information on this topic. For example how much similarity two FH2 domain must to be able to dimerize? In mammals, there is at least 15 distinct formins in 7 clades (Higgs, 2005, Grunt et al., 2008), which is still fewer than the 21 known forms in *Arabidopsis* (Grunt et al., 2008, Blanchoir and Staiger, 2010, Cvrčková et al., 2014). By simple mathematical operation, we come to 225 in and 441 possible combinations of FH2 heterodimers in *Mus musculus* and *Arabidopsis thaliana* respectively.

The conserved process of nucleation and elongation of actin is depending on formation of dimer of FH2 domain as described in previous chapter. It has been shown by Moseley et al. (2004) that mutation of yeast Bni1 FH2 domain, which normally forms a stable dimer, disrupts formation of dimer and abolishes FH2 domain activity, confirming the need of a stable dimer for the process, but this does not bring any information on the composition of the FH2 dimer.

One of the few available studies regarding this topic brings some interesting results. Lu et al. (2007) proposed that despite the overall similarity within the dimeric FH2 domain among different organisms, some crucial structural differences might occur between clades. For example the human formin Daam1 FH2 domain, compared to yeast formin Bni1p, has a number of differences in secondary structure elements and in the “lasso/post” dimerization interface that may be functionally important. The human Daam1 FH2 domain follows conserved pattern and consists of an N-terminal “lasso” segment, a flexible, somehow more disordered linker and a part formed by three sub-domains termed the “knob”, “coiled-coil”, and “post”. As in the Bni1 FH2 domain (Xu et al., 2004), the Daam1 domain forms a head-to-tail dimer stabilized by the N-terminal lasso segment in each subunit. Although the overall “tethered dimer” architecture is quite similar to that of the yeast Bni1 and mouse mDia1 FH2 domains, the biggest differences lie within the knob sub-domain and in its orientation relative to the rest of the domain and such differences may be functionally important. In particular, divergence in the lasso/post dimerization interface (but not the lasso/post structure which is preserved) may prevent heterodimerization of Daam1 with other formins (Lu et al. 2007). Even though this has not been

confirmed experimentally, it might be a key for answering the question of dimerization of formins´ FH2 domains in plants as well.

Furthermore, Lu et al. (2007) showed in the structure/function studies of the Daam1 FH2 domain that the wild-type domain has only weak actin assembly activity in comparison with other mammalian formins (mDia1 and mDia2 respectively), but mutations that disrupt its putative auto-inhibitory interactions increase actin assembly about tenfold. These "derepressed" mutants have activity similar to other formins studied formins.

Other interesting evidence came from study of *Plasmodium falciparum* formins (Ignatev et al., 2012). In this malaria-causing apicomplexan parasite, formins are the only present actin nucleators. Two formins are present, both with conserved FH2 domain. The structure of FH1 domain has been determined in presence and absence of the lasso segment. Samples of cloned recombinant formins were measured via synchrotron radiation circular dichroism, synchrotron small-angle X-ray scattering (SAXS) and molecular weight measured by the mean of multi-angle static light scattering. Size exclusion chromatography of three constructs (FH1-FH2, longer FH2 with lasso and shorter FH2 without lasso) revealed two sized dimers for two long versions of protein whereas short version without the lasso was mainly monomeric. Data from SAXS then indicated that the construct with lasso and without FH1 domain was the most compact. Results given together confirm that the FH2 domain dimer, mediated by the lasso region, is both necessary and sufficient for the nucleation activity and also support the notion that formation of higher-order stable FH2 oligomers is not likely (Ignatev et al. 2012). Given the importance of lasso for dimer formation, the sequence difference among formin isoforms might inhibit formation of certain heterodimers.

1.5. Plant formin interactors

There are many formin interactors and an exhaustive discussion would exceed the scope of this work, and those discussed below should be understood as examples. The most notorious formin "partner" is actin since formins work as nucleators of actin. They also stabilize actin filaments at the +end but not very firmly, and continuous polymerization and depolymerisation of microfilaments takes place; when the formin is attached, such exchange is slower (Pring et al., 2003). More importantly, formins prevent capping proteins from binding (Zigmond, 2004). The role of domains is besides FH2 considerable since for instance formin Cdc12 can fully inhibit actin polymerization (Higgs, 2005), and formins can also bundle the actin cytoskeleton (Evangelista et al., 2003).

Evidence that formins interact with microtubules is less frequent. The interactions of formins with mictorubules occur in animals (Bartolini et al., 2008), yeasts (Martin et al., 2005) but it has been proven that such interaction is common also in plants (Deeks et al., 2010). Most often such interaction stabilizes the microtubules and formins coordinate the responses of the cytoskeleton in diverse regulated and homeostatic processes (Bartolini and Gundersen, 2010). In plant formin AtFH4, domain GOE is responsible for the attachment to the microtubules (Deeks et al., 2010).

In plants, additional formin-interacting proteins have been discovered. GEM-like protein 1 and 2 (or Forming homology-interacting protein 1 and 2, FIP1, Banno and Chua, 2000). A partial cDNA of AtFH1 was used as a probe in yeast two-hybrid assay and *Arabidopsis* cDNA library was screened. One of the isolated cDNAs encoded a novel protein, FIP2. The amino acid sequence of FIP2 has partial homology to bacterial putative membrane proteins and animal A-type K+ ATPases. AFH1 may form a membrane anchored complex with FIP2, which might be involved in the organization of the actin cytoskeleton (Banno and Chua, 2000). Additional protein identified in the same screen, FIP1, turned out to be a GEM-like protein, i.e. a putative GTPase, though not a member of the RHO family (see annotation of UniProt entry Q9SE96).

However, here we shall focus especially on plant candidate interactors predicted on the basis of homology with formin-binding proteins described in metazoans.

1.5.1. Potential selected interactors of plant formins

Plant formins are often associated with membranes; especially for Class I formins, this has been experimentally proved (for example Favery et al., 2004, Martiniere et al., 2011; reviewed e.g. in van Ginsbergen and Bezanilla, 2013, Liu et al., 2015). More specifically, Class I formins are directly inserted in membranes, most often in the plasmalemma (Banno and Chua, 2000). In the Class II formins, the possession of PTEN domain is most probably responsible for the peripheral association with membranes (see chapter “Structure and composition of formins”).

Based on animal and yeast examples, it has been proposed (Cvrčková, 2013) that plant formins might have some membrane-associated interactors (table 1) enabling them to bind membranes via a different mechanism then the above mentioned direct insertion and peripheral association. Known and proposed mechanism are visualized in scale and explained in figure 6.

Table 1 Candidate plant membrane-associated formin interactors

Only results where land plant candidates were found are shown. Adjusted from Cvrčková, 2013

Protein or domains	Non-plant query	Land plant candidates	Notes
Other FYVE	Cd00065 (FYVE domain	At4g33240, FAB1A At3g14270, FAB1B	Many plant FYVE domain protein exist
Other BAR-SH3	Cd07607 (BAR domain of the plant SH3 domain-containing proteins)	At1g31440, AtSH3P1 At4g34660, AtSH3P2 At4g18060, AtSH3P3	No additional Arabidopsis paralogs identified by Blast with AtSH3P3

First group of proposed possible interactors are characterized by possession of the FYVE domain, which is named based on the first letters of four proteins containing same genuine zinc binding domain (Stenmark et al., 1996). FYVE are membrane-targeting domains specific for phosphatidylinositol 3-phosphate (PtdIns (3P)), meaning they are mostly found in proteins involved in diverse cell trafficking pathways. The conserved sequence of amino acid in classic FYVE domain proteins is R-[RK]-H-H-C-R-x-C-G (Stenmark and Aasland, 1999, Jensen et al., 2001).

Arabidopsis thaliana has 15 FYVE-domain containing proteins divided into 5 groups based on their domain architecture (Wyvial and Singh, 2010). It is known that the *vacuolar fusion defective 1* (*vfd1*) mutant is defective in FYVE domain-containing protein, named FYVE1. FYVE1 has been found to localize on late endosomes and its mutants are defective in ubiquitin-mediated protein degradation, vacuolar transport and autophagy (Kolb et al., 2015). However, in Arabidopsis, only two experimentally characterized FYVE-domain containing proteins are members of type III phosphatidylinositol 3-phosphate 5-kinase (or PIKFYVE) family, namely FAB1A and FAB1B.

In metazoan cells, PIKFYVE family members are implicated in membrane trafficking, endocytosis and actin dynamics (Shisheva, 2008). In *Saccharomyces cerevisiae,* Arabidopsis FAB1 is involved in dehydration stress tolerance (López-Martinez et al., 2015). Nevertheless, there is no evidence for involvement of formins.

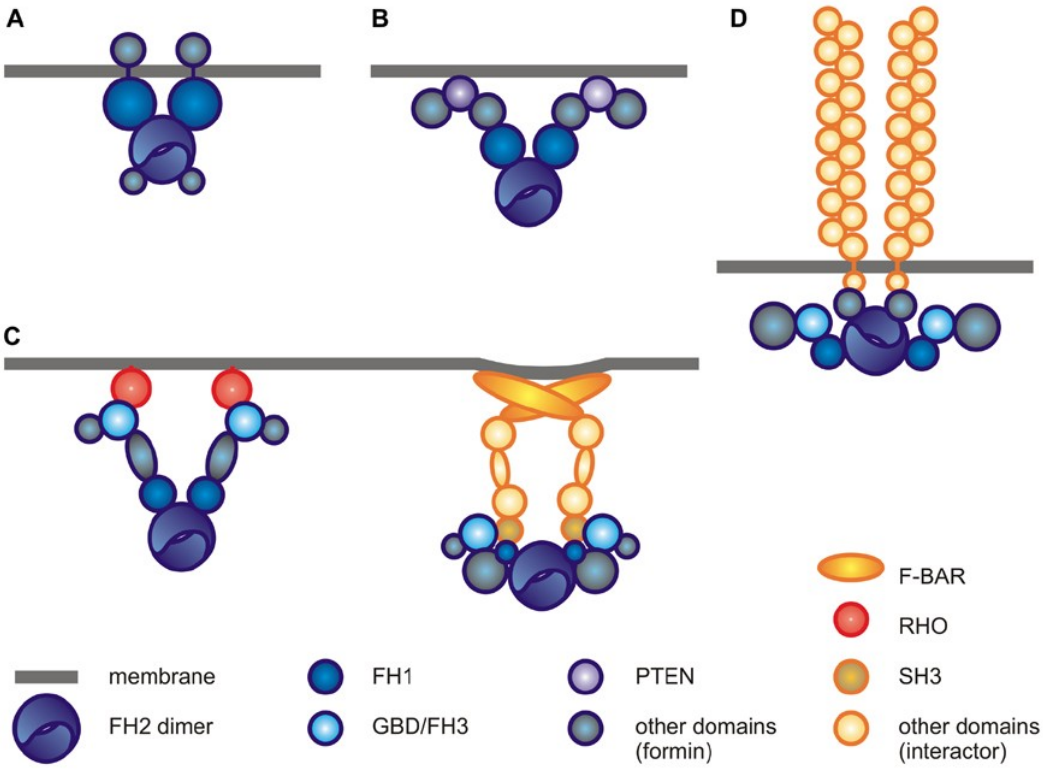


Figure 4 Mechanisms of formin-membrane attachment

A) Direct insertion as in plant Class I formins
B) Peripheral membrane binding as in plant Class II formins
C) Interaction with a *peripheral membrane protein*, such as a RHO GTPase or F-BAR (left: mouse mDia1 and Cdc42, right: human DAAM and FBP17)
D) Interaction with an *integral membrane protein,* as in mammalian formins binding to CD21 (here human FHOS and CD21)
Adapted from Cvrčková, 2013

The results up to date concerning *Arabidopsis thaliana* proteins FAB1A and FAB1B are very limited. Like the above mentioned protein FYVE1, FAB1 is crucial for vacuole homeostasis (Hirano et al., 2011). The protein seems to be localized to the endosomes of root epidermal cells. Gene knockout via RNA interference impaired vacuolar acidification and endocytosis and expression of AtFAB1A/B in fission yeast *Schizosaccharomyces pombe fab1* mutant led to complementation of the vacuole morphology phenotype. The developmental phenotypes observed in mutant plants are related to the auxin signalling, which is depending on endomembrane trafficking (Rakusova et al., 2015).

Other role of FAB1 proteins lays in the signalling pathway involving PtdIns(3,5)P(2) whose production is up-regulated during osmotic stress. There is no double mutant of FAB1A and FAB1B because the pollen carrying both mutated alleles is defective in vacuolar reorganisation following the first mitotic division and thus loses its viability (Whitley et al., 2009). However, double mutant of FAB1A and a related protein FAB1D (which, however, is not

a member of the PIKFYVE family) was viable and pollen viability, germination and tube morphology were not significantly affected. However, on the cellular level, fab1b and fab1d single mutant pollen both exhibited abnormal membrane recycling and vacuolar acidification. Also the production of reactive oxygen species was decreased (Serrazina et al., 2014).

Other group of potential interactors share possession of SRC homology 3 domain (SH3). SH3 is a conserved, noncatalytic domain approximately 60 amino acids long, found in proteins of signalling pathways regulating the cytoskeleton, in proteins modulating membranes and some proteins serving as adaptors for linking tyrosine kinases to specific target proteins (Koch at el., 1991). The domain is a compact beta-barrel made of five antiparallel beta-strands (Musacchio et al., 1992). In addition to the SH3 domain, members of the coiled-coil-SH3 containing family of AtSH3Ps also share N-terminal Bin/amphiphysin/Rvs (BAR) domain, which is a shorter, plant specific version of F-BAR (or FCH-BAR, or EFC for extended FCH homology) domain. BAR domain is very well conserved and its crescent-shaped dimer binds preferentially to highly curved membranes (Peter et al., 2004). Dawson et al., 2006, reviewed role of BAR domain in actin assembly in clathrin-mediated endocytosis.

