Mammalian cells in the service of plant science; a
different way to isolate new cytoskeletal proteins

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Abstract

Based on the high conservation of cytoskeleton building blocks between the plant and animal kingdoms, we have developed a functional genomic screen for the isolation of new plant cytoskeleton-binding proteins in mammalian cells. A YFP-fusion cDNA library from Arabidopsis was inserted into rat fibroblasts and screened for fluorescent chimeras localizing to cytoskeletal structures. The high-throughput screen was performed by an automatic microscopic device. An initial set of candidate genes identified in the screen were isolated, sequenced, their full length cDNAs were synthesized by RT-PCR and tested by biochemical approaches to verify the ability to bind actin directly. Alternatively, indirect binding via the interaction with other actin-binding proteins was studied. The full-length cDNAs were transferred back to plants as a YFP chimera behind the 35S promoter. We give here two examples of new plant cytoskeletal proteins identified in the pilot screen. ERD10, a member of the dehydrin family of proteins, was localized to actin stress fibers in rat fibroblasts. Its direct binding to actin filaments was confirmed by several biochemical approaches. Touch-induced calmodulin-like protein, TCH2, was also localized to actin stress fibers in fibroblasts, but was unable to bind actin filaments directly in vitro. Nevertheless, it did bind to the IQ domain of Arabidopsis myosin VIII in a calcium-dependent manner. Interestingly, when the cDNAs of these two proteins were expressed in plants they were diffusely localized in the cytoplasm suggesting that a similar screen of a YFP-fused cDNA library in plant cells would not reliably identify these proteins as cytoskeleton-associated partners.
Introduction

The cytoskeleton evolved before plants diverged from animals, and the main features of the cytoskeleton have been conserved in both, although specialized cytoskeletal structures and cytoskeleton-mediated mechanisms evolved separately. Indeed, there is recent evidence for a prokaryotic origin of microtubule- and actin-based cell skeletons (reviewed in (Moller-Jensen and Lowe, 2005). For several cytoskeletal proteins the similarity in the amino acid sequence has been well conserved between the two kingdoms. For example, the amino acid similarity between actin or tubulin from Arabidopsis and human is 80-90% (Meagher and Williamson, 1994). In addition, some of the actin-associated proteins are conserved between plants and animals, these include profilin (30% identity in amino acid sequence and the three dimensional structure is well conserved; (Thorn et al., 1997), coflin or actin depolymerizing factor (ADF) (30% identity; (Carlier et al., 1997; Dong et al., 2001), unconventional myosins (80% identity in the S1 domain; (Kinkema and Schiefelbein, 1994)) fimbrin (40% overall similarity and up to 74% similarity in the actin-binding domain; (McCurdy and Kim, 1998), villin (80% similarity in the G1 domain; (Klahre et al., 2000; Vidali et al., 1999) and ARP2 and ARP3 from Arabidopsis have 62% and 60% sequence identity to human Arp2 and Arp3, respectively (McKinney et al., 2002). Other components of the ARP2/3 complex as well as ARP2/3 activators from the WAVE complex were recently characterized in plants and show high homology to their mammalian counterparts (Li et al., 2003; Mathur, 2005). Moreover, some plant cytoskeletal binding proteins recognize animal actin filaments or microtubules; for
example, Arabidopsis villin-GFP fusions decorate actin filament stress fibers and membrane protrusions in mammalian Vero cells (Klahre et al., 2000), MAP65 from carrot co-polymerizes with porcine brain microtubules (Chan et al., 1999), myosin from Chara translocates muscle F-actin in an *in vitro* motility assay at a similar velocity (60µm/sec) as cytoplasmic streaming in this alga (Higashi-Fujime et al., 1995) and birch pollen profilin binds to human VASP directly and stabilizes microfilaments in animal cells (Reinhard et al., 1995; Rothkegel et al., 1996). Despite the similar sequence and structure of the cytoskeleton building blocks, there are still missing protein mediators for known plant cytoskeletal functions; for example, cortical microtubules are connected to the plasma membrane by unidentified protein links as shown by electron microscopy (Gunning and Hardham, 1982; Vesk et al., 1996). Nevertheless, there is no evidence that any of the identified proteins in plants play a role in cytoskeleton-plasmamembrane-cell-wall interactions. In addition, some of the actin-binding proteins, like profilin, cofilin and capping protein are stimulus responsive, but many of the proteins delivering the stimulus signaling are still missing (Deeks et al., 2002). Extensive searches of the completed Arabidopsis genome database (AGI, 2000) reveal the presence and elaboration of several classes of actin- and microtubule-binding proteins. However, many of the more than 70 classes of eukaryotic actin-binding proteins (Kreis and Vale, 1999) are apparently absent or poorly conserved in Arabidopsis (Assaad, 2001; Drøbak et al., 2004; Hussey et al., 2002; Staiger and Hussey, 2004), including spectrin, α-actinin, filamin, thymosin β4, and others. Plant analogues of these proteins may exist but they may share tertiary structure rather than primary structure resemblance (Deeks and Hussey, 2003). This strongly
suggests that further screens should be based on function rather than on bio-informatics or on cross-reaction of antibodies.

