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Formins control dynamics of F-actin in the central cell of *Arabidopsis thaliana*.

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Abstract:

In the female gamete of flowering plants, sperm nuclear migration is controlled by a constant inward movement of actin filaments (F-actin) for successful fertilization. This dynamic F-actin movement is ARP2/3-independent, raising the question of how actin nucleation and polymerization is controlled in the female gamete. Using confocal microscopy live-cell imaging in combination with a pharmacological approach, we assessed the involvement of another group of actin nucleators, formins, in F-actin inward movement in the central cell of *Arabidopsis thaliana*. We identify that the inhibition of the formin function, by formins inhibitor SMIFH2, significantly reduced the dynamic inward movement of F-actin in the central cell, indicating that formins play a major role in actin nucleation required for F-actin inward movement in the central cell.

Keywords: Formin, SMIFH2, Fertilization, Female gamete, and F-actin.

Fertilization consists of a series of steps to blend parental genomes for the initiation of the next generation.[1–3] In most animals, after the gamete fusion, both male and female pronuclei move toward each other within the fertilized egg for nuclear fusion. The movement of pronuclei is regulated by microtubules that assemble the sperm aster from the centrosome.[4,5] Unlike animals, flowering plants have lost the centrosome, and instead, have established a filamentous actin (F-actin) based sperm nuclear migration system for successful double fertilization.[6–9] Prior to fertilization, the female gamete forms a mesh-like structure of F-actin that shows constant inward movement from the plasma membrane periphery to the center of the cell where the female gamete nucleus resides (Fig. 1A).[6,10] The movement of the sperm nucleus coincides with the inward F-actin movement[6], and this F-actin movement is required for sperm nuclear migration in *Arabidopsis thaliana* (Arabidopsis), *Oryza sativa* (rice), *Nicotiana tabacum* (tobacco), and *Zea mays* (maize).[6,8,10]

In Arabidopsis, a member of the plant-specific Rho-GTPases family, ROP8, the Wiskott–Aldrich syndrome protein family verprolin-homologous/suppressor of cAMP receptor (WAVE/SCAR) complex protein, SCAR2, and the plant-specific class XI myosin, XI-G, play positive roles in the constant inward F-actin movement in the central cell for fertilization.[6,11] ROPs at the plasma membrane interact with SCARs[12], relaying the signal to the actin nucleator ACTIN RELATED PROTEIN 2/3 (ARP2/3) for F-actin dynamics in somatic cells such as trichomes and cotyledon pavement cells.[13,14] Contrary to the situation in somatic cells, neither the application of ARP2/3 inhibitor CK-666, nor ARP2/3 complex mutants such as *arp2-1* (*ARP2*), *dis2-1* (*ARPC2*), and *arpc4-t2* (*ARPC4*), affects the F-actin dynamics in the central cell.[11] These results raised the question of what actin nucleator is involved in this dynamic F-actin movement in the central cell for fertilization. In plants, there are other actin nucleators, named formins. Formins contain the conserved formin-homology (FH) domains, FH1 and FH2, and the FH2 domain is the active domain for actin nucleation.[15] In this experiment, to identify the involvement of formins in the female gamete F-actin inward movement for sperm nuclear migration, we performed pharmacological analyses in the Arabidopsis central cell. The small molecule SMIFH2 is an effective inhibitor of the FH2 domain both in animals and plants[16–19], and reduces actin nucleation and assembly.[17,18,20]

