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Running head: Cytoskeleton dynamics in the plant stigma

Cytoskeleton remodelling in *Arabidopsis* stigmatic cells following pollination

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Abstract:

In plants, the first interaction that occurs between the male gametophytes (pollen grains) and the stigmatic epidermis of the female organ is crucial for successful reproduction. The stigma consists of a dome of flask-shaped cells specialised in pollen capture. In these stigmatic cells, the cytoskeleton network (cortical microtubules and actin microfilaments) actively responds to pollen contact and undergoes dynamic remodelling required for successful pollen acceptance to occur. Here, we have designed several microscopy mountings to monitor stigmatic cytoskeleton dynamics. These designs are based on the constraints linked to the tightly regulated pollen-stigma interaction and depend upon the experimental goal, either a static view or live-cell imaging.

Key words: stigma cytoskeleton, pollen, confocal microscopy, live imaging

1. Introduction

The plant cytoskeleton is a filamentous network consisting of cortical microtubules (CMTs) and actin microfilaments (AMFs). This network undergoes dynamic changes in response to environmental stimuli and plays key roles in both biotic and abiotic responses [1]. During reproduction, when the pollen grains land on the stigma surface, the cytoskeleton of the stigmatic cells actively responds to pollen arrival. CMT breakdown and polymerisation of AMFs occur in these cells at the pollen attachment sites and such cytoskeleton reorganisation is required for successful pollen acceptance [2–4]. Capturing these dynamic events by imaging the stigmatic cell is not trivial. In *Arabidopsis*, the whole stigma measures around 3 mm and consists of a 3-dimensional dome composed of hundreds of flask-shaped cells (papillae, Figure 1). Each papilla cell is covered by a thick cuticular layer that generates background autofluorescence and prevents absorption of conventional plant cell dyes and drugs [4, 5]. In addition, monitoring cytoskeleton dynamics is challenging, since CMTs are, for instance, highly sensitive to pressure and can be destabilised in contact with the microscope coverslip [5]. Finally, the pollen-stigma interaction is tightly regulated. Very soon after contact with the papilla surface, the incoming pollen grain triggers water fluxes from the stigmatic cell to hydrate and reach a critical water content required for germination of a pollen tube [4, 6, 7]. It has been shown that a high humidity environment can stimulate pollen hydration bypassing the stigma control [4, 8]. Then, controlling the humidity at the vicinity of the stigma is necessary to properly image the pollen-stigma interaction.

Considering these physiological constraints, we have designed different microscopy mountings to monitor the stigmatic cytoskeleton, by either static viewing or live-cell imaging. We also describe a procedure to quantify fibril orientation in papillae using Fibriltool [9] as well as a method to destabilise the papilla cytoskeleton using drugs.

2. Materials

2.1. *Plant material*

1. *Arabidopsis thaliana* (Col-0)
2. pSLR1::MAP65.1-citrine transgenic *Arabidopsis*: stigma-expressed marker for CMTs [5]
3. pSLR1::LifeActin-Venus transgenic *Arabidopsis*: stigma-expressed marker for AMFs [4]
4. pACT11::RFP transgenic *Arabidopsis*: pollen grain/tube-expressed construction [10].

2.2 Media

1. Medium A: ½ Murashig and Skoog (MS) medium. Weight 0.22 g MS basal salt mixture and 1g of sucrose. Add water to a volume of 90 ml and mix. Adjust pH to 5.7 +/- 0.1 with KOH and make to 100 ml with water. Sterilise by autoclaving. Keep at room temperature.
2. Medium B: MS Solid medium. Weight 0.44 g MS basal salt mixture and 1g of sucrose. Add water to a volume of 90 ml and mix. Adjust pH to 5.7 +/- 0.1 with KOH and make up to 100 ml with water. Transfer to an autoclavable bottle and add 0.7 g of plant agar. Sterilise by autoclaving and let dry at room temperature. Keep at room temperature. When needed, warm for a few minutes in a microwave oven until the medium has melted.

2.3 Microscopes

Different microscopes were used dependant on the experiment and these are listed below:

1. Upright Leica SP8 microscope using a 25x objective (numeric aperture 0.95, water immersion),
2. Inverted Zeiss LSM800 microscope (AxioObserver Z1) using a 40x PlanApochromat objective (numerical aperture 1.3, oil immersion)
3. Inverted Zeiss microscope (AxioObserver Z1) equipped with a spinning disk module (CSU-W1-T3, Yokogawa) using a 40x PlanApochromat objective (numerical aperture 1.1, water immersion).