Based on the high conservation of the cytoskeletal building blocks between the animal and plant kingdoms we developed a method of screening an Arabidopsis cDNA library fused to YFP in rat fibroblasts. Using this method we were able to find new cytoskeletal associated proteins, which were not discovered so far by other screens. We give here two examples of plant genes, ERD10 and TCH2 that were localized to actin stress fibers in Ref52 cells and give biochemical evidence that they bind actin either directly or indirectly in vitro.

Results

The library

A library of partial Arabidopsis cDNAs fused to Venus YFP was constructed as described in materials and methods (Figure 1). In order to verify the quality of the library we sequenced 30 random clones. It was found that among the 30 clones, none were an empty vector and all clones were oriented 5’ to 3’. Furthermore, no clones corresponding to ribosomal RNA were found. Transforming the library into XL1 Blue E. coli competent cells was highly efficient resulting in more than 100,000 colonies, ~60,000 of which were collected. In a control experiment, DNA made from a pool of 2500 clones and a mixture of the empty vectors was transfected into 293T cells. It was found that while transfection of the library resulted in many fluorescent cells, the empty vectors gave only background fluorescence (Figure 2), proving that each fluorescent clone observed in the screen contained an Arabidopsis cDNA insert in frame with YFP.
The screen

The cells were plated in 384-well plates, and infected. After 48 h when expression had peaked, the culture was already confluent and covered the whole well. At this stage the cells were fixed. For the automatic high resolution high throughput screen we used a X60/0.9 NA objective yielding images with field dimensions of 111 X 111 μm. The size of a Ref52 cell is approximately 60 X 60 μm. Thus it was possible to allow 20 μm gaps between the acquired fields and still sample all cells in each well. In addition, a rim of 4 fields thick around the well periphery was not acquired because of poor optical conditions near the well edges. Altogether, the screen included 256 images from each well. The time for each image acquisition (auto focusing, exposure of 1 sec, and the movement of the stage to the next field) was 4 sec. Thus a full plate screen required 110 h or 4.5 d. The computer memory required for storing this information is approximately 80 Gigabyte. The infection efficiency was calibrated to 30-60% in order to make sure that no more than 1-3 retrovirus integrations will occur which might result in masking of specific localized clone by a diffused cytoplasmic localized one. It was found in a pilot screen that the number of fluorescent cells matched well the expected efficiency of infection (10-20% fluorescent cells indicate a 30-60% efficiency because only one third of the clones are in frame and fluorescent). Most of the fluorescent cells exhibited diffuse cytoplasmic fluorescence. About 5% displayed staining patterns with subcellular localization such as to vesicles, nucleus, nucleoli, or mitochondria and 1% localized to cytoskeletal structures (Figure 3).
The isolation and characterization of ERD10 and TCH2 as new plant cytoskeleton-associated proteins

The clones showing cytoskeletal localization were isolated. Among them were fragments belonging to ERD10, a member of the dehydrin family (Kiyosue et al., 1994), and touch-induced calmodulin-like protein (Braam and Davis, 1990). The full length cDNAs were isolated, fused to YFP and retransfected into Ref52 cells. Fixation was done in the presence of 0.5% Triton X-100 in order to wash cytoplasmic soluble proteins and double immuno-staining for actin and vinculin was performed. It is shown in Figure 4 that the fibers decorated by the YFP chimeras of the control AtFIMBRIN1 actin-binding domain 2 (FABD2), ERD10, or TCH2 were stained by phalloidin (an F-actin binding reagent) and that their ends were marked by vinculin labeling (a marker for focal contacts). This proves that the structures labeled by ERD10-YFP and TCH2-YFP are genuine actin stress fibers (Figure 4).

