Protoplasma (2011) 248:239–250
DOI 10.1007/s00709-010-0202-0

REVIEW ARTICLE

Development and application of probes for labeling the actin cytoskeleton in living plant cells

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Received: 31 May 2010 / Accepted: 14 August 2010 / Published online: 28 August 2010
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Abstract The actin cytoskeleton is one of the most important components of eukaryotic cytoskeletons. It participates in numerous crucial procedures of cells and has been studied by using various methods. The development and application of appropriate probes for actin visualization is the first and foremost step for functional analysis of actin in vivo. Since the actin cytoskeleton is a highly dynamic and sensitive structure, methods previously used to visualize actin often harm cells and cannot reveal the native state of the actin cytoskeleton in living cells. The development of labeling technologies for living plant cells, especially the emergence and application of green fluorescent protein-tagged actin markers, has provided new insights into the structure and function of the actin cytoskeleton in vivo. There has been a number of probes for actin labeling in living plant cells though they each present different advantages and defects. In this review, we discuss and compare those widely used methods for actin visualization and analysis.

Keywords Actin cytoskeleton · Labeling method · Fluorescent probes · Living plant cell

Introduction

Actin filaments (AFs) or filamentous actins (F-actin) are formed from global actin (G-actin) monomers, which associate to form fibrous structures approximately 7 nm in diameter. These structures can be further organized into complex and dynamic networks in vivo through cross-linking, bundling, or other associations. As an important part of the cytoskeleton system, the actin cytoskeleton participates in cell morphogenesis, endocytosis, intracellular transportation, cytokinesis, cell mobility, and signal transduction (Pollard and Cooper 2009; Staiger et al. 2010).

The use of appropriate probes to label the actin cytoskeleton and obtain fine images of its native state is crucial for investigating actin function. Labeling techniques used in previous studies include immunofluorescence using an actin antibody (Cande et al. 1977), labeling of heavy meromyosin and observation by electron microscopy (Kersey and Wessells 1976), and staining by fluorescent phallotoxin or fluorescent phalloidin, which specifically bind to actin filaments (Wulf et al. 1979). Although these methods have provided considerable information about the organization of the actin cytoskeleton, they have some limitations. These techniques are technically demanding, and chemical treatments such as fixation and permeabilization can destroy native actin organization, leading to inaccurate labeling. Most importantly, none of these methods can be used to observe the dynamics of actin filaments in living cells (Lloyd and Traas 1988; Kovar et al. 2001; Higaki et al. 2006).

Imaging in living cells allows the visualization of cytoskeletal structures as they change in response to growth conditions (Vidali et al. 2009) and thus provides an opportunity to visualize the organization of native actin filaments. Microinjection of both fluorescent phalloidin and fluorescent actin analogs allow living observations of actin filaments in plant cells (Schmit and Lambert 1990; Zhang et al. 1993; Ren and Yuan 2000). Whereas phalloidin can disturb the dynamic balance of actin filaments, fluorescent

Handling Editor: David Robinson

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actin analogs do not have this effect and therefore are a better choice for tagging as they reflect the true state of the actin cytoskeleton. For this reason, fluorescent actin analogs have been used for research on actin functions in vivo (Ren 1999; Ren and Yuan 2000). However, fluorescent dyes such as FITC and Oregon Green are susceptible to photobleaching. Recent developments in fusion technologies for green fluorescent protein (GFP) and other fluorescent proteins (FPs) have enabled more convenient and reliable observations of the dynamic behaviors of actin filaments in living plant cells while minimizing negative impacts. These live probes usually use a fluorescent protein-tagged actin monomer (GFP-actin) and a full-length protein or protein domain that binds specifically to actin filaments (Wasteneys and Yang 2004; Wang et al. 2007). Using these probes has provided new insights into some special dynamic behaviors of actin filaments during many cellular processes. In this review, we discuss the recent developments in cellular probes used to determine the structure and function of the actin cytoskeleton in living plant cells. This information will further our understanding of labeling technologies for actin filaments and provide references for discovery of novel approaches.

Microinjection into plant cells for the visualization of actin structures

Microinjection of fluorescent phalloidin

Phalloidin, which specifically binds to actin, is a fungal toxin isolated from the poisonous mushroom *Amanita phalloides*. Most of the existing knowledge about the actin cytoskeleton has been obtained through fluorescent phalloidin staining after chemical treatments of cells. As actin is highly sensitive to fixation and permeabilization (Lloyd and Traas 1988), such procedures may result in artificial rearrangements of actin filaments (Schmit and Lambert 1990). Although some progress has been made in the development of in vivo staining methods that do not involve fixation (Tewinkel et al. 1989; Olyslaegers and Verbelen 1998), diverse permeabilization treatments may still destroy the cell membrane and membranous organelles.

