PLANT signaling & behavior

Plant Signaling & Behavior

ISSN: (Print) 1559-2324 (Online) Journal homepage: https://www.tandfonline.com/loi/kpsb20

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To cite this article: Ján Jásik, Karol Mičieta, Wei Siao, Boris Voigt, Stanislav Stuchlík, Elmon Schmelzer, Ján Turňa & František Baluška (2016) *Actin3* promoter reveals undulating F-actin bundles at shanks and dynamic F-actin meshworks at tips of tip-growing pollen tubes, Plant Signaling & Behavior, 11:3, e1146845, DOI: 10.1080/15592324.2016.1146845

To link to this article: https://doi.org/10.1080/15592324.2016.1146845

PLANT SIGNALING & BEHAVIOR
2016, VOL. 11, NO. 3, e1146845 (4 pages)
http://dx.doi.org/10.1080/15592324.2016.1146845

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SHORT COMMUNICATION

Actin3 promoter reveals undulating F-actin bundles at shanks and dynamic F-actin meshworks at tips of tip-growing pollen tubes

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ABSTRACT
The dynamic actin cytoskeleton of pollen tubes is both the driver of the tip growth and the organizer of cell polarity. In order to understand this fast re-arranging cytoskeletal system, we need reliable constructs expressed under relevant promoters. Here we are reporting that the Lifeact reporter, expressed under the pollen-specific *Actin3* promoter, visualizes very dynamic F-actin elements both in germinating pollen grains and tip-growing pollen tubes. Importantly, we have documented very active actin polymerization at the cell periphery, especially in the bulging area during pollen germination and in the apical clear zone. Expression of the Lifeact reporter under control of the pollen-specific *Actin3* promoter revealed 2 new aspects: (i) long F-actin bundles in pollen tube shanks are dynamic, showing undulating movements, (ii) subapical 'actin collars' or 'fringes' are absent.

ARTICLE HISTORY
Received 7 December 2015
Revised 17 January 2016
Accepted 19 January 2016

KEYWORDS
Actin cytoskeleton; *actin3* promoter; lifeact construct; polarity; pollen tubes

Organization of F-actin in polarly growing pollen tubes, especially, at their growing tips is still not understood. The problem is that all the methods, published and used so far, are having some drawbacks. In order to understand the dynamic nature of the actin cytoskeleton, we need reliable constructs driven via relevant promoters. For example, clearly improved distributions of the actin cytoskeleton elements have been achieved in the ABD2 Arabidopsis reporter lines by replacing the strong and constitutive 35S promoter with *Ubiquitin10* (*Ubq10*) promoter.[1]

Moreover, also nature of the reporter construct affects the F-actin distributions. In Arabidopsis, the first actin-specific construct was the *35S::GFP-Talin* which often shows intense diffuse cytoplasmic labeling especially prominent at root hair tips.[2,3] Later, Fimbrin 1 Actin Binding Domain 2 (ABD2) was selected to generate the *35S::GFP-ABD2* transgenic lines cells of which showed F-actin distributions closer to the native situation.[4-7] However, the *35S::GFP-ABD2* has the tendency to over-bundle F-actin bundles and, moreover, the constitutive *35S* promoter can cause aberrant expression levels.[1] The Lifeact is the newest one in this series of F-actin constructs, and is based on a 17 amino acid sequence derived from budding yeast actin-binding protein Abp140.[8-17] But also this newest reporter is showing some aberrant actin organization if its expression is driven by the cauliflower mosaic virus *35S* promoter.[1] In order, to avoid these problems and uncertainties, it is essential to express these constructs under the proper endogenous promoters. In this Short Communication, we have tested 3 pollen-specific promoters and have chosen the *Actin3* promoter for expression of the Lifeact-GFP in pollen grains / tubes of Arabidopsis.

From three pollen-specific promoters tested: *Synaptotagmin2* (*Syt2*), *Armadillo Repeat Only 1* (*ARO1*), and *Actin 3* (*Act3*), the last one was selected for expression of the Lifeact-GFP fusion. Transgenic plants with the Lifeact-GFP driven by the *Syt2* promoter showed no or minimal signal, plants with the *ARO1* promoter showed intermediate expression, whereas the *pACT3::Lifeact-GFP* transgenic plants exhibited sufficient fluorescence of F-actin and similar strength of the promoter through the investigated transgenic lines. *ARO1* promoter was active also in somatic cells of the carpel in some transgenic lines. The low activity of the *Syt2* promoter is in agreement also with the Arabidopsis microarray database (https://www.genevestigator.ethz.ch/[18]), and with our recent study.[19] As the *Act3* promoter is specific only for pollen, and showed the highest strength among studied promoters, we have chosen *pACT3::Lifeact-GFP* lines for our investigations.