To examine whether the decoration of stress fibers in Ref52 cells is due to the ability of these putative actin-interacting proteins to bind actin filaments directly, we expressed recombinant proteins as fusions with GST in bacterial cells. Fusion proteins were isolated by affinity chromatography, the GST-tag removed by thrombin cleavage, and the resulting purified, recombinant protein tested for binding to actin filaments prepared from muscle α-actin isoform. Initially, high-speed cosedimentation assays were performed to detect interactions between side-binding proteins and pre-formed actin filaments (Huang et al., 2004; Kovar et al., 2000; Michelot et al., 2005). ERD10, but not TCH2, demonstrated binding to actin filaments in a dose-dependent manner (data not shown). Compared with Arabidopsis FIMBRIN1 (AtFIM1; \( K_d \) for actin filaments ~ 0.5 µM; (Kovar et al., 2000),
the binding of ERD10 appeared to be somewhat weaker. Unfortunately, a substantial amount of ERD10 also sedimented in the absence of actin filaments, making accurate determination of a $K_d$ impossible.

To confirm the binding of ERD10 to actin filaments, several kinetic or steady-state assays for actin polymerization were performed using pyrene-labeled actin. At steady state, the monomer binding protein profilin increased the critical concentration ($C_c$) for actin assembly by sequestering actin subunits and preventing their polymerization (Figure 5A). ERD10 had the opposite effect on actin polymerization, markedly lowering the $C_c$ value at equilibrium (Figure 5A) and is consistent with stabilization of actin filaments. When actin polymerization from monomers was monitored as a function of time, a typical curve for assembly shows a lag period for actin filament nucleation, a period of rapid growth, and ultimately a plateau of polymer formation (Figure 5B). ERD10 inhibited actin polymerization in a dose-dependent manner, extending the period required for nucleation and reducing the rate of polymerization (Figure 5B). The filament side-binding and crosslinking protein AtFIM1 had similar effects in this assay (data not shown). Also like AtFIM1 (Figure 5D), ERD10 inhibited actin depolymerization following dilution (Figure 5C). Increasing amounts of ERD10 reduced the rate and extent of actin disassembly in a dose-dependent manner, but was not quite as potent as AtFIM1 under the identical conditions. The collective results strongly support the notion that ERD10 is capable of binding to the side or ends of actin filaments in vitro and altering actin dynamics.

As for TCH2, it was still plausible that it bound Ref52 actin stress fibers indirectly by associating with another actin-binding protein. Calmodulin and calmodulin-like proteins can be myosin light chains through binding to the IQ domain(s) on the heavy chains of
both conventional and unconventional myosins (reviewed in (Bahler and Rhoads, 2002; Cyert, 2001). Therefore, we considered the possibility that over-expressed plant TCH2 was recognizing and binding the IQ domain of non muscle rat myosin II, and thereby associating with the actin stress fibers of Ref52 cells. In order to address the question whether TCH2 has the potential to be a plant myosin light chain, a fragment of cDNA corresponding to the four IQ domains of myosin VIII from Arabidopsis (ATM1) was fused to GST. It was found that TCH2-YFP was pulled down by GST-IQ in a calcium-dependent manner (Figure 6).

Expressing the TCH2-YFP and ERD10-YFP chimeras in plants

The fluorescent chimeras of ERD10 and TCH2 were transiently expressed in *N. benthamiana* leaves. In both cases fluorescence distribution in cells was cytoplasmic (figure 7). This might indicate that over-expression under the 35S promoter led to mislocalization, or that abundant cytosolic YFP-fusion protein hinders the visualization of cytoskeletal elements. It also suggests that these genes could not be identified as cytoskeleton related if the screen would have been performed in plant cells using a fluorescent-protein-fused library under the control of the 35S promoter (Cutler et al., 2000) or under strong sub-genomic promoters of TMV (Escobar et al., 2003).