Fluorescent phalloidin is not membrane permeable, and therefore, the most appropriate delivery technique is microinjection of living cells with minute amounts of this drug (Olyslaegers and Verbelen 1998). Since microinjection of fluorescent phalloidin was first used in higher plants (Schmit and Lambert 1990), this method has shed new light on observation of the dynamics of F-actin in vivo, especially the role of actin in plant mitosis and cytokinesis. However, the method is technically demanding and its application is restricted to certain types of plant cells such as endosperm cells of *Haemanthus katherinae* (Schmit and Lambert 1990), *Tradescantia virginiana* L. stamen hair cells (Cleary et al. 1992), living guard cells of *Commelina communis* L. (Kim et al. 1995), pollen tubes of *Lilium longiflorum* (Miller et al. 1996) and isolated egg cells of wheat (Pónya and Barnabás 2001). However, since the phalloidin injection method does not include a fixation step, the dynamics of the actin cytoskeleton are severely altered by the injection process (Ren and Yuan 2000). Whether used as a stain or in microinjection, phalloidin itself stabilizes F-actin, and thereby alters the dynamic activity of the actin cytoskeleton. It is generally acknowledged, therefore, that phalloidin can give artificial results in terms of actin structure. Therefore, neither staining nor microinjection by fluorescent phalloidin is an ideal method to study actin filaments in living cells (Estes et al. 1981; Sampath and Pollard 1991; Kovar et al. 2001; Wang et al. 2007; Zhang et al. 2009). To minimize these detrimental effects, one can reduce the concentration of phalloidin and improve the sensitivity of imaging.

Microinjection of fluorescent actin analogs

Fluorescent analog cytochemistry is a more suitable method for studying the dynamics of the actin cytoskeleton, because it uses the normal components of the actin cytoskeleton and has little effect on its dynamic movements. By labeling actin with fluorescent dyes and introducing the labeled actin into living cells by microinjection, the endogenous cytoskeletal arrays can incorporate the labeled actin through the turnover of the cytoskeleton system (Ren and Yuan 2000). Previous in vitro experiments have generally used actin from animal muscles. However, it has been found that the fluorescent analogs of these animal actins cannot be incorporated into the plant cytoskeleton system when they are injected into living plant cells (Ren 1999).

Ren et al. (1997) reported a method for purifying actin from maize pollen that enabled more effective and convenient preparation of fluorescent plant actin analogs. The double injection of purified pollen actin and fluorescent phalloidin into *T. virginiana* L. stamen hair cells demonstrated that exogenous actin did not perturb cytoskeletal organization or function in live cells. Ren and coworkers first labeled plant actin with fluorescent dye and then introduced these fluorescent actin analogs into living plant cells (Ren 1999; Ren and Yuan 2000). The prepared actin fluorescent analog was able to incorporate into the actin cytoskeleton via turnover. Compared with the actin cytoskeleton visualized by microinjection of rhodamine–phalloidin, the actin filaments revealed using fluorescent actin were finer and thinner. This result suggested that the microinjection of fluorescent actin analogs might not cause

actin assembly and therefore may reflect the true state of actin dynamics in living plant cells (Ren and Yuan 2000). Thus, the microinjection of fluorescent actin analogs is more reliable than that of fluorescent phalloidin, but the same limitations exist; that is, the method is technically demanding, and is limited to certain cell types.

Labeling of actin by expression of fluorescent fusion protein in living plant cells

Expression of GFP-actin

Green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a fluorescent protein that has been widely used in investigations of almost all biological processes in cells. GFP has many advantages as a marker. The formation of its chromophore is cell-autonomous in that it requires only molecular oxygen. In addition, GFP is photostable, being strongly resistant to photobleaching and other stresses, and the quality of its fluorescence is not affected by its fusion to other proteins (Chiu et al. 1996; Chytilova et al. 1999). These advantages make GFP an attractive fluorescent tag for actin cytoskeleton labeling. GFP imaging does not require staining or other harmful procedures and allows for analysis of cells in a relatively undisturbed, living state.

GFP directly fused to actin has been used in many studies. Expression of GFP-actin fusion proteins potentially labels F-actin and pools of G-actin. GFP-actin labeling has been used to observe actin localization in cells of *Dictyostelium discoideum* (Aizawa et al. 1997; Westphal et al. 1997), yeast (Doyle and Botstein 1996), *Drosophila melanogaster* (Verkhushaa et al. 1999), and mammals (Ballestrem et al. 1998; Fischer et al. 1998). However, expression of GFP-actin can have severe impacts on cell morphogenesis and function. For example, expression of GFP-actin severely affected the morphology and function of *D. discoideum* cells (Aizawa et al. 1997), possibly as a result of the large increase in the number of actin monomers that occurs during overexpression of GFP-actin fusion proteins. These monomers play important roles for the maintenance of cell shape and function (Yoneda et al. 2007).

Expression of GFP-ABPs

GFP-mTalin tag

The organization and dynamics of actin filaments are regulated by various actin-binding proteins (ABPs) that can bind to monomeric actin, filamentous actin, or both. Therefore, it is feasible to label the actin cytoskeleton using fusion proteins of GFP fused to certain ABPs. In fact, that method has provided a vast quantity of useful information about the structures and functions of the actin cytoskeleton during plant development (Wang et al. 2004; Ye et al. 2009).

