Visualization and analysis of actin cytoskeleton organization in plants

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Abstract

The plant cytoskeleton is a highly dynamic system that consists of two components: microfilaments and microtubules. Actin microfilaments are essential for polar growth, cytoplasmic streaming, directing polar growth, anchoring the nucleus, gravity sensing, signalling pathway integration and a number of other functions. Actin morphology and dynamics are orchestrated by a variety of small actin binding proteins, and some of them have become a source of actin interaction domains widely used as markers for microfilaments in fusions with fluorescent reporter proteins. However, older techniques are still employed for actin visualization. In this short review, we will focus on the diversity of fluorescent reporter fusions for F-actin and on approaches and existing free software for the analysis of cytoskeleton organization, mainly in *Arabidopsis*.

Keywords: cytoskeleton, actin, microfilament, plants, cell biology, visualization, microscopy, fluorescent protein, fluorescent dye.

Introduction

Every plant cell includes a number of different organelles, such as a nucleus, an endoplasmic reticulum, ribosomes, a Golgi apparatus, plastids, mitochondria, etc. However, these are not simply contained in the cellular envelope but are suspended in a network of fine filamentous structures — the cytoskeleton. The plant cytoskeleton consists of two main components, both built from protein polymers: *microfilaments* built from actin, and *microtubules* built from tubulin. The existence of *intermediate filaments* in plants is doubtful except for nuclear lamins, which exist but have sequences distinct from their animal analogues (Menzel, 1993). Microtubules (MTs) and microfilaments (MFs) are among the key elements of cytoarchitecture that define polarity in the plant cell (Medvedev, 2012) and direct the development of plant cell shape (Mathur, 2004; Klyachko, 2004). Microtubules and microfilaments feature intrinsic polarity since these are built from polymers of asymmetrical protein momomers. Although the very term 'cytoskeleton' suggests a rigid and stable thing, in reality the plant cytoskeleton is equal to the animal one in its dynamics (Steinborn, 2002; Paradez, Wright, and Ehrhardt, 2006). Both MTs and MFs are highly dynamic in vivo and are constantly rebuilt and rearranged. Furthermore, microtubule and microfilament subsets of the cytoskeleton mutually interact (Shevchenko, Kalinina, and Kordyum, 2007; Sampathkumar et al., 2011). However, functions of MTs and MFs differ in plant cells — MTs are involved in the formation of the mitotic spindle, cell shape establishment on par with MFs, and cell wall organization (Steinborn et al., 2002; Paradez, Wright, and Ehrhardt, 2006; Gutierrez et al., 2009; Sampathkumar et al., 2011). The actin microfilament network is essential for polar growth and intra-

cellular communication: MFs mediate vesicle transport, cytoplasmic streaming, extension growth, anchoring of the nucleus, orientation of the cell division plane, gravity sensing, signalling of pathway integration, defense signalling, etc. (Baluška et al., 2001; Ketelaar et al., 2002; Klyachko, 2004; Bannigan and Baskin, 2005; Blancaflor, Wang, and Motes, 2006; Morita, 2010; Yoo et al., 2012; Blancaflor, 2013; Janda, Matoušková, Burketová, and Valentová, 2014). The actin cytoskeleton could be considered as a finer and more dynamic structure than MTs. In this review we will focus on MF organization, visualization approaches and the analysis of its organization.

Actin cytoskeleton organization

Microfilaments are ≈ 7–8 nm thick (Egelman, 1985; Holmes, Popp, Gebhard, and Kabsch, 1990) and are built from dimers of actin, which is found in the cytoplasm in two states, globular G-actin and polymeric F-actin, concentrations of which are in dynamic equilibrium. Microfilament is found in a steady state, at which the dissociation of G-actin from the "−" end and G-actin attachment to the "+" end are balanced (Staiger, Gibbon, Kovar, and Zonia, 1997; Blanchoin et al., 2010).

The actin cytoskeleton organization is vital for the establishment of cell shape, and its disruption leads to an irregular trichome shape, curvy root hairs, epidermal cell shape anomalies and an irregular shape of the hypocotyl. MFs carry the cytoplasmic strands and are responsible for cyclosis (Valster et al., 1997; Ketelaar et al., 2002). In the stele, root hairs and trichomes, actin microfilaments are assembled into thick bundles oriented along the cell's long axis. Thin actin microfilaments end next to the membranes and make contact with organelles (Ketelaar and Emons, 2001). Apical meristem cells lack thick bundles; their nuclei are located in the centre and are surrounded with a dense network of thin actin microfilaments. In the epiderm, a developed thin actin network underlies the plasma membrane (Mathur, J., Mathur, N., Kernebeck, and Hülskamp, 2003). Similarly, in root cap statocytes, the actin cytoskeleton lacks a developed fine MF network and is composed of short F-actin strands found near the plasma membrane or around organelles (Baluška and Hasenstein, 1997). The *Arabidopsis* genome consists of 10 genes of actin, and the majority of vegetative cells express ACT2 (Meagher and Fechheimer, 2003). However, various types of actin organization and its dynamics rely on a number of actin-binding proteins.