Potential interactors of plant formin, proteins AtSH3P1, AtSH3P2 and AtSH3P3, has not been studied extensively. According to few published studies, AtSH3P1 is involved in in trafficking of clathrin-coated vesicles, which is in accordance with experimentally proved presence of protein in the endomembrane system, more specifically on or adjacent to the plasmalemma and associated vesicles, endomembranes and vesicles of trans-Golgi network. Furthermore, on all these locations, strong colocalisation with clathrin is evident. Even more interestingly, immunohistochemical studies with actin binding assays indicated that AtSH3P1 can also regulate vesicle trafficking along the actin cytoskeleton (Lam et al., 2001). Important role of clathrin in plant endocytosis is described by Holstein, 2002.

AtSH3P2, which is upregulated in pollen tubes (Wang et al., 2008), is involved in vesicle trafficking via processes important for proper formation of autophagosomes and interacts with autophagy-related protein 8 (ATG8). This protein, AtSH3P2, also actively participates in the membrane deformation process. The knockout of AtSH3P2 is developmentally lethal and leads to suppression of formation of autophagosomes (Zhuang et al., 2013). Recently, AtSH3P2 has been shown as interactor of a FYVE domain protein required for endosomal sorting 1 (FREE1), together, they mediate the interplay between endosomal sorting complex required for transport (ESCRT) and autophagy pathways (Gao et al., 2015).

2. Aims of thesis

This work has two aims; they are both dealing with protein-protein interactions of the FH2 domain of plant formins. According to literature, FH2 domain is well known for creating dimers, which are then responsible for nucleation of actin. However, there is general lack of knowledge whether that happens only between identical formins (homodimerization) or if dimerization is possible between different formins (heterodimerization). If the latter is true, heterodimerization might occur between plant formins from class I , between members of class II, or perhaps even between formins from both classes II and I. One of the aims is therefore to test possible dimerization between FH2 domains, presented by at least one formin from each class, by yeast two-hybrid assay.

The second aim is based on animal model-based bioinformatic prediction of interactions of formins with different membrane bounded proteins (Cvrčková, 2013). Two groups of proteins have been selected, BAR domain containing SH3 proteins and FYVE domain containing FAB proteins. To test whether these proteins do interact with formins or not, two hybrid yeast assay will be used using the FH2 domain as a bait and selected proteins as preys.

Last but not least, to support laboratory results, in-silico models of proteins were created and possible models of protein-protein docking were suggested.

3. Materials and methods

3.1. Biological material

- Plants

Plant material was used only for isolation of genomic DNA and RNA (see section RT-PCR). Mgr. Lenka Stillerová provided young leaves from *Arabidopsis thaliana*, ecotype Columbia. Plants were cultivated on peat pellets (Jiffy) in cultivation chamber under stable conditions: temperature of 20°C, humidity 40%, light conditions 16 hours light/8 hours dark. The cultivation cycle was approximately 3 weeks.

- Bacteria

For multiplication of plasmids, bacterial strains DH5α or TOP10 of *Escherichia coli* (*E. coli* further in text) have been used. Preparation of competent cells

E. coli from laboratory collections were inoculated in sterile conditions onto plate with MPA medium. The plate was incubated overnight in 37°C. As next, 10 ml of MPB was inoculated by a single colony and incubated overnight in 37°C while shaking. The following day in the morning, prepared one litre of MBA medium was inoculated by approximately 1/10 of well-grown inoculum. The culture was left to grow in 37°C while shaking to reach $O.D._{600}$ between 0,5 and 0,8, measured with clean MPB as control. Such prepared culture was redistributed into 50 ml test-tubes, chilled on ice for 15 min and then centrifuged 15 min on 3600 x g in 4°C. Sedimented pellet was resuspended in one litre of H_2O and centrifuged in same conditions as mentioned. Pellet was resuspended again in 0,5 l of H_2O and again centrifuged as above. Formed pellet was dissolved in 20 ml of 10% glycerol. A sample was taken from this suspension to measure $O.D._{600,}$ clean water being the control. The rest of the suspension was centrifuged 15 min on 4600xGVE in 4°C and sedimented pellet resuspended in 2-3 ml of 10% glycerol. Specific volume of glycerol was calculated according to previously measured $O.D._{600}$, so that final concentration of cells was higher that $3x10^{10}$ of cells conditioned by assumption that $O.D._{600}$ 0,1 corresponds 108 cells. This suspension was distributed as 50 µl into micro-centrifuge tubes and frozen in liquid nitrogen. Such prepared competent cells are stored at -80°C.

- Yeasts

For co-transformation of yeasts with prepared constructs, the strain of *Saccharomyces cerevisae* AH109 (BD Biosciences Clontech Inc.) with genotype: *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ* was used. (Kindly provided by Mgr. Ivan Kulich PhD.)

- Media

YEPD medium: 2,75g yeast extract, 5,5g peptone a 5,5mg adenine, filled by H_2O up to 250ml, after autoclaving 10ml 50% sterile glucose was added

SD medium: 0,67g yeast nitrogen base without amino acids, 2g plant agar, filled to 85ml by H_2O, after autoclaving 4ml 50% sterile glucose was added. If SD dropout medium prepared, corresponding dropout media supplements were used (-Leu/-Trp or –Leu/-Trp/-His/-Ade)

MPA medium: 23g nutrient agar (n. 1, Imuna®Pharm) and 10g NaCl was filled to 1l by sterile H_2O.

MPB medium: 25g nutrient agar n. 2 (Imuna®Pharm) was filled to 1l by H_2O and pH was adjusted to 7,5.

Media were autoclaved in 121°C, 0,144MPa, 20min on the autoclave OmegaTMMedia.

Water in protocols was deionized distilled water.

3.2. Primers

Based on knowledge of genomic and cDNA sequences of formins, their FH2 domains respectively and SH3 proteins (available at https://www.arabidopsis.org/), primers for their cloning were designed. For verification of the length, sequence and exact position of various FH2 domains, database UniProt (The UniProt Consortium, 2015) was used.

Web-based tool for finding specific primers NCBI Primer Blast (Ye et al., 2012) was used for the primer design. Suitable restriction places were added into primers for following restriction and insertion in the donor vectors pGBKT7 and pGADT7 AD (numbers in collection FD451 and FD452, Clontech Laboratories, Inc). Primers were ordered from company Sigma-Aldrich®.

For sequencing of inserted fragments in the donor vectors, primers originally designed by Mgr. Ivan Kulich PhD and available at laboratory collection were used. For sequencing of FAB1A in the vector pGADT7, additional simple sequencing primers were designed due to sequence length.

Names of primers, their sequences, orientation and added restriction places are specified in table 3. Summary of most crucial combinations of used primers, annealing T_m and estimated length of obtained fragments are presented in table 2.

Table 2 Adjustments of PCR reactions for cloning of below listed sequences

Primers were used as combination of forward and reverse, in table 3

For cloning: (Combination of forward and reverse primer used)	Annealing T_m in °C	Estimated product length in bp, genomic DNA	Estimated product length in bp, cDNA	Time of extension in PCR in seconds (Only cDNA)
FH1(FH2 domain)	63,6	From plasmid	1819	40
FH5(FH2 domain)	67,4	From plasmid	1904	45
FH8(FH2 domain)	63,8	From plasmid	1839	45
FH13(FH2 domain)	61	2717	1481	60
FH14(FH2 domain)	67,4	From plasmid	1475	45
SH3P1	623	2880	1375	35
SH3P2	64,5	3257	1290	35
SH3P3	66	2420	1163	30
FAB1A	62	From plasmid	5654	150

Table 3 List of primers used for cloning and sequencing

For cloning (sequencing) of:	Direction:	Sequence: From 5' to 3'	Restriction site:	Comment:
AtFAB1A	Forward	TTCCCGGGGAAGTTTGAGAGTGACCCAG	XmaI	pGADT7
AtFAB1A	Reverse	TTCTCGAGTGATAAAAACATAAGTTACGACGA	XhoI	pGADT7
AtSH3P1	Reverse	TTCTCGAGAAAGGGCATCACTGTTGCTT	XhoI	pGADT7
AtSH3P1	Forward	TTGGATCCGGTACTGAAGAAACCGACTTGA	BamHI	pGADT7
AtSH3P2	Reverse	TTGGATCCGCAGAAACCCGCATCAACTC	BamHI	pGADT7
AtSH3P2	Forward	TTGAATTCTGTGAATAGCTCTTGATCTGGTGA	EcoRI	pGADT7
AtSH3P3	Reverse	TTCTCGAGGTAGTTTGTTGGGGACAGGACA	XhoI	pGADT7
AtSH3P3	Forward	TTGAATTCGGGAAATCAAGAGGAGAAGTGAC	EcoRI	pGADT7
FAB1A	Forward	ACTTTGGTCTGAATCTCGG		Sequencing
FAB1A	Reverse	AACACTGCTTCTATCTTTTAGTGTTT		Sequencing
FAB1A	Forward	TACCTTCGCCCATCAAATAG		Sequencing
FAB1A	Forward	CTGCCCGAAACCTTTGGGTT		Sequencing
FAB1A	Forward	TCAGATGCTGCGTGGGGCC		Sequencing
FAB1A	Forward	ATGGAGTTCGACCCTAGTGA		Sequencing
FAB1A	Forward	TGCAAAAAGTTTGGATGACC		Sequencing
AtFH13(FH2 domain)	Forward	TTGAATTCGTTAATTTAAAGAATAGTCCAGCC	EcoRI	pGBKT7/pGADT7
AtFH13(FH2 domain)	Reverse	TTGGATCCGTTCTTTCCTTTAGTCGGTCAC	BamHI	pGBKT7/pGADT7
AtFH14(FH2 domain)	Forward	TTGGATCCGGTTGGGTGCTCCCCCT	BamHI	pGBKT7 only
AtFH14(FH2 domain)	Reverse	TTCTGCAGCTGCTGGATAAGATCGTTGTCGTT	SalI	pGBKT7 only
AtFH14(FH2 domain)	Forward	TTGAATTCAGGTTGGGTGCTCCCCCT	EcoRI	pGADT7 only
AtFH14(FH2 domain)	Reverse	TTCCCGGGTCTGCTGGATAAGATCGTTGTCGTT	XmaI	pGADT7 only
AtFH5(FH2domain)	Forward	TTCATATGTACTCTGTTGGTTCCTCCATCAA	NdeI	pGBKT7 only
AtFH5(FH2domain)	Reverse	TTCTGCAGAACTAGACTGATCCACGCGTCT	PstI	pGBKT7 only
AtFH5(FH2domain)	Forward	TTCATATGTACTCTGTTGGTTCCTCCATCAA	NdeI	pGADT7 only
AtFH5(FH2domain)	Reverse	TTCCCGGGAACTAGACTGATCCACGCGTCT	XmaI	pGADT7 only
AtFH1(FH2 domain)	Forward	TTCATATGTTACGTTCTCGTTCACCGTCG	NdeI	pGBKT7/pGADT7
AtFH1(FH2 domain)	Reverse	TTGGCATCCCAACATGCACAGACAAAGCTGAG	BamHI	pGBKT7/pGADT7
AtFH8(FH2 domain)	Forward	TTCATATGAGGTTTGGTGGTGTGAAAGGT	NdeI	pGBKT7/pGADT7
AtFH8(FH2 domain)	Reverse	TTGGATCCCGACCCACCAGAATCACTC	BamHI	pGBKT7/pGADT7
AtSH3P2	Forward	TTGGATCCTTTGTGAATAGCTCTTGATCTGGTGA	BamHI	For pENTR1A
AtSH3P2	Reverse	TTGAATTCAGAAAACTTCGGACACTTTGCTAGC	EcoRI	For pENTR1A
AtSH3P3	Forward	TTCTCGAGCCTCGTCATTGTTCTCGTT	EcoRI	For pENTR1A
AtSH3P3	Reverse	TTCTCGAGAGTAAACTTCAGCAGCAAAGTT	XhoI	For pENTR1A
AtFH5(FH2domain)	Forward	TTGGATCCATATGACCAATCACGGGCTTCCAC	BamHI	For pENTR1A
AtFH5(FH2domain)	Reverse	TTCTCGAGGACTGATCCACGCGTCTCTC	XhoI	For pENTR1A

3.3. Cloning

- Isolation of plant genomic DNA

Young leaves of *Arabidopsis thaliana* Col-0, approximately 1 cm big were frozen in liquid nitrogen and homogenized by homogenization pestle in sterile 1,5 eppendorf test-tube. Into crushed plant material, 400 µl of extraction buffer (200mM Tris-HCl, pH 7,5, 250 mM NaCl, 25 mM EDTA, 0,5% SDS) and 300 µl chloroform was added. Mixture was vortexed (Vortex2 Genie, Scientific Industries) 1 min followed by centrifuging (Eppendorf Microcentrifuge 5415R) for 3 minute on 13000 rpm. Formed supernatant was transferred (300 µl) into new sterile 1,5 eppendorf test-tube and 300 µl of isopropanol was added, gently mixed and let on ice to precipitate for 10 min. Suspension was centrifuged on 13000 rpm, supernatant was discarded and the test-tube was left opened in a room temperature for 10 min to dry out. The Sedimented DNA was dissolved in 100 µl 2mM TRIS (pH 8,8). Isolated DNA was kept for immediate use in fridge and served for optimisation of PCR protocols.

- Polymerase chain reaction

PCR reactions composition while using polymerase Phusion® High-Fidelity (Finnzymes, concentration 2u/µl) was as following:

Volume in µl:	Component:
12,8	Sterile distilled water
4	5x Phusion® HF reaction buffer
1	DNA template
1	Premix of primers, 1µM, see table 3
0,6	DMSO
0,4	dNTP 10mM
0,2	Phusion® High-Fidelity polymerase, 2u/µl

General reaction conditions were in accordance with those cycles: step 1) 98°C 30s step 2) 98°C 10s step 3) 60°C 30s step 4) 72°C 1 min step 5) 72°C 10 min, with repetition of steps 2. - 4. 30x.