The first tagged actin-binding protein used in plants was the talin protein from mouse (mTalin or mTn) (Kost et al. 1998). The actin-binding domain (ABD) located in the C-terminal of the talin protein is highly conserved among a diverse group of ABPs. It binds actin filaments with a higher affinity than the full-length protein but, in contrast to the latter, does not nucleate actin polymerization (McCann and Craig 1997). Therefore, this domain appears to be an ideal tag to fuse with GFP for actin labeling. Kost et al. constructed a fusion protein of GFP-mTalin that was stably expressed in *Arabidopsis thaliana* plants and transiently expressed in tobacco BY-2 (*Nicotiana tabacum* L. cv Bright Yellow) cells and tobacco pollen tubes. The patterns of green GFP fluorescence and red rhodamine–phalloidin fluorescence emitted from double-labeled BY-2 cells were essentially identical, indicating that GFP-mTalin can specifically label the plant actin cytoskeleton. Transient expression of GFP-mTalin did not affect morphology or mitosis in BY-2 cells. In *A. thaliana*, constitutive expression of GFP-mTalin labeled actin filaments in diverse cell types, including epidermal cells of hypocotyl, root, and cotyledon, with no detrimental effects on morphology or development (Kost et al. 1998).

Using GFP-mTalin and fusion proteins of mTalin with other fluorescent tags (e.g., YFP-mTalin) gives a new approach for observing the actin cytoskeleton. Such strategies have been widely used in diverse tissues of various plants including *A. thaliana* epidermal and trichome cells (Mathur et al. 1999; Mathur and Chua 2000; Ilgenfritz et al. 2003; Li et al. 2003), root hairs (Ketelaar et al. 2004; Yi et al. 2005), and pollen tubes (Ketelaar et al. 2004; Yi et al. 2005); tobacco BY-2 cells (Yu et al. 2006), leaf pavement cells (Brandizzi et al. 2002), and pollen tubes (Wilsen et al. 2006); *Lilium* pollen tubes (Wilsen et al. 2006); rice coleoptiles (Holweg et al. 2004); onion epidermal cells (Xu and Scheres 2005); and cells of the moss *Physcomitrella patens* (Finka et al. 2007). The quality of GFP-mTalin labeling relies on cell types; the labeling of actin arrays is more subtle in certain cell types, such as various epidermal cells and pollen tubes, than in others (Wasteneys and Yang 2004; Voigt et al. 2005).

Nevertheless, it has been reported that GFP-mTalin might cause some problems. High levels of GFP-mTalin expression cause thick actin cables and slow cytoplasmic streaming in tobacco pollen tubes and result in premature cessation of growth (Kost et al. 1998). Using an ethanol-inducible system, Ketelaar et al. (2004) found that over-expression of GFP-mTalin destroyed actin organization in

A. thaliana root hairs and consequently led to changed cell shapes, inhibition of growth, and even cell death. However, this may have been due, at least in part, to the effect of transient expression by the ethanol-inducible system because these severe phenotypes were not observed in lines constitutively expressing GFP-mTalin. Voigt et al. (2005) also showed that GFP-mTalin seedlings have slightly decreased hypocotyl growth and a significantly slower gravitropic response. These phenotypes may be due to the high level of GFP-mTalin expression, which affects the natural dynamics of actin filaments in vivo. For instance, the universally increased actin bundles in GFP-mTalin transgenic lines may result from competition of this marker with endogenous actin depolymerizing factor (ADF) and even other ABPs because experiments in vitro have revealed that GFP-mTalin can inhibit the actin depolymerizing activity through hindering the interaction of ADF with F-actin (Ketelaar et al. 2004). However, the excessive bundling effect caused by overexpression of YFP-mTalin in tobacco BY-2 cells and rice has been used to determine the biological functions of actin configuration in the context of auxin transport. The studies suggest that auxin regulates its own transport by changing the state of actin filaments (Maisch and Nick 2007; Nick et al. 2009). Besides, this undesired side effect of GFP-mTalin differs in its severity among different cell types. Some are more sensitive to this fusion tag, while others are less so (Kost et al. 1998).

GFP-fimbrin/plastin tag

A. thaliana fimbrin1 (AtFim1) was the first plant actin-binding protein used for tracking the spatial and temporal distribution of actin filaments in vivo (Kovar et al. 2001). AtFim1 is a member of the fimbrin/plastin family of actin filament bundling or cross-linking proteins (Kovar et al. 2000). Two conservative actin-binding domains (ABD1 and ABD2) are localized in the single peptide chain of AtFim1, and each contains a 27-amino-acid fragment (BD1 and BD2, respectively) (McCurdy and Kim 1998; Wang et al. 2004). K_d values in vitro showed that ABD2 has stronger affinity than ABD1 for F-actin (Kovar et al. 2000; Nakano et al. 2001). There have been many studies of AtFim1 labeling, in which full-length or truncated AtFim1 proteins fused to GFP were used as probes. The results of these studies indicate that these probes have different labeling features (Kovar et al. 2001; Wang et al. 2004; Sheahan et al. 2004; Bannigan and Baskin 2005).