Discussion

Several screens of GFP-fusion cDNA libraries expressed in plants and animal cells have been reported (for example (Cutler et al., 2000; Escobar et al., 2003; Rolls et al., 1999;
Tian et al., 2004). However our system is unique by the fact that it combines a plant YFP-fusion library with a host of mammalian cells serving as the screening system. We believe that our system is advantageous for plants for the following reasons:

1. **Optical limitations:** Adhesion to the cover-slide substrate, flattening and single layer growth of adherent animal cells in tissue culture makes microscopy easier when compared with large, cell wall encased, and thick plant cells. High-resolution microscopy is dramatically limited by the short working distance of high-magnification high-numerical aperture objectives.

2. **Signal-to-noise limitations:** Auto-fluorescence of the cell wall, phenols and chlorophyll is often very high in plant cells. In addition, the cytoplasm is a thin layer pressed between the vacuole and cell wall. The absorption and emission spectra of auto-fluorescence is very broad, leaving no "clean" window in the visible range for specific labeling. The microscopy images suffer from both a very high background and a strong absorption of the specifically labeled fluorescence.

3. **Cell biological limitations:** Transformation efficiency and rate of growth has been optimized for mammalian cell lines, ensuring that the number of individual infected cells screened will represent a full expression library. This is extremely important in this “post genome” era when we design sophisticated functional genomic, high-throughput-screens to look for new, low abundance proteins which have so far escaped other “conventional” and bioinformatic screens.

4. **Automization of microscopy** facilitates a comprehensive high-throughput screening.
5. Ref52 fibroblasts are specifically big and flat cells with prominent stress fibers, thus serving as an optimal platform for detecting cytoskeletal components under the microscope.

Using our system we were able to find that the dehydrin ERD10 is associated with actin stress fibers in rat fibroblasts and binds directly to F-actin in vitro. Interestingly, bioinformatic predictions suggesting a cytoskeleton-association for group 2 and 3 LEA proteins was published previously (Wise, 2003). In addition, TCH2 a calmodulin like protein was found to interact with the IQ domains of myosin VIII (ATM1) in a calcium-dependent manner. Importantly, the subcellular localization of these proteins when overexpressed as YFP chimeras in leaves of *N. benthamiana* was diffuse and typical of cytoplasmic labeling and not cytoskeletal association. This suggests that our system is potent in revealing new plant cytoskeletal proteins which could not be revealed by the same screen performed in plants. A full characterization of the new cytoskeleton proteins and the full list of genes identified in this preliminary screen will be published elsewhere.

**Experimental procedures**

*The microscope*

A microscope system was developed including a computer controlled stage for multi-well plate scanning, fast laser auto-focusing for high magnification imaging, extremely sensitive back-illuminated digital CCD camera (quantum efficiency is >95% throughout the visible and IR wavelengths) and modular controlling software for fully automated
operation. The system acquires 20 Gbytes of image data per day, and via a client-server
link saves and analyzes the data practically on-line (Kam et al., in preparation).

Constructing the library

A cDNA library was prepared from two-week-old Arabidopsis thaliana type Columbia
seedlings. The library was constructed using the Smart cDNA library construction kit of
Clontech, CA, USA (Cat No. K1051-1). The expression vector used for fusion to YFP is
the Clontech retroviral vector pLPCX that was modified as follows: a cDNA of the
improved, higher fluorescent Venus version of YFP (Nagai et al., 2002), without its ATG
was inserted between the NotI and Clal of the pLPCX multiple cloning site. A BamHI
site and a linker of Ser-Gly-Thr-Gly-Ser in three reading frames were added upstream to
the YFP (figure 1). The full length Arabidopsis cDNAs were first digested with SfiI and
then partially digested with Sau3A which is a four cutter enzyme compatible with
BamHI. The cDNA fragments were ligated between SfiIA and BamHI (figure 1).