Temperatures T_m in step 3 were adjusted accordingly to the T_m of primers (see tab. 2) and time of annealing in step 4 according to the expected length of product.

All the reactions were carried out in gradient termocycler TGradient (Biometra) or multi block TProfessional TRIO 48 Thermal Cycler (Biometra) in the 200 µl volume test-tubes.

For the separation, visualization and purification of DNA fragments, the agarose gel electrophoresis was used. Powdered agarose was diluted in TBE buffer (10 mM Tris, 20 mM boric acid, 1 mM EDTA, pH 8,0). For visualization agarose concentration was 1%, for

isolation and purification 1,5%. The mixture was carefully heated in microwave oven for few so that agarose could completely melt down and the liquid was clearly transparent. The fluorescent color GelRed (Biotium, diluted 10 000x in H_2O) was added in the solution to the final concentration (1 µl of stain for 10ml of gel). The solution was poured into the sealing container with plastic comb. After solidification of the gel (approx. 25 min), the comb was removed and the gel was placed into the electrophoretic chamber filled by TBE buffer. Every sample was mixed with 6x Loading Dye (Fermentas) in a ratio 1:5. For easier analysis of fragments, marker GeneRuler DNA Ladder Mix (Fermentas) was pipetted in the first pit. Used volume was 2 µl (0,5 µg/µl). Electrophoresis was carried out at 90 V.

For visualization and evaluation of results, the digital visual system G-BOX (Syngene) and software GeneSnap were used.

For isolation of DNA fragments from agarose gel, gel was taken into the dark room under the UV lamp. The right size fragment was selected and cut away with sterile blade. The sample was placed in sterile 1,5ml eppendorf test-tube. The DNA was isolated from gel by a kit MinElute Gel Extraction Kit (250) Cat. no. 28606 (Quiagen). The result of procedure was 10 µl or 20 µl elute dissolved in elution buffer. Samples were used immediately or stored in -20 °C

- RT-PCR

For isolation of RNA, plants of *Arabidopsis,* cultivated on ½ MS medium were used. Five days old seedlings were weighted and frozen in liquid nitrogen. The total RNA was isolated by kit RNeasy Plant Mini Kit (50) Cat. No. 74904 (Quiagen) resulting in 30 µl elute in H_2O.

Samples were quantified spectrophotometricaly and 1µg of isolated RNA was for used for DNAsing via 1 unit of DNAse and RNase-free (Fermentas). Inactivation was done in 30 minutes by adding of 50mM EDTA and heating to 65 °C. Resulting RNA free of contamination by DNA was used as input for Transcriptor High Fidelity cDNA Synthesis Kit Cat. No. 05 081 955 001 (Roche) with the use of random hexamer primers. The result was sample of 20 µl cDNA. Samples were stored in -20°C.

cDNA was used for amplifying sequences of FH2 domain of AtFH13 and sequences of SH3P2 and SH3P3 by PCR as described above.

- Restriction

Presence of desired fragments in amplified cDNA or amplified genomic DNA were verified by control restriction. Suitable restriction sites were found according to analysis

through software SMS manipulation suite (Stothard, 2000). Each restriction reaction was composed of 16,8 µl sterile distilled water, 2 µl suitable restriction buffer (found according to manufactures website), 1 µl DNA, 0,2 µl restriction enzyme (enzymes and buffers by Fermentas). Reaction was running 1 hour in 37°C and result evaluated on agarosis gel.

cDNA of FAB1A has been acquired from RIKEN, Japan (RAFL09-68-K04 in RIKEN Arabidopsis full length clone database search). Numbers of plasmid from laboratory collections used for cloning FH2 domains of formins were as following: AtFH1-FD233, AtFH5-FD234 AtFH8-FD195 and AtFH14-FD503, provided by Mgr. Lenka Stillerová.

Preparation of fragments for ligation was as following. First, the PCR product was cleaned via High Pure PCR Cleanup Micro Kit (Roche) to give 30 µl eluate. Such cleaned DNA (insert) and plasmid (donor vector) were restricted by restriction enzymes. Restriction reaction for insert DNA contained 11,6 µl sterile distilled H_2O, 6 µl DNA (PCR product or source plasmid), 2 µl restriction buffer, 0,2 µl restrictase A (10 u/µl), 1µl restrictase B (10 u/µl) (Fermentas). Vector was restricted in 40 µl sterile distilled H_2O, 5 µl plasmid DNA, 4 µl restriction buffer, 0,5 µl restrictase A (10 u/µl), 0,5 µl restrictase B (10 u/µl) (Fermantas). Both reaction were left in 37°C overnight. Reactions were mixed with loading dye, loaded on the agarose gel and fragments visualized and isolated as described in paragraph "polymerase chain reaction".

- Ligation

Amount of DNA in samples was verified by loading 1 µl of DNA mixed with 1 µl of loading dye. According to the results, the ratios of vector to insert in the ligation mixture were adjusted. The basic non-adjusted reaction mixture was composed of 4 µl vector DNA, 8µl insert DNA, 2 µl 10x T4 ligation buffer (Fermentas), 0,5 µl T4 ligase (Fermentas) and 5,5 µl H_2O. Ligation mixture was incubated one hour at 20°C and then left at 16°C overnight. Inactivation was done by leaving reaction mixture 10 min in 65°C. Reaction was running in termoblock BioSan CH -100.

Concentration of vector and insert DNA were always adjusted based on analysis described previously. If the ligation reaction was not used immediately, it was stored at -20°C.

- Transformation of bacteria by electroporation

Mixture of *E. coli* and 2 µl construct were brought into well-chilled electroporation cuvette. Cuvette was placed in electroporation system Gene Pulser Xcell Electroporation

System (Bio-Rad) and electroporated under the voltage 2,5kV, reaching pulse time of about 5ms. Volume of 500 µl of sterile MPB (see supplemental data) medium was added in the cuvette and left shaking in 37°C the laboratory shaker GFL 3032 (GFL, 180 rev./min). In sterile conditions in laminar box ALPINA, bacteria were placed on Petri dish with MPA medium containing antibiotics matching the plasmid resistance. Concentrations of used antibiotics were: ampicilin 100mg/ml, kanamycin 50mg/ml. Cultures were left overnight in 37°C v thermo regulator Q-CELL. For further processing and working, cultures were stored in basic fridge.

3.4. Plasmids

- Vectors

All designed primers contained artificially inserted restriction site so that it was possible to insert restricted fragment (FH2 domains of plant formins, FAB and SH3P) of DNA into destined vector.

Vector pGBKT7 (number in collection FD451, Clontech Laboratories, Inc) is a yeast expression vector designed to constitutively (due to *ADH1* promoter) express protein of interest fused to a GAL4 binding domain thus serving as bait in yeast two hybrid-screening protocols. This vector carries resistance to kanamycin.

Vector pGADT7 (no. FD452 in collections, Clontech Laboratories, Inc), similarly, expresses protein of interest, under same promotor as pGBKT7, fused with GAL4 activation domain. This vector serve as pray in yeast two hybrid assays and carries resistance to ampicillin.

- Isolation of plasmid DNA

One colony of bacteria caring the plasmid was inoculated in laminar box to 2ml of sterile MPB medium with corresponding antibiotics. They were left to grow overnight in 37°C. Following day in the morning, bacteria were centrifuged 2min on maximum speed using centrifuge Eppendorf Microcentrifuge 5415R. Isolation was done according to protocol by Maniatis, 1982. Solution of GTE was with added RNAse and for higher purity of isolated DNA other step was included. For removal of RNA, before washing the pellet by 70% ethanol, the pellet was dissolved in 40 µl TE and 40 µl 5M LiCl added and mixed. Mixture was left approximately 15min on ice followed by centrifugation on maximal speed for 5min. The supernatant was taken into new test-tube and 200 µl 95% EtOH was added to precipitate

the DNA. Reaction test-tube was vortexed and centrifuged 3min on maximum speed. Next steps were according to protocol.

Isolated DNA was verified by restrictions and evaluated on agarosis gel (see chap. 3.3.)

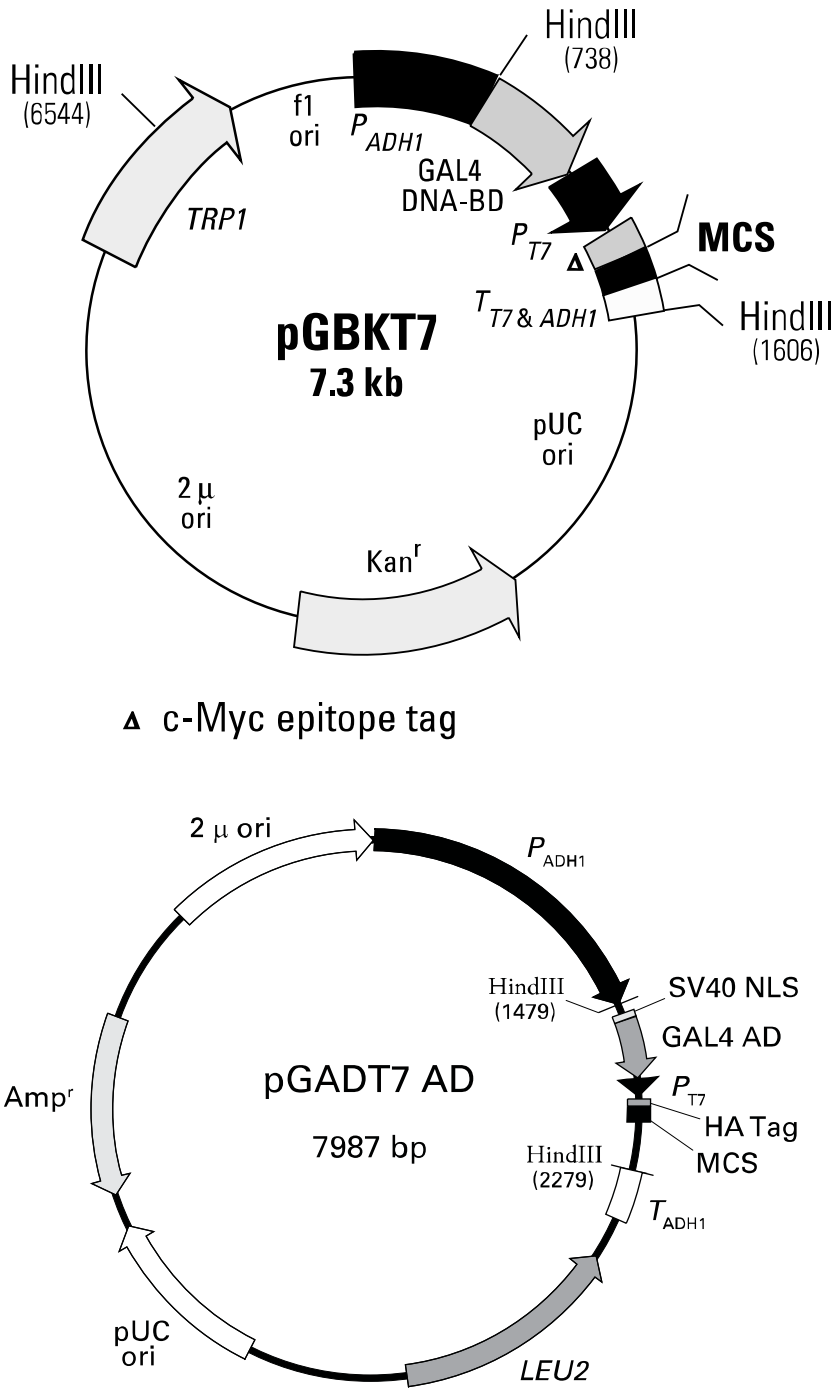


Figure 5 Maps of used plasmids for yeast expression

- Sequencing

Concentration of sequenced DNA was measured spectrophotometrically using the NanoDrop spectrophotometer. Sequenceing mixture contained 5pmol-sequencing primer; plasmid DNA in the volume 4ng/100bp and total volume was completed by H_2O into 8 µl.

Sequencing itself was done at the service laboratory of Faculty of Science of Charles University. They own two sequencing machines-four capillary 3130 Genetic Analyzer (Applied Biosystems) and 16 capillary 3130xl Genetic Analyzer (Applied Biosystems). Sequencing kit they use is from Applied Biosystems: BigDye®Terminator v3.1 Cycle Sequencing Kit (further details, reaction cycle and other can be found: http://web.natur.cuni.cz/~seqlab/)

Sequences were controlled using software Geneious (Kearse et al., 2012). From database TAIR (Rhee et al., 2003), full-length coding DNA sequences were downloaded and compared in Geneious with sequences obtained from service laboratory.

3.5. Yeast two hybrid system

- Yeast transformation and screening of transformants

Modified protocol of yeast transformation mediated by LiAc from Yeast Protocols Handbook (Clontech Laboratories, Inc., 2009) was used for cotransformation.

Several colonies of *Saccharomyces cerevisiae* AH109 were inoculated into 1ml of YEPD medium, resuspended and filled with YEPD to volume of 5ml. Such inoculum was incubated overnight (16-18 hours) in 30°C while shaking (250 rev/min). Following day, the mixture was complemented with another 45ml of YEPD medium and left in same conditions next 3-5 hours so that final $O.D._{600}$ was 0,4-1,1. Yeast culture was poured into cuvette suitable for centrifugation and centrifuged in room temperature 5min at 1000xg. Supernatant was disposed and pellet was resuspended in H_2O to make final volume of 50ml. The centrifugation was repeated at same conditions as above and the supernatant was disposed. Sedimented yeasts were resuspended in 1,5ml of freshly prepared sterile solution of TE/LiAc. Meanwhile, the plasmids destined for transformation were prepared. In 1,5ml eppendorf test-tube, 0,1µg of each plasmid DNA was mixed with 0,1mg herring testes carrier DNA (prepared by boiling for 10min and chilled on ice afterwards). In every test-tube with prepared plasmids, 100 µl of yeast in TE/LiAc was added. Mixture was well vortexed and 600 µl of sterile solution of PEG/LiAc was added followed by 10s long vortexing of each sample. Samples were incubated 30 min in 30°C and stirred (200 rev/min). After incubation,

70 µl of DMSO was added into each sample and placed in water bath heated on 42°C for 15 min. After second incubations, samples were chilled on ice for 2 min causing heat shock to yeasts. Test-tubes were then centrifuged 5s on 14000xg and supernatant discarded. Sedimented yeasts were resuspended in 250 µl TE buffer. Final mixture was immediately spread on Petri dish with SD medium (with suitable auxotrophic selection- SD/–Leu/–Trp double dropout medium). Plates with yeasts were incubated in 30°C 2-4 days until colonies appeared.