Microinjection of Oregon Green-labeled AtFim1 into *T. virginiana* L. stamen hair cells or transient expression of AtFim1-GFP in epidermal cells of tobacco and onion can decorate intricate and dynamic cortical networks of actin filaments, but both methods result in a fluorescence signal in the cytoplasmic background (Kovar et al. 2001; Wang et al. 2004; Sheahan et al. 2004). This is also observed during constitutive expression of AtFim1-GFP in various cell types of *A. thaliana* root. AtFim1-GFP also shows mostly diffuse fluorescence and few actin filaments in meristem cells and growing root hairs (Wang et al. 2004; Voigt et al. 2005).

Truncated AtFim1 proteins, including ABD1-GFP and its more conserved segment BD1-GFP, label diffuse fluorescence rather than filamentous structures in leaf epidermal cells of tobacco. This is a result of the lower affinity of AtFim1 for F-actin in vitro (Wang et al. 2004; Sheahan et al. 2004). However, ABD2-GFP and the segment containing the two conserved BD domains (BD1/2-GFP) can decorate more sophisticated and dynamic actin networks than GFP-mTalin in various cell types. ABD2-GFP also labeled more subtle actin filaments in the transition zone between the meristem zone and the elongation zone, and its expression did not result in fluorescent aggregates in specific cell regions. By contrast, plants with extremely high levels of BD1/2-GFP showed stunted growth compared with the wild-type, and plant lines expressing BD1/2-GFP showed silenced and weak fluorescence in the meristem but thick fluorescent aggregates in cells at the root–hypocotyl junctions and hypocotyls (Wang et al. 2004; Voigt et al. 2005).

Plastin proteins belong to another subfamily of the fimbrin/plastin family. One plastin tag used as a probe in living plant cells is the C terminus of truncated human T-plastin fused with GFP. Plastin-GFP labeled thick filament bundles and nuclei in *Medicago* root epidermal cells but presented a diffuse signal rather than fibrous structures in *Medicago* root hairs (Voigt et al. 2005).

GFP-fABD2 tag

Sheahan et al. (2004) fused GFP with the N-terminal of ABD2 of AtFim1. Like ABD2-GFP, the GFP-fABD2 fusion protein labeled highly dynamic and dense actin networks in diverse species and cell types. These results demonstrated that GFP fused to either the N- or C-terminal has no obvious influence on the labeling characteristics. Compared with full-length AtFim1, microfilament bundling is avoided by trimming the gene to encode only one of fimbrin's actin-binding domains (Wasteneys and Yang 2004). In addition, GFP-fABD2 labeling reveals structural details not seen with the commonly used GFP-mTalin labeling method and has no detectable adverse effects on plant morphology or development. In contrast, however, plants expressing GFP-mTalin showed reduced cell elongation, resulting in plants with stunted growth and reduced seed set (Sheahan et al. 2004). Voigt et al. (2005) showed that GFP-fABD2 labeling was superior for labeling actin filaments of deeply embedded cells, especially stelar and statocyte cells and cells in the root meristem. However,

GFP-mTalin was more suitable for labeling in cell layers close to the organ's surface and resulted in only diffuse or no fluorescence from deeper cell layers. Another disadvantage of GFP-mTalin is that it induces rearrangements of the actin cytoskeleton, such as shorter, more branched, and more convoluted filament networks with increased bundling and greatly reduced dynamism (Sheahan et al. 2004; Sano et al. 2005; Ketelaar et al. 2004). When expressed at high levels, GFP-mTalin can result in transverse hoops and small mobile rings of actin in live cells (Wilsen et al. 2006).

Holweg (2007) reported that GFP-fABD2 slightly affected the motility of organelles and the transport of auxin in plant cells. Other groups have obtained consistent results that GFP-fABD2 or ABD2-GFP expressed in *Arabidopsis* seedlings result in slightly extended primary roots and hypocotyls because of an increase in the average length of epidermal cells (Sheahan et al. 2004; Voigt et al. 2005; Wang et al. 2007). In addition, root hairs of seedlings expressing GFP-fABD2 or ABD2-GFP showed slightly higher growth rates than those of wild-type roots (Voigt et al. 2005; Wang et al. 2007). Thus, it is clear that GFP-fABD2 can accelerate the growth of both diffusely and tip-growing cell types to some extent. Despite the fact that those phenotypes are subtle and harmless to plant morphogenesis, the expression level still should be controlled in transgenic seedlings. If expressed at high levels, either GFP-mTalin or GFP-fABD2 would change the original actin arrays. In contrast to GFP-mTalin, which was expressed at high relative levels but continued to label highly bundled actin structures, GFP-fABD2-expressing cells at similar levels of GFP fluorescence always caused cytoplasmic accumulation (Sheahan et al. 2004; Wilsen et al. 2006). This finding may reflect differences in the affinity of the C-terminal actin-binding domain of talin and ABD2 of AtFim1 for binding to plant actin filaments. Alternatively, it could reflect a reduced number of available binding sites for GFP-fABD2, given the presence of endogenous fimbrins. A low level of GFP-fABD2 expression may be vital for its accurate labeling of actin networks in live plant cells (Sheahan et al. 2004). Inducible promoters could be used to control the expression level of these probes.