We investigated the effect of a dose series of 10, 20, and 50 µM SMIFH2, on F-actin dynamics in the Arabidopsis central cell, expressing the F-actin marker *(proFWA::Lifeact-Venus)*[6] (Fig. 1B-E). One hour after incubation, we did not observe any significant difference in the F-actin inward movement of mock and 10 µM SMIFH2 application (Fig. 1B, C, and F). By contrast, 20 and 50 µM SMIFH2 applications reduced the F-actin inward movement (Fig. 1D, E and F), indicating that formins regulate F-actin inward movement in the Arabidopsis central cell, and that 20 µM SMIFH2 is required to observe inhibition of formin's function in the Arabidopsis central cell F-actin dynamics. The effects of SMIFH2 have been investigated in different tissue types of Arabidopsis, and the application of 20-30 µM SMIFH2 has been found to inhibit formin's function.[17–19] We noticed bundle-like F-actin structures in the central cell when treated with 50 µM SMIFH2 (Fig. 1E). In vegetative tissues, longer incubation of SMIFH2 increased microfilament bundles,[17] and this structural alteration may cause changes in F-actin dynamics. Thus, we also investigated F-actin dynamics 20 min, 40 min, and 60 min after incubation with 20 µM SMIFH2 as well as the recovery of F-actin dynamics after SMIFH2 removal (Fig. 1G). 20 min incubation already showed a significant reduction of the F-actin movement without changing the overall F-actin structure compared to the mock. The inward F-actin movement recovered to the mock treatment level after SMIFH2 removal. Taken together, our results show that formins play an essential role in actin nucleation that is required for the inward movement of F-actin in the central cell.

The actin polymerization process generates force in the elongating F-actin[21], and this force is required for buckling of F-actin from the plasma membrane periphery towards the opposite direction.[22] In Arabidopsis, *AtFH1, 4, 5, 14, 16,* and *20* show relatively high expression compared to other formins in the central cell.[23,24] Among them, *AtFH1* and 5 have been functionally studied; they have membrane anchoring peptides that associate with the endomembrane and plasma membrane for actin polymerization at the plasma membrane.[25,26] The active generation of actin cables occurs at the plasma membrane periphery in the Arabidopsis central cell[6], and it is possible that these plasma membrane-anchored formins play an essential role in actin nucleation and polymerization in the Arabidopsis central cell and generate part of the force for this dynamic F-actin movement. We did not observe F-actin marker

accumulations around the central cell nucleus (Fig. 1B-E)[6]. This result indicates that active F-actin depolymerization occurs around the nucleus. Together with the formin-involved actin nucleation, treadmilling may also support the dynamic inward movement of F-actin in the central cell for successful sperm nuclear migration.

Not only formins (Fig. 1), but also ROP, WAVE/SCAR, and the class XI myosin play pivotal roles in F-actin inward movement in the central cell.[11] ROP8, which is present at the plasma membrane, likely interacts with SCAR2 and positively regulates the F-actin inward movement.[11] How ROP8-SCAR2 and formins coordinately control the F-actin movement in the central cell still remain unknown. In *Drosophila,* WASH (related to the plant WAVE/SCAR family) becomes activated by the RHO GTPase, Rho1, and interacts with the formin, cappuccino, for actin nucleation.[27] Revealing whether ROP8-SCAR2 and formins are on the same pathway like in *Drosophila* or regulate F-actin dynamics in parallel is one of the key questions to be addressed next.

Materials and Method:

Arabidopsis thaliana (Columbia-0) ecotype was used in all experiments. Seeds were first germinated in soil and seedlings were grown for three weeks under short-day conditions (8 h light, 22°C and 16 h dark, 18°C). Plants were then shifted to 22°C with continuous light. The *proFWA::Lifeact:Venus*[6] line has been described previously. Pistils, from flowers emasculated two days before the experiment, were dissected out by a sharp knife and mature ovules from two-three pistils were collected into 200 µL assay medium (2.1 g/L Nitsch basal salt mixture, 5% w/v trehalose dehydrate, 0.05% w/v MES KOH (pH 5.8), and 1x Gamborg vitamin) in a glass-bottom dish as described previously.[28] Formin inhibitor, SMIFH2 (stock, 10 mM in DMSO; Sigma-Aldrich, MO, USA), was prepared before the experiment and kept at -80°C. Working concentrations of 10, 20, and 50 µM were prepared freshly before each experiment. To remove SMIFH2, ovules were washed out 5 times with the assay medium and imaging was performed 1h after the removal of SMIFH2. An Olympus laser scanning confocal system (FV1200) equipped with 515-nm, and the GaAsP detection filter was used to illuminate Lifeact:Venus. Time-lapse (1 min interval) images

with z-planes (15-20 µm total, 3-4 µm each slice) were acquired using FV10-ASW 4.2 software. Laser 2-3%, HV 500, gain 1 and Kalman 2 options were used to capture images. All images were processed using Fiji (ImageJ) software. F-actin dynamics quantification was performed as described previously.[11]

