BIOLOGIA PLANTARUM 56 (3): 431-440, 2012

Expression of GFP-mTalin reveals an actin-related role for the *Arabidopsis* Class II formin AtFH12

F. CVRČKOVÁ[1]*, M. GRUNT[1] and V. ŽÁRSKÝ[1,2]

Department of Experimental Plant Biology, Faculty of Sciences, Charles University, Viničná 5, CZ-12843 Prague, Czech Republic[1]
Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, CZ-16502 Prague, Czech Republic[2]

Abstract

Formins (FH2 proteins) are implicated in F-actin nucleation and other aspects of cytoskeletal organization. Plants possess two formin clades, relatively well-described Class I formins and so far poorly characterized Class II formins. Comparison of Class II formin genes of two *Arabidopsis* species, *A. thaliana* and *A. lyrata*, indicates dynamic evolution within the Class II formin clade. Disruption of an outlier *A. thaliana* Class II formin gene, *AtFH12* (At1g42980), whose expression is induced by NaCl, produced only negligible phenotypic effects under a variety of conditions, including salt stress, suggesting functional redundancy among Class II formins. However, the same mutation massively aggravated toxic effects of the expression of a fluorescent actin marker, GFP-tagged mouse talin (GFP-mTalin), known to interfere with normal actin dynamics. Abnormal actin structures were observed in *atfh12* mutants expressing GFP-mTalin as compared to wild type. This not only demonstrates an actin-associated function for AtFH12, but also documents the feasibility of using the heterologous actin marker to "stress-test" the actin cytoskeleton in phenotyping "weak" actin related mutant alleles.

Additional key words: FH2 proteins, genetic redundancy, salt stress, synthetic lethality.

Introduction

Formins are a large family of eukaryotic proteins sharing the evolutionarily conserved formin homology 2 (FH2) domain involved in actin organization, including but not limited to filament nucleation by a mechanism independent of the Arp2/3 complex. Some formins also participate in the regulation of the microtubular cytoskeleton and many signaling processes (for review, see Paul and Pollard 2009, Bartolini and Gundersen 2010, Blanchoin and Staiger 2010). Most eukaryotes have multiple formins that can be classified into distinct clades based on FH2 domain sequences and overall domain composition (Higgs and Peterson 2005, Grunt *et al*. 2008, Chalkia *et al*. 2008).

Seed plants possess two clades of FH2 proteins (Deeks *et al*. 2002, Cvrčková *et al*. 2004). Almost all experimental work reported so far has focused on members of the *Arabidopsis thaliana* [L.] Heynh. Class I, possibly due to the combination of very low expression levels, complex locus structure and large cDNA size of Class II formin genes that may hamper cDNA cloning. Only 3 out of the 11 *Arabidopsis* Class II loci are currently associated with experimentally characterized full-length cDNA sequences listed in the *SIGnAL* database (Yamada *et al*. 2003; http://signal.salk.edu), compared to 8 of 11 Class I formins, sometimes represented by multiple cDNAs.

Several *Arabidopsis* Class I formins have been so far shown to affect the organization of the actin cytoskeleton *in vivo* or other aspects of cell morphogenesis, and/or to nucleate actin *in vitro*. This is the case of AtFH1 (Banno and Chua 2000, Cheung and Wu 2004, Michelot *et al*. 2005), AtFH3 (a formin predominantly expressed in pollen, whose antisense inhibition blocked both pollen tube growth and cytoplasmic streaming; Ye *et al*. 2009), AtFH4 (Deeks *et al*. 2005), AtFH5 (participating in cell division; Ingouff *et al*. 2005), AtFH6 (induced in giant

Received 4 April 2011, *accepted* 21 June 2011.
Abbreviations: FH2 - formin homology 2, LatB - latrunculin B.
Acknowledgements: We appreciate the possibility to use *Arabidopsis lyrata* sequence data produced by the US Department of Energy Joint Genome Institute in collaboration with the user community. We thank Dr. Tijs Ketelaar for the GFP:mTalin line and Marta Čadyová for technical assistance. This work has been financially supported by the Ministry of Education of the Czech Republic MSM 0021620858, Charles University SVV 263210 and the Grant Agency of the Czech Republic P305/10/0433 projects.
* Corresponding author; fax: (+420) 221951704, e-mail: fatima@natur.cuni.cz

cells of nematode-induced galls; Favery *et al*. 2004), and AtFH8 (associated with the cell plate; Xue *et al*. 2011). Overexpression of AtFH8 was found to stimulate ectopic tip growth in root hairs, while a dominant-negative mutation inhibited root hair development (Deeks *et al*. 2005, Yi *et al*. 2005). Moreover, microtubule binding, well documented for some fungal and metazoan formins (reviewed in Bartolini and Gundersen 2010), was recently described also for AtFH4 (Deeks *et al*. 2010). Since typical Class I formins carry transmembrane domains, they are good candidates for serving as "anchors" tethering the cortical cytoskeleton to the cell wall, as recently shown for AtFH1 (Martinière *et al*. 2011).

The only angiosperm Class II formins analyzed in detail so far are *Arabidopsis* AtFH14, which binds and bundles both actin and microtubules and participates in cell division (Li *et al*. 2010), and rice FH5 with a pleiotropic phenotype (Yang *et al*. 2011, Zhang *et al*. 2011). In addition, RNAi-mediated suppression of two Class II formin genes in the moss *Physcomitrella patens* [Hedw.] Bruch & Schimp. in B.S.G.led to pronounced cell polarity defects and actin pattern disruption (Vidali *et al*. 2009).

Here we report a detailed bioinformatic analysis of the Class II formin family in *Arabidopsis* and phenotypic characterization of a T-DNA mutant disrupting the atypical (outlier) Class II formin gene *AtFH12*. Our observations confirm expected functional redundancy among Class II formins and are consistent with participation of AtFH12 in actin-dependent cell and organ growth and morphogenesis. We also demonstrate the feasibility of using overexpression of a moderately toxic heterologous actin-binding protein *in planta* to elicit an observable "synthetic" phenotype in an otherwise phenotypically silent mutation.

Materials and methods

Bioinformatics: For phylogenetic analyses, selection studies and sequence similarity estimations, sequences from a previously published collection of almost 300 FH2 domains were used (Grunt *et al*. 2008; see there for sequences and accession numbers). Subsets of at least 9 sequences from well-defined plant or metazoan formin clades, exhibiting approximately similar level of within-clade divergence, were chosen for selection analysis. Corresponding nucleotide sequences were aligned on protein alignment templates using *RevTrans* (Wernersson and Pedersen 2003; http://cbs.dtu.dk). Site-specific non-synonymous to synonymous substitution rates (Ka/Ks) were calculated from resulting nucleotide alignments using *Selecton* (Stern *et al*. 2007) with default settings. The same sequence collection has been used to identify closest homologues by standalone *BLAST* (McGinnis and Madden 2004); hits with highest percentage of identical amino acids across the whole length of the FH2 domain are reported.