Mammalian tissue culture, Infections and transfections

Ref52 and 293T cells were cultured in DMEM supplemented with 10% fetal calf serum,
in a 37°C / 5% CO₂ incubator. For infection 293T cells were transfected with 10μg of
library DNA and 10 μg of helper virus using the Ca-phosphate method in the presence of
25μM chloroquine (C-6628 Sigma, Rehovot Israel). After two days the virions were
collected from the medium, filtered through a 0.45μm nitrocellulose filter and added to
Ref52 culture with 8μg/ml polybrene (H-9268 Sigma, Rehovot Israel ). The latter step
was repeated 3 times. Ref 52 cells were fixed with 3% paraformaldehyde in PBS 48 h
after infection and examined by the microscope. Transfection to Ref52 cells was carried out using TransIT reagent (Mirus, WI, USA) using the manufacturer instructions.

**The screen**

Bacterial colonies containing the Arabidopsis cDNA clones were individually collected into sixty 96-well plates (5-10 clones per well). DNA was prepared from two pools of 2500 clones (five 96-well plates). The pooled DNA was infected into rat fibroblasts, screened with the microscope and scored for potential clones that showed interesting subcellular localization patterns. The fibroblast cells were infected in 384-well plates (#781091 greiner bio-one, Frickenhausen, Germany). Each well was seeded with 300-500 cells that were infected, cultured for 2 d and screened. Each pool yielding cytoskeleton localizing clones was sub-divided into smaller pools of 500 clones (stored in one 96-well plate). From the positive 96-well plates we prepared 8 pools from the rows and 12 pools from the columns. These were screened and positive clones were localized in the wells at the crossing of a positive row and column.

**Immunostaining**

Single clones localizing to actin stress fibers were isolated from the wells and purified DNA was re-transfected into Ref52 cells cultured on a cover slip. The verification of their localization was carried out by double labeling with specific markers such as coumarine- phalloidin (P-2495 Sigma, Rehovot Israel) for actin filaments and anti-vinculin antibody (H Vin) a marker for focal contacts, and a fluorescent secondary antibody conjugated to Cy3 (115-165-072, Jackson ImmunoResearch PA, USA).
**RT-PCR and plasmids**

Full-length cDNAs for Arabidopsis ERD10 (accession number NM_180616) and TCH2 (accession number NM_123136) were isolated from Arabidopsis RNA using RT-PCR and the following primers: ERD10 forward primer containing an *Eco*RI site 5’-CGGAATTCATGGCAGAAGAGTACAAGAACACGGTCCA-3’ and a reverse primer with *Bam*HI site 5’-CGGGATCCATCATCACAGACACTTTTTTC

TTTCTTCTTTCTC-3’. TCH2 forward primer containing *Hind*III 5’-CCCAAGCTTATGTTCATCGAAGAAGGAGTT-3’ and a reverse primer containing *Bam*HI 5’-CGGGATCCAGGACACCACCACCATCTCAT-3’. The PCR products were ligated upstream to YFP in one of the vectors prepared for the library according to the reading frame. The fluorescent chimera was transferred to plant expression vector pART7 containing the 35S promoter and as a full expression cassette to the binary vector pART27. For GST fusion ERD10 was amplified using the following primers 5’-CGGGATCCATGGCAGAAGAGTACAAGAACACGGTCCA-3’ and 5’-CGGAATTCTTAATCAGACACTTTTTCTTTCTC-3’ and ligated to the *pGEX*-KG plasmid (Guan and Dixon, 1991) between *Bam*HI and *Eco*RI. The 4XIQ domain of myosin VIII (ATM1) from nucleotide 2651 to 2929 was isolated by PCR from a cDNA clone kindly provided by Dieter Volkmann (University of Bonn) and fused to GST between *Bam*HI and *Eco*RI using the following primers: 5’-CGGGATCCCTCCACCGCATTTTACGT-3’ and 5’-CGGAATTCTTTACCCTGAACATCTCTAAC-3’.
**Actin Purification**

Actin was purified from rabbit skeletal muscle acetone powder (Spudich and Watt, 1971), and monomeric Ca-ATP-actin was purified by Sephacryl S-300 chromatography (Pollard, 1984) in G buffer (5 mM Tris-HCl, pH 8, 0.2 mM ATP, 0.1 mM CaCl$_2$, 0.5 mM DTT, 0.1 mM azide). Actin was labeled on Cys-374 with pyrene iodoacetamide (Pollard, 1984).