GFP-fABD2 labeling has revealed some special actin structures that could not be seen using GFP-mTalin labeling. Sano et al. (2005) observed a “twin peaks” structure of actin filaments in the metaphase cell cortex in a stably transformed BY-2 cell line (BY-GF11). The “twin peaks” of actin filaments are thought to play a role in guiding the movement of cell plates. In our own laboratory, the precise structure and dynamics of AFs during the initiation and late lateral expansion of the phragmoplast have been studied in a tobacco BY-2 cell line stably expressing GFP-fABD2. This has revealed new information that was not evident in our previous studies using GFP-mTalin for labeling (Yu et al. 2006; Zhang et al. 2009). This may be due to the nuclear localization of GFP-mTalin. That is, GFP-mTalin shows intense fluorescence in nuclei at interphase and around the nuclear zone during mitosis; hence, some subtle and dynamic structures near the nuclei might be obscured (Zhang et al. 2009).

GFP-fABD2-GFP tag

Wang et al. 2007 used an improved method in which a construct containing GFP fused to both the C- and N-terminals of ABD2 of AtFim1 under the control of the cauliflower mosaic virus (CaMV) 35S promoter (35S::GFP-ABD2-GFP) was expressed in plant cells, resulting in enhanced in vivo F-actin imaging. Compared with the original ABD2-GFP approach, 35S::GFP-ABD2-GFP resulted in superior quality of F-actin labeling in specific regions of tissues and cells, such as in the root meristem and lateral roots of *A. thaliana* seedlings. In cells of the root meristem, it highlights the fine F-actin networks surrounding the nuclei during interphase and phragmoplasts during cytokinesis without affecting cell division. In cells or tissues with highly dynamic actin filaments, such as guard cells and pollen tubes, GFP-ABD2-GFP does not disturb the F-actin dynamics or cellular responses to environmental cues. In addition, GFP-ABD2-GFP is a superior probe for visualizing actin filaments of root vasculature and lateral roots, and therefore, is an important tool for studying actin dynamics during the initiation of lateral roots (Wang et al. 2007).

Although this approach has not been widely used, the quality of F-actin labeling and the ease of imaging provided by GFP-ABD2-GFP exceed those of the original ABD2-GFP lines. The addition of extra GFP molecules can boost fluorescence spectral yield for F-actin labeling while reducing the confocal microscope excitation setting required for data collection (Wang et al. 2007). GFP-ABD2-GFP has been used successfully to acquire fine actin cytoskeleton images in several different systems. In studies of plant development, Bernal et al. (2008) expressed GFP-ABD2-GFP in *A. thaliana* mutants defective in several different cellulose synthase-like genes and observed diverse structures of actin filaments located in the apex zone of root hairs. The ARP2/3 complex (actin related protein 2/3 complex), a highly conserved nucleator of F-actin polymerization, and its activator, the SCAR complex, play important roles in leaf epidermal cell morphogenesis in *Arabidopsis*. Using a GFP-ABD2-GFP tag, Dyachok et al. (2008) showed that cortical F-actin accumulation in root tip cells was reduced in *brk1* mutants (mutants defective in the expression of one of the SCAR complex subunits, BRK1). This was possibly because the loss of BRK1 affected the function of the SCAR complex in activating the downstream ARP2/3 complex, thereby reducing the number of

nucleation sites in cells. In a plant–rhizobium system, Yokota et al. (2009) found that two genes, *NAP1* and *PIR1*, were essential for *Mesorhizobium loti* to infect and colonize *Lotus japonicus* roots through disturbing actin cytoskeleton organization.

Certain cell types in plants expressing the 35S::GFP-ABD2-GFP construct show slight growth defects. Overexpression of GFP-fABD2-GFP reduced the average length of some root hairs and trichomes by inhibiting cell expansion (Wang et al. 2007). This may have been because binding of GFP-ABD2-GFP interferes with the loosening of F-actin networks, which is thought to be required for rapid cell expansion (Holweg et al. 2004).

GFP-ADF tag

Although mTalin and fABD2 are still the most commonly used and widely accepted probes to date, several other actin-binding proteins have been used as actin probes. Such proteins include ADF (Chen et al. 2002; Cheung and Wu 2004; Wilsen et al. 2006; Cheung et al. 2008) and some pollen-specific LIM domain proteins (Thomas et al. 2006; Cheung et al. 2008), which have been used as actin probes in pollen tubes.