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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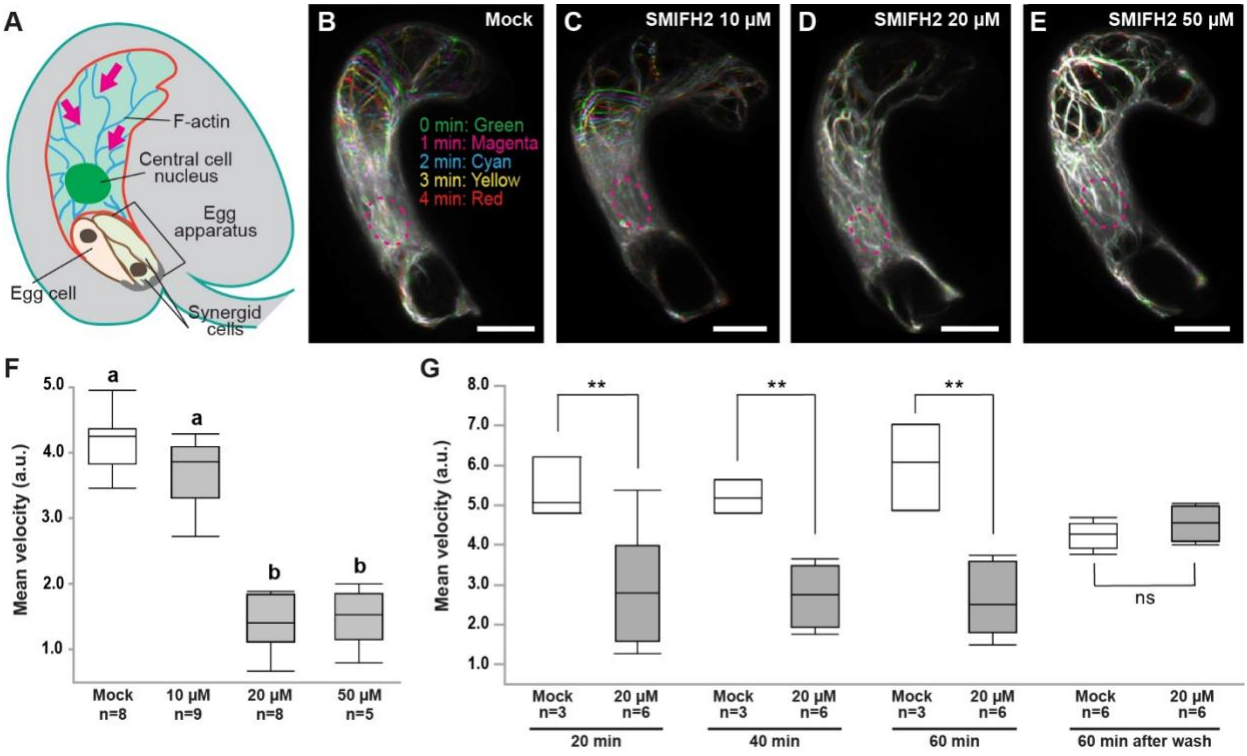

Figure 1: Application of SMIFH2 reduced F-actin inward movement in the Arabidopsis central cell. (A) Scheme of Arabidopsis mature ovule. Arrows indicate the inward movement of F-actin from plasma membrane towards central cell nucleus. (B-E) Time-lapse stacks of Z-projected central cell F-actin images (1-min interval images, marked by five different colors) of mock (B), SMIFH2 10 µM (C), 20 µM (D), and 50 µM (E). Dashed circles indicate the position of the central cell nucleus. F-actin marked by different colors denotes F-actin inward movement. White results from overlapping of all colors, representing less or no movement. (F-G) Average velocity of F-actin in the central cell. Levels not connected by the same letter (a-b) are significantly different ($P < 0.001$; Tukey-Kramer HSD test) (F). **, $P < 0.001$; ns, not significant; Tukey-Kramer HSD test (G). (Scale bar, 20 µm).