F-actin forms mobile and unwinding ring-shaped structures in germinating *Arabidopsis* pollen expressing Lifeact

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The flowering plant pollen tube is the fastest elongating plant cell and transports the sperm cells for double fertilization. The highly dynamic formation and reorganization of the actin cytoskeleton is essential for pollen germination and pollen tube growth. To drive pollen-specific expression of fluorescent marker proteins, commonly the strong *Lat52* promoter is used. Here we show by quantitative fluorescent analysis that the gametophyte-specific *ARO1* promoter from *Arabidopsis* drives an about 3.5 times weaker transgene expression than the *Lat52* promoter. In one third of the pollen of F-actin-labeled *ARO1p:tagRFP-T-Lifeact* transgenic lines we observed mobile ring-shaped actin structures in pollen grains and pollen tubes. Pollen tube growth, transgene transmission and seed production were not affected by tagRFP-T-Lifeact expression. F-actin rings were able to integrate into emerging actin filaments and they may reflect a particular physiological state of the pollen or a readily available storage form provided for rapid actin network remodeling.

With a growth speed up to 1 cm/h the pollen tubes of flowering plants are the fastest elongating plant cells and they can reach up to 30 cm in length. Their enormous cell elongation is depending on a well-organized and highly dynamic actin cytoskeleton that organizes in thick longitudinal actin bundles in the shank, as a dense subapical actin fringe and as a highly dynamic pool of short actin filaments at the very tip of the growing tube. Recently, we reported that the actin cytoskeleton undergoes characteristic reorganizations during pollen rehydration and germination. Our time-lapse live imaging of F-actin in in vitro germinating pollen revealed the accumulation of F-actin at the periphery of the pollen grain vegetative cell opposite to the future germination site and the massive assembly of longitudinal actin bundles at the germination site of the pollen tube when rapid tip growth is initiated. To visualize F-actin in in vitro germinating *Arabidopsis* pollen, we employed the first 17 amino acid N-terminal sequence of the yeast Abp140 protein, termed Lifeact, and fused it to the highly photostable monomeric tagRFP-T fluorophore. To drive the expression of marker proteins in pollen, usually the strong pollen-specific *Lat52* promoter is used. However, as the strong overexpression of fluorescent marker proteins can cause problems and evoke artifacts, we intended to use a weaker promoter to drive the actin marker expression in pollen grains and pollen tubes.

To quantitatively assess the strength of the commonly used *Lat52* promoter and the gametophyte specific *ARO1* promoter, we used both promoters to drive the expression of an *ARO1-GFP* fusion protein and quantitatively compared the GFP fluorescence intensities of 30 pollen grains each, derived from transgenic lines homozygous for *Lat52p:ARO1-GFP* and *ARO1p:ARO1-GFP*, respectively (Figs. 1A–B). We found that the *ARO1* promoter is about 3.5 times weaker than the *Lat52* promoter and therefore it is well suited to achieve moderate levels of transgene expression in pollen.

To date, Lifeact is considered as the marker of choice to visualize pollen tube F-actin and it has already been employed...
to study the actin cytoskeleton in a wide range of plant species and cell types. As it has been demonstrated that high expression levels of Lifeact can reduce the dynamic reorganization of F-actin in Arabidopsis root epidermal cells, we used the ARO1 promoter to drive moderate expression of tagRFP-T-Lifeact in pollen and evaluated whether the presence of the tagRFP-T-Lifeact fusion protein reduces plant fertility. We were able to generate stable homozygous lines and did not observe a reduction in seed set in self-pollination experiments (Fig. 1C). This indicates no significant negative effect of marker expression on in vivo pollen tube growth. Moreover, in vitro pollen tube growth rates were not affected.

Notably, we observed quoit-like F-actin structures in the cytoplasm of 33% of all ARO1p:tagRFP-T-Lifeact pollen analyzed by live cell imaging (Fig. 2). These F-actin structures were of variable size (1.42 to 4.33 μm) with a mean diameter of 2.92 ± 0.15 μm and they moved, both inside the rehydrating pollen grain and the growing pollen tube. The number of ring-shaped actin bundles per pollen vegetative cell was generally low: 84.2% of the pollen with circular actin bundles contained 1 to 3 rings. In two exceptional cases we observed 10 and 12 F-actin rings per cell. Live cell imaging revealed that the ring-shaped F-actin is able to dynamically integrate into existing actin filaments during pollen germination and rapid tip growth (Figs. 2A and B).