Shortly after placing pollen on the germination medium, they showed irregular shaped short and thick F-actin ribbons or rings randomly distributed throughout the pollen grains (Fig. 1A, Movie 1). At the beginning of pollen germination, randomly arranged F-actin networks are more obvious and located mainly at the pollen grain periphery (Figs. B, C – right image). In the central zone of pollen grain, F-actin filaments

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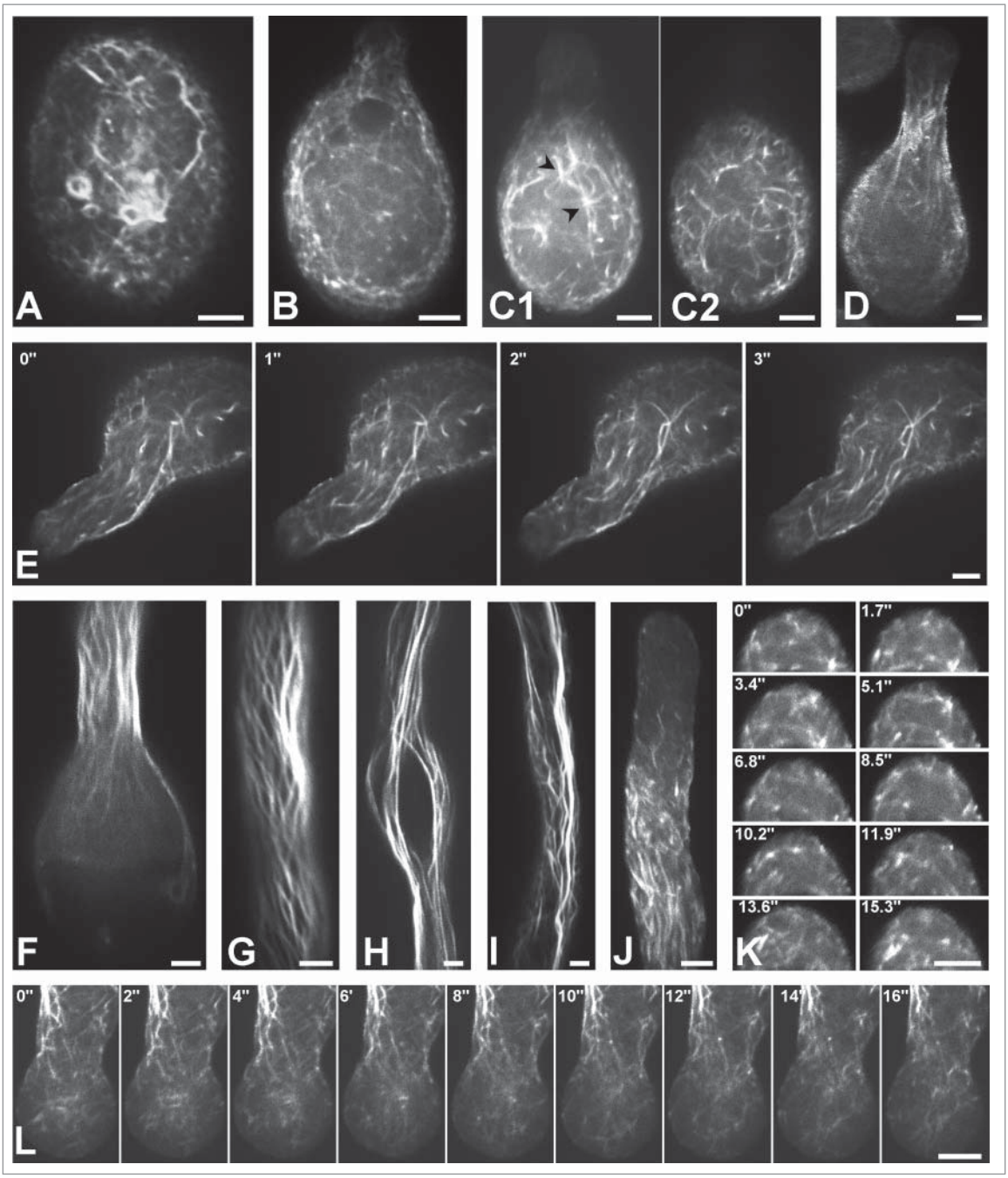

Figure 1. F-Actin arrays in the germinating pollen grains and in the tip-growing pollen tubes. A/ F-actin forms sparse meshworks and thick ribbons or rings in pollen grains before their germination. B/ Early germinated pollen grain showing the F-actin network mainly at its periphery. C/ Early germinated pollen grains. The left image is Z projection of 5 serial slices through central zone of grain showing stellate F-actin bundles, the right image represents Z projection of 5 slices taken from grain periphery and shows randomly arranged actin network. D/ F-actin bundles have tendency to be oriented along the emerging tube when pollen tubes reach about the length of the pollen grain. E/ Quicly changing actin pattern in the grain of similar stage as in D. Images were taken from Movie 3. F/ F-actin cables emerging from tubes seems to end bluntly in pollen grain. G/ Dense actin network at the pollen tube periphery in a well developed pollen tube. H and I/ F-actin cables of different thickness in the central part of the pollen tube. J/ Pollen tube tip. Notice F-actin cables ending bluntly in the tip and short F-actin profiles within the tip. The images represent a projection of 60 Z stacks of longitudinal slices covering whole volume of the tube. K/ Dynamic rearrangements of F-actin elements within the pollen tube tip. L/ Dynamic F-actin meshworks in the pollen tube tip. Notice absence of longitudinally arranged F-actin filaments (actin collars) and dynamic rearrangements of F-actin arrays (in seconds). In C1, the "stellate F-actin" arrangements are marked with arrowheads. Bars = 5 μm.