- Testing of protein-protein interactions in yeast two hybrid essay

Yeast two hybrid protein is method used for testing protein-protein interactions. First tested protein is inserted in vector resulting in protein fused with binding domain (BD) of transcription factor GAL4. The vector also contains reporter gene(s). The other protein is inserted in "matching" vector resulting in protein fused with activation domain (AD) of transcription factor GAL4. The vector also contains reporter gene(s). Tested proteins are expressed as fusion proteins and if they interact, they approach physically each other so that the fused domains AD and BD can react. Such connected domains are then capable of activation of transcription of reporter genes. Reporter genes confer resistance to selection to yeasts (Gietz et al., 1997).

In this work, vectors pGBKT containing BD and reporter gene TRP1 allowing yeasts to grow on selection medium without tryptophan and vector pGAD containing AD and reporter gene LEU2 allowing yeasts to grow on selection medium without leucin were used. Transformed yeasts containing tested proteins were as a consequence selected on Petri dishes with SD double dropout –Leu/-Trp.

Interactions of proteins were tested by droplet dilution-series experiment. Several colonies of transformed yeast were transferred to 500 µl sterile H_2O with by sterile pick and mixed well. $O.D._{600}$ was measured at each sample and adjusted to three variants of concentration for each sample. Three concentrations of each sample were with $O.D._{600}$ as following: $2x10^{-2}$, $2x10^{-4}$ and $2x10^{-6}$. Such diluted solutions were then dripped (10 µl of each) onto quadruple dropout SD medium (-Ade/–His/–Leu/-Trp) for strong selection. Weaker selection was performed onto medium –His/–Leu/-Trp +10 mM triaminotriazol (3AT). Cultivation of prepared plates was in 30°C approximately 5 to 7 days maximum.

3.6. In-silico protein modeling:

- Building protein models

All the models were based on amino acid sequence of cloned segment.

The main program used for modelling was server-based Protein Homology/analogY Recognition Engine V 2.0 (Phyre2) (Kelley et al., 2015, available http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). For comparison, some models were also built in the SWISS-MODEL (Biasini et al. 2014, available http://swissmodel.expasy.org/).

All models were visualized and colored in open source software PyMOL (version for educational use, Version 1.7.4 Schrödinger, LLC, 2010)

- Protein body docking

For protein body docking several programs were used. Mainly, the HADDOCK software portal (available http://haddock.science.uu.nl/) was used. Cport (de Vries and Bonvin, 2011), an algorithm for the prediction of protein-protein interface residues, which combines six interface prediction methods into a consensus and was used for prediction of active and passive residues of given sequences. Predictions from Cport were used for High Ambiguity Driven protein-protein DOCKing (HADDOCK) itself (de Vries et al., 2010). Haddock is an information-driven flexible docking approach for the modeling of biomolecular complexes which encodes information from identified or predicted protein interfaces in ambiguous interaction restraints (AIRs) to drive the docking process. Such approached was used for docking FH2 domain with protein SH3P3 and FAB1A.

For model of FH2 dimers, GRAMM-X Protein-Protein Docking Web Server v.1.2.0 (Tovchigrechko and Vakser, 2006, available http://vakser.compbio.ku.edu/resources/gramm/grammx/) was used. Rosetta Online Server that Includes Everyone, ROSIE (Lyskov et al., 2013, available http://rosie.rosettacommons.org/), a web front-end to the Rosetta 3.x software suite, a molecular modeling software package could not be used for dimerization modeling since the length restriction for symmetric docking is 400 amino acids and the only suitable sequence was FH2 domain of AtFH13.

All proposed models of docking were also visualized in open source software PyMOL.

4. Results

4.1. RT-PCR

RNA has been isolated from wild type *A. thaliana* leaves in sufficient amount and tested on the agarose gel to estimate its quality (figure 8). Loaded volume was 1 µl of each sample, samples marked by numbers one to seven. Such RNA, considered suitable for RT-PCR, then served for preparation of cDNA. cDNA was tested by using proved actin primers and then stored+used for cloning of AtFH13 (FH2 domain), AtSH3P1, AtSH3P2 and AtSH3P3.

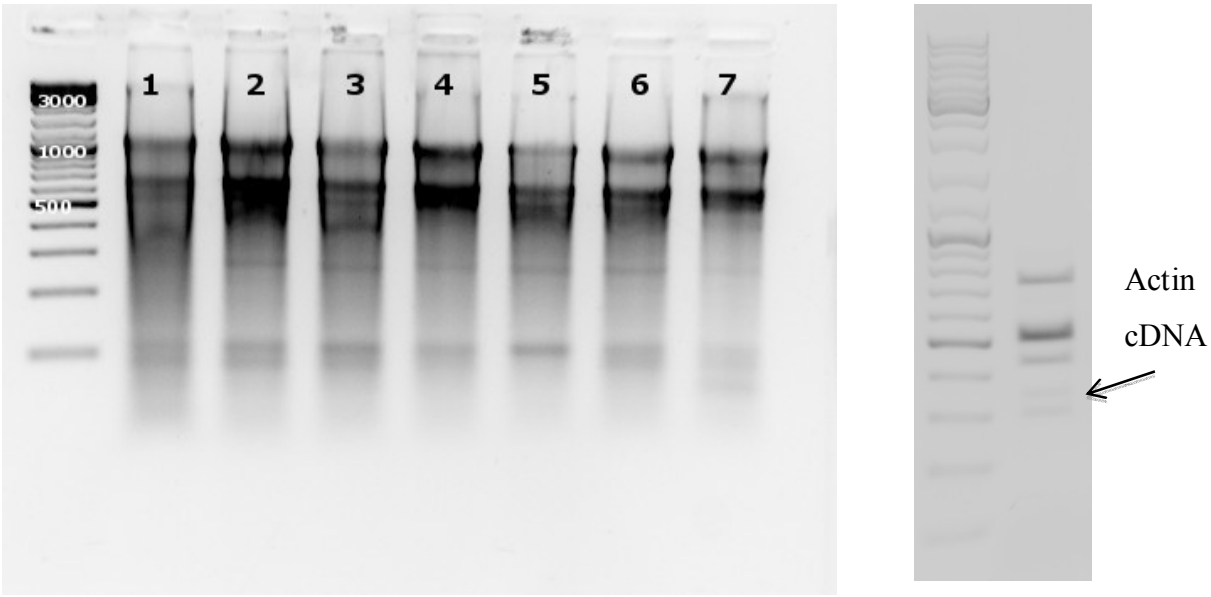


Figure 6 Visualisation of gel with loaded RNA samples and actin control

Left: samples 1-7 are independent RNA preparations; Right: Control actin fragment amplified on RNA sample No1.

4.2. Cloning

4.2.1. Cloning of AtFAB1A (At4g33240)

cDNA of AtFAB1A was obtained by PCR amplification from plasmid with catalogue number RAFL09-68-K04 in the RIKEN database. Primers were designed to cover beginning and end of AfFAB1A coding sequence. The estimated length of product was 5786 bp. However, the visualization on agarose gel showed it to be rather bigger that estimated length (see figure 9) The PCR was then repeated with different conditions. The product of PCR with following conditions T_m=62°C, Annealing time=190 s and polymerase Phusion (Thermo Scientific^{TM})

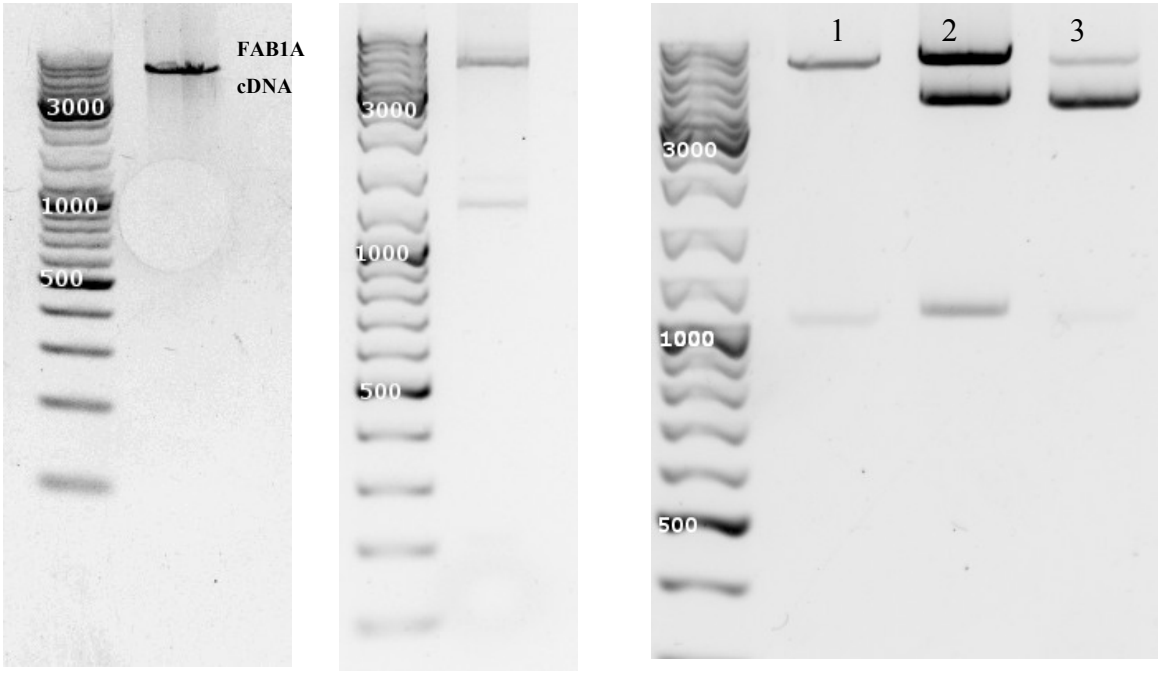


Figure 7 Visualisation of partial steps of cloning of AtFAB1A

Left-cDNA, expected size 5786 bp, appears bigger

Middle-restricted cDNA, expected size 1213 bp+4573 bp

Right-Restriction of isolated plasmids (pGADT7 with AtFAB1A), expected size 1072bp+ 5389bp+7143bp, samples numbered 1 to 3 isolated from different colonies, restriction enzymes was SphI

Scale of marker in base pairs

(rest same as described in chapter 3.3 PCR) was then used for control restriction by restriction enzyme BamHI (restriction site G↓GATCC).

The restrictions corresponded to prediction (restriction analysis via Addgene, available online, Kamens, 2015) thus the cDNA fragment was considered good for further work. Total amount 6 µl of cDNA and 5 µl of vector pGADT7 was then restricted by restriction enzymes Cfr9I (isoschizomer of XmaI , restriction site C↓CCGGG) and XhoI (restriction site C↓TCGAG) in 1X Tango buffer (low salt concentration buffer) with 4-fold excess of Cfr9I (XmaI) and incubated at 37°C for 1 hour. When the first digestion was complete, 10X concentrated Tango buffer was added to a final 2X concentration (high salt concentration buffer) and finally added restriction enzyme XhoI for an additional hour. Unsuccessful attempts at cloning have necessitated optimization of the restriction protocol., and buffer SdaI (in which both enzyme perform 50-100% activity) has been finally used for successful cloning. After transformation, bacteria were grown on MPA medium supplemented with ampicillin. From nicely grown colonies, several were selected to multiply for plasmid isolation. Isolated plasmids were verified together with empty pGADT7 vector by restriction enzyme SphI (synonym PaeI, restriction site GCATG↓C) in “B” buffer. One corresponding sample was selected and reamplified.

Samples were sent to the service laboratory for sequencing. Sequencing primers were taken from laboratory collection (primers for sequencing vector pGADT7). After obtaining results, few places had to be fixed according to DNA chromatogram however; the sequenced reads were to short, covering only small part about 500bp from both edges. For that reason, other five primers were design for the objective for sequencing the rest of sequence. After complementation, fixing based on chromatogram and comparison with sequence of AtFAB1A from The Arabidopsis Information Resource (TAIR, tair.org, Rhee et al., 2003) in Geneious software; the cloned AtFAB1A was endorsed for further experiments.