**Critical concentration determination at steady state**

The critical concentration ($C_c$) for actin polymerization was determined as described by (Brenner and Korn, 1983) (1983). Increasing concentrations of actin (5% pyrene-labeled) were polymerized in 1x KMEI (50 mM KCl, 1 mM MgCl$_2$, 1 mM EGTA and 10 mM imidazole-HCl, pH 7.0) in the absence or presence of 2 μM ERD10 or 1 μM HPRO1 (human profilin I) for 16 h at room temperature in the dark. Human plasma gelsolin at 1:200 stoichiometry was used to cap the barbed ends of actin filaments. Fluorescence measurements were performed at room temperature using a spectrofluorometer (Quantamaster 2000-SE; Photon Technology International, South Brunswick, NJ) with excitation set at 365 nm and emission detected at 407 nm. Linear best fit of the data, plotted as arbitrary fluorescence units versus actin concentration, was used to determine the intercept with the x axis ($C_c$).
Kinetic studies of actin polymerization and depolymerization

Kinetic analysis of actin assembly and disassembly was monitored by changes in pyrene fluorescence according to assays described previously (Huang et al., 2005). The polymerization of monomeric actin in the presence of various concentrations of ERD10 was measured over time. Actin monomers (2 μM, 5% pyrene-labeled) were polymerized with the addition of 0.1 volume of 10x KMEI and the change in fluorescence was followed for 30 min. To document effects on actin depolymerization, pyrene-actin filaments (5 μM; 100% labeled) were assembled and then pre-incubated for 5 min in the presence or absence of various amounts of actin-binding proteins (ERD10 and FIMBRIN1). Depolymerization was monitored by fluorimetry following dilution of the sample by 25-fold with Buffer G.

GST pull down assay

Plasmids with the GST-IQ or GST alone were transformed to BL21 E. coli cells and expression was induced with 0.4 mM IPTG for 4 h. After precipitation, bacterial cells were re-suspended in Perp. Buffer (100 mM NaCl, 100 mM Tris HCl pH 8, 50 mM EDTA, 2% Triton X-100, 2 mM DTT) supplemented with protease inhibitors cocktail for bacterial cells (P 8465 Sigma, Rehovot Israel) and 10 mg/ml lysozyme (L-7651 Sigma, Rehovot Israel) and incubated 30 min on ice. Debris was precipitated at 14,000 rpm at 4°C for 10 min and the extracts were incubated with 20 μl of glutathione-agarose beads (G-4510 Sigma, Rehovot Israel) for 1 h at room temp. The glutathione beads were washed with the same buffer and incubated with cell lysate of 293T expressing TCH2-
YFP. Cell culture of 293T was transfected with TCH2-YFP and 48 h later harvested in a buffer containing 40 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM MgCl$_2$, 4% glycerol, 0.5% Triton X-100, 5 mM DTT, 1 mM PMSF and either 5 mM EGTA or 5 mM CaCl$_2$. After 2 h incubation at 4°C the beads were washed 6 times with washing buffer containing 20 mM Tris, pH 8, 150 mM NaCl, 0.5% NP40 and either 5 mM EGTA or 5 mM CaCl$_2$ respectively. The beads were boiled in sample buffer and bound proteins were separated on an SDS-PAGE. TCH2-YFP was detected using anti-GFP antibody (1 814 460 Roche Basel, Switzerland).

**Transient expression in N. benthamiana leaves**

*Nicotiana benthamiana* plants were grown in peat in a greenhouse at 25°C with optimum light for 16 h. The fluorescent chimeras were expressed using Agrobacterium infiltration. Briefly: *Agrobacterium tumefaciens* strain GVE3101 were transformed with the plasmid and grown at 28°C for 48 h. The culture was precipitated and dissolved to OD$_{600}$ = 0.5 in the following buffer: 50 mM MES, pH 5.6, 0.5% glucose, 2 mM NaPO$_4$ and 100 μM acetosyringone (Sigma Aldrich D13440-6). Leaves of 3-week-old *N. benthamiana* plants were infiltrated with the bacterial culture using a syringe. Expression of the fluorescent chimera in the leaf cells was detectable after 24 h, peaking at 48 h and still observable after 5 d.
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References