The neighbor-joining tree of *A. thaliana* Class II FH2 domain sequences was constructed as described (Grunt *et al*. 2008), using 500 bootstrap replicates, and rooted with a Class I formin outgroup. Shortest proposed splicing variants were included for AtH15 and AtFH20. Repeats outside FH2 have been identified visually.

Formins of *Arabidopsis lyrata* [L.] O'Kane & Al-Shehbaz were identified by *BLAST* searches of the *A. lyrata v. 1.0* genome assembly (http://genome.jgi-psf.org/Araly1/Araly1.info.html) and re-checked against the version of the *A. lyrata* genome draft in *GenBank* in March 2011. Automated gene predictions have been modified where necessary, taking into account homologous sequences as described previously (Cvrčková *et al*. 2004).

Phylogenetic trees of *A. thaliana* and *A. lyrata* FH2 domains were constructed using the maximum likelihood method implemented in the *PhyML* program *v3.0 aLRT* (Guindon and Gascuel 2003, Anisimova and Gascuel 2006) at *Phylogeny.fr* (Dereeper *et al*. 2008), using the default substitution model, assuming an estimated proportion of invariant sites of 0.000 and 4 γ-distributed rate categories to account for rate heterogeneity across sites. The γ shape parameter was estimated directly from the data (γ = 1.171). Internal branch reliability was assessed using the aLRT test (SH-like support values are shown).

***Arabidopsis* lines, crosses and genotyping:** The kanamycin-resistant homozygous T-DNA insertion line SALK_004741.53.50 carrying the *atfh12-1* allele, derived from *A. thaliana* ecotype Columbia, has been obtained from the SALK Institute collection (Alonso *et al*. 2003) *via* the European *Arabidopsis* Stock Center (NASC). Wild type Columbia-0 plants have been used for characterization of gene expression.

The transgenic *Arabidopsis* line expressing a green fluorescent protein-mouse talin fusion under the 35S promoter, GFP:mTalin (Ketelaar *et al*. 2004), was kindly provided by Dr. T. Ketelaar. Presence of the transgene was followed in progeny of crosses by low magnification fluorescent microscopy of roots of young agar-grown seedlings. Allelic status of GFP-positive plants was determined by visual examination of the progeny of selfed plants (absence of negatives among 20 seedlings was considered as an evidence of homozygosity with χ^2 at $P = 0.01$).

Plants were crossed as described previously (Cole *et al*. 2005). Allelic status of *AtFH12* in the progeny was determined by PCR. The gene-specific primer FH12_SALK_1: ATGCAAATGTTACAGATTTTCGGGG was used together with the T-DNA-specific primer LbB1: GCGTGGACCGCTTGCTGCAAC to detect *atfh12-1*, and FH12_LP: TCCTTCAAGGTCTAACCGAGAG together with FH12_RP: TATGCATAGAGACAG

ACCCGG to detect the wild type allele (all primer sequences are in the 5´ to 3´ direction). Leaf DNA for genotyping was isolated using *Plant DNAzol* (*Invitrogen*, Carlsbad, CA, USA) according to manufacturer´s instructions. PCR conditions have been optimized for each set of primers. Selection against the T-DNA marker was performed to confirm T-DNA presence where possible (the kanamycin marker suffered somewhat by silencing; however, its Mendelian segregation, as well as co-segregation with the PCR marker, indicated the absence of distant second-site T-DNA insertions). Plants from the 3rd or 4th generation grown from the original stock were used for experiments, with sister insertion-free segregants used as controls.

Plant cultures and phenotype characterization: Plants for seed production, crossing and *ex vitro* experiments were grown either in soil or in peat pellets (*Jiffy*, Moerdijk, Netherlands). For *in vitro* cultures, seeds were surface-sterilized, stratified at 4 °C for 2 - 4 d, and planted on Murashige and Skoog (MS) growth media (0.5 MS salts and vitamins, 1.6 % agar, 2 % sucrose); NaCl or latrunculin B (LatB) have been added where required (see Results). All cultivations were at ambient temperature and 16-h photoperiod (irradiance of 100 μmol m^{-2} s^{-1}), unless stated otherwise.

For root observation and measurements, seedlings were grown on near-vertical plates, photographed using a digital camera, and individual roots were traced and measured with the aid of the *ImageJ* software (Abramoff *et al*. 2004). In quantitative experiments, approximately equal numbers of seedlings originating from the progeny of at least two sister segregants have been measured for each genotype.

For salt challenge experiments under non-sterile conditions, about 50 stratified seeds per pot were planted on a fully hydrated 30 mm peat pellet (containing 25 cm^3 of water), and from the time of germination, regularly watered with a defined volume of either deionized water (controls) or 100 mM NaCl solution, raising thereby gradually the concentration of salt up to 460 mM across 6 weeks.

For salt stress experiments on agar media, germinated seedlings with fully expanded cotyledons were aseptically transferred from standard MS to plates containing 100 mM NaCl at day 4 after sowing, *i.e*. approx. 3 d after germination, and cultivated for further 7 d; control seedlings were transferred at the same time to standard MS plates.

Semiquantitative RT-PCR: Wild type plants for RNA analysis have been grown in liquid MS media on plastic rafts in *Magenta* jars (*Sigma-Aldrich*, St. Louis, MO, USA) for 3 weeks. Salt treatment was performed by addition of sterile 2.5 M NaCl solution to the media to final concentration of 150 mM for the specified time.

RNA was isolated from leaves and roots of stressed plants with the aid of the *RNeasy Plant Mini* kit (*Qiagen*, Hilden, Denmark). First strand cDNA synthesis and semiquantitative RT-PCR (with *GAPC* primers for control) has been performed as described previously (Dvořáková *et al*. 2007) except that 30 cycles with *DreamTaq*DNA polymerase (*Fermentas*, Graciuino, Lithuania) and *AtFH12*-specific primers (AGCAAGGAA GAAATGGATCG and GCCCACAAAATTAGCAC CAT, both 5´to 3´) have been used.

Microscopy: Confocal microscopic images were obtained using the *TCS SP2* microscope (*Leica Microsystems*, Wetzlar, Germany), using the Ar/Kr laser for fluorescence excitation. Stacks of images were taken at an interval of 0.4 - 0.6 μm, with up to 4 × line averaging where necessary. Maximum intensity projections are shown in all figures.

For quantitative evaluation of the GFP:mTalin transgene expression, a simple ratiometric assay using the chlorophyll autofluorescence as the endogenous standard has been employed. Wide field fluorescence snapshots of expanded cotyledons of 8-d-old seedlings have been taken using a *RGB* video camera (*Sony*, Tokyo, Japan) attached to a *Provis AX70* fluorescence microscope (*Olympus*, Tokyo, Japan) with a 4 × objective and the wide blue (*WIB*) filter set. Intensity profiles were determined with the aid of the *NIS Elements* software (*Laboratory Imaging*, Praha, Czech Republic) in each of the three channels (red, green and blue) for 100 to 300 pixels long line segments selected within a well-focused part of each image; green to red (G/R) ratios have been calculated for each pixel of the segment and averaged across the segment using MS Excel. Statistical significance of observed differences between lines was determined using the Student *t*-test.

