Chapter 3

Optimized Method for Growing In Vitro *Arabidopsis thaliana* Pollen Tubes

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Abstract

Pollen tubes elongate by tip growth toward the ovule to deliver the sperm cells during fertilization. Since pollen tubes from several species can be grown in vitro maintaining their polarity, pollen tube growth is a suitable model system to study cell polarity and tip growth. *A. thaliana* pollen tubes germinated in vitro for 6 h can reach up to 800 μm. By studying the phenotype of mutants of T-DNA insertion lines, genes involved in pollen tube growth can be identified. Moreover, components involved in the regulation of pollen tube growth such as calcium ions and reactive oxygen species (ROS) can be analyzed.

Key words Pollen tube growth, Cell expansion, Arabidopsis pollen

1 Introduction

In Angiosperms, development of pollen grains (male gametophytes) is carried out inside the anther and is a tightly controlled process because it directly affects fertilization. When Arabidopsis mature pollen grain reaches the stigma surface, and after a positive recognition, starts pollen hydration after which a pollen tube emerges from the pollen grain. Pollen tubes are single cells that elongate within the pistil to deliver the sperm cells into the ovule to achieve fertilization. In this process pollen tubes grow rapidly and they are guided by endogenous signals as well as signals coming from the female tissues, which need to go through the cell wall to reach their targets.

The elongation of the pollen tube is driven by a process known as tip growth, in which all growth is restricted to the apex of the cell and the cytoplasm remains confined to the most proximal region of the tube by the formation of callose plugs. In order to transport materials for the plasma membrane expansion and cell wall assembly, pollen growth requires an active membrane trafficking system

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and a dynamic actin cytoskeleton [1, 2]. The organelles and vesicles are transported by the F-actin filaments along the flank of the tube and recycled back along the centre of the tube. This process known as reverse-fountain streaming pattern is typically observed in elongating pollen tubes. As a consequence, four zones can be distinguished inside the pollen tube: an apical zone where the secretory vesicles accumulates, a subapical zone rich in organelles, a nuclear zone, and a vacuolated zone.

During the polarized growth of pollen tubes the cell wall plays a fundamental role not only being the surface of communication between the pollen tube cell and the female tissues (or culture medium) but also in the control of cell shape, in the protection of the sperm cells, and in the resistance against turgor pressure [3]. The later is achieved by a rigorous control of cell wall deposition and remodeling to modify its mechanical properties.

Pollen tubes of many species, including *A. thaliana*, have the capacity to grow in vitro maintaining the polarity and the ability to respond to molecular cues, such as guidance molecules. For this reason in vitro germinated pollen is used as an experimental system to examine the control of cell expansion, directionality of cell growth, and signaling mechanisms. In addition, it can be used to identify mutations in genes involved in pollen tube growth (e.g., genes involved in cell wall biosynthesis, in pollen–pistil interactions, in cell wall remodeling) that could cause an abnormal growth in pollen tubes.

2 Materials

2.1 Plant Material

Seeds of *Arabidopsis thaliana* Col-0 and those from mutants of T-DNA insertion lines which can be obtained from ABRC (Arabidopsis Biological Resource Center) or NASC (Nottingham Arabidopsis Stock Centre).

2.2 Plant Growth Conditions

Sterilized seeds were sown/plate in 0.5× Murashige and Skoog (1962) medium supplemented with 0.8–1 % agar and the corresponding antibiotic. Seeds were cold stratified 2–4 days in the dark at 4 °C and germinated and grown for 10 days at 22 °C under continuous light. Seedlings were then transferred to soil or peat, mixed with vermiculite and perlite (2:1:1), and grown in chamber at 22 °C under long day (16/8 h light/dark) photoperiod and 60 % relative humidity.

2.3 Equipment

1. Plant growth chamber.
2. Bright field microscope.
3. Vortex mixer.

2.4 Reagents Required

1. The following stock solutions must be prepared with MilliQ H_2O:
   (a) 1 M KCl.
   (b) 1 M $CaCl_2$.
   (c) 1 M $MgSO_4$.
   (d) 1 % H_3BO_3.
2. Other reagents:
   (a) Sucrose.
   (b) 1 M KOH (for adjusting pH).
   (c) Low melting agarose.

2.5 Pollen Growth Medium (PGM)

To prepare 30 ml of liquid PGM (*see* **Note 1**):

1. Weigh 3 g of sucrose (final concentration of 10 %). Pour it into a 50 ml Falcon conical tube and add 25 ml of MilliQ H_2O to dissolve the sucrose.
2. Add the necessary volume of each stock solution to the sucrose solution to reach the final desired concentrations of each reagent (1 mM KCl, 1 mM $CaCl_2$, 5 mM $MgSO_4$, and 0.01 % H_3BO_3) (*see* **Note 2**).
3. Add the rest of the MilliQ H_2O to reach the final volume of 30 ml and vortex.
4. If necessary add 0.1 M KOH to adjust the pH 7.5 (*see* **Note 3**).