Figure 1. Schematic representation of the strategy applied for constructing the YFP cDNA library. cDNA was synthesized, flanked by two restriction sites of SfiI A and B. It was then digested by SfiI followed by partial digestion with sau3A and ligated to SfiIA – BamHI sites fused to YFP in three reading frames.
**Figure 2. 293T cells transfected with either the empty vectors or the library.** A mixture of the empty vectors (upper panel) and a sample of the library (lower panel) were transfected into 293T cells and examined in the microscope. It is demonstrated that the empty vectors yielded only background fluorescence, whereas the library contained many clones inserted in frame to the “ATG-less” YFP and activated its expression.
Figure 3. Different subcellular localizations of Arabidopsis cDNAs in Ref52 rat fibroblasts. Rat fibroblasts (Ref52) were infected with DNA prepared from pools of 2500 different Arabidopsis cDNAs. A collection of different subcellular localization patterns is displayed.
Figure 4. The full length cDNA of ERD10 and TCH2 were fused to YFP and transfected to Ref52 fibroblasts. Upper pane: YFP chimeras. Lower panel: double immuno-staining of the fluorescent chimera expressing cells for actin (red) and vinculin (blue) to prove genuine actin localization. FIM1 ABD2 fused to YFP and expressed in Ref52 fibroblasts serves as a positive control showing a known plant actin binding protein localizing to mammalian actin stress fibers. Scale bar = 20μm.
Figure 5. ERD10 affects actin assembly and disassembly directly.

A. Determination of the critical concentration ($C_c$) for actin assembly in the absence and presence of ERD10 or human profilin, HPRO1. Increasing concentrations of actin were incubated in the absence (open squares) or presence of 2 μM ERD10 (open circles) or 1 μM HPRO1 (closed circles) and allowed to polymerize for 16 h at room temperature. To cap filament barbed ends, a 1:200 ratio of human plasma gelsolin to actin was included in all reactions. Fluorescence emission from pyrene-actin assembly was recorded with the fluorimeter. The $C_c$ values (x-axis intercept of each regression line) for this representative
experiment were 0.63 µM for actin alone, 0.38 µM for ERD10, and 0.77 µM for HPRO1.
a.u., arbitrary units.

**B.** ERD10 inhibits actin polymerization. ERD10 at various concentrations was incubated
for 5 min with 2 µM actin (5% pyrene-labeled) before polymerization. Pyrene
fluorescence is plotted versus time after the addition of KMEI buffer to initiate
polymerization. ERD10 extended the lag period or nucleation phase and reduced the
initial rate of actin assembly in a dose-dependent manner. a.u., arbitrary units.

**C & D.** ERD10 and AtFIM1 stabilize actin filaments and inhibit depolymerization. 5
µM actin in polymeric form (100% pyrene labeled) was incubated with various
concentrations of ERD10 (**C**) or AtFIM1 (**D**) for 5 min before dilution of the solution 25-
fold in Buffer G. Concentrations given are for each protein prior to dilution. Both actin
binding proteins reduced the rate and extent of actin filament depolymerization in a dose-
dependent fashion. However, FIM1 appeared to be more potent, almost completely
preventing depolymerization at 1 µM, when compared with ERD10 at the same
concentration.
Figure 6. Pull down assay of GST-IQ and YFP-TCH2. The 4XIQ domain from ATM1 (Arabodopsis myosin VIII) was fused to GST and collected from bacterial extract using glutathione agarose beads. These beads were incubated with extracts from 293T cells expressing TCH2-YFP in the presence or absence of free Ca$^{2+}$. Beads were precipitated, washed and boiled in sample buffer and bound proteins were separated on SDS-PAGE and detected by anti GFP antibody. Upper panel; pull down assay, lower panel: total 293T cell extracts.
Figure 7. ERD10-YFP and TCH2-YFP transiently expressed in epidermis cells of leaves of *N. benthamiana*. The fluorescent chimeras of the full length cDNAs of ERD10 or TCH2 with YFP were transferred to plant expression vectors and transiently expressed in *N. benthamiana* leaves using Agrobacterium infiltration. Scale bar = 20μm.