2.4 Other materials

1. Forceps (tip thickness 0.01 mm).
2. Cellulose filter paper.
3. Slide, small coverslip (22 x 22mm), large coverslip (22 x 60 mm)
4. 0.2 mL PCR tube
5. High vacuum Grease.
6. Lanolin.

2.5 Computer Programs required

1. Image J software (<https://imagej.nih.gov/ij/>) to process images and movies.
2. Image J Fibriltool plugin [9].

3. Methods

Plants were grown in a growth chamber under a long day cycle of 16h light/8h dark at 21°C/19°C with a relative humidity ca. 60 %. All procedures should be carried out at room temperature unless otherwise stated.

3.1. Static view of the stigmatic cytoskeleton: analysis of cytoskeleton fibre orientation

The mounting shown in Figure 2 can be used to observe stigmas at all developmental stages from stage 12 to 15 as described in [5]. We are equipped with an inverted microscope, but this mounting can also be used with an upright microscope.

1. Emasculate floral buds from the MAP65.1-citrine line (to visualise the CMTs) or LifeActin-Venus line (to visualise the AMFs) [].
2. In the middle of the microscope slide, deposit four small plugs of grease.
3. With a scalpel, cut the pistil just beneath the style transverse to its length (Figure 2A).
4. Gently deposit the cut pistil in between the four grease plugs.

5. Rapidly place a small microscope coverslip on the top of the grease plugs and gently press downwards (Figure 2B). The coverslip needs to touch the papillae without damaging the cells (see **Note 1**).
6. Add a droplet of *medium A* to fill the space between the slide and the coverslip (see **Note 2**).
7. Observe under an inverted Zeiss LSM800 microscope using a 40x Plan-Apochromat objective (oil immersion).
8. From here (step 8 to 14), we describe the procedure for quantifying the CMTs anisotropy as an example, but the same procedure can be applied to the AMFs. Quantitative analyses of the average orientation and anisotropy of fibres can be assessed from images obtained with the methodology described in step 1 to 7 (Figure 2D, E).
9. Install the Fibriltool plugin on ImageJ (instructions can be retrieved from [9]).
10. Open your confocal image.
11. Create a maximum projection of half the volume of the stigmatic cell, and only measure the fibre organisation of the upward face of the papilla (close to the objective lens).
12. Double click on the Tool symbol and choose the channel corresponding to the fibril signal.
13. Select your region of interest (ROI) using the Polygon tool.
14. Click on Fibriltool and then on your ROI (Figure 3A). A segment with a specific orientation appears. This corresponds to the average orientation of your fibril network within your ROI. A window also opens (Figure 3C), with a series of numbers in order of appearance (Figure 3B): file name, ROI number, x-coordinate of ROI centre, y-coordinate of ROI centre, ROI surface area, average fibril orientation, anisotropy, coordinate of ROI vertices. Anisotropy values range from zero to one; zero indicates pure isotropy and one complete anisotropy.

3.2. Static view of the stigmatic cytoskeleton: cytoskeleton destabilisation

To perform drug delivery to papilla cells without applying liquid directly on the stigma, we applied lanolin paste around the style as described in [5].

1. Prepare the lanolin paste containing the oryzalin as follows: add 1 volume of the oryzalin stock solution (888 $\mu\text{g}/\text{mL}$) to 2 volumes of melted (55°C) lanolin and mix thoroughly until the formation of a homogeneous emulsion.
2. Under a stereomicroscope, apply the oryzalin containing lanolin just beneath the stigmatic cells, around the style (Figure 4A, B). Be careful not to directly touch the stigmatic cells with lanolin.
3. Wait for 4 hours at 21°C.
4. After 4 hours, observe the stigma using the mounting described in 3.1. We observed that lower oryzalin concentrations produce incomplete CMT destabilisation (Figure 4C).
5. Here, we have described the procedure to deliver oryzalin but the same method could be applied to other drugs.

3.3. Dynamic view of the stigmatic cytoskeleton remodelling upon pollination, using an inverted microscope.

To visualise stigmatic CMT and AMF remodelling in response to pollen arrival, it is necessary to avoid liquid excess since it can perturb the control of pollen hydration and germination at the stigma surface. In addition, to visualise the cytoskeleton in papilla cells, the stigma should be maintained alive throughout the experiment.