In plant cells, circular conformations of actin filaments have been observed in several studies using either fixation and staining techniques or by expressing fluorescent protein-tagged actin-binding domains or proteins such as WLIM1-GFP, GFP-Talin, GFP-PLIM2a and GFP-PLIM2b, Lifeact-Venus, GFP-Lifeact, or GFP-fABD. Strong ubiquitous (such as CaMV35S) or strong cell-type specific (such as Lat52 for pollen tubes) promoters have been used to drive expression of fluorescently tagged actin binding domains or proteins. Overexpression-induced artifacts due to the competition with native actin binding proteins and artifacts because of fixation and staining were discussed as an explanation for the occurrence of coiled-like actin filaments.

However, the appearance of ring-shaped actin bundles in pollen expressing moderate levels of tagRFP-T-Lifeact (Fig. 1) do not attribute these structures to strong overexpression but rather suggests them to be cytoskeletal components that occur either at a certain physiological state or when the pollen prepares for a rapid reorganization of the microfilament network. The fact that we observed the circular conformations of actin filaments only in every third pollen grain or tube nevertheless implicates that they do not play an essential role in the organization of the actin cytoskeleton, or in rapid actin network remodeling.

Actin quoit-like structures, termed ‘acquosomes’, have also been described in transgenic tobacco BY-2 cell lines and in tissues of transgenic Arabidopsis plants expressing GFP-Lifeact and GFP-fABD. Acquosome diameters were variable, with 3.5 ± 1.2 μm in BY-2 cells and 1.6 ± 0.3 μm in root hairs and frequencies were highest in actively dividing BY-2 cells cultivated in the presence of 2,4-dichlorophenoxy acetic acid (2,4-D). The number of acquosomes was reduced by 98.9% after cells were cultured in 2,4-D-free medium,

Figure 1. Expression of the ARO1-GFP fusion protein in Arabidopsis pollen is significantly lower under control of the ARO1 promoter compared to the commonly used Lat52 promoter. (A) Pollen grains from plants homozygous for ARO1p:ARO1-GFP and Lat52p:ARO1-GFP, imaged under identical conditions and microscope settings. Images are displayed with identical brightness and contrast settings using the smart LUT in ImageJ. GFP fluorescence is false-colored according to the signal intensities (from low to high: purple-blue-white-green-red). (B) Signal quantification of 30 pollen grains per homozygous line reveals that expression driven by the Lat52 promoter is approximately 3.5 times stronger than under the ARO1 promoter. (C) Plants homozygous for the ARO1p:tagRFP-T-Lifeact construct display full seed set. Bar = 5 μm in (A) and 1 mm in (C).
suggesting a positive correlation between the formation of F-actin rings and cell proliferation.\textsuperscript{16} After fixation and staining of the actin cytoskeleton, the formation of actin coils or rings with a mean diameter of 1.7 µm was occasionally observed in heterotrophic, chloroplast-free cell cultures of \textit{Chenopodium rubrum} and their number significantly increased by heat shock at 37°C.\textsuperscript{18} The authors attributed the formation of actin rings to stress conditions such as temperature or extracellular pH, as well as to the availability of Ca\textsuperscript{2+}-ions. The use of ion transport affecting drugs furthermore implied the involvement of antiporters for monovalent cations in the formation of actin rings.\textsuperscript{18}

Pollen released from the anther of a flowering plant germinates on the receptive papillae of the stigma and the pollen tube grows through the female reproductive tissues to fertilize the ovule. It is evident that flowering is a very sensitive developmental stage, as environmental stress at microsporogenesis and anthesis dramatically reduces anther dehiscence, pollen viability, pollen germination and pollen tube growth rates.\textsuperscript{22} In vitro pollen germination assays it has been observed that pollen germination rates, pollen tube growth rates, and final tube lengths are far lower than those observed in planta. Especially for \textit{Arabidopsis}, the pollen quality can highly vary among different plants and even among flowers from the same plant\textsuperscript{23,24} It is therefore conceivable that ring-shaped F-actin structures form intermittently in vitro pollen germination assays under adverse conditions, but are able to come reintegrated into emerging actin filaments (Figs. 2A-B). It may, however, be possible that the appearance of ring-shaped F-actin in germinating pollen indicate a certain physiological state of the cell that is not restricted to in vitro conditions. Live imaging of F-actin dynamics of in vivo germinating pollen will be necessary to address the question whether F-actin rings have a transient physiological function in germinating pollen.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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