Chapter 10

***Arabidopsis thaliana* Pollen Tube Culture for Multi-omics Studies**

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Abstract

Pollen tubes have been key models to study plant cell wall elongation. *Arabidopsis thaliana*, although small, is a nice model, easy to grow and with a large set of studies to simplify result integration and interpretation. Pollen tubes may be used for gene expression essays, but also for biochemical characterization of the cell wall composition. However, pollen tube culture methods though seemingly straightforward have often a multitude of small technical details crucial for success, quickly deterring the more inexperienced and setting back experiments for months at the time. Here we propose a detailed method to set up easily a pollen tube culture routine in any lab, with a minimal set of equipment, to isolate and process pollen tubes for gene expression and/or cell wall biochemistry studies.

Key words Pollen tube, Easy method, Protein extraction, Cell wall, RNA extraction

1 Introduction

Reproduction in higher plants relies on the efficient delivery of the male gametes (sperm cells) to the receptive ovule by a specialized type of tip-polarized apical growth structure, the pollen tube [1].

This specialized growth type is present in nerve cells, root hairs, fungal hyphae, and pollen tubes and studies have shown that apical growth in all these models share common physiological characteristics [2, 3]. Animal neurons show growth rates of 7–13 nm/s [4], root hairs grow at rates of 10–40 nm/s [5, 6], and pollen tubes can achieve growth rates of 250 nm/s [7]. As the heterotrophic pollen tube creeps toward the receptive ovule guided by molecular cues to adjust the tip elongation direction [8, 9], an intense metabolism must fuel this growth, with a rapid turnover of cytoskeleton, proteins and an accelerated vesicle traffic to deliver membrane and cell wall material to the growing tip [10].

Due to this growth type, fast metabolism, and responsiveness to environmental cues, the pollen tube has been suitable for studies on polarization, signal transduction, cell-cell communication, ion

channel and ion flux activity, gene expression, cytoskeleton, cell wall structure and membrane dynamics, among others [11–14]. Knowing that about 35% of Arabidopsis cell wall synthesis genes are expressed in the pollen and pollen tube [15, 16], the pollen tube is a major model for the study of hormonal, enzymatic, or chemical effects on the plant cell walls [17]. In addition, an ever-increasing availability of cell wall mutant lines makes the pollen tube model furthermore appealing.

Arabidopsis thaliana, although being the leading model in plant science, is not the most frequently used species for studies of pollen germination and pollen tube growth [18]. Like other tricellular pollen species, Arabidopsis in vitro pollen germination has proven difficult [19]. Several more or less complex protocols for pollen tube culture media have been described [20–22] and even though some showed promising results with germination rates of 60–80%, they lacked reproducibility and consistency [23]. In addition, the lack of a standardized culture medium and growth method renders it difficult to isolate variables and compare the different studies. As shown by Costa in 2013 [24] the nutrient composition of the pollen germination medium and culture conditions influence pollen tube gene expression and metabolism to a significant extent. Therefore, the need for a standardized culture medium for the study of Arabidopsis pollen tube is evident. The optimization of composition, pH, and temperature of the culture medium together with the selection of the best flower stage for pollen harvest (best pollen density of the culture) by Boavida and McCormick [25], significantly improved the germination rate. Further improvement was introduced by an optimized pollen harvest method in liquid medium by Dardelle in 2010 [26].

The protocol proposed here describes a simple method to harvest Arabidopsis pollen and to culture pollen tubes in liquid medium. Additionally, several simple yet helpful details for characterizing the pollen tube growth are proposed. These detailed protocols can be combined to provide a more complete data set, or used individually. In vitro pollen tube germination and pollen tube elongation rates are considered the best indicator of pollen viability [27] and an important tool to detect physiological impairments that may interfere with pollen gamete delivery [28]. Gene expression studies by qPCR or RNAseq are excellent tools to help pinpoint the genetic origin of physiological disorders. Additionally, protein and cell wall composition analysis may provide the necessary complementary evidence to understand and describe the mechanics behind the observed physiological disorders of the pollen tube. The following protocol proposes a holistic in vitro study approach of the pollen tube, with optimized culture and harvest conditions for maximum purity and yields.

2 Materials

2.1 Equipment

1. 1.5 mL Eppendorf tubes.
2. 400 mm² flat bottom scintillation flasks with lid or parafilm.
3. Fine tip forceps.
4. Centrifuge.
5. 22 °C refrigerated incubation chamber.
6. Pestles.
7. 60 µm nylon mesh filter sheets.
8. 10 cm funnel.
9. Microscope.
10. Neubauer counting chamber.

2.2 Plant Material

Whether working with wild-type or mutant lines, plant material should be of the best quality possible and grown under stable conditions. For the purpose of pollen production, healthy plants with a good vegetative growth are strongly recommended. Plants grown under short day conditions for at least a few weeks are preferred. The plants should grow at 22 °C and relative humidity of 60%. Stress will affect pollen yields and introduce undesirable variables in the study. Sowing a maximum of five plants per 10 cm² pots. *Arabidopsis thaliana* grows best in well