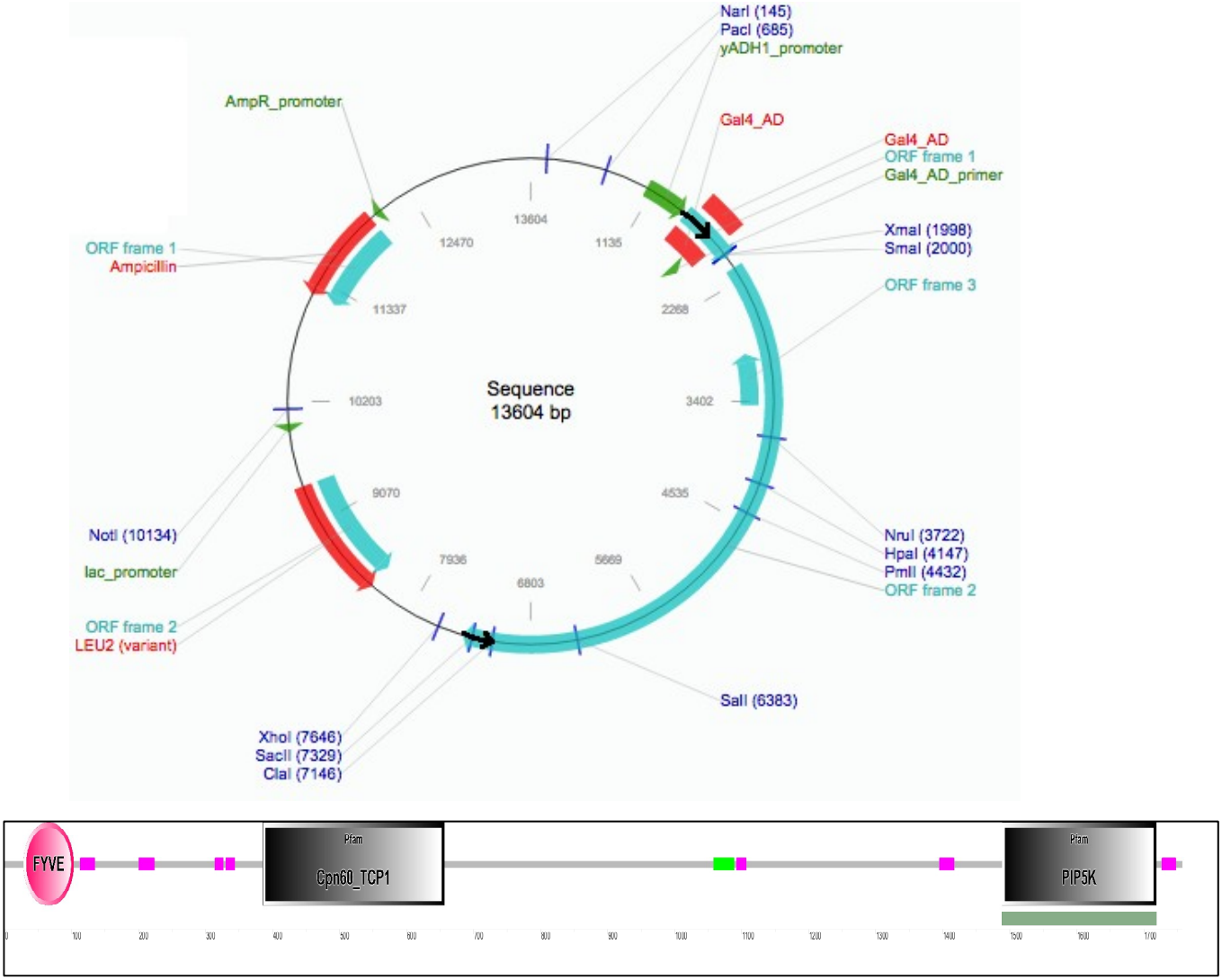


Figure 8 Restriction map and visualisation of cloned AtFAB1A

Restriction map shows most important features of plasmid. Protein visualization based on SMART prediction (Letunic et al., 2015). Green regions=coiled-coiled region as detected by COILS programme (Lupas et al., 1991). Violet regions=regions of low compositional complexity, as detected by the SEG program (Wooton and Federhen, 1996). Characteristic domain: FYVE, PIP5K, Cpn60_TCP1 domain in the middle, Pfam domain (E value 2.1e-35). Total length 1757 amino acids.

4.2.2. Cloning of AtSH3P1 (At1g31440)

The cloning of AtSH3P1 was not successful. The fragment of AtSH3P1 has never been amplified neither from genomic DNA neither from cDNA. Even though many variations of PCR reaction were tried (temperature gradient, different annealing time, different polymerases, various concentrations of reaction mixture). Most probably, the primers were self-complementary or complementary between themselves despite the use of primer design tool (see methods). Since meanwhile cDNA of AtSH3P2 and AtSH3P3 were obtained, additional attempts of obtaining cDNA of AtSH3P1 were abandoned.

4.2.3. Cloning of AtSH3P2 (At4g34660)

Primers for amplification of AtSH3P2 were first tested on genomic DNA and after optimization of conditions (annealing time=90 s, T_m=65,5°C and 0,5 µl of template DNA) they were used on cDNA. Unfortunately, the product corresponded by its size to the genomic sequence (expected 3257bp), rather than product from cDNA (expected 1290 bp). That was most probably caused by contamination of DNA by genomic DNA. However after several attempts with when all reagents were exchanged, fairly visible band corresponding to the cDNA size appeared. This band was cut out, frozen and centrifuged on maximum speed. 1 µl of the resulting liquid was then used as template to amplify AtSH3P2 cDNA in sufficient amount for restrictions. Restriction was done by restriction enzymes BamHI (restriction site G↓GATCC) and EcoRI (restriction site G↓AATTC) in buffer EcoRI. The subsequent procedure was same as with AtFAB1A. Verification of isolated plasmids was by restriction enzyme XhoI to give two segments (935bp+8321bp) and corresponding sample was multiplicated and sent to the service laboratory. The reading from forward side had to be sent totally 3 times due to wrong readings. Finally, full sequence was compared to sequence of AtSH3P2 from TAIR and once, considered without mistakes, the plasmid was stored for subsequent use.

Figure 9 Visualization of AtSH3P2

Picture created at MyDomains - Image Creator (PROSITE datebase, Sigrist et al., 2013) and based on results of InterPro database search (Hunter et al., 2009). Total length 368 (AtSH3P2). Visible features BAR domain and SH3 domain AtSH3P3 is not shown.

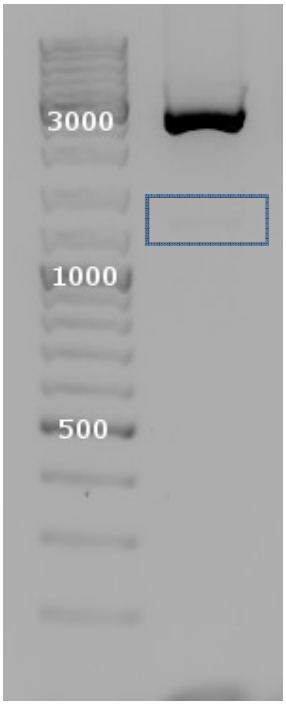


Figure 12 Agarose gel with product of PCR with primers for AtSH3P2 on cDNA

The band corresponding to size 1290 bp faintly visible, bordered by blue rectangle.

4.2.4. Cloning of AtSH3P3 (At4g18060)

Cloning of AtSH3P3 was almost identical to the cloning of AtSH3P3. The same problem with contamination of cDNA by genomic DNA occurred, and the same method was used to recover the right fragment. When a sufficient amount of cDNA was reamplified, samples were restricted before ligation. Restriction enzymes used were EcoRI and XhoI in buffer "R". The volume of cDNA used for restriction reaction was 6 µl and that of vector pGADT7 DNA was 5 µl. However, the control restriction of isolated plasmids did not fit restriction analysis from Addgene thus another attempt of cloning was performed, unfortunately also negative. Third attempt at cloning brought expected results of restriction analysis. Selected sample was reamplified, checked again and sent to the service laboratory for sequencing. As in previous processing of sequenced fragments, the results obtained from laboratory was controlled against chromatogram and unclear positions repaired. The sequence was corresponding to the sequence of AtSH3P3 from TAIR database and was without significant insertion or deletion. Such sequence was then stored with others for following experiments.

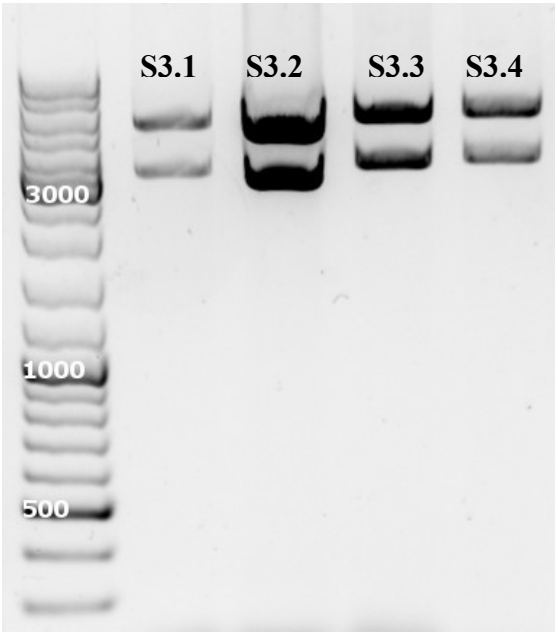


Figure 13 Restriction of isolated plasmids pGADT7 with AtSH3P3, third attempt

Restriction enzyme used EcoRV (synonym Eco32I,, restriction site ATA↓TCG). Expected size of bands 3423 bp and 5694 bp.. Samples named S3.1 to S3.4.

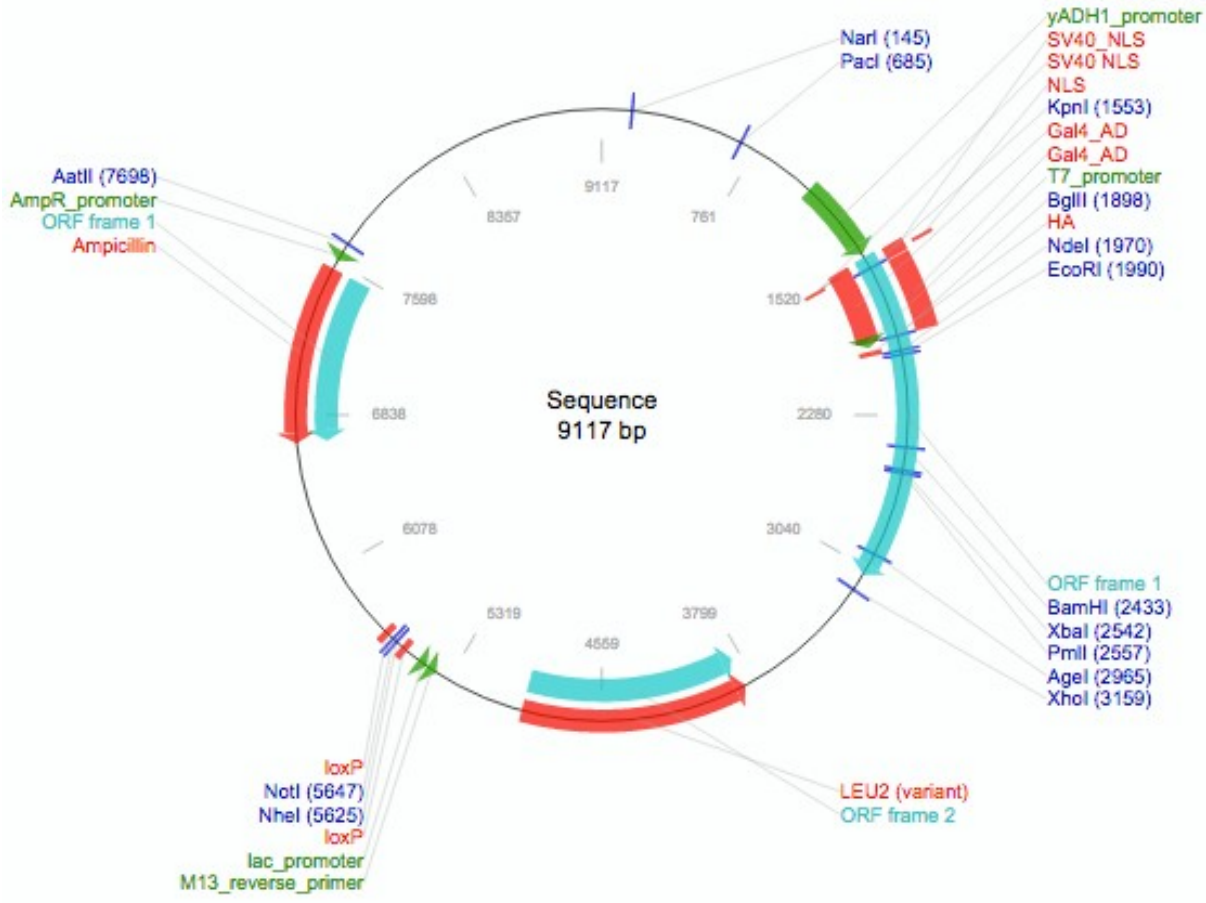


Figure 14 Restriction map of AtSH3P3

Main features of plasmid are shown, AtSH3P3 correspond to the light blue mark in open reading frame 1 beteen EcoRI and XhoI.

4.2.5. Cloning of AtFH13, At5g58160

The first FH2 domain cloned was that of AtFH13. The primers were designed to define the boundaries exactly at the beginning and the end of the FH2 domain thus specific for cloning only the core part of formin (see summary of cloning of FH2 domain of plant formins, table 5). Primers (see table 3) were suitable for cloning into "bait" vector pGBKT7 as well as "prey" vector. The specific conditions of PCR reaction were tested on genomic DNA first and after the finding of convenient conditions, primers were tested on cDNA (0,5 µl used). A band of corresponding size appeared on agarose gel and has been cut out for isolation. After isolation, cDNA of AtFH13 has been used for restriction reaction (6 µl of isolated cDNA used) by restriction enzymes EcoRI and BamHI. The buffer used in reaction was BamHI. Same restriction enzymes were used for restriction of 5 µl of vector (pGBKT7, collection number FD451). The cloning into pGADT7 happened later; since the restriction sites are the same for both vectors, same cDNA stored in -20°C was used. Both vector and insert were cleaned by running through agarose gel, isolated by centrifugation as described above and electroporated. Bacteria were grown on MPA medium supplemented with

kanamycin (ampicillin for pGADT7). Nicely grown, numerous colonies were sampled into 12 test tubes. After isolation of plasmids, control restriction for verification was done (Addgene). Restriction enzyme XhoI (restriction site C↓TCGAG) in R buffer was used. Samples corresponding to the digesting analysis were kept and representative sample sent to the service laboratory for sequencing. Primers for sequencing vectors pGBKT7 and pGADT7 were from laboratory collection.