1. Emasculate floral buds from the MAP65.1-citrine line at the end of stage 12 [11].
2. Detach a mature anther from a stage 14 flower of pACT11::RFP Arabidopsis.
3. Under a stereomicroscope, pollinate the stigma surface by gently brushing the stigma with the dehiscent anther (Figure 5A). Ideally, cover the stigma with a single layer of pollen grains (see **Note 3**).
3. Immediately after pollination, cut the pistil in the middle of the ovary, transverse to its length.
4. Deposit the pollinated pistil upside down in the centre of a large coverslip.
5. Place four grease plugs around the cut pistil.

6. Cover the cut part of the ovary with a strip of cellulose paper (0.5cm x 3cm) humidified with medium A. Be careful to avoid medium excess on the cellulose strip (see **Note 2**)
7. At the opposite side of the strip, place a block of *medium B* (square of 0.5 cm²), to maintain a constant humidity (Figure 5B).
7. Gently place a small coverslip on the top of the grease plugs and gently press downwards; not too much so as to avoid papilla damage and water release (see **Note 1**). Do not add any medium between the coverslips (see **Note 2**).
8. Observe under a Zeiss microscope equipped with a spinning disk module using a 40x Plan-Apochromat objective (numerical aperture 1.1, water immersion).
9. Acquire serial-confocal images of the entire volume of the stigma every μm and every minute. This mounting coupled with a spinning confocal microscope is particularly adapted for high-speed optical sectioning over short timescales to image the CMT remodelling in stigmatic cells along the pollen tube path [5], (Figure 5C).
10. The protocol described above can also be used to study AMFs.

3.4. Dynamic view of the stigmatic cytoskeleton remodelling upon pollination, using an upright microscope.

1. A small humidity chamber is designed to control the humidity in the vicinity of the stigma. In the centre of a microscope slide, deposit a droplet of medium B and let this dry for at least 30 minutes.
2. A PCR tube is cut in half and its conical extremity is perforated with a needle. The pierced and cut PCR tube is embedded into the solid droplet of medium B (Figure 6 A, B).
3. Place four grease plugs around the humidity chamber.
4. Emasculate floral buds from the LifeActin-Venus line at the end of stage 12 [11].
5. Cut the pistil in the middle of the ovary, transversally to its length, and introduce vertically into the pierced PCR tube of the humidity chamber.
5. Detach a mature anther from a stage 14 flower of pACT11-RFP Arabidopsis.

6. Under a stereomicroscope, pollinate the stigma surface by carefully brushing the stigma with the dehiscent anther. Ideally, cover the stigma with a single layer of pollen grains (see **Note 3**).
7. Immediately after pollination, gently place a small coverslip on top of the grease plugs and gently press downwards (Figure 6C). The coverslip needs to touch the papillae without damaging the cells and avoid any water release (see **Note 1**). Do not add any medium between slide and the coverslip (see **Note 2**).
8. Observe under an inverted Zeiss LSM800 microscope using a 40x Plan-Apochromat objective (oil immersion). Acquire serial-confocal images of the entire volume of the stigma every μm and every two to five minutes (Figure 6D). As pollination is performed just before observation and directly in the humidity chamber, this mounting is particularly adapted to catch the very early cellular changes occurring in the stigmatic cells after pollen attachment. Moreover, contrary to the previous mounting, the humidity level is better controlled which is suitable for assessing pollen hydration and germination [4].
9. The protocol described above can also be used to study CMTs.

4 Notes

1. A better visualisation of the fluorescent signal is obtained from papillae just beneath the coverslip and almost perpendicular to the confocal laser path. To obtain accurate images of papillae, we use grease plugs around the stigma to cushion the coverslip and reduce cellular damage. We gently press on it to lay down papillae at the top periphery of the stigma dome so that they are as perpendicular as possible to the laser path (Figure 2B).
2. When non-pollinated papillae are imaged, mounting in a liquid medium is suitable. However, we observed that pollen germination and pollen tube growth are inhibited in liquid, thus avoid using wet mounting for time lapse imaging. To capture dynamic events occurring in stigmatic cells in response to pollen, a dry mounting is more adapted. Be careful not to press too much on the coverslip on top of the stigma in order to avoid cell damage and water release.