3 Methods

3.1 Pollen Growth Conditions

1. Put 15–20 flowers of each genotype in a glass vial (*see* **Note 4**).
2. Add 200 µl of PGM and cover the tubes with parafilm.
3. Vortex the tubes for 1–2 min (*see* **Note 5**).
4. Incubate the pollen grains for 4–6 h at 22 °C in light conditions (*see* **Note 6**).

3.2 Pollen Assays

1. Put 15 µl of the liquid PGM (using a cut-off pipette tip) containing the germinated pollen grains (Fig. 1a) on a glass slide and place a cover slip (*see* **Notes 7–9**).
2. Analyze pollen germination and pollen tube length under an Olympus BX43 microscope using bright field (10×, 0.25 NA; 20×, 0.5 NA). Take images of the pollen tubes (*see* **Note 10**).
3. Perform at least three independent experiments with a minimum of three replicates (three samples incubated in different glass tubes under the similar conditions) for each genotype and/or treatment (Fig. 1a, b).

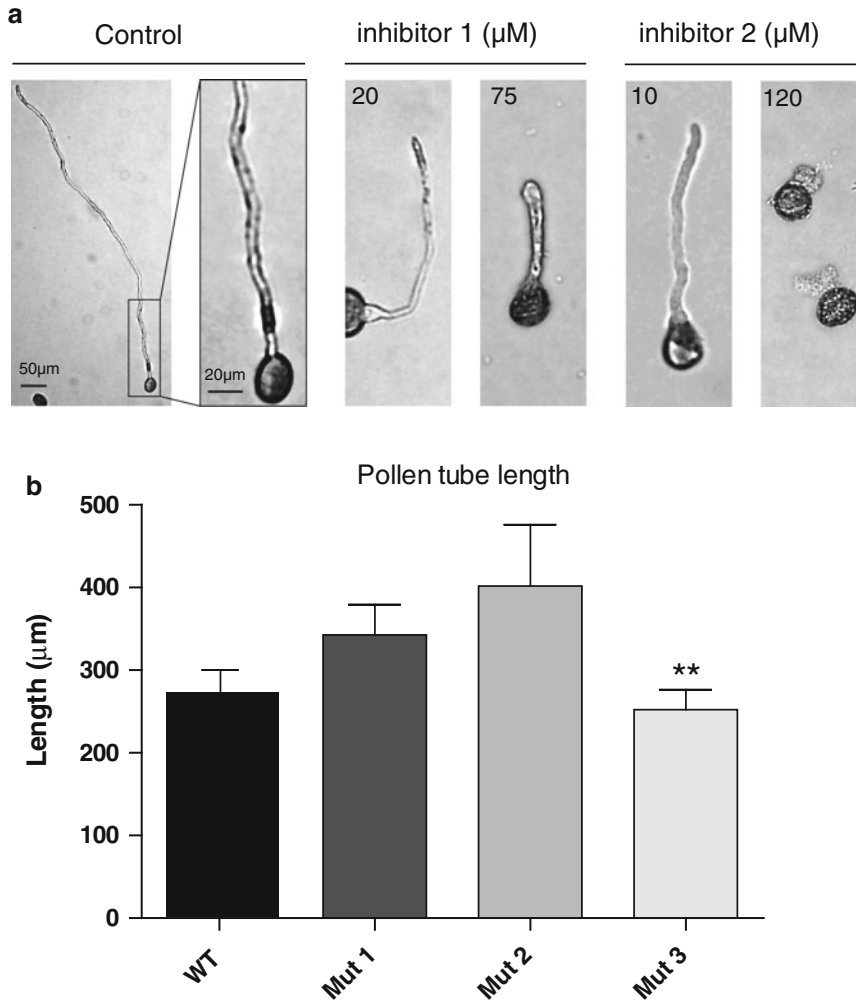


Fig. 1 (**a**) WT Col-0 pollen tube after 4 h in vitro germination (*left*) and in presence of two different inhibitors (*right*). (**b**) Pollen tube length of three different T-DNA mutants compared to WT Col-0. Data are presented as the mean ± SEM from the averages from duplicate samples from one representative experiment; ** statistically significant differences with $p < 0.05$

3.3 Percentage of Germinated Pollen Grains (ImageJ)

1. Open the ImageJ application. In "File," go to "import"-"image sequence" and find the location of the file. Click open (this will open the file using the ImageJ software).
2. Go to "Plugins"-"Analyze"-"Cell Counter".
3. Click on "Initialize" to start the analysis (*see* **Note 11**).
4. Choose a counter of the list for each "cell type" (*see* **Note 12**).
5. Once all pollen grains were classified click on "results" (*see* **Note 13**).
6. Copy and paste the results to an Excel document for analysis.