Results

AtFH12 is an outlier of the dynamically evolving plant Class II formin family: The *A. thaliana* genome contains 11 Class II formin genes, denoted *AtFH12* to *AtFH21*, encoding up to 13 proteins. In phylogenetic analyses, the Class II FH2 domains cluster consistently together and apart from other well-defined formin clades (Grunt *et al.* 2008). *Arabidopsis* Class II formins share at most 29 % of identical amino acids within the FH2 domain with their Class I counterparts (AtFH16 *vs*. AtFH11), at most 31 % identical amino acids with Daam formins (AtFH13 *vs*. *Daphnia pulex* Leydig DappuDaam), and no more than 30 % identical amino acids with the well-characterized Diaphanous-related formins (AtFH20 *vs*. mouse MmDia3). Similar figures have been obtained also for Class I formins (30 % identity between AtFH4 and *Lottia gigantea* Sowerby LotgiDia). Analysis of position-specific

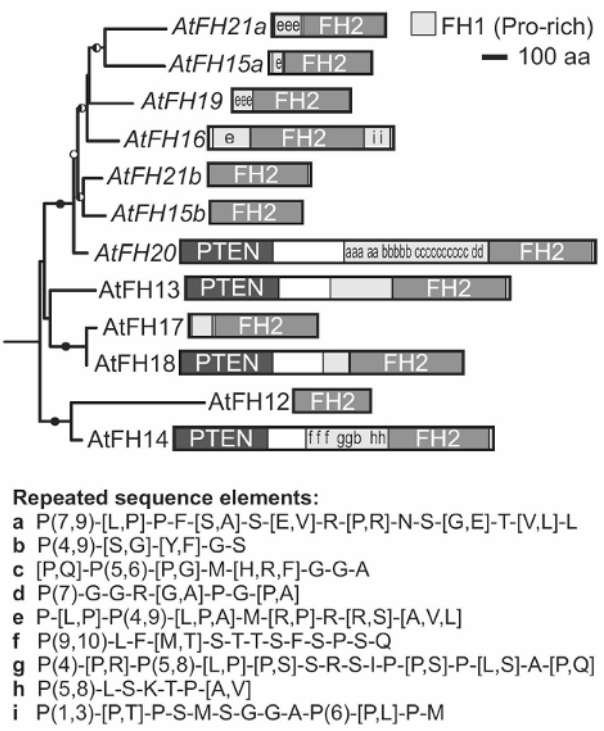

Fig. 1. Domain structure of *A. thaliana* Class II formins mapped on a neighbor-joining tree of their FH2 domain sequences. Letters within the Pro-rich (FH1) region denote repetitive peptides whose *PROSITE* patterns are shown at the bottom (number of letters corresponds to the number of repeats). Loci clustered on chromosome 5 are shown in italics. *Dots* denote bootstrap values (*filled* - more than 90 %; *half-filled* - more than 75 %; *open* - more than 50 %; branches without dots had bootstrap support below 50 %).

nonsynonymous to synonymous nucleotide substitution ratios (Ka/Ks) of seven major formin clades (plant Class I and Class II formins and metazoan Fmn, FRL, Dia, Daam and FHOD) revealed in all cases strong purifying selection across most of the FH2 domain (not shown).

FH2 domains of plant Class II formins are conserved to a degree comparable with other formin clades; however, outside of FH2, these proteins exhibit dramatic diversity in domain composition. Only four *Arabidopsis* proteins (AtFH13, AtFH14, AtFH18 and AtFH20) exhibit the typical domain organization with a N-terminal domain related to the PTEN antioncogene, followed by a proline-rich FH1 domain and C-terminal FH2. Outside of the FH2 domain, several repetitive proline-rich motifs are shared by six out of the seven genes of the previously reported cluster of formin-encoding loci on chromosome V (Cvrčková *et al*. 2004), which also group together in the phylogenetic tree (Fig. 1). The seventh member of this cluster, AtFH20, has an extraordinarily long and repetitive FH1 domain devoid of the particular motifs found in the remaining six genes. This is suggestive of an increased frequency of sequence duplication in the area of chromosome V encoding the formin cluster, both on the scale of whole genes (generating the paralogous loci), gene fragments (resulting in the complex loci *AtFH15* and *AtFH21* that may encode multiple formins by alternative splicing), and short sequence motifs (responsible for the proline-rich repeats).

To gain further insight into the microevolution of Class II formins, we compared the sequences of the *A. thaliana* formins with those from the publicly available draft genome sequence of a closely related species, *A. lyrata*. We could easily distinguish

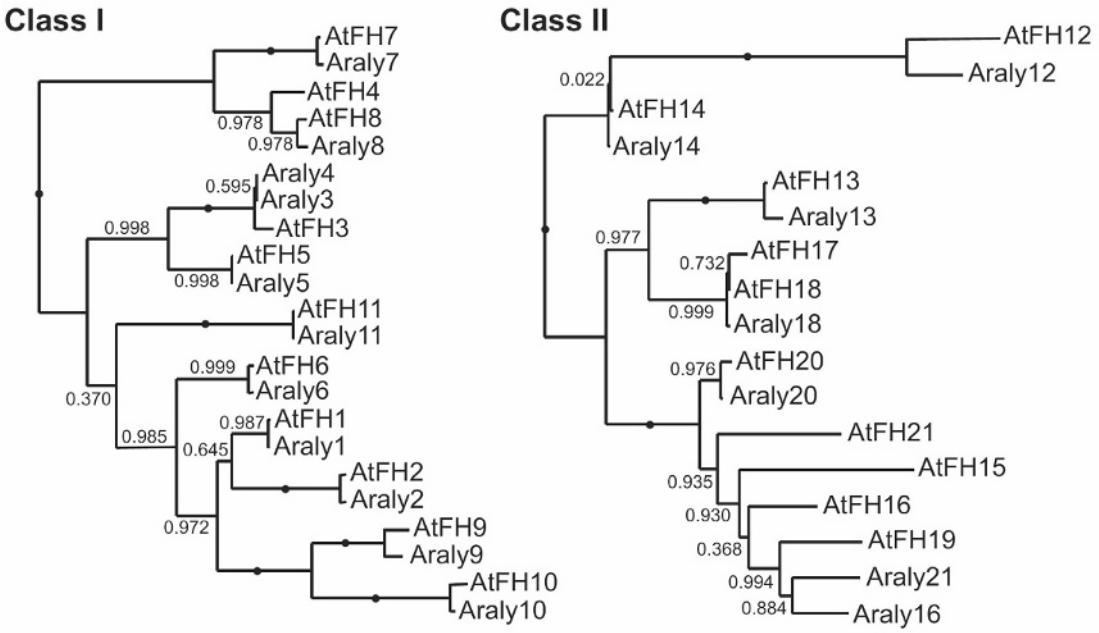

Fig. 2. Maximum likelihood trees of *A. thaliana* and *A. lyrata* FH2 domains. Partial FH2 domains of AtFH15a and AtFH21a have been left out to preserve alignment length. Numbers at branches denote aLRT SH-like support values (with *dots* for values greater than 0.999).