3. To avoid too much pollen grain deposition, under a stereomicroscope, start by brushing the dehiscent anther on a tissue to release excess pollen and then gently brush the stigma to deposit a single layer of pollen grains. Do not use a paintbrush or another instrument (forceps, needle...) to deposit pollen. We observed that touching pollen grains with a paintbrush or another instrument to transport them, drastically reduces their hydration rate on the stigma surface.

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Figures

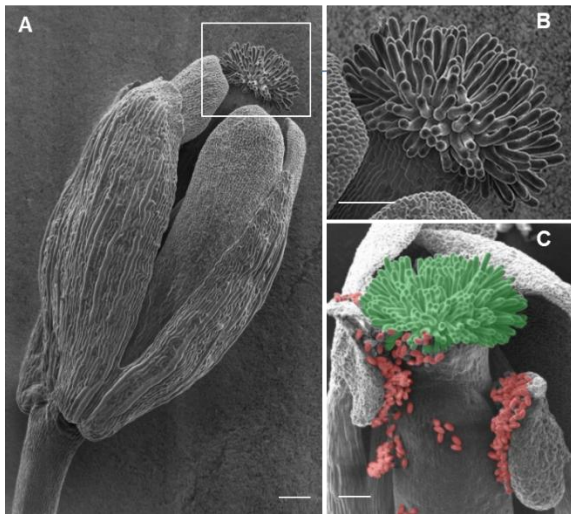


Figure 1: Pollination in Arabidopsis. (A) An Arabidopsis flower bud at the end of stage 12, observed by scanning electron microscopy (SEM). The stigma is highlighted by a white square. (B) Enlarged view of the stigma, composed of elongated papilla cells. (C) During pollination, pollen grains released from the anthers land on the stigmatic cells. Pollen grains have been highlighted in red and papilla cells in green for a better visualisation. Scale bars, 50 μm .

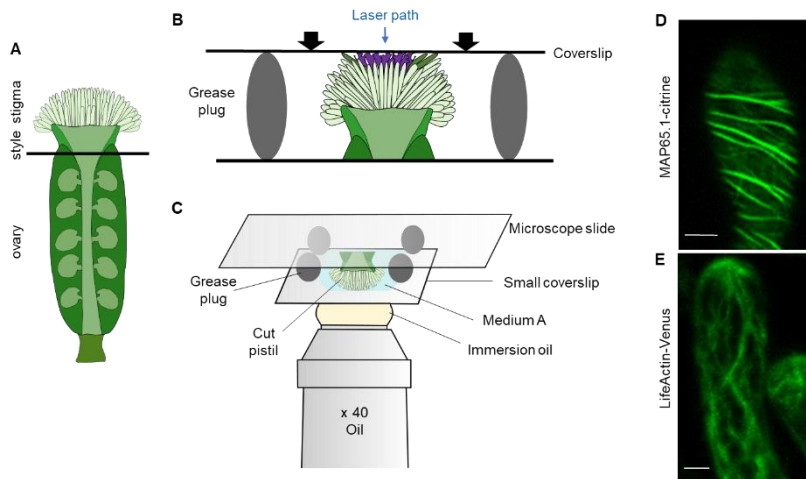


Figure 2: Static view of the stigmatic cytoskeleton. (A) The pistil is cut beneath the style transverse to its length (black line) before mounting. (B) To get the most suitable papillae to image, deposit grease plugs around the stigma, place a microscope coverslip on these plugs and gently press downwards (black arrows) to lay down the top periphery papillae. Some papillae, directly in contact with the coverslip will be damaged (highlighted in purple). The non-damaged cells just beneath the

coverslip (highlighted in dark green) and almost perpendicular to the confocal laser path (blue arrow) are imaged. (C) Confocal mounting in *medium A*. (D, E) Confocal images of papilla cells expressing MAP65.1-citrine (D) or LifeActin-Venus (E). Scale bars, 5 μm .