show a tendency to form stellate F-actin bundles (Fig. 1 C left image). At the grain pole, with the emerged bulge, the F-actin network is less prominent and formed predominantly by short and randomly oriented F-actin bundles (Figs. 1B-C, Movie 2). In very short pollen tubes, F-actin still forms tiny and numerous bundles at the periphery, but also within the bulge (Figs. 1B-C). When pollen tubes reach about the length of the pollen grain, F-actin bundles are still short but they have the tendency to be oriented along the emerging tube (Fig 1D). At the same time they are very dynamic, changing their positions within seconds (Fig. 1E). Time lapse imaging experiments showed very quick movements of F-actin bundles within tubes and these movements are more dynamic than the movement of F-actin bundles in the rest of pollen grains (Movie 3). As the

pollen tubes grow further, the longitudinal F-actin cables became very prominent. They are ending bluntly within pollen grains (Fig. 1F, Movie 4). In fast growing pollen tubes, F-actin assembles into dense dynamic networks at the pollen tube periphery (Fig. 1G). More centrally F-actin formed longitudinally oriented cables of different thickness (Fig. 1H-I). F-actin bundles are very dynamic throughout the pollen tubes, they are branching and moving (Movie 5, Movie 6, Movie 7). On the opposite site, near the pollen tube tip, F-actin cables also finish bluntly and actin becomes visible as short bundles (Fig. 1J). In the pollen tube tips, there are numerous short and very dynamic F-actin elements organized as dense, rapidly moving meshworks (Fig. 1K, Movie 8). Importantly, however, we have never observed so-called 'actin collars' or 'actin fringes' formed by longitudinally oriented actin profiles closely behind the tube tips (Fig. 1L, Movie 8).

Recently, better properties of the *UBQ10::GFP-ABD2-GFP* construct in the visualization of F-actin arrays in root cells and root hairs were associated with lower expression levels of this construct in cells.[1] Similarly, lower expression levels of the Lifeact construct driven under the moderate *Aro1* promoter resulted in new aspects of pollen tube actin cytoskeleton, not visible when the strong *35S* and *Lat52* promoters were used.[9,15-17] Moreover, so-called 'actin collars' behind tips of pollen tubes are visible only if the Lifeact construct is expressed under the strong *Lat52* and *35S* promoters[10,14] but not when the Lifeact is expressed under the moderately strong *Act3* promoter (this study). At any stage of the pollen tube germination and the tip-growth, no trace of 'actin collar' or 'actin fringe'[9,14-17,20-27] could be detected behind the clear zone if the *Act3* promoter is used for the Lifeact expression. Moreover, spinning disk confocal microscopy demonstrated that F-actin elements in the tube tip are fastly moving, and arrangement of F-actin is changing quickly. Finally, we have observed many dynamically moving F-actin profiles also within the clear zone. These obervations suggest that it is very important not only to use the endogenous promoters but also to choose those promoters which have proper expression levels. This conclusion is supported by the visualization of the 'actin fringes' also with the phalloidin labeling.[23,26] We have also confirmed the F-actin circles of variable size (Fig. 1A) within pollen grains, reported recently using the Lifeact expressed under the *ARO1* promoter.[16,17] The most surprising observation of the spinning disc microscopy of the *pACT3::Lifeact-GFP* pollen tubes are the wavelike undulating movements of long F-actin cables (Movies 5, 6 and 7). It emerges that both the subapical 'actin fringes' and the static F-actin bundles are side effects of the actin overpolymerization. However, more studies are needed to settle this issue finally.

When the Lifeact is expressed under the *Act3* promoter, very prominent feature is the dynamic nature of the undulating thinner F-actin bundles, while the thicker F-actin cables were more stable (Movies 5, 6 and 7). Some lateral movements of F-actin cables were reported also for tip-growing root hairs expressing the Lifeact under the *35S* promoter.[10] These findings suggest that F-actin cables of tip-growing pollen tubes and root hairs are more dynamic than it is generally assumed. Their undulating movements might have a role in the actin polymerization-driven tip growth of pollen tubes[27] and root hairs.[28,29] Interesting, dynamic F-actin bundles are important for the penetration of phytopathogenic fungi into plant tissues and cells.[30] Longitudinal F-actin cables are generally considered to serve only as tracks for cytoplasmic streaming in tip-growing cells. However, they might serve also as 'pushing devices', contributing to the invasion of the pollen tubes into pistil tissues.[31-33] Future studies should focus on these undulating F-actin bundles and their roles in pollen tube growing through pistil tissues during plant sexual reproduction.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

J.J., K.M., S.S. and J.T. were supported by the Research and Development Operational Program "Comenius University Science Park" (ERDF, ITMS 26240220086). K.M. and J.T. were also supported by the VEGA 1/0885/16 research grant. W.S. and F.B. were supported by German Academic Exchange Service (DAAD).

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