Table 1. List of *Arabidopsis thaliana* formins and their *A. lyrata* homologs ([a] - genes probably encoding multiple products, [b] - original protein predictions manually modified, n.a. - not available).

Class	*A. thaliana* gene	*A. thaliana* locus	*A. thaliana* protein accession (*Uniprot*)	*A. lyrata* gene	*A. lyrata* protein accession (*GenBank*)	Identity *A. t.* - *A. l.* [%]
I	AtFH1	At3g25500	Q9SE97	Araly1	XP_002885725	94
I	AtFH2	At2g43800	O22824	Araly2	XP_002880068	93
I	AtFH3	At4g15190,At4g15200	O23373	Araly3	XP_002868221	91
I	AtFH4	At1g24150	O48682	Araly4	XP_002890610	94
I	AtFH5	At5g54650	Q94B77	Araly5	XP_002866054	95
I	AtFH6	At5g67470	Q9FJX6	Araly6	XP_002866702[b]	95
I	AtFH7	At1g59910	Q9XIE0	Araly7	XP_002886649	90
I	AtFH8	At1g70140	O04532	Araly8	XP_002888757	84
I	AtFH9	At5g48360	Q8GX37	Araly9	XP_002863891	91
I	AtFH10	At3g07540	Q9SRR2	Araly10	XP_002884656	92
I	AtFH11	At3g05470	Q9MA60	Araly11	XP_002884514	94
II	AtFH12	At1g42980	Q9C7S1	Araly12	XP_002891246	75
II	AtFH13	At5g58160	Q9LVN1	Araly13	XP_002864551[b]	85
II	AtFH14	At1g31810	Q9C6S1	Araly14	XP_002893687[b]	91
II	AtFH15[a]	At5g07645,At5g07650	P0C5K2, P0C5K3	n.a.	n.a.	n.a.
II	AtFH16	At5g07770	Q9FF15	Araly16	XP_002871289	59
II	AtFH17	At3g32400	Q9LH02	n.a.	n.a.	n.a.
II	AtFH18	At2g25050	Q9SK28	Araly18	XP_002880647	94
II	AtFH19	At5g07780	Q9FF14	n.a.	n.a.	n.a.
II	AtFH20	At5g07740, At5g07750	Q9FLQ7	Araly20	XP_002873323	88
II	AtFH21 [a]	At5g07760, At5g07765	P0C5K4, P0C5K5	Araly21	XP_002886678	52

orthologues of most Class I formins, with the notable exception of AtFH4, whose closest relative (according to overall sequence identity), Araly4, appears to possess a FH2 domain closely related to members of the AtFH3/AtFH5 subgroup of Class I. However, the situation is somewhat less clear for Class II proteins, consistent with gene duplications taking place even after separation of the *A. thaliana* and *A. lyrata* lineages (Table 1, Fig. 2).

The numerous Class II formins are likely to exhibit functional redundancy. Study of genes with unique features (outliers) might uncover non-redundant functions. *AtFH12* (At1g42980), which has not only diverged far from its closest relative, but even lost part of the conserved FH2 domain including the "lasso" portion of its dimerization interface (see Xu *et al*. 2004), is clearly such an outlier, and we therefore focused our experimental attention on this gene.

Mutants lacking *AtFH12* exhibit negligible phenotypic effects: We obtained a T-DNA disruption allele of *AtFH12*, *atfh12-1* (Fig. 3*A*), from a public collection. In crosses with *AtFH12* plants, *atfh12-1* segregated in Mendelian ratios, suggesting no impairment of viability or fertility. *atfh12-1* homozygotes did not exhibit any readily observable differences from wild type in terms of appearance, size, time course of development (including timing of flowering and senescence), fertility and seed yield under standard culture conditions in soil (data not shown). In agar cultures of young seedlings grown under standard conditions or in darkness (etiolation), no consistent major differences between wild-type and mutant plants were observed, except of minor but significant increase in root length in dark-grown plants. As mutants with a putative cytoskeleton-related defect may exhibit increased sensitivity to sublethal doses of cytoskeletal drugs, we germinated and grew wild type and *atfh12-1* seedlings also on media containing up to 0.1 μM latrunculin B (LatB), a specific inhibitor of actin polymerization. No additional significant differences between wild type and mutant plants were found, though the increase in the length of dark-grown roots persisted (Fig. 3*B*).

Microarray data from the *Genevestigator* database (Zimmermann *et al*. 2004) suggest that *AtFH12* is expressed only at very low levels under standard culture conditions but strongly induced in plants challenged by NaCl but not under other osmotic stress. We indeed confirmed increased levels of *AtFH12* mRNA in wild type roots after several hours of exposure to 150 mM NaCl (Fig. 3*C*), while no expression was detected in leaves under either non-inducing or inducing conditions. However, in soil cultures we found no phenotypic difference between wild-type and *atfh12-1* mutant plants challenged with slowly increasing NaCl concentration. Both genotypes gradually exhibited symptoms of salt stress (growth inhibition, anthocyanin accumulation and later leaf death) at the same time and NaCl concentration (starting from 100 mM NaCl at 12 d) and subsequently died upon reaching 460 mM NaCl at 6 weeks, while salt-free controls vigorously developed and set flowers. Phenotype of *in vitro* grown *atfh12-1* mutants on media with 100 mM NaCl did not significantly differ from wild type plants (Fig. 3*B*). Thus, the *atfh12-1* mutation

exhibits at best subtle effects on *Arabidopsis* development, consistent with functional redundancy within the extensive formin family.

Expression of a heterologous actin-binding protein reveals actin defects in *atfh12* mutants: To study the effects of formin mutations on actin organization, we crossed the *atfh12-1* mutant to a transgenic line expressing the GFP:mTalin fusion protein that can act as an *in vivo* actin marker. Surprisingly, homozygous formin mutants were significantly underrepresented in the F2 and F3 progeny expressing the fluorescent transgene, regardless of the direction of the cross, suggestive of either synthetic lethality with incomplete penetrance (due to either bilateral gametophytic insufficiency or zygotic lethality), or elimination (silencing) of GFP:mTalin expression in *atfh12-1* mutants. No aborted seeds in siliques have been observed in the progeny of the cross,