Results obtained from service laboratory where compared in Geneious software with cDNA sequence of AtFH13 obtained from TAIR. The sequence did not have any mutations. A few places had to be compared with chromatogram of DNA sample due to high amount of mistakes from bad reading. Thus, the cloned sample was considered as good for further use since the sequence corresponded to the sequence in TAIR database.

4.2.6. Cloning of AtFH1, AtFH5, AtFH8, AtFH14

The cDNA of FH2 domains of Arabidopsis formins FH1, FH5, FH8 and FH14 were obtained by reamplification with specific primers (see chapter 3.2 Primers). All sequences were reamplified without any complication since templates for PCR reaction were already cloned into vectors and not raw cDNA. The informations of origin of template sequences are gathered into the table 4. For restrictions, following restriction enzymes were used:

AtFH1: NdeI (restriction site CA↓TATG) and BamHI in "R" buffer (for both pGADT7 and pGBKT7)

AtFH5: for pGBKT7: NdeI and PstI (restriction site CTGCA↓G) in buffer "O"
for pGADT7: NdeI and XmaI in SdaI buffer

AtFH8: NdeI and BamHI in "R" buffer (for both pGADT7 and pGBKT7)

AtFH14: for pGBKT7: BamHI and SalI in BamHI buffer
for pGADT7: EcoRI and XmaI in SdaI buffer

The process of cloning was more or less identical to those mentioned previously, however cloning AtFH1 and AtFH8 was not achieved until very late date and the resulting constructs have not been confirmed by sequencing and neither was used for transformation of yeasts.

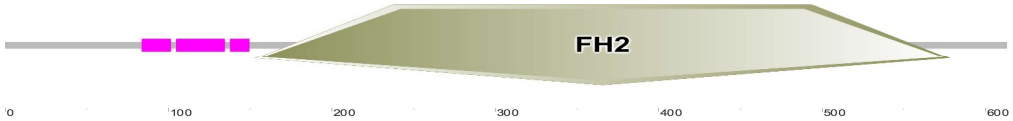
Summary of structure of cloned FH2 domains of selected plant formins is in the table 5.

Table 4 Informations about vectors of origin for cloning FH2 domains

Note: parenthesis in destination vectors denote unsuccessful cloning in that vector

Name	Collection number	Note	Destination vectors	Size of cloned fragment of FH2 domain in bp
AtFH1	FD233	cDNAGenBank AV544190, tvarin8 (old nomenclature)	pGADT7 (pGBKT7)	1819
AtFH5	FD234	cDNAGenBank AV548458 tvarin5 (old nomenclature)	pGADT7 pGBKT7	1904
AtFH8	FD195	AtFH8 (tvarin1, by old nomenclature) made by triple ligation assembled in pGEX	pGADT7 (pGBKT7)	2268
AtFH14	FD503	From cDNA sequence in pENTR1a	pGADT7 pGBKT7	1475

Table 5 Summary of cloning of FH2 domains of plant formins Visualisation based on SMART prediction (Letunic et al., 2015). Violet regions= regions of low compositional complexity, as detected by the SEG program (Wooton and Federhen, 1996).

Name	Locus	Graphic visualization	Length (in amino acids)
AtFH1	At5g25500		595
AtFH5	At5g54650		634
AtFH8	At1g70140		613
AtFH13	At5g58160		493
AtFH14	At1g31810		491

4.3. Yeast two hybrid assays

The transformation of yeasts was done according to available constructs. Most often, enough colonies grew on selection plates –Leu-Trp however some of the co-transformation failed even after repeated attempt. All transformations are summarized in table 6. Construct of AtFH16 in pGAD was acquired from laboratory collections, where it is stored under number FD316 and contains AtFH16 fragment (FH2 domain) (prepared by Mgr. Denisa Oulehlová PhD.). As negative control, empty pGADT7 vector was used. Bc. Denisa Rácová provided positive control and the construct was Exo70h4_pGBKT with exo84b c-term_pGAD.

Table 6 Summary of transformed yeasts.

For specification of constructs, see previous chapters. Failed=transformation of yeasts failed after several attempts.

pGADT7 / pGBKT7	FH1	FH5	FH8	FH13	FH14	FH16	FAB1A	SH3P2	SH3P3	Empty pGAD
FH1	Missing construct AtFH1(FH2domain) in pGBKT7									
FH5	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
FH8	Missing construct AtFH8(FH2domain) in pGBKT7									
FH13	failed	Yes	Yes	Yes	Yes	Yes	Yes	failed	Yes	Yes
FH14	failed	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

All transformed yeasts were tested via dilution series experiment on quadruple dropout SD medium (-Ade/–His/–Leu/-Trp). Some were tested also on weaker selection medium –His/–Leu/-Trp +10 mM triaminotriazol. The transformants who were evaluated as interacting were transformed independently second time to confirm the result. Strong interaction was detected between FH2 domain of AtFH5 and AtSH3P3, between FH2 domains of AtFH14 and AtFH13 and also between FH2 domains of AtFH13 and AtFH13.

Photos of representative dilution series experiments with comments are on pictures 15 and 16.

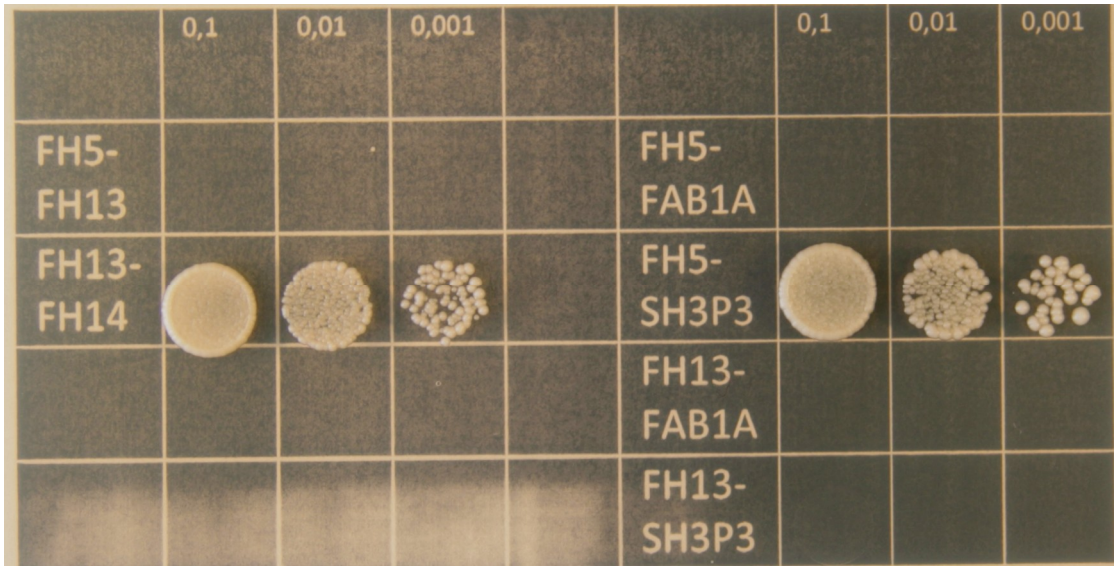


Figure 15 Results of yeast two hybrid assay

Medium(-Ade/–His/–Leu/-Trp) for strong selection was used.

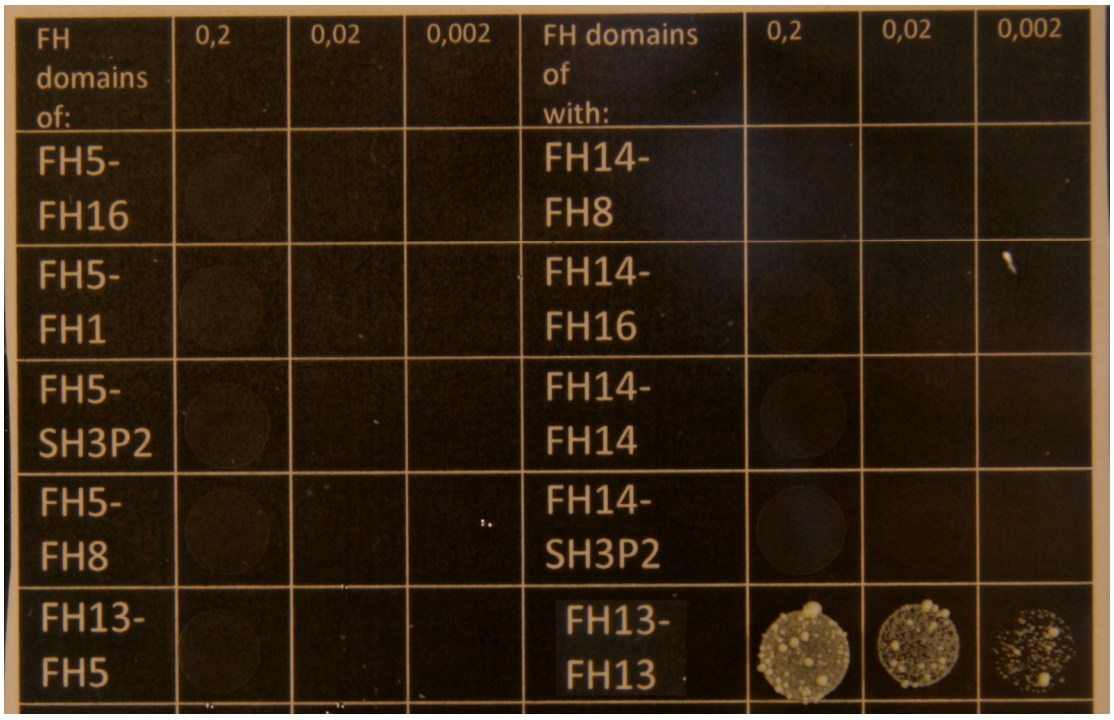


Figure 16 Results of yeasts two hybrid

Weaker selection, medium –His/–Leu/-Trp +10 mM triaminotriazol (3AT) was used.

4.4. In-silico modeling

Models of all cloned FH2 domain were prepared for comparison among themselves and possible docking (modelling dimerization). From the group of potential interactors, AtFAB1A and AtSH3P3 were selected, AtSH3P3 already as confirmed interactor of AtFH5 worth of deeper investigation.

From models proposed by phyre2, only the top models were selected. In all cases, the template was crystal structure of complex between amino and carboxy terminal2 fragments of mdia1 (PDB code ID 3O4X)

Accuracy correspond as following (table 7).

Table 7 Accuracy of protein models

Formin	Percentage of residues modelled	Confidence	Library id of template
AtFH1	88%	>90%	c3o4xE
AtFH5	78%	>90%	c3o4xE
AtFH8	84%	>90%	c3o4xF
AtFH13	84%	>90%	c3o4xE
AtFH14	100%	>90%	c3o4xE

For modelling of AtSH3P3, an alternative method was used since neither Phyre2 nor Swiss-Model managed to build a complete model. Phyre2 proposed only the SH3 barrel, and Swiss-Model, on the other hand proposed exclusively BAR domain. Eventually, RaptorX (Peng and Xu, 2011) proposed a model including both properties. The input predicted as **2** domain(s). Model was based on template 4IGZ (SH3 domain of human sorbin and SH3 domain-containing protein 2) and p-value of model was 7.46e-07 with 94% residues modelled (for evaluation of RaptorX models, see http://raptorx.uchicago.edu/documentation/#goto2)

Protein docking brought varying results. In attempts to model FH2 domain dimerization, HADDOCK refused to perform docking of two identical proteins (dimerization) even after change of various parameters. Results from Rosie (see chapter…) did not fit any of the known (experimentally determined) structures of FH2 dimers. For that reason, HADDOCK and Rosie were rejected for modelling of dimers. Only Gramm-X was able to dock the proteins in more

realistic way. However, from 35 proposed models, merely a few complied with the contact of lasso/post subdomains contact (figure 19)

Docking of AtSH3P3 with AtFH5 seemed to be more successful. HADDOCK calculated the results with best score in every proposed cluster and most importantly the place of interaction was the same in all cases.

Models of AtFH5 and AtSH3P3 separately and selected models (chose based on the score) of possible protein-protein interaction are in following pictures (figures 17 and 18):

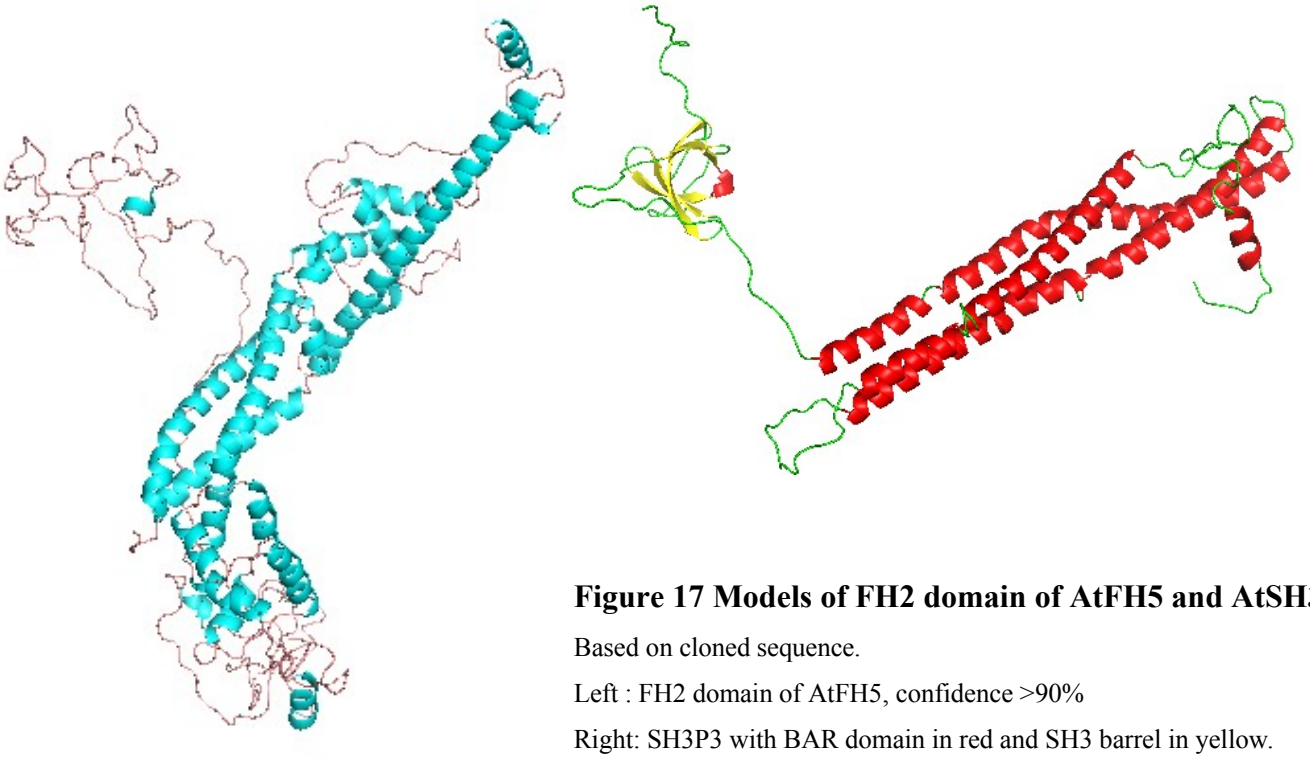


Figure 17 Models of FH2 domain of AtFH5 and AtSH3P3

Based on cloned sequence.