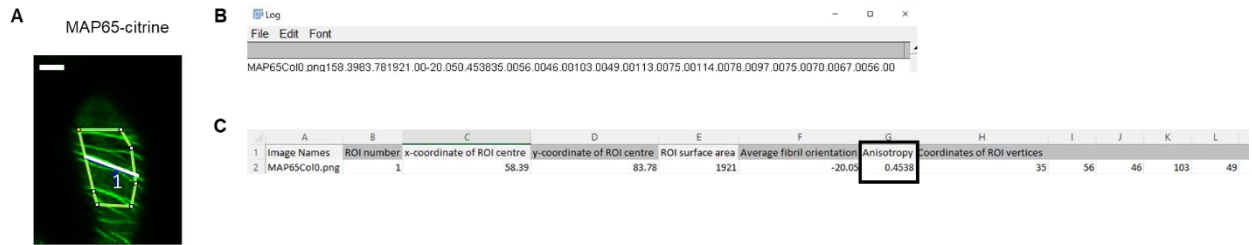


Figure 3: Quantification of the cytoskeleton anisotropy using FibrilTool. (A) A region of interest (green polygon) is defined to estimate the CMT anisotropy in papillae expressing MAP65-citrine. The white line corresponds to the average orientation of the fibril network estimated by FibrilTool. The number corresponds to the region of interest (number 1). Scale bars, 5 μm . (B) Result window obtained from ImageJ using FibrilTool plugin. (C) To ease the identification of the results, data obtained are analysed in an Excel file sheet, with their corresponding column names. Anisotropy value is framed in black.

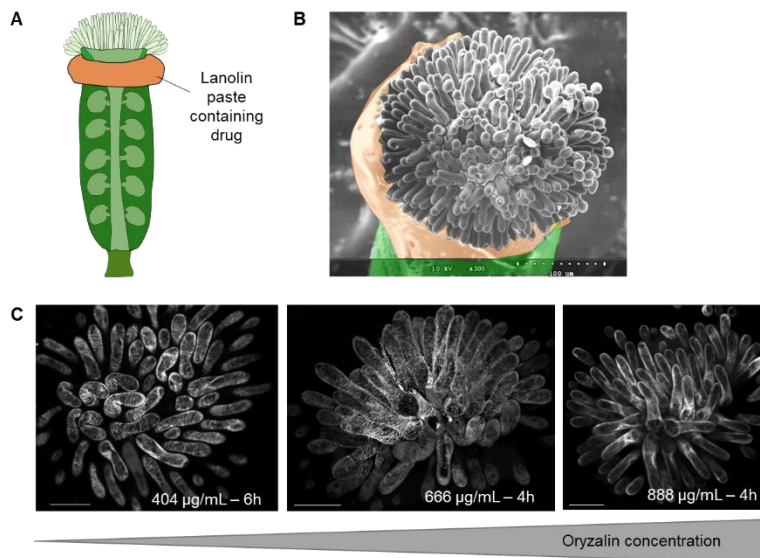


Figure 4: Application of cytoskeleton destabilisation drugs. (A) Schematic view of a treated pistil showing the position of the lanolin paste (orange ring). (B) Top view of the treated stigma, observed by SEM. The lanolin paste is highlighted in orange, the basal part of the pistil in green for a better visualisation. (C) Papillae expressing MAP65-citrine after 4 or 6 hours of oryzalin treatment. CMT

depolymerization is complete and homogeneous after 4 hours of treatment at 888 $\mu\text{g}/\text{mL}$. At 404 $\mu\text{g}/\text{mL}$ and 666 $\mu\text{g}/\text{mL}$, CMTs are not completely destabilized. Scale bars, 50 μm . Reproduced from Riglet et al., 2020 with permission from eLife, under the Creative Commons Attribution 4.0 International Public License (CC-BY 4.0).