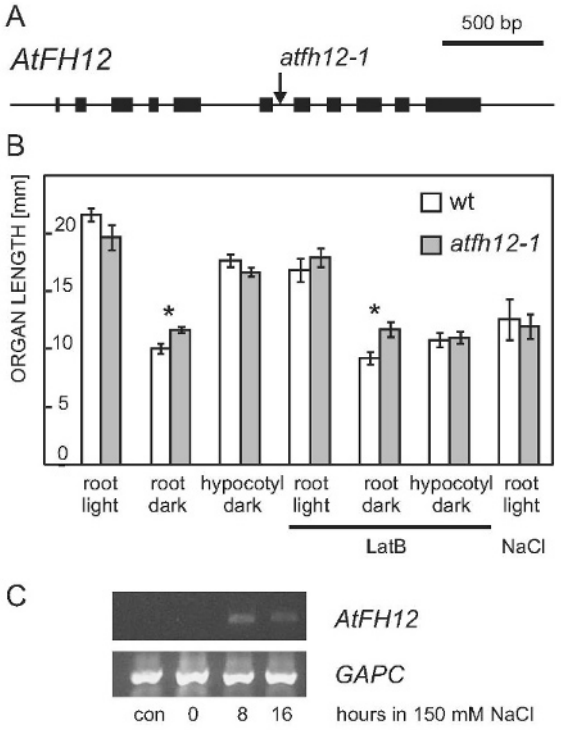

Fig. 3. Structure of the *AtFH12* locus (*A*), including position of the T-DNA insertion in *atfh12-1* (*boxes* denote coding exons), and effect of the *atfh12-1* mutation on primary root and etiolated hypocotyl growth under standard conditions and in the presence of 100 mM NaCl or 0.1 μM LatB (*B*). Average organ lengths of 7-d-old seedlings (± SE) are shown; *asterisks* denote a significant difference between mutant and wild type plants (*t*-test, $P < 0.01$). Results shown are from a representative experiment with $n \geq 14$; consistent results have been obtained repeatedly for 40 to 100 plants per genotype and treatment. *C* - *AtFH12* mRNA level is increased during salt treatment. Products of RT-PCR with *AtFH12*-specific and control (*GAPC*) primers on total root RNA from wild type plants subjected to varying duration of salt stress are shown (con – untreated control).

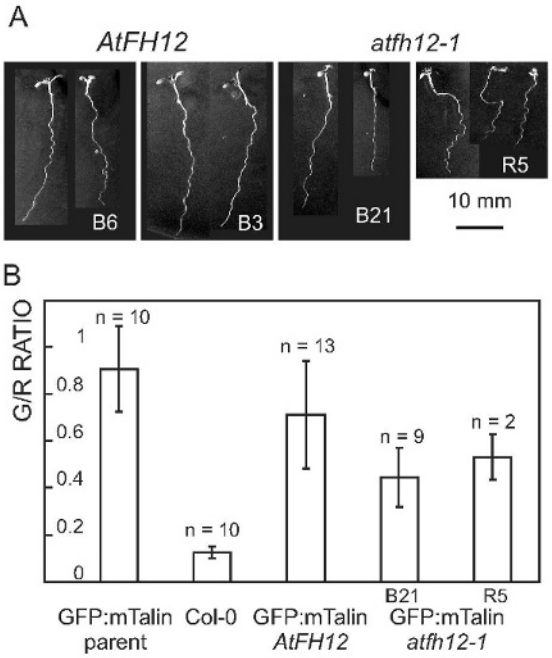

Fig. 4. Representative 8-d-old *AtFH12* wild-type and homozygous *atfh12-1* seedlings in a homozygous *GFP:mTalin* transgenic background (*A*). *Left* - two F2 segregants (B3 and B6) carrying wild-type *AtFH12*, *right* - the only two surviving F_2 segregants homozygous for both *GFP:mTalin* and *atfh12-1* (R5 and B21). *B* - The effect of *AtFH12* genotype on expression of GFP:mTalin in cotyledons of 4-d-old seedlings, expressed as ratio of green (G, GFP) to red (R, chlorophyll) fluorescence intensity (Means ± SD; see Methods). Non-transgenic wild type Col-0 plants are included as a control. The GFP:mTalin *AtFH12* plants are pooled B3 and B6 lines (see *A*). Differences between GFP:mTalin *AtFH12* and *atfh12-1* are highly significant (*t*-test $P < 0.005$ for both wild-type *versus* B21 and wild-type *versus* pooled B21 and R5), while the difference between parental transgenic line and the *AtFH12* wild-type segregant pool is marginally significant ($P < 0.05$). All lines carrying *GFP:mTalin* differ from non-transgenic wild type ($P < 10^{-4}$).

and no decrease in seed germination efficiency was observed, consistent with pre-zygotic lethality. Genetic linkage between wild type *AtFH12* and the site of transgene insertion is unlikely, since transgene-free wild-type segregants were found relatively frequently (8 out of 76 F2 plants, while less than five were expected for Mendelian segregation of the two markers). Moreover, most of the GFP-positive *atfh12-1* homozygotes exhibited conspicuously low fluorescence, suggesting that their survival may depend on silencing of the *GFP:mTalin* transgene, known to be somewhat toxic even in wild type plants, at least in part due to perturbation of the actin network (Ketelaar *et al*. 2004).
Only twelve plants homozygous for *atfh12-1* have been found among 90 GFP:mTalin-positive F2 and F3 segregants (expected 22; $\chi^2 = 0.011$), and only two out of them were homozygous for both the GFP:mTalin marker and the formin mutation. One of these (line B21) exhibited a practically normal phenotype and very low

level of GFP:mTalin fluorescence both in the original F2 plant and in its progeny. The other one (line R5) was a small, stunted plant with a high anthocyanin content that produced only few seeds which gave rise to seedlings exhibiting slow growth, delayed cotyledon expansion, and a possible gravitropic defect (Fig. 4*A*). Nevertheless, they subsequently recovered, albeit younger tissues (*e.g.* true leaves) showed substantially lower GFP fluorescence, *i.e.* loss of expression of the toxic transgene. Quantitative measurements of GFP:mTalin fluorescence indeed confirmed that in particular the healthier double mutant (B21) exhibits lower GFP fluorescence in cotyledons than its *AtFH12* wild type siblings of equal age (Fig.4*B*).

Upon microscopic examination, tissues of GFP:mTalin-positive seedlings homozygous for *atfh12-1* often exhibited reduced or non-detectable transgene expression, or a mosaic of expressing and non-expressing cells, especially in younger tissues (*e.g.* true leaves or younger parts of the root). In such tissues with low GFP:mTalin levels, the actin cytoskeleton appeared normal. Occasionally, cells exhibiting a diffuse florescence across the cytoplasm, with enrichment in the nucleus, were observed, suggesting degradation of the fusion protein with release of free GFP (not shown); rare frequency of such events did not allow further biochemical analysis. However, in tissues formed early during or after germination (such as the hypocotyl or the base of the root), cells showing dramatic abnormalities of the actin cytoskeleton, excessive formation of actin rings or fragmented thick actin bundles have been regularly observed in *atfh12-1* homozygotes, while no such structures were found in controls carrying wild-type *AtFH12* (Fig. 5; note that actin rings are occasionally observed also in GFP:mTalin-expressing controls, but never to such extent as in the mutants).

Discussion

Formins are a highly diversified large family of cytoskeletal organizers, represented in seed plants by two clades, Class I and Class II (Deeks *et al*. 2002, Cvrčková *et al*. 2004, Grunt *et al*. 2008). In *Arabidopsis*, both formin classes have expanded to families of more than ten genes, making mutational analyses difficult due to apparent functional redundancy. Experimental characterization of angiosperm formins (see Introduction) so far focused mainly on members of the *Arabidopsis* Class I family, which is expressed more abundantly than Class II according to publicly available microarray data (Zimmermann *et al*. 2004, Winter *et al*. 2007, Hruz *et al*. 2008).