As a depolymerizing ABP of actin, ADF decreases the size of actin cables when it is overexpressed (Dong et al. 2001; Chen et al. 2002). However, since the N-terminal of ADF is important for its actin depolymerization activity, fusion of a GFP at its N terminus compromises its depolymerization activity while preserving its actin-binding activity, thereby making it suitable for use as an actin probe (Chen et al. 2002; Cheung et al. 2008). ADF has been used as an actin marker in research on pollen grains and pollen tubes. The two main pollen-specific ADF components used for probing are from tobacco and lily, named NtADF1 (Chen et al. 2002) and LIADF1 (Allwood et al. 2002), respectively. Both GFP-NtADF1 and GFP-LIADF1 label the actin cytoskeleton structure, showing long actin cables aligned with the tube axis in the shank and a prominent mesh- or basket-like actin structures at the subapical region of transformed pollen tubes (Chen et al. 2002; Cheung and Wu 2004; Wilsen et al. 2006; Cheung et al. 2008). That pattern is similar to the actin organization in rapidly frozen and immunolabeled pollen tubes (Lovy-Wheeler et al. 2005) and co-localizes with the phalloidin-labeled actin structure (Chen et al. 2002). Compared with some other actin probes such as GFP-talin and GFP-fimbrin, GFP-ADFs are the most well tolerated by pollen tubes and seldom result in actin rearrangement (Wilsen et al. 2006). On the other hand, however, GFP-ADFs are more deficient in labeling actin bundles or cables along the shank of pollen tubes than GFP-talin and GFP-fimbrin, except when they are expressed at high levels (Chen et al. 2002; Wilsen et al. 2006). Using GFP-NtADF1, Cheung and Wu (2004) reported that overexpression of the *Arabidopsis* formin AFH1 in pollen tubes induced the formation of supernumerary actin cables from the cell membrane.

GFP-LIM tag

Recently, the actin-labeling abilities of members of the LIM protein family have been investigated. LIM proteins are characterized by LIM domains—cysteine–histidine-rich, zinc finger-containing domains that are present in a wide variety of eukaryotic proteins and typically mediate protein–protein interactions and subcellular targeting (Cheung et al. 2008; Staiger et al. 2010). A GFP-fused tobacco LIM protein, WLIM1-GFP, associated prominently with the actin cytoskeleton in BY2 cells and in *Nicotiana benthamiana* leaf cells (Thomas et al. 2006). Because WLIM1 can stabilize the actin cytoskeleton by promoting actin bundling, expression of WLIM1-GFP in *N. benthamiana* leaves induced fewer and thicker actin bundles than in control cells expressing fABD2-GFP and YFP-mTalin. This result suggested that WLIM1-GFP may not be an ideal marker due to its induced disorganization of original actin arrays and its inability to label thinner actin structures. Two pollen-specific LIM proteins, LILIM1 and NtPLIM2b, were recently identified from lily (Wang et al. 2008) and tobacco (Cheung et al. 2008), respectively. Both GFP-LILIM1 and GFP-NtPLM2b can decorate fine axial actin cables in pollen tubes, giving clearer and more elaborate results than those obtained using GFP-ADFs as a label (Cheung et al. 2008). In the subapical regions of pollen tubes, GFP-NtPLM2b labeled a discernable but subtle subapical actin structure that was more frequently basket-shaped, whereas that labeled with GFP-ADFs was mesh-shaped (Cheung et al. 2008). GFP-LILIM1 showed better decoration of fine and short filaments in this region compared with mTalin labeling (Wang et al. 2008). However, overexpressed GFP-LlLIM1 induced novel asterisk-shaped actin aggregates and disturbed vesicle trafficking and oscillatory pollen tube growth, while none of the GFP-NtPLM2b-transformed plants showed noticeable fertility defects, suggesting that pollen tube growth in vivo is quite tolerant of GFP-NtPLM2b (Wang et al. 2008; Cheung et al. 2008).

GFP-Lifeact tag

Recently, a short peptide named “Lifeact” has emerged as a novel and versatile tool for visualizing actin organization in vitro and in vivo (Riedl et al. 2008). Lifeact comprises the first 17 amino acids of the yeast protein Abp-140. To our knowledge, it is the smallest protein tag for actin labeling.

The superiority of Lifeact is due to its small size and the lack of homologous sequences in higher organisms (Riedl et al. 2008; Era et al. 2009). The small size of Lifeact presents little competition against major endogenous actin-binding proteins, while the existence of homologous sequences only in close relatives of *Saccharomyces cerevisiae* makes it an attractive actin marker for higher

eukaryotes. To date, Lifeact is the only protein domain that can be used either in a transformation construct or as a stain to visualize actin. That is, it can be fused with fluorescent proteins and transformed into cells to label actin, or it can be used as a chemical staining marker in vitro, like phalloidin. It has been reported that fine and distinct actin structures could be visualized by using chemically synthesized FITC-Lifeact for staining in fixed or living mammalian cells (Li et al. 2008; Riedl et al. 2008).

As a new actin probe, Lifeact was rapidly adopted by researchers and has been widely used in studies of animal cells (Li et al. 2008; Riedl et al. 2008; Renkawitz et al. 2009; Kardash et al. 2010; Riedl et al. 2010). Lifeact-GFP or other fluorescent proteins present no sign of cytotoxicity, do not affect cell growth, and do not influence normal actin-dependent processes (Riedl et al. 2008; Era et al. 2009). Although it is less commonly used in plants, the results obtained in animal studies suggest that Lifeact could be a useful tool for studying the actin cytoskeleton in a wide range of plant lineages (Era et al. 2009). Lifeact was used to label a distinct and highly dynamic actin meshwork in various cell types of *A. thaliana* and some bryophytes. In epidermal cells of *A. thaliana*, the networks of fine and dense filaments visualized using Lifeact were quite similar to those observed with GFP-ABD2 (Era et al. 2009). In tip-growing plant cells such as pollen tubes of *Lilium formosanum* and *Nicotiana tobacum*, *A. thaliana* root hairs, and protonemata of the moss *P. patens*, Lifeact labeled distinct and dynamic actin arrays and consistently revealed a dynamic apical network of F-actin (Era et al. 2009; Vidali et al. 2009). In the liverwort *Marchantia polymorpha* expressing a Lifeact probe, the branching of actin bundles was clearly observed for the first time in plant cells (Era et al. 2009). Using GFP-Lifeact for labeling, Deeks et al. (2010) showed that the plant formin AtFH4 interacted with both microfilaments and microtubules.