Left : FH2 domain of AtFH5, confidence >90%

Right: SH3P3 with BAR domain in red and SH3 barrel in yellow.

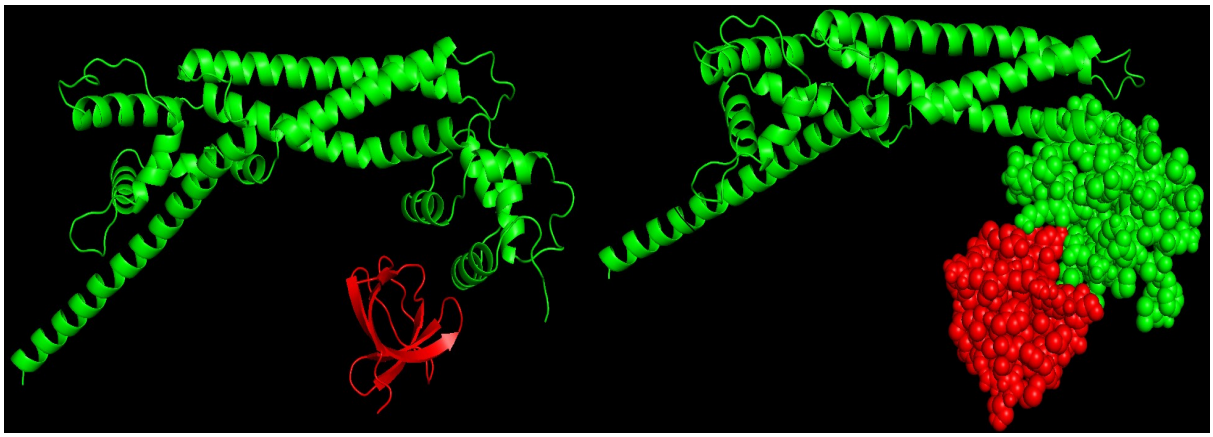
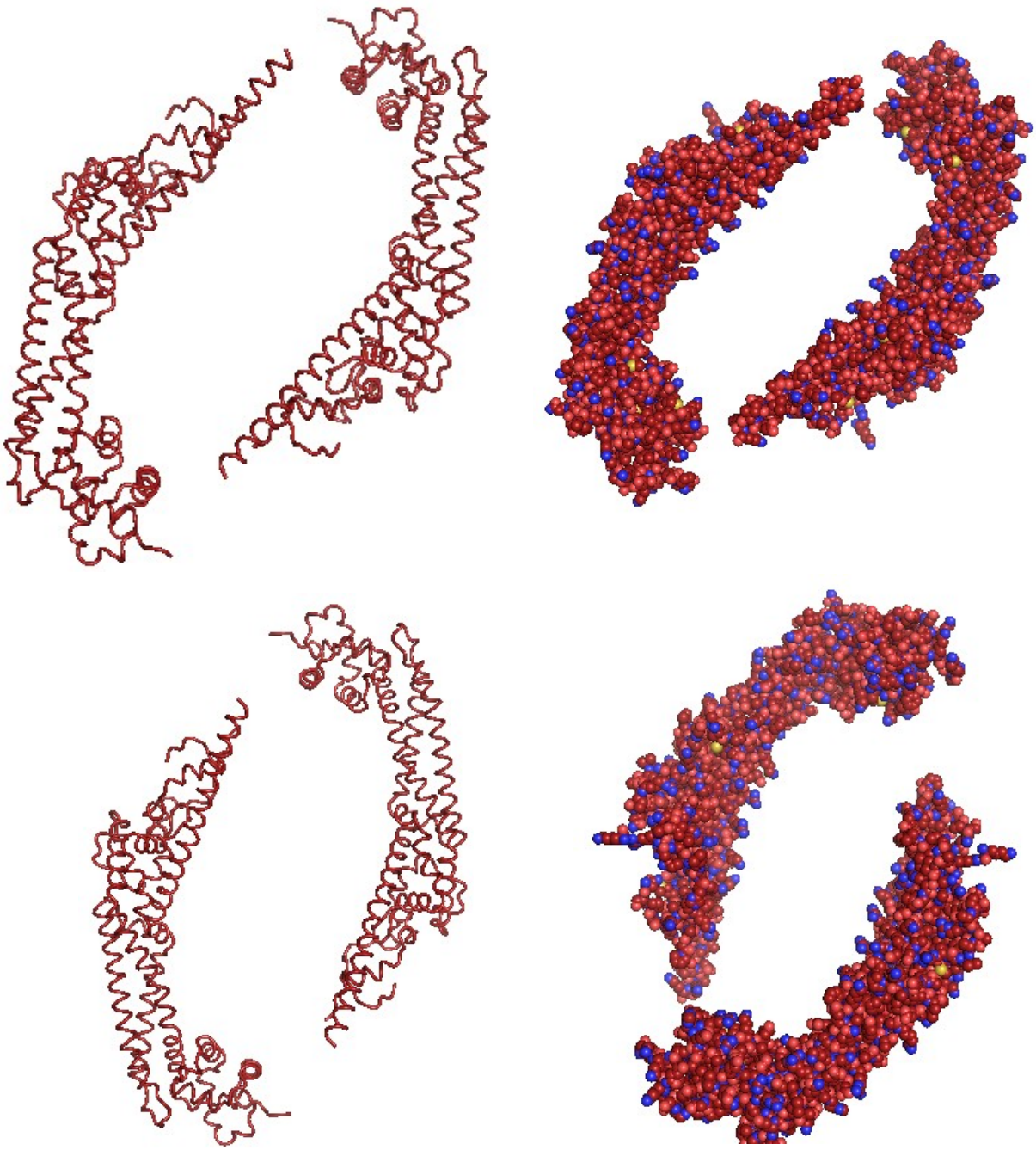


Figure 18 Proposed docking for AtFH5 and SH3P3

FH2 domain of AtFH5 I in green, SH3 barrel of AtSH3P3, BAR domain is not shown.



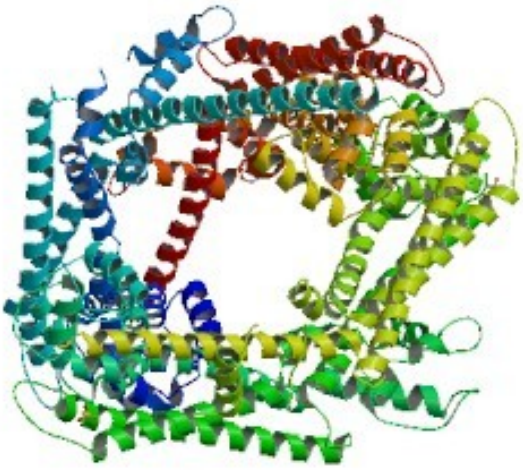


Figure 19 Examples of proposed docking for AtFH13 x AtFH13

Cluster of proposed 35 variants of docking. Only few (example shows number 10 and 33) kept the "head-to-tail" (or lasso/post) dimerization interface.

Left corner: Crystal structure of the core FH2 domain of mouse mDia1 for comparison.

5. Discussion

This work is dedicated to the FH2 domain(s) of *Arabidopsis thaliana* formins from both classes. Main goals were (1) to determine whether plant formins interact with predicted potential interactors and (2) to test the possibility or homo- and heterodimerization of FH2 domains.

The discussion is thus divided into two parts, dealing with these two questions.

5.1. Results of interaction experiments

Results of yeast two hybrid experiments clearly showed strong interaction between AtFH5 and AtSH3P3 on both strong and weaker selection. Thus, at least some plant Class I FH2 domains are capable of interacting with AtSH3P2, which might mediate a connection between formins and vesicular trafficking. Unfortunately, interactions of additional candidates, AtSH3P2 and AtFAB1A, were not proven for any of the tested FH2 domains of several formins (notably AtFH5, AtFH13, AtFH14).

Several studies investigating interaction of formins and Src homology proteins are available (see Introduction). However, none of them studies plant proteins, and thus the conclusions deduced from animal analogy might not be significant. Human SLIT-ROBO Rho GTPase-activating protein 2 (srGAP2) forms a complex with formin-like 1 (FMNL1); binding of the SH3 domain of srGAP2 to the FMNL1 may inhibit formin-mediated actin severing (Mason et al., 2011). The recruitment of srGAP2 is mediated by activation of FMNL by the Rac signalling pathway, thus very unlikely in plants since plant formins are missing the GBD domain (Grunt et al., 2008). In fact, the interaction of various mammalian diaphanous-related formins with various SH3 domain-containing proteins seems to be rather common feature and part of the Rho GTPase signaling pathway (Young et al., 2010).

The role of the protein AtSH3P3 in *Arabidopsis* is even less studied than that of its relative AtSH3P2, which is known to interact with FREE1, which is FYVE domain protein required for endosomal sorting 1 and plant-specific ESCRT component essential for multivesicular body biogenesis and plant growth (Gao et al., 2015). The protein AtSH3P2 is also a regulator of autophagosome biogenesis (Zhung and Jiang, 2014). All AtSH3Ps also participate in clathrin-mediated vesicle trafficking (Lam et al., 2001). AtSH3P3 has been shown to interact in yeast two-hybrid system with the proline-rich domain of DRP2A (dynamin-related protein), which in Arabidopsis most probably participates in clathrin-mediated trafficking of vesicles originating from the Golgi (Lam et al., 2001, reviewed in Fujimoto and Tsutsumi, 2014).

Its formin interaction partner AtFH5 localizes to the cell plate and its loss of function compromises cytokinesis in the seed endosperm and perturbs proper morphogenesis of the endosperm posterior pole (Ingouff et al., 2005). It also stimulates actin assembly from the subapical membrane, provides actin filaments for vesicular trafficking to the apical dome, and mediates assembly of the subapical actin structure (Cheung et al., 2010). Both pollen tube growth and formation of cell plates are processes where heavy vesicular trafficking occurs and where cytoskeleton involvement plays particularly important role.

The function and importance of interaction between formin and SH3 barrel containing protein AtSH3P3 remains to be investigated; but their confirmed interaction suggest recruitment of formin in the processes of vesicular trafficking with need of precise organization of cytoskeleton.

The results from protein docking proposed by HADDOCK suggest that the binding may take place between the SH3 barrel and lasso/knob/linker of the FH2 domain. Nearly the same interaction site was proposed for all protein-protein interaction models with reasonably good scores. If such proposition is realistic, it would then mean that the interaction between AtFH5 and AtSH3P3 cannot occur simultaneously with dimerization of AtFH5, since the lasso/knob/linker interface is necessary for successful creation of dimer, and binding of the SH3 barrel of AtSH3P3 would interfere with this interface; however, the BAR domain of AtSH3P3 remains accessible and available for membrane or other protein interaction, allowing speculation about the intriguing possibility that the formin might be able of switching between an actin-binding dimer and a SH3-BAR-bound monomer.

5.2. Results of formin dimerization experiments

Intriguingly, the hypothesis that FH2 domain should form dimers has only been fully confirmed for the homodimerization case. Therefore we used the yeast two-hybrid system to investigate homo-and heterodimerization of representative Class I and Class II formins. The FH2 domains of AtFH13 interacted with themselves on weaker selection. Surprisingly homodimerization did not occur between FH2 domains of AtFH5 and neither AtFH14 did not interact with itself even though interactions were expected.

Heterodimerization of FH2 domains between classes has not been confirmed in any case. Nevertheless, results proved that heterodimerization in at least closely related formins is possible since strong interaction between AtFH13 and AtFH14 occurred. However, when FH2 domains were cloned in opposite vectors, the interaction did not appear.

Such results might have different explanation. One of the problems might lie in the subdomain organization and structure differences. It has been shown that the lasso segment at the N-terminal end of FH2 is required for accurate dimerization of FH2 domains (Xu et al, 2004, Ignatev et al., 2012). A 'lasso' extends from the knob of one monomer and wraps around the 'post' of the other monomer to stabilize this dimeric configuration. The lasso–post interactions in human DAAM1 FH2 dimer are mostly hydrophobic as well as in yeast Bni1p FH2 dimer (Yamashita et al., 2007).