So far, little is known about Class II formins. Phylogenetic evidence suggests that they may represent an ancestral group of FH2 proteins in the plant kingdom; their relation to Class I formins remains unresolved. Their hallmark architecture, characterized by the presence of a domain related to the metazoan *PTEN* antioncogene (Cvrčková *et al*. 2004), is shared also by some algae (Grunt *et al*. 2008).

Comparison of Class II formin genes in two closely related *Arabidopsis* species, *A. thaliana* and *A. lyrata*, estimated to share a common ancestor approximately 5 million years ago (Koch *et al*. 2000), indicates that even on the scale separating these two species, Class II formins evolved much more rapidly than their Class I counterparts. However, we found that their FH2 domains were subjected to strong purifying selection, in good agreement with recently published similar results for Class I formins (Martinière *et al*. 2011). Along with the diversification of their FH2 domains, *A. thaliana* Class II formins underwent extensive rearrangements, in particular at least two separate incidents of PTEN domain loss, as well as repetitive sequence diversification and amplification in the proline-rich regions including the FH1 domain. Propensity to sequence amplification, both on the scale of gene segments and whole genes, which tend to form clusters of loci (as in the case of the chromosome V cluster of *A. thaliana* formins), might be a common feature of plant proteins containing repetitive segments. Similar behavior has been documented, *e.g.*, for the proline-rich extensin-like kinases (PERKs; Nakhamchik *et al*. 2004), leucine-rich receptor-like proteins (RLPs; Mondragon-Palomino and Gaunt 2005) or hybrid proline-rich proteins (HyPRPs) of the cell wall (Dvořáková *et al*. 2007).

We focused our experimental study on AtFH12, an outlier of the extensive *A. thaliana* Class II family lacking the hallmark PTEN domain. We have reasoned that its unique domain structure might facilitate observation of mutant phenotypes due to loss of functions that cannot be supplied by other members of the gene family. However, under a variety of conditions, we could find only relatively weak, if any, phenotypic changes in *atfh12* mutants, even after perturbation of actin by Latrunculin B (LatB), known to enhance subtle phenotypic defects of cytoskeleton-related mutations (*e.g.* Collings *et al*. 2006), including, among others, also loss of AtFH8 (Xue *et al*. 2011). Such an effect, were it observed, would have been an example of a "synthetic phenotype", or "synthetic sickness", occurring when simultaneous perturbation of a cellular function by combination of a mutation with a specific drug or condition (or second mutation) causes defects that neither treatment alone would elicit (an extension of the general concept of "synthetic lethality"– *e.g.* Hartman *et al*. 2001, Ooi *et al*. 2006, Nijman 2011). The only measurable effect of AtFH12 loss was a slight increase in primary root length, observed only in dark-grown but not light-grown seedlings. *Arabidopsis* root growth is stimulated by light in a phytochrome-dependent manner (Kurata and Yamamoto 1997). Thus, this phenotype might be due

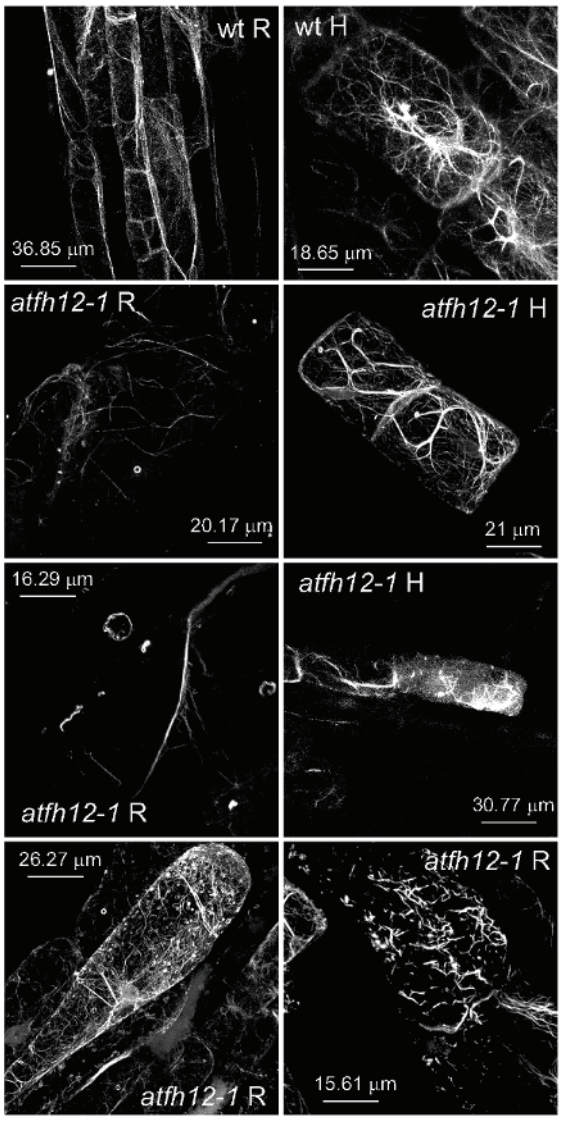

Fig. 5. Abnormal actin structures in rhizodermis (R) and hypocotyl epidermis (H) of *atfh12* mutants expressing GFP:mTalin. Stacks of confocal images from 4 to 7-d-old F2 segregants homozygous for *atfh12-1* and expressing GFP:mTalin (either homo- or heterozygous) are shown, with typical controls (sister segregants with wild-type formin genotype) included for comparison.

either to perturbation of signalling, or to alteration of the effector mechanisms responsive for cell growth and division; the latter is somewhat more likely given the known participation of formins in cell growth (see Introduction). Lack of observable phenotypes in light-grown seedlings may be due to increased expression of other Class II formins, such as, *e.g.*, AtFH14 (upregulated in light-grown roots according to *Genevestigator* and *eFP* browser data; Hruz *et al*. 2008, Winter *et al*. 2007), that may override the effects of the *atfh12* mutation.

We have confirmed that the level of *AtFH12* mRNA is noticeably increased in the presence of NaCl, as previously observed in a systematic gene expression study (Ma *et al*. 2006); at present, it is not clear whether this is a consequence of transcriptional induction or changes in mRNA stability. Based on this observation, we also subjected the *atfh12* mutants to salt stress, but observed no additional phenotypic differences between the mutant and wild type plants, similar to situation in LatB-treated plants.