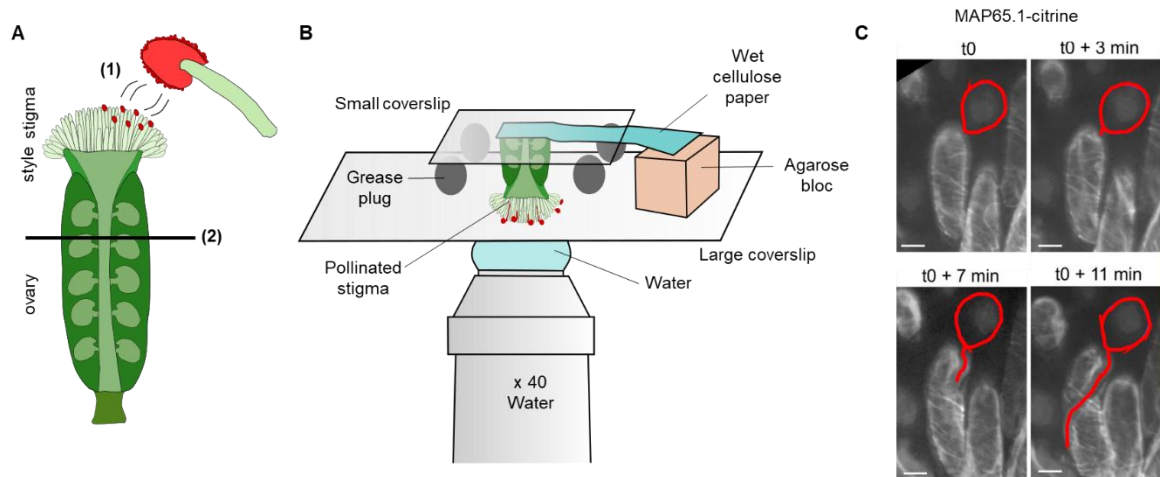


Figure 5: Live imaging of cytoskeleton dynamics upon pollination (inverted mounting). (A) pACT11-RFP pollen grains are deposited on a MAP65-citrine stigma (1). Pollinated pistil is cut in the middle of the ovary transverse to its length (black line) (2). (B) The cut pistil is mounted without any medium and observed under confocal microscopy. (C) Time-lapse imaging of stigmatic CMT dynamics upon pollination. t_0 corresponds to the pollen germination time point. Pollen outline and pollen tube path are highlighted in red for a better visualisation. No major alteration of stigmatic CMT arrays is visible. Scale bars, 10 μm . Reproduced from Riglet et al., 2020 with permission from eLife, under the Creative Commons Attribution 4.0 International Public License (CC-BY 4.0).

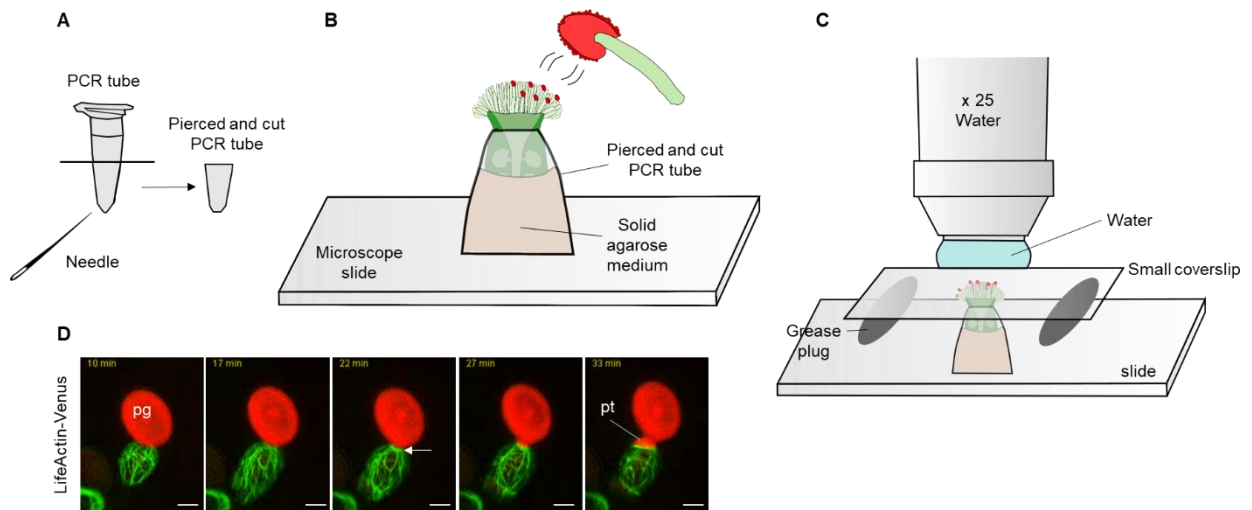


Figure 6: Live imaging of cytoskeleton dynamics upon pollination (upright mounting). (A) To prepare the humidity chamber, pierce the bottom of the PCR tube with a needle and cut the tube in half (black line). (B) A lifeActin-Venus pistil is cut in the middle of the ovary and introduced in the pierced and cut PCR tube embedded into a droplet of solid *medium B*. Then, pACT11-RFP pollen grains are deposited on the stigma. (C) Confocal mounting. (D) Time-lapse imaging of stigmatic AMF dynamics upon pollination. The first image was recorded 10 min after pollen deposition. Actin filaments focus towards the pollen grain contact site. 22 min after pollen deposition, a ring of AMFs around the emerging pollen tube is visible (white arrow). Pg: pollen grain, pt: pollen tube. Scale bars, 10 μm .