7. The percentage of geminated pollen grains is calculated for each replicate as (*see* **Note 14**).

$$\frac{\text{no of germinated pollen grains}}{\text{no of pollen grains}} \times 100.$$

3.4 Pollen Tube Length (ImageJ)

1. Open the ImageJ application. In "File", go to "import"-"image sequence" and find the location of the file and open it. Click "ok" on the Sequence Options window.
2. To set the scale:
   (a) In the ImageJ toolbar, right click on the button with the diagonal straight line. Click the option "straight line".
   (b) Trace a line over/upon the scale bar.
   (c) Go to "analyze"-"set scale".
   - Type in the known distance (*see* **Note 15**).
   - Click the "global" button. This will keep the scale bar set while ImageJ is open.
3. Go to the ImageJ toolbar and right click on the button with the diagonal straight line and choose the option "freehand line".
4. Trace the pollen tube from the edge of the pollen grain to the tip of the tube.
5. Press the letter M to measure the length of the pollen tube.
6. All the measurements made on the image sequence will be shown up in a new window called "Results".
7. Copy and paste the results to an Excel document for analysis.

3.5 Statistical Analysis

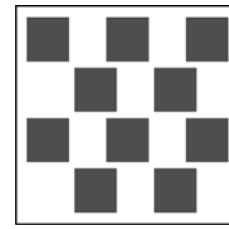
Statistical analysis of results and further processing can be performed using GraphPad Prism version 5 or a similar program such as Excel. Once the data analysis is finished, a statistical test must be performed to evaluate the differences between the *wild type* and the mutants of interest (Fig. 1b).

4 Notes

1. Agarose is required to prepare solid PGM. When making solid media 1–2 % of low melting agarose is added to PGM containing 20 % sucrose and incubated at 65 °C water bath until all the agarose is completely dissolved. Add the H_3BO_3 after dissolving the agarose. Then, it is poured into small Petri dishes or onto microscope slides and quickly stored in a wet chamber to avoid drying. Once the PGM solidifies take the flowers and gently brush them across the surface of the PGM to transfer the pollen grains. Incubate the Petri dishes or slides in a transparent wet

chamber [4] for 4–6 h at 22 °C in light conditions. To analyze pollen germination using a solid PGM an inverted microscope is recommended.

2. The PGM can be stored at −20 °C without the H_3BO_3. The H_3BO_3 should be added the same day of the experiment.
3. For better results employ younger plants, however the first few flowers of the primary bolt are often infertile. Look for flowers above siliques that have started to lengthen. In general, mature pollen is present in the flower once white petals are visible, coinciding with stage 13 of flower development. If the plants have been grown in a plant growth chamber, then avoid the flowers that have been growing very close to the lights because heat stress can negatively affect pollen development.
4. Vortexing the tubes facilitates the release of pollen grains. Make sure most of the flowers stay submerged in the PGM.
5. Temperature is a determinant factor for pollen tube growth [4]. The glass vials can be incubated in a plant growth chamber under controlled temperature conditions.
6. A cut-off pipette tip is used to avoid the rupture of the pollen tubes.
7. Up to three samples can be placed on the same glass slide.
8. Once the cover slip is placed over the sample it can be sealed with clear nail polish to avoid the evaporation of water. This is recommended if the assay takes too long, otherwise it is not necessary.
9. To take images, choose areas that include the central areas as well as the corners and borders of the cover slip, because the latter are the zones where the germinated pollen grains tend to accumulate.



10. Click “Initialize” each time a new set of images is opened.
11. For example, select “type1” each time you click on nongerminated pollen grains and “type2” for germinated pollen grains.
12. A pollen grain is classified as germinated if the pollen tube length was equal to or greater than the pollen grain diameter. A minimum of 100 pollen grains are classified per replicate.
13. No less than 100 pollen grains must be counted. The normal percentage of germination observed for WT pollen grains is

about 70–80 %. Any value below 50 % will cause the assay to be discarded, because no reliable comparison can be done.

14. For example, if your scale bar represents 200 μm, type that in *known distance*, and type in "micrometer" in the *unit of length*.
15. A minimum of 100 pollen tubes are measured for each replicate. The pollen tube length value is obtained by averaging the mean of each replicate. In case that collected data do not fit in a normal distribution then median instead of the mean should be used.

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