To study possible effects of formin mutations upon the organization of the actin cytoskeleton, we crossed the *atfh12* mutant with an *Arabidopsis* line carrying a fluorescent *in vivo* actin marker, the GFP:mTalin fusion protein. This protein is known to inhibit actin depolymerization *in vitro*, affect somewhat actin organization *in vivo*, and even cause cell death in root hairs under some circumstances (Ketelaar *et al*. 2004). While this may be perceived as a drawback of the GFP:mTalin marker, it also allowed us to observe the consequences of the *atfh12* disruption under conditions that could be understood as "stress testing" the actin cytoskeleton. Indeed, we found unusual actin structures, in particular extensive bundling or formation of actin rings and clusters, often accompanied by reduction or absence of normal filamentous structures, in mutant plants. At the moment, we can only speculate about the involved molecular mechanisms, since plant formins can directly participate in actin-related processes ranging from *de novo* nucleation through capping to bundling (*e.g*. Michelot *et al*. 2005, Xue *et al*. 2011). Moreover, actin aggregation and ring formation may be in plant cells elicited by a variety of conditions including, *e.g*., mechanical isolation of mesophyll cells, as described for *Zinnia elegans* (Frost and Roberts 1996).

We had major difficulties isolating homozygous *atfh12* mutants expressing high levels of GFP:mTalin, suggesting synthetic lethality with incomplete penetrance. Detailed observation of the few *atfh12* homozygotes, which expressed GFP:mTalin, showed rapid silencing of the fluorescent transgene during seedling development, confirmed also by ratiometric fluorescence measurements. The only mutant line with high GFP:mTalin expression exhibited severe impairment of seedling growth, in spite of expressing *less* of the fluorescent marker that its normally growing wild-type siblings.

Thus, we can conclude that loss of AtFH12 perturbs some cellular functions, and this effect is aggravated by imposing additional stress on the actin cytoskeleton by means of expressing a heterologous protein that interferes with normal actin dynamics. This not only documents participation of AtFH12 in actin organization, but also presents a good example of the applicability of the "synthetic phenotype" concept in functional analysis of large and degenerate ("redundant") gene families in plants.

References

Abramoff, M.D., Magelhaes, P.J., Ram, S.J.: Image processing with ImageJ. - Biophotonics Int. **11**: 36-42, 2004.

Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby, W.L., Berry, C.C., Ecker, J.R.: Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. - Science **301**: 653-657, 2003.

Anisimova, M., Gascuel, O.: Approximate likelihood ratio test for branches: a fast, accurate and powerful alternative. - Syst. Biol. **55**: 539-552, 2006.

Banno, H., Chua, N.H.: Characterization of the *Arabidopsis* formin-like protein AFH1 and its interacting protein. - Plant Cell Physiol. **41**: 617-626, 2000.

Bartolini, F., Gundersen, G.G.: Formins and microtubules. - Biochim. biophys. Acta **1803**: 164-173, 2010.

Blanchoin, L., Staiger, C.J.: Plant formins: diverse isoforms and unique molecular mechanism. - Biochim. biophys. Acta **1803**: 201-206, 2010.

Chalkia, D., Nikolaidis, N., Makalowski, W., Klein, J., Nei, M.: Origins and evolution of the formin multigene family that is involved in the formation of actin filaments. - Mol. Biol. Evol. **25**: 2717-2733, 2008.

Cheung, A.Y., Wu, H.: Overexpression of an *Arabidopsis* formin stimulates supernumerary actin cable formation from pollen tube cell membrane. - Plant Cell **16**: 257-269, 2004.

Cole, R.A., Synek, L., Žárský, V., Fowler, J.E.: SEC8, a subunit of the putative *Arabidopsis* exocyst complex, facilitates pollen germination and competitive pollen tube growth. - Plant Physiol. **138**: 2005-2018, 2005.

Collings, D.A., Lill, A.W., Himmelspach, R., Wasteneys, G.O.: Hypersensitivity to cytoskeletal antagonists demonstrates microtubule-microfilament cross-talk in the control of root elongation in *Arabidopsis thaliana*. - New Phytol. **170**: 275-290, 2006.

Cvrčková, F., Novotný, M., Pícková, D., Žárský, V.: Formin homology 2 domains occur in multiple contexts in angiosperms. - BMC Genomics **5**: 44, 2004.

Deeks, M.J., Cvrčková, F., Machesky, L.M., Mikitová, V., Ketelaar, T., Žárský, V., Davies, B., Hussey, P.J.: *Arabidopsis* group Ie formins localize to specific cell membrane domains, interact with actin-binding proteins and cause defects in cell expansion upon aberrant expression. - New Phytol. **168**: 529-540, 2005.

Deeks, M.J., Fendrych, M., Smertenko, A., Bell, K.S., Oparka, K., Cvrčková, F., Žárský, V., Hussey, P.J.: The plant formin AtFH4 interacts with both actin and microtubules, and contains a newly identified microtubule-binding domain. - J. cell. Sci. **123**: 1209-1215, 2010.

Deeks, M.J., Hussey, P., Davies, B.: Formins: intermediates in signal transduction cascades that affect cytoskeletal reorganization. - Trends Plant Sci. **7**: 492-498, 2002.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.M., Gascuel, O: Phylogeny.fr: robust phylogenetic analysis for the non-specialist. - Nucl. Acids Res. **36**: W465-W469, 2008.

Dvořáková, L., Cvrčková, F., Fischer, L.: Analysis of the hybrid proline-rich protein families from seven plant species suggests rapid diversification of their sequences and expression patterns. - BMC Genomics **8**: 412, 2007.

Favery, B., Chelysheva, L. A., Lebris, M., Jammes, F., Marmagne, A., De Almeida-Engler, J., Lecomte, P., Vaury, C., Arkowitz, R. A., Abad, P.: *Arabidopsis* formin AtFH6 is a plasma membrane-associated protein upregulated in giant cells induced by parasitic nematodes. - Plant Cell **16**: 2529-2540, 2004.

Frost, A.O., Roberts, A.W.: Cortical actin filaments fragment and aggregate to form chloroplast-associated and free F-actin rings in mechanically isolated *Zinnia* mesophyll cells. - Protoplasma **164**: 195-207, 1996.

Grunt, M., Žárský, V., Cvrčková, F.: Roots of angiosperm formins: the evolutionary history of plant FH2 domain-containing proteins. - BMC Evol. Biol. **8**: 1-19, 2008.

Guindon, S., Gascuel, O.: A simple, fast an accurate algorithm to estimate large phylogenies by maximum likelihood. - Syst. Biol. **52**: 696-704, 2003.

Hartman, J.L., Garvik, B., Hartwell, L.H.: Principles for the buffering of genetic variation. - Science **291**: 1001-1004, 2001.

Higgs, H.N., Peterson, K.J.: Phylogenetic analysis of the formin homology 2 domain. - Mol. Biol. Cell **16**: 1-13, 2005.

Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W., Zimmermann, P.: Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. - Adv. Bioinformat. **2008**: ID 420747, 2008.

Ingouff, M., Fitzgerald, J.N., Guerin, C., Robert, H., Sorensen, M.B., Van Damme, D., Geelen, D., Blanchoin, L., Berger, F.: Plant formin AtFH5 is an evolutionarily conserved actin nucleator involved in cytokinesis. - Nat. cell. Biol. **7**: 374-380, 2005.

Ketelaar, T., Anthony, R.G., Hussey, P.J.: Green fluorescent protein-mTalin causes defects in actin organization and cell expansion in *Arabidopsis* and inhibits actin depolymerizing factor's actin depolymerizing activity *in vitro*. - Plant Physiol. **136**: 3990-3998, 2004.