Except for the report that high expression of Lifeact-mEGFP slightly affected moss protonemal growth (Vidali et al. 2009), there are no reports of negative effects of Lifeact on morphogenesis and growth of plant cells. It is likely that the small size of Lifeact allows for effective imaging and normal cell growth, even when it is expressed at high levels.

Other fluorescent protein-ABPs tags

DsRed-ABP tag

Since the discovery of GFP, genetic manipulations have generated GFP variants that are better suited than wild-type GFP for fluorescence microscopy or other purposes. These variants include enhanced green fluorescent protein, blue fluorescent protein, cyan fluorescent protein, yellow fluorescent protein (YFP), and so on. Red fluorescent proteins (RFPs), which originate from different *Anthozoa* species, extend the spectrum of fluorescent tags, making multispectral imaging very feasible (Schwartz and Patterson 2003; Ivanchenko et al. 2005). These RFPs have been widely adopted as an alternative for GFP in labeling of actin filaments.

DsRed or drFP583, the most commonly used RFP from *Discosoma* species, shares only 23% sequence identity with GFP, but the amino acids next to its chromophore are highly conserved. These amino acids may be crucial for chromophore formation (Matz et al. 1999). DsRed is a photoconvertible fluorescent protein that the GFP chromophore forms initially, and subsequently, the Cα–Nα bond of the first amino acid in the tripeptide oxidizes, thereby extending the π-conjugated electron system along the backbone and shifting the fluorescence emission to red (Nienhaus et al. 2005). In contrast to GFP, DsRed shows an excitation maximum at 558 nm, which does not overlap with that of lignin, a cell wall polymer that hinders GFP detection in the green range (~440 to ~540 nm), thereby providing a lower cellular autofluorescence background. Moreover, the longer wavelength excitation emitted by DsRed is less cytotoxic, and its fluorescence is much more intense than that of GFP due to its high fluorescent quantum yield. However, DsRed has several limitations, including slow protein maturation and a strong tendency to form tetramers both in vitro and in vivo (Baird et al. 2000; Patterson et al. 2001; Ivanchenko et al. 2005). Engineered variants of DsRed have effectively solved the above two problems (Campbell et al. 2002; Yanushevich et al. 2002) and have been used to construct markers for labeling of acting filaments. Such variants include DsRed-talin (Blancaflor 2002; Liu et al. 2005; Harries et al. 2009) and DsRed-ABD2 (Abied et al. 2006), which have been used in plant pathology studies. In addition to DsRed, there is another important RFP; the fluorescent protein mCherry. This protein has been used to label cellular organelles such as the ER (Bracha-Drori et al. 2008), the Golgi (Müller et al. 2010), and peroxisomes (Prokhnevsky et al. 2008), and also plant microtubules (Gutierrez et al. 2009) and actin (Wightman and Turner 2008). By labeling with mCherry-fABD2, Wightman and Turner (2008) observed the distribution of actin in developing xylem cells of *Arabidopsis*. Since mCherry is brighter and shows greater photostability than other RFPs (Shaner et al. 2005), it may be widely adopted in the future, and could replace DsRed for the labeling of actin in living plant cells.

EosFP-ABP tag

Recently, other green-to-red photoconvertible fluorescent proteins have become available, including EosFP, Kaede, DendFP, mcavRFP, and rfloRFP. In contrast to DsRed, their green-to-red photoconversion is driven by light, rather than chemical