The plant formins have never been experimentally investigated on the structural level, nor they have been ever studied from the point of view of dimerization. Important major differences between plant formins of class I and class II are surprisingly within the well-conserved FH2 domain (Cvrčková et al., 2004). Class I formins, compared to yeast Bni1p, have small or non-polar amino acid at the position corresponding to lysine residue K1639, which is contributing to the actin-binding site. Most impotent difference of class II compared to Bni1p is insertion of 15-43aa in the post region. Such insertion is not in all class II formins, but it can be found in AtFH13, AtFH15 and AtFH16. Importantly, AtFH13 has insertion also in the lasso region/subdomain. Insertion in this region might have consequences on the dimerization interface.

Yamashita et al. (2007) were comparing orientation of FH2 dimer rings between Bni1p and mDia and found a structural difference in the linker region and also the lasso orientation. They concluded that the linker length, but not specific amino acids in the region, affects the actin assembly activity and that the linker sequence and length are divergent in the formin family. The difference in the actin assembly activity might reflect a physiological role of each formin protein. Such structural differences as length the linker subdomain or lasso segment orientation might

play a role also in a dimerization interface. The subdomain organization of FH2 domain is on the figure 20.

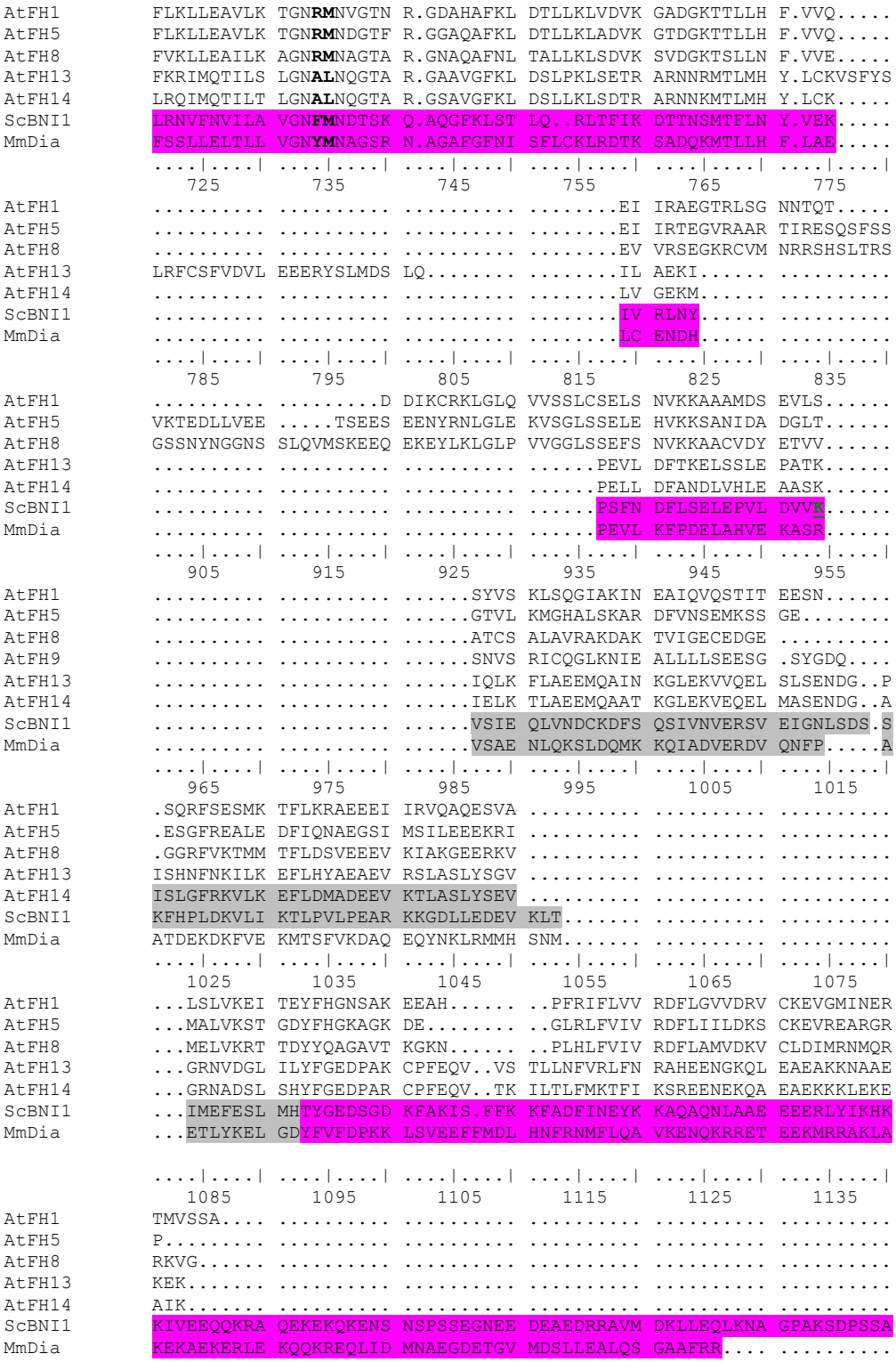


Figure 20 Sequence alignment of FH2 domains of formins

Only cloned formins (AtFH1, AtFH5, AtFH8, AtFH13 and AtFH14) together with yeast Bni1p and mouse mDia whose structure has been experimentally determined are shown. The highlighted subdomains in the sequences of these structurally characterized proteins are as following: Lasso region in yellow, Linker region in light blue, Knob region in green, Coiled-coil region grey and Post region in magenta. In bold, the amino acids, which are crucial for dimerization in the Bni1p and in the mDia (the link between the lasso–post) are highlighted (based on Yamashita et al., 2007). Alignment adapted from Cvrčková at al. 2004.

What can be concluded from the alignment of FH2 domain is that in the sequence of lasso where are crucial amino acids necessary for dimerization, Bni1 has leucine and mDia has proline, AtFH13 has tyrosine and AtFH5 has phenylalanine. Human DAAM formin (not in the picture) also has phenylalanine on that place so such exchange should not impose difference in dimerization (Yamashita et al., 2007). The other position, tryptophan, is same in all protein within alignment. The most variable is thus AtFH13, not only it has different amino acid on place responsible for the link between the lasso–post interface but it also has considerably longer whole lasso region. However, the post region, where the amino acids responsible for interface are found, is quite variable, not only between Bni1 and mDia but also between classes of plant formins. This could have an impact on possibility of plant formins to dimerize between classes.

The results of yeast two hybrid are also indicating that outcome of the two-hybrid experiment might be affected by the positioning of GAL4 binding and GAL4 activation domains with respect to the FH2 domains. In pGADT7 and pGBKT7 vectors by Clontech, only N terminal fusion is possible, and in some cases GAL4-BD and GAL4-AD might not come close enough to be able to activate transcription because of sterical hindrances, even if the "bait" and "prey" proteins themselves do exhibit an interaction. Especially the shape of FH2 domain, which can be compared to an "L" shape in a tetris game, is rather problematic for possible orientation of GAL domain parts. FH2 domain dimer positions the two subunits in "reverse", head-to-tail position (Xu et al., 2004) meaning than that GAL4-BD and GAL4-AD end up on different "ends" or sides of the dimer, and their ability to activate transcription may depend on the length and flexibility of the protein chain connecting the GAL4 domains to the FH2 domain core.

Unfortunately, Clontech Matchmaker system does not allow any other than N-terminal fusion; otherwise it would be very interesting to perform the same experiments with one FH2 domain as N-terminal fusion and the other as C-terminal fusion.

Looking at the alignment of cloned FH2 domains, it is visible that the region preceding FH2 domain in not very conserved and that it is variable among cloned plant formins. Although they have one characteristic in common, they region before FH2 domain is remarkably rich in prolin. Since AtFH13 was missing this region, it might be possible that this region has negative influence on dimerization. In figure 21, the distance between GAL activating domain and beginning of the lasso region is visible.

The fact the "prolin-rich" might also explain why the dimerization occurred between AtFH14_pGBKT7 and AtFH13_pGADT7 but not in the opposite direction-the beginning of the FH2 domain of AtFH13 is just the GAL4 domain thus when cloned into "prey" vector pGBKT7, there is and AtFH14 had "prolin-rich" region shorter, compared to cloned formins from class I.

Figure 21 Alignment of beginnings of cloned FH2 domains together with GAL4 binding domain

First 112 amino acids belong to the GAL4 activation domain (first full consensus). The beginning of the FH2 domain is highlighted in bold. Note especially the beginning of FH2 domain of AtFH13 close behind the GAL domain. Red box indicates approximate location of the lasso subdomain. Colors of alignment are according to consensus: BAD AVG GOOD, consensus not shown. Alignment prepared in TCoffee (Notrdame et al., 2000).

The models produced by Phyre2 correspond to the general structure of FH2 domain however the software did not generated very well the transition between knob through linker to the lasso. That can be due to higher overall variability of the region of linker and lasso (figure 22)

The lasso segment plays important role in the dimerization process thus the reason why protein docking was not so successful can be due to low specificity in the proposed models.

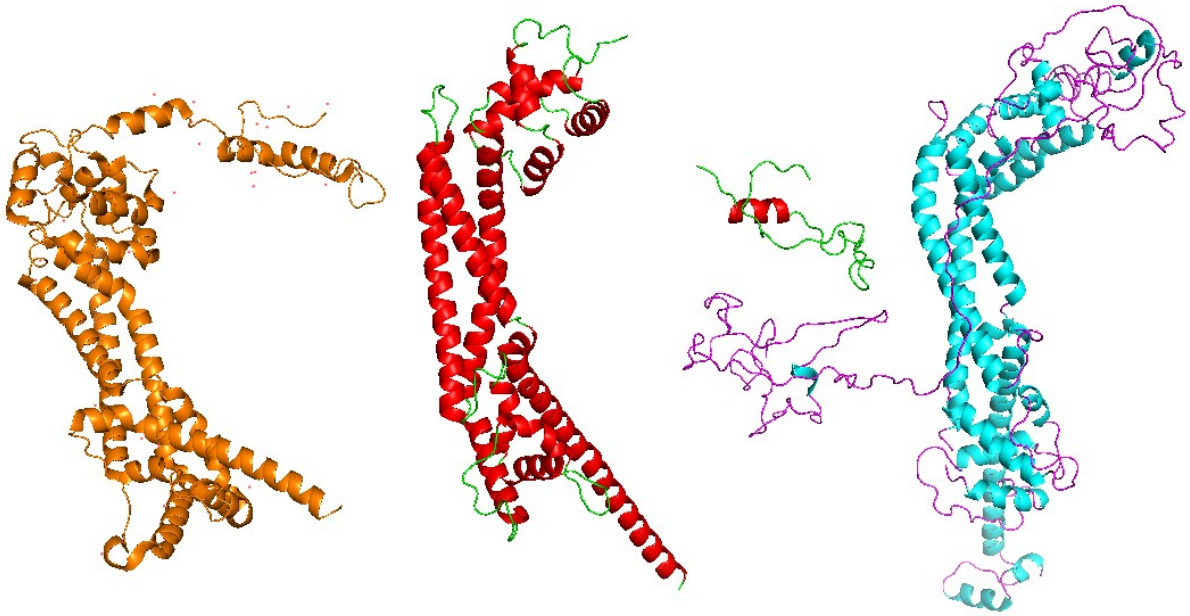


Figure 22 Comparison of models of FH2 domain

Orange: monomer of FH2 domain of Bni1p

Red: Class II formin AtFH13, monomer of its FH2 domain

Light blue: Class I formin AtFH5, monomer of its FH2 domain

6. Conclusions

Most of the aims specified at the beginning were fulfilled, however some partial experiments were not finished (such as cloning of AtFH1_FH2 domain and AtFH1_FH2 domain into vector pGBKT or transformation of yeasts by certain constructs), so some of the results should be considered preliminary.

Potential interactor AtFAB1A was amplified from vector obtained from RIKEN plant division and successfully cloned into vector pGADT7. Sequences of other potential interactors, proteins AtSH3P2 and AtSH3P3 were obtained from cDNA and cloned also into vector pGADT7. All cloned sequences were matching sequences available at the TAIR database.

Co-transformation of yeasts and subsequent dilution series experiment revealed interaction of AtFH5 (FH2 domain) with one of the selected interactors, protein AtSH3P3 however other interactions were not proven in the experimental set-up.

This work is also trying to understand the problem of dimerization of plant formins. Despite the common known fact that FH2 domain dimerizes, it is unclear, whether only homodimerization occurs or if heterodimerization is also possible. Plant FH2 domains served, as a good model since in *Arabidopsis*, there are 21 different isoforms falling in two families.

FH2 domains of five different formins (AtFH1, AtFH5, AtFH8, AtFH13, AtFH14) from both families were successfully amplified and cloned into vector suitable for expression in yeasts pGBKT7 with GAL4 DNA-binding domain and pGADT7 with GAL activation domain with exception of AtFH1 and AtFH8 which were not cloned successfully into pGBKT7. Sequences matched with predicted sequences in the TAIR database. While additional experiments will be required to complete the is study, and interactions should be also confirmed by additional methods such as in vivo co-localization or co-purification, the example of interaction of AtFH13 with AtFH14 FH2 domains clearly shows that at least closely related FH2 domains can heterodimerize. Also, the fact that two proteins interact together in a specific two-hybrid setup does not mean they will also interact when cloned in switched vectors. The explanation of the interaction between AtFH13 and AtFH14 might be based on the structure differences. The construct with AtFH13 was missing non-conserved, prolin rich region while AtFH14 had short one. It is speculative whether the prolin rich region itself might inhibit dimerization. However, the potential interactors should be tested by cloning also into “bait” vector pGBKT7, with formins as the “prey”. To finish the whole picture, the rest of planned yeast two hybrid interactions should be tested, including the constructs remaining to clone or transform into yeast. Also, development of protocol for protein docking in software Rosetta or PyRosetta would be

convenient because a docking protocol adjusted for specific demand will be more accurate then currently used less specific web-based docking tool, which, however, has already provided results compatible with the experimental findings.

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8. Other

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