Koch, M.A., Haubold, B., Mitchell-Olds, T.: Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in *Arabidopsis*, *Arabis*, and related genera (*Brassicaceae*). - Mol. Biol. Evol. **17**: 1483-1498, 2000.

Kurata, T., Yamamoto, K.T.: Light-stimulated root elongation in *Arabidopsis thaliana*. - J. Plant Physiol. **151**: 346-351, 1997.

Li, Y., Shen, Y., Cai, C., Zhong, C., Zhu, L., Yuan, M., Ren, H.: The type II *Arabidopsis* formin14 interacts with microtubules and microfilaments to regulate cell division. - Plant Cell **22**: 2710-2726, 2010.

Ma, S., Gong, Q., Bohnert, H.J.: Dissecting salt stress pathways. - J. exp. Bot. **57**: 1097-1107, 2006.

Martinière, A., Gayral, P., Hawes, C., Runions, J.: Building bridges: formin1 of *Arabidopsis* forms a connection between the cell wall and the actin cytoskeleton. - Plant J. **66**: 354-365, 2011.

McGinnis, S., Madden, T.L.: BLAST: at the core of a powerful and diverse set of sequence analysis tools. – Nucl. Acids Res. **32**: W20-W25, 2004.

Michelot, A., Guerin, C., Huang, S., Ingouff, M., Richard, S., Rodiuc, N., Staiger, C.J., Blanchoin, L.: The Formin Homology 1 domain modulates the actin nucleation and

bundling activity of *Arabidopsis* FORMIN1. - Plant Cell **17**: 2296-2313, 2005.

Mondragon-Palomino, M., Gaunt, B.S.: Gene conversion and the evolution of three leucine-rich repeat gene families in *Arabidopsis thaliana*. - Mol. Biol. Evol. **22**: 2444-2456, 2005.

Nakhamchik, A., Zhao, Z., Provart, N.J., Shiu, S.H., Keatley, S.K., Cameron, R.K., Goring, D.R.: A comprehensive expression analysis of the *Arabidopsis* proline-rich extensin-like receptor kinase gene family using bioinformatic and experimental approaches. - Plant Cell Physiol. **45**: 1875-1881, 2004.

Nijman, S.M.: Synthetic lethality: general principles, utility and detection using genetic screens in human cells. - FEBS Lett. **585**:1-6, 2011.

Ooi, S.L., Pan, X., Peyser, B.D., Ye, P., Meluh, P.B., Yuan, D.S., Irizarry, R.A., Bader, J.S., Spencer, F.A., Boeke, J.D.: Global synthetic-lethality analysis and yeast functional profiling. - Trends Genet. **22**: 56-63, 2006.

Paul, A.S., Pollard, T.D.: Review of the mechanism of processive actin filament elongation by formins. - Cell Motility Cytoskeleton **66**: 606-617, 2009.

Stern, A., Doron-Faigenboim, A., Erez, E., Martz, E., Bacharach, E., Pupko, T.: Selecton 2007: advanced models for detecting positive and purifying selection using a Bayesian inference approach. - Nucl. Acids Res. **35**: W506-W511, 2007.

Vidali, L., Van Gisbergen, P.A., Guerin, C., Franco, P., Li, M., Burkart, G.M., Augustine, R.C., Blanchoin, L., Bezanilla, M.: Rapid formin-mediated actin-filament elongation is essential for polarized plant cell growth. - Proc. nat. Acad. Sci. USA **106**: 13341-13346, 2009.

Wernersson, R., Pedersen, A.G.: RevTrans - constructing alignments of coding DNA from aligned amino acid sequences. - Nucl. Acids Res. **31**: 3537-3539, 2003.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., Provart, N.J.: An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. - PLOS One **2**: e718, 2007.

Xu, Y., Moseley, J.B., Sagot, I., Poy, F., Pellman, D., Goode, B.L., Eck, M.J.: Crystal structures of a formin homology-2 domain reveal a tethered dimer architecture. - Cell **116**: 711-723, 2004.

Xue, X.H., Guo, C.Q., Du, F., Lu, Q.L., Zhang, C.M., Ren, H.Y.: AtFH8 is involved in root development under effect of low-dose Latrunculin B in dividing cells. - Mol. Plant **4**: 264-278, 2011.

Yamada, K., Lim, J., Dale, J.M., Chen, H., Shinn, P., Palm, C.J., Southwick, A.M., Wu, H.C., Kim, C., Nguyen, M., Pham, P., Cheuk, R., Karlin-Newmann, G., Liu, S.X., Lam, B., Sakano, H., Wu, T., Yu, G., Miranda, M., Quach, H.L., Tripp, M., Chang, C.H., Lee, J.M., Toriumi, M., Chan, M.M., Tang, C.C., Onodera, C.S., Deng, J.M., Akiyama, K., Ansari, Y., Arakawa, T., Banh, J., Banno, F., Bowser, L., Brooks, S., Carninci, P., Chao, Q., Choy, N., Enju, A., Goldsmith, A.D., Gurjal, M., Hansen, N.F., Hayashizaki, Y., Johnson-Hopson, C., Hsuan, V.W., Iida, K., Karnes, M., Khan, S., Koesema, E., Ishida, J., Jiang, P.X., Jones, T., Kawai, J., Kamiya, A., Meyers, C., Nakajima, M., Narusaka, M., Seki, M., Sakurai, T., Satou, M., Tamse, R., Vaysberg, M., Wallender, E.K., Wong, C., Yamamura, Y., Yuan, S., Shinozaki, K., Davis, R.W., Theologis, A., Ecker, J.R.: Empirical analysis of transcriptional activity in the *Arabidopsis* genome. - Science **302**: 842-846, 2003.

Yang, W., Ren, S., Zhang, X., Gao, M., Ye, S., Qi, Y., Zheng, Y., Wang, J., Zeng, L., Li, Q., Huang, S., He, Z.: BENT UPPERMOST INTERNODE1 encodes the class II formin FH5 crucial for actin organization and rice development. - Plant Cell **23**: 661-680, 2011.

Ye, J., Zheng, Y., Yan, A., Chen, N., Wang, Z., Huang, S., Yang, Z.: *Arabidopsis* formin3 directs the formation of actin cables and polarized growth in pollen tubes. - Plant Cell **21**: 3868-3884, 2009.

Yi, K., Guo, C., Chen, D., Zhao, B., Yang, B., Ren, H.: Cloning and functional characterization of a formin-like protein (AtFH8) from *Arabidopsis*. - Plant Physiol. **138**: 1071-1082, 2005.

Zhang, Z., Zhang, Y., Tan, H., Wang, Y., Li, G., Liang, W., Yuan, Z., Hu, J., Ren, H., Zhang, D.: RICE MORPHOLOGY DETERMINANT encodes the type II formin FH5 and regulates rice morphogenesis. - Plant Cell **23**: 681-700, 2011.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., Gruissem, W.: GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. - Plant Physiol. **136**: 2621-2632, 2004.