Table 1 Actin cytoskeleton probes used in living plant cells

Probe	Description	Expression	Advantages	Disadvantages
Fluorescent phalloidin	Specific drug of actin filaments	Microinjection	Decorates prominent actin filaments and bundles	Alters actin cytoskeleton dynamics by stabilization
Fluorescent actin analogs	Abstracted actin from plant tissues	Microinjection	Incorporates into actin cytoskeleton as functional components	Competes with and disturbs endogenous actin
GFP-actin	Actin monomers	Transient expression	Labels actin filaments in BY-2 cells	Inhibits tobacco pollen tube growth
GFP-mTalin (GFP-mTn)	Actin-binding domain of mouse talin	Transient expression and stable transformation	Decorates prominent actin filaments and bundles in various cell types	Induces rearrangement of actin cytoskeleton and plant growth inhibition
AtFim1-GFP	*Arabidopsis* fimbrin1 protein	Transient expression and stable transformation	Decorate intricate and dynamic cortical networks of actin filaments	Labels few actin filaments in meristem cells and root hairs with a diffuse signal
Plastin-GFP	C terminus of truncated human T-plastin protein	Stable transformation	Labels thick filament bundles in root epidermal cells	Presents a diffuse signal in root hairs
GFP-fABD2 (GFP-ABD2)	Second actin-binding domain of *Arabidopsis* fimbrin1	Transient expression and stable transformation	Decorates fine and dynamic actin filaments in nearly all cell types	Slight effect on motility of organelles and transport of auxin in plant cells
GFP-ABD2-GFP	Two GFP tagged ABD2	Stable transformation	Enhanced quality and ease of F-actin imaging; decorates more details in root meristem and lateral roots	Slight defects in plant growth and inhibition of cell expansion
GFP-NtADF1 GFP-LIADF1	ADF protein from tobacco pollen ADF protein from lily pollen	Transient expression Transient expression	Decorates subtle actin cytoskeleton structures in shanks and subapical regions of pollen tubes	Labels few actin bundles or cables along the shanks except for high expression
GFP-LILIM1	Pollen-specific LIM protein from lily	Transient expression	Decorates fine axial actin cables in pollen tubes	Induces asterisk-shaped actin aggregates, disturbs vesicle trafficking
GFP-NtPLM2b	Pollen-specific LIM protein from tobacco	Transient expression	Decorates fine axial actin cables in pollen tubes and presents no evident dwarfing phenotypes	Labels discernable but not prominent subapical actin structures
GFP-Lifeact	Truncated yeast Abp-140 protein	Transient expression and stable transformation	Labels distinct and highly dynamic actin meshwork in various cell types; small size and absence of homologs in higher organisms	Slight defects in plant growth at high expression level
DsRed-ABPs	Engineered red fluorescent protein	Transient expression and stable transformation	Extends the spectrum of fluorescent tags and provides lower cellular autofluorescence background	Slow protein maturation and a strong tendency to form tetramers
mEosFP-ABPs	Monomeric photoconvertible fluorescent protein	Transient expression	Offers both global and localized color discrimination in a cell through its easy and rapid photoconvertibility	Technical demanding and cell type limitation

oxidation (Nienhaus et al. 2005). EosFP from the scleractinian coral *Lobophyllia hemprichii* is a novel fluorescent protein that switches its fluorescence emission from green (516 nm) to red (581 nm) upon irradiation with ≈400 nm light. The mechanism of that photoconversion is a switch in structure that changes the green chromophore into the red chromophore. This property enables localized tagging of proteins and thus provides a valuable tool for tracking protein movements within live cells (Mizuno et al. 2003; Wiedenmann et al. 2004; Nienhaus et al. 2005). Because the wild-type of EosFP is a tetramer, the relatively smaller EosFP monomer (mEos) is used for merging with other proteins. Schenkel et al. (2008) constructed a fusion gene containing the mEos coding gene and the filament-actin-binding domain of the mammalian talin gene (mEos::FABD-mTalin). When used to label the actin cytoskeleton in onion epidermal cells, mEos::FABD-mTalin presented perfect imaging. Moreover, it can stably exist in multicolor-labeled cells and reveal the interaction of actin filaments with other organelles such as peroxisomes. Compared with GFP-mTalin and GFP-fABD2, mEosFP::FABD-mTalin is potentially the most versatile probe, offering both global and localized color discrimination in a cell through its easy and rapid photoconvertibility.

The properties of all actin probes used to date are summarized in Table 1.

Summary and outlook

The structure and dynamics of actin in living plant cells are complicated and change over time. Effective probing techniques should reflect these changes sensitively without affecting the process. Although it has several limitations, probing based on microinjection of fluorescent phalloidin or actin analogs can be applied in those plants or plant cells for which transformation technologies have not been established.

GFP and other fluorescent proteins provide powerful tools for actin filament labeling and give new insights into the rules of actin distribution and its dynamics in living cells. However, there remains the inevitable problem that whenever we label in vivo, we will always interfere with the biology of the labeled structures. As a central organelle, the actin cytoskeleton interacts with numerous molecules that regulate its dynamics and signaling. Any marker will interfere with those biological processes by occupying the limited binding sites along the actin cytoskeleton. If those binding sites are saturated with markers, they will not bind the factors that regulate actin structure, and therefore, the markers will interfere with the function of the visualized structures. Since we cannot escape this fundamental limitation, we can only try to minimize it by increasing the sensitivity of our observations and by decreasing the expression level of the markers.

Although many methods based on fluorescent proteins have been established, it is difficult to demonstrate which one has the best labeling efficiency and universality. Therefore, existing markers should be compared on the basis of their suitability for each cell type and their expression level in live cells. The most informative images of actin structure will be obtained by selecting the most appropriate probe for each cell type and system.

Acknowledgments This work was supported by the National Natural Science Foundation of China (Grant No. 30325005, 30870211, 30970174 to H.Y.R.) and the National Basic Research Program of China (Grant No. 2007CB108700, 2006CB100100 to H. Y.R.). The authors have no conflicts of interest to declare.

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