Actin microfilament branching at the angle of 70° is facilitated by the **ARP2/3 complex**. It is essential for the development of trichome shape and epidermal cell lobes (Mathur, 2005). In these cells, the ARPC2 component of ARP2/3 mediates the interaction with microtubules (Havelková et al., 2015). Actin polymerizes into long MFs due to the **formins** (AtFH1) activity. Group I of these actin-binding proteins also enables actin cytoskeleton interaction with the plasma membrane and extracellular signals (Deeks et al., 2005). **Fimbrins** (AtFIM) carry two actin-binding domains (ABD1 and ABD2), and each of them contains an EF-hand Ca^{2+}-binding motif. Therefore, fimbrins are capable of MF cross-linking and inhibit their Ca^{2+}-independent polymerization regulated by profilins (Higaki, Sano, and Hasezawa, 2007). Based on the second actin-binding domain of fimbrin (fABD2), various GFP fusions were developed to enable actin cytoskeleton in vivo visualization (Voigt et al., 2005). **Vilins** belong to the gelsolin family of actin-interacting proteins and facilitate actin bundling into thick bundles (Klahre et al., 2000). **Profilins** are small (12–15 kDa) proteins that prevent the spontaneous polymerization of actin (Valster et al., 1997). **Actin-depolymerizing factors** (ADF) are also small (15–20 kDa) proteins that are active in complex with actin-interacting protein 1, severe MFs and further facilitate actin depolymerisation by making free ends in MFs (Ketelaar et al., 2004; Ketelaar, Anthony, and Hussey, 2004). **Myosins** are various motor proteins that enable organelle movement along the actin cytoskeleton and are essential for vesicular transport, assembly of transvacuolar actin cable, plasmodesmata pore adjustment, chloroplast positioning, and interaction between MTs and MFs (Šamaj, Peters, Volkmann, and Baluška, 2000; Meagher and Fecheimer, 2003; Grebe et al., 2003; Šamaj, Peters, Volkmann, and Baluška, 2006). **Small GTP-binding proteins** (GTPases) are also involved in the regulation of MF morphology in control of vesicular transport, polar growth and the development of complex cell morphology (Valster et al., 2000; Li et al., 2001; Vernoud et al., 2003; Berken et al., 2005; Li, Xu, J., Xu, Z., and Xue, 2005) as well as in reproduction development (Kawashima et al., 2014). ROPs convert external signals to the microfilament branching state by interaction with ARP2/3 (Klyachko, 2004; Xu and Scheres, 2005; Hussey, Ketelaar, and Deeks, 2006; Nagawa et al., 2012).

Rapid and dynamic rearrangement of the actin cytoskeleton is required for a number of cellular processes, e.g., gravitropism (Morita, 2010). For instance, SGR9 (SHOOT GRAVITROPISM 9) E3 ligase mediates the interaction of F-actin with amyloplasts (Nakamura et al., 2011). Vesicles carrying auxin efflux carriers of the PIN family are retargeted in a polar manner to a specific side of the cell membrane (Boutté et al., 2006). In this process of retrograde endocytosis, GNOM (ADP-ribosylation factor — guanine nucleotide exchange factor) acts specifically at the assembly of vesicles containing PIN1 (Geldner et al., 2004), and GNL1 (GNOM-LIKE1) controls the trafficking of PIN2 (Kleine-Vehn et al., 2008). Both GNOM and GNL1 control auxin polar transport and at the same time are auxin-dependent (Dhonukshe et al., 2008; Nick, Han, and An, 2009).

Clearly, various proteins interact with actin and modulate its morphology, dynamics and interaction with cell organelles and compartments. Therefore, molecular probes allowing the visualization of actin MFs, especially in vivo, and software tools facilitating the analysis of cytoskeleton organization, are required for a complete understanding of complex development patterns and responses to external stimuli.
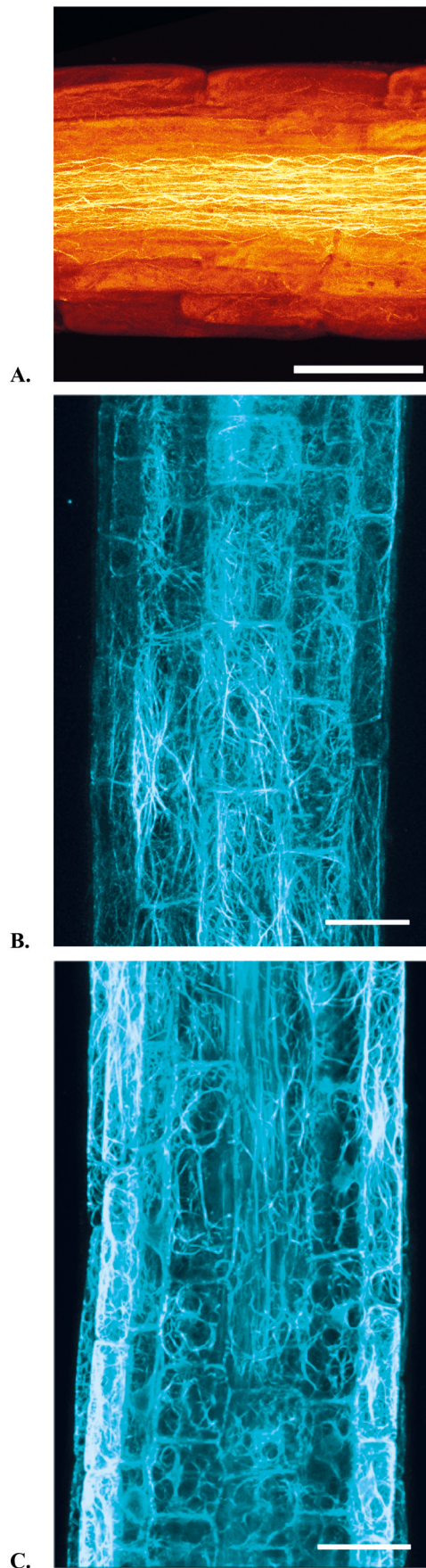
Actin cytoskeleton visualization

Historically, the first methods used to visualize the actin cytoskeleton in plants under an optical microscope were staining of plant tissues with fluorescently labelled actin-binding phallotoxins after chemical fixation (Parthasarathy, Perdue, Witztum, and Alvernaz, 1985; Kakimoto and Shibaoka, 1987; Traas et al., 1987), and direct microinjection of such probes into cells (Cleary, 1995). In contrast to microtubules, which may be visualized by injection of fluorescently labelled tubulin, the applications of G-actin chemically derivatized in the same manner were not successful. However, a natural bicyclic hexapeptide phalloidin (Fig. 1A) from poisonous mushroom *Amanita phalloides* is capable of binding to actin — with a higher affinity to F-actin than to G-actin (Cooper, 1987). Therefore, upon addition to the cytoplasm, phalloidin selectively binds to actin MFs. A fluorescent dye such as rhodamine (Fig. 1B) or fluorescein (Fig. 1C), chemically bound to phalloidin, allows for fluorescent imaging of actin microfilaments in the cell in vivo or after chemical fixation (Fig. 2A). The choice between rhodamine and fluorescein (commonly fluorescein isothiocyanate, FITC) in a particular experiment is based on the fluorophores' excitation/emission properties: 540/565 nm for rhodamine phalloidin, and 496/516 nm for fluorescein phalloidin (Johnson, 2010); the set of available lasers for confocal imaging or filters for fluorescence imaging; as well as possible fluorescence cross-talk between fluorescent F-actin probe and autofluorescence of endogenous cellular structures such as the secondary cell wall. Importantly, phalloidin affects the G-/F-actin dynamic equilibrium (Dancker, Löw, Hasselbach, and Wieland, 1975). Its binding to F-actin stabilizes microfilaments and promotes G-actin polymerization, followed by the depletion of the G-actin pool. Therefore, when using phalloidin conjugates with fluorophores, it is important to keep its concentration in the staining solution minimal (or at least below the threshold level), otherwise the visualization itself may introduce artefacts to MF organization and/or induce the formation of F-actin filaments of abnormal length. Since microinjection is not possible for tissues with small and numerous cells, a chemical fixation step (with formaldehyde and/or paraformaldehyde) is commonly added prior to staining to prevent phalloi-
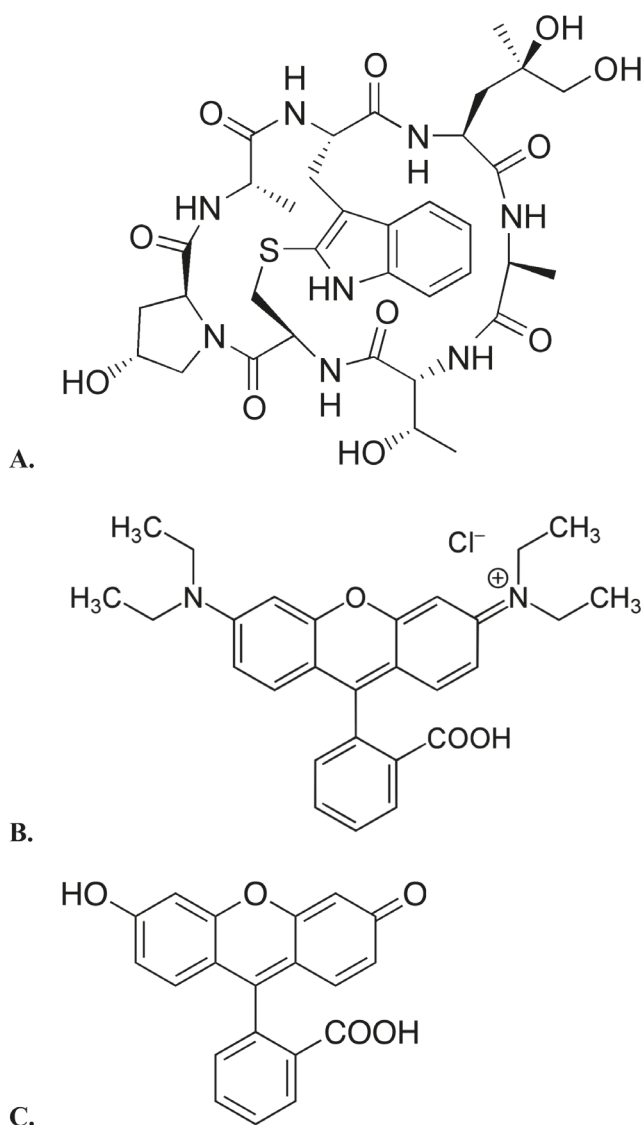
Fig. 1. Chemical structures of phalloidin and fluorescent labels commonly used for F-actin visualization. **A.** Phalloidin from Amanita phalloides. **B.** Rhodamine. **C.** Fluorescein.

din-induced actin polymerization or actin cytoskeleton rearrangement resulting from the staining process. Typically, the sample preparation for MF phalloidin-based visualization includes chemical fixation, permeabilization of membranes with a detergent, and staining with rhodamine-phalloidin, for example (Van Gestel, Le, and Verbelen, 2001). Rhodamine-phalloidin is advantageous for labelling the actin cytoskeleton in plants where genetic transformation is hard or impossible, despite the rather long staining procedure it requires. For plant species in which genetic transformation is hard or unreliable, this method of actin MF visualization is still the only choice: actin has been visualized in lily pollen tubes (Hörmanseder, Obermeyer, and Foissner, 2005), tomato (Humbert et al., 2015), wheat (Khokhlova and Makarova, 2006) and other species.

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Fig. 2. Actin visualization in *Arabidopsis* root elongation zone. **A.** Staining with rhodamine-phalloidin. **B.** GFP-fABD2 reporter. **C.** Lifeact-Venus reporter. Scale bar: A, 50 μm; B,C: 20 μm.

Immunofluorescence labelling of the actin cytoskeleton became an appropriate technique for studying MFs in plant tissues or organs that are too thick or too large in diameter to be observed under a fluorescent or confocal microscope due to emission and fluorescence light absorption (Śniegowska-Świerk, Dubas, and Rapacz, 2015; Dyachok et al., 2016). In immunofluorescence labelling methodology, primary anti-actin antibodies are responsible for the specificity of binding, and secondary antibodies conjugated with fluorescent dye provide effective visualisation in situ. The procedure requires several additional steps, including obtaining tissue sections, cell wall digestion, permeabilization, incubation with primary antibodies, incubation with secondary antibodies, washing and mounting. Consecutively, the procedure of immunolabelling is complicated and may introduce artefacts as well, but for a number of plant objects it serves as the only option available. For an overview and a detailed protocol of visualization with immunofluorescence, please refer to the excellent paper by Dyachok and colleagues (Dyachok et al., 2016).

Any chemical fixative requires several minutes to penetrate the cells and react with protein polymers and halt the dynamic cytoskeleton. To overcome this problem, a cryofixation was developed (Lovy-Wheeler, Wilsen, Baskin, and Hepler, 2005). In this method, a plant specimen is first rapidly frozen in liquid gas such as propane (at –180°C), then freeze-substituted in dry acetone containing anhydrous glutaraldehyde, followed by further rehydratation and immunolabelling. In lily pollen tubes, this approach revealed a fringe of thin actin microfilaments near the pollen tube apex followed by numerous MFs in the pollen tube shank. The apical fringe of actin was otherwise destroyed by conventional chemical fixation methods (Lovy-Wheeler, Wilsen, Baskin, and Hepler, 2005). Then, using this image of the actin cytoskeleton organization as a reference, paper authors adjusted the chemical fixation method to room temperature to keep these pollen tube actin structures intact.

In plants where genetic transformation is well established or transient transformation is possible, a real breakthrough in in vivo cytoskeleton imaging started with the introduction of genetically encoded fusions of fluorescent proteins with natural actin-binding proteins from eukaryotic cells. A chimeric fusion protein composed of GFP (Green fluorescent protein) or its derivatives such as CFP (Cyan-), YFP (Yellow-), mOrange, mCherry, etc., fused with a whole actin-binding protein or its specific actin-binding domain, is constitutively expressed in transgenic plants, thus allowing direct visualization under a fluorescent or laser scanning confocal microscope without any general sample preparation steps (Kost, Spielhofer, and Chua, 1998; Schenkel et al., 2008; Dyachok et al., 2014). In **GFP-mTn** construct,

the C-terminal actin-binding domain of mouse talin is responsible for the association of the probe with actin filaments (Kost, Spielhofer, and Chua, 1998). Unlike the full-length talin, its actin-binding protein does not trigger G-actin nucleation and was considered a robust marker tool. Indeed, colocalization of GFP-mTn-labelled actin with MFs labelled by rhodamine-phalloidin showed significant overlap in transiently transformed tobacco BY-2 cells (Kost, Spielhofer, and Chua, 1998). The introduction of GFP-mTn was encouraging. Its signal was stable and bright, thus facilitating live observation of actin organization and dynamics in transformed cells. Tobacco pollen tubes transiently transformed with *GFP-mTn* revealed fine microfilaments in tube shank, and these pollen tubes featured the same growth rate and morphology as the control. However, it was demonstrated later that in transgenic *GFP-mTn Arabidopsis* lines, GFP-mTalin caused aberration in root hair growth (growth termination, swelling or thickening) and competed with ADF for the binding sites on MFs, thus inhibiting physiological depolymerisation of F-actin by ADF (Ketelaar, Anthony, and Hussey, 2004). The **GFP-FABD2** construct was created to provide an alternative to GFP-mTn. In GFP-FABD2, GFP's C-terminus was fused to the second (C-terminal) F-actin binding domain of fimbrin, an actin-binding protein native to *Arabidopsis* (Sheahan, Staiger, Rose, and McCurdy, 2004; Voigt et al., 2005). This construct was active in different cell types of *Arabidopsis* ranging from root cap columella cells, rhizoderm to epidermal cells (Fig. 2B), mesophyll cells and trichomes, thus allowing research to overcome the limitations of full-length fimbrin fusions to GFP (Voitg et al., 2005). Double GFP — FABD2 fusion protein — **GFP-FABD2-GFP** — features an even stronger signal for actin cytoskeleton visualization (Wang, Yoo, and Blancaflor, 2008). A distinct actin-binding peptide sequence from yeast — **ABP140** (Actin-binding peptide 140) — is another potent marker for actin cytoskeleton visualization. The essential part of ABP140, a peptide containing the first 17 amino acid residues, called **Lifeact**, is sufficient to replicate the F-actin affinity of the full-length ABP140 (Riedl et al., 2008). Lifeact fusions to GFP and, later, to Venus — an improved version of YFP with enhanced brightness and stability — which makes it possible to reveal fast F-actin dynamics in *Arabidopsis* (Fig. 2C) and *Marchantia polymorpha* moss in vivo (Era et al., 2009).

Since all GFP fusions developed to visualize actin in vivo are based on F-actin binding or interacting proteins, one could suspect that they somehow affect the normal physiology of actin microfilaments. Simply, the surface of an actin microfilament is finite, as well as the number of binding sites on it; therefore, if at least some of them are occupied by an effectively binding and constitutively expressed fluorescent protein fusion, the poten-

tial for interactions of actin with its native cytoplasmic binding proteins becomes limited. In addition to that, GFP and its derivatives are capable of generating ROS under continuous light. For instance, drawbacks of GFP-mTn application were already mentioned above. Similarly, other actin-binding fluorescent protein fusions were shown to imply some developmental or physiological alterations in their carrying plant. The **GFP-FABD2-GFP** fusion construct is bright and capable, but its use caused altered embryonal development, shorter and abnormally positioned root hairs and shorter branches in trichomes (Wang, Yoo, and Blancaflor, 2008). **Lifeact-Venus** was widely used for live F-actin observations, and it was generally believed to be safe for the dynamics of actin polymerization and depolymerisation (van der Honing, van Bezouwen, Emons, and Ketelaar, 2011). Surprisingly, Lifeact-Venus was recently demonstrated to interfere with nuclear actin (Du, Fan, Chen, and Feng, 2015). Also, Lifeact-Venus was found to delay actin bundles relocation and to reduce the cytoplasmic strand reorganization rate in epidermal cells of *Arabidopsis* (van der Honing, 2011), and, supporting this, in the same study Lifeact was shown to exchange more rapidly from actin MFs than FABD2. Dyachok et al. studied various protein fusion-based F-actin reporters for their potential impact on *Arabidopsis* development and morphology (2014). Their study showed that in general, FABD2-based fusions were less likely to cause abnormal F-actin bundling, which mTn-fusions were prone to do. Interestingly, the substitution of a strong constitutive 35S promoter with UBQ10 promoter for GFP-FABD2-GFP probe prevented loss of fluorescence in the subsequent generations of transgenic plants and helped to avoid growth inhibitory effects of 35S constructs. The expression level of marker fusion proteins appears to be very important for the correct development of polarly growing cells that have a shape highly dependent on the actin cytoskeleton, such as pollen tubes or root hairs. High expression levels of GFP-talin or GFP-fimbrin in tobacco pollen tubes led to the formation of abnormal transverse cortical hoops of actin, moving rings of F-actin or its large aggregates (Wilsen et al., 2006). Our own data show that, for instance, *GFP-FABD2 Arabidopsis* plants exhibit slightly slower root growth pace but faster gravitropic bending development. Their metabolite profiles also differ from the background ecotype and accumulate higher levels of amino acids while being deficient in fatty acids, monoacyl glycerols and monosaccharides (unpublished data). Therefore, before making conclusions based on fusion fluorescent reporter protein visualization, related physiological parameters should be carefully considered and compared with results obtained by alternative reporters.

In addition to the actin cytoskeleton visualization techniques discussed above, a new and promising approach has recently emerged. Silicone-containing

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rhodamine derivatives such as SiR-methyl and SiR-carboxyl are bright, biocompatible and highly permeable (Lukinavičius et al., 2013). These fluorescent dyes may be easily conjugated with a marker protein or protein of interest, and since their emission spectra lies in near-infrared, they are excellent for super-resolution microscopy techniques. Later, SiR-actin and SiR-tubulin were generated and demonstrated to be effective in animal fibroblasts (Lukinavičius et al., 2014). However, no attempt has yet been made to apply these labels to plant systems.

Analysis of actin cytoskeleton organization

Given that the actin cytoskeleton plays a vital role in a number of physiological and developmental processes at the cellular level, it is important to qualitatively and quantitatively analyse and describe its organization. This includes the abundance of MFs, their preferential localization within the cell, length and angular distribution, thickness or diameter of bundles, branching, the presence and the number of contacts with the plasma membrane, etc. In a very simple approach, microfilaments are detected manually 'by eye' based on a fluorescence or confocal image and counted to provide quantitative data. Software filters such as threshold level in ImageJ (Schneider, Rasband, and Eliceiri, 2012) may provide aid in automated detection of microfilaments. Similarly, the thickness or diameter of actin cables could be measured indirectly in pixels based on a confocal image, and then converted to physical units, e.g., micrometers. This simple approach is robust yet time-consuming and is still employed (Zhang, Colyvas, Patrick, and Offler, 2017). However, sharp confocal images of a reasonable logical size are required for this approach. Predominantly longitudinal microfilaments found in root stele are not straight, however, and for their curvature assessment a simple index of curvature was suggested (Pozhvanov, Suslov, and Medvedev, 2013). This metric assumes that a curved microfilament will intersect a virtual longitudinal line grid more often than a straight one. However, the number of total intersections with longitudinal lines has to be normalized by the overall microfilament abundance in the cell, which is given by the number of intersections with a grid of transversal, or radial lines (Fig. 3):

$$i_{curv.} = \frac{\sum_i n_{axial}}{\sum_j n_{radial}} \qquad (1)$$

In the living cell, the actin cytoskeleton is dynamic; it undergoes rearrangement to adjust to current cellular activity. Using fluorescent protein fusions to actin-binding proteins, MFs are trackable in real time. Captured time-course fluorescence images can be analysed further to quantify the actin cytoskeleton dynamics. This
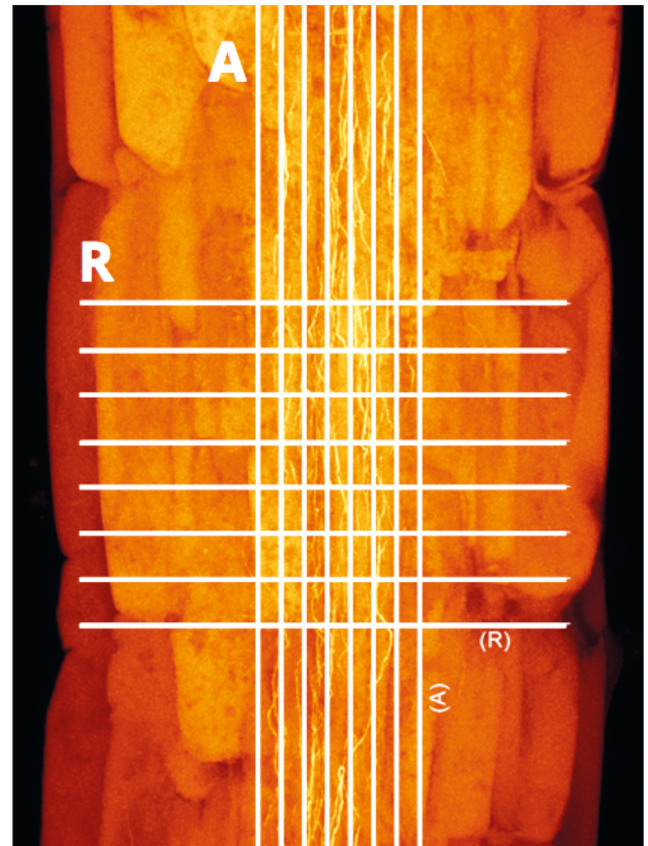
Fig. 3. Calculation of curvature index ($i_{curv.}$) for axially oriented microfilaments in root stele (wild-type *Aranidopsis* root stained with rhodamine-phalloidin). The number of actin microfilament intersections with axial grid lines is normalized by the number of intersections with radial grid lines (density of microfilaments).

type of analysis is commonly performed by automated pixel-to-pixel comparison of numerical fluorescence levels, $f_{x,y}$. If the given microfilament remains stable over time between two sequential key frames, its position in the digital image is the same, hence there is no significant shift in the level of signal: $f_{x,y}(t_2)-f_{x,y}(t_1)=0$. If a microfilament moves over time, the signal is lost at its initial position: $f_{x,y}(t_2)-f_{x,y}(t_1)<0$. Conversely, in case of a microfilament de novo assembly, a signal appears in previously dark pixels: $f_{x,y}(t_2)-f_{x,y}(t_1)>0$. The overall statistics of fluorescence shift gives an overview picture of actin dynamics within the cell (Nick, Han, and An, 2009).

To quantify the angular distribution, length and density of fibrillary structures such as MFs or MTs, a free dedicated software tool called Microfilament Analyzer (MFA) was developed (Jacques et al., 2013). Its analysis workflow is based on the concept of 'rotating polarisator'. Virtual lines at a specific angle are projected on the image, and the luminosity (fluorescence signal) of adjacent pixels is compared along these lines. When the number of sequential pixels with brightness exceeding the threshold level reaches the minimal required length,
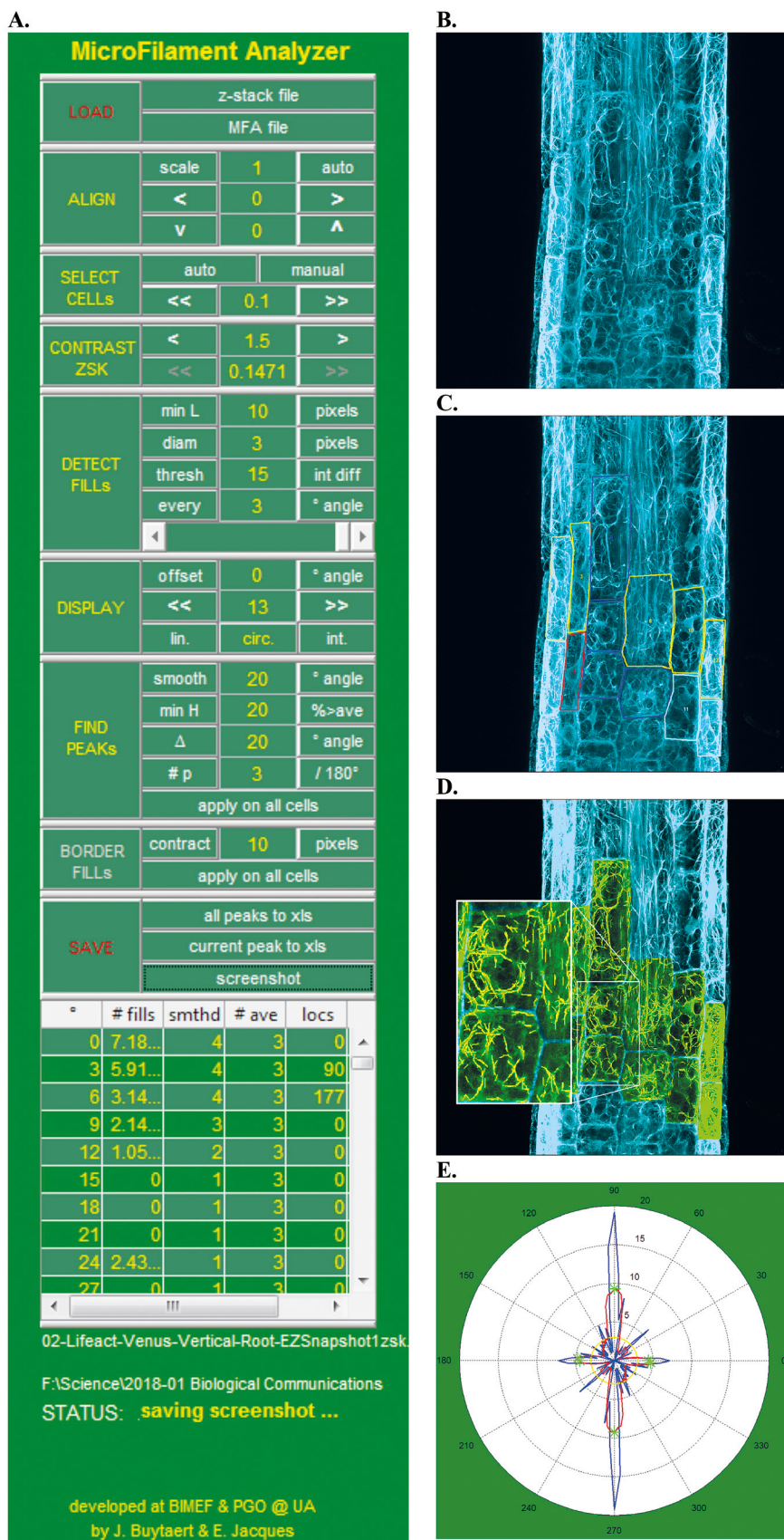
Fig. 4. Actin cytoskeleton analysis in Microfilament Analyzer software tool (Jacques et al., 2013). **A.** Analysis workflow: load z-stack file, adjust contrast for detection, apply settings for automatic filament detection, display analysis results. **B.** Image of *Lifeact-Venus* line of *Arabidopsis thaliana* loaded for analysis. **C.** Manual selecton of cell boundaries. **D.** Automatically detected microfilaments are highlighted in yellow. Inset: enlarged view of filaments detected in cells 5–6. **E.** Analysis results: microfilament angular distribution in a given cell.

these count as a segment of microfilament of a specific length and orientation. After the detection of microfilaments oriented at a specific angle is completed, the virtual grid is rotated in a given angular step, and the detection process repeats (Fig. 4). MFA allows for the adjustment of image contrast, manual or automated cell boundary detection (based on image mask or other fluorescence channel, i.e., propidium iodide), and minimal length of MFs; their minimal thickness, threshold value and angular step are also adjustable parameters in the resolution of an image being analysed. The output of MFA is full statistics of MF angular distribution, number of filaments per cell, length of each detected filament, and the detection of predominant MF orientation angles. These statistics may be further classified or analysed to give numerical features of cytoskeleton rearrangement under stress or environmental response (Pozhvanov, Suslov, and Medvedev, 2016). The drawbacks of the current MFA version are its inability to batch process image and its operating system restriction (PC only).

An approach similar to MFA was introduced by Matoušková et al. (2014) to analyse actin cytoskeleton involvement in salicylate signalling. Confocal images were batch processed in ImageJ using a set of image masks of a given angular sector as low pass FFT filter for the detection of filamentous structures within the image (Matoušková et al., 2014, S1). The resulting images were converted to binary and subjected to particle analysis, resulting in statistics of length and number of filaments of a given angle.

Currently, a wide range of approaches and tools are available for various analyses of cytoskeleton organization and dynamics, and a number of new open source software will possibly emerge in the near future to describe and quantify the complex actin cytoskeleton features such as branching and interactions with actin-binding proteins or membranes.

Conclusions

The plant cytoskeleton is a dynamic system that is involved in various processes, ranging from cell housekeeping and division to complex responses to environmental stimuli. Over the past two decades, a number of visualization tools have been developed that literally cover the visible spectrum for fluorescence colour and allow us to reveal in vivo the structure and dynamics of the cytoskeleton using fluorescence and confocal microscopy. Since fluorescent protein fusion reporters utilize actin binding domains in their marker part, corresponding transgenic lines should be carefully checked for developmental and/or morphological anomalies. For plant objects unsuitable for transformation, other visualization methodology still exists, including staining with phalloidin-coupled dyes or immunofluorescence labelling. A number of software tools and approaches to quantify the cytoskeleton organization have been developed that allow for characterization of cytoskeleton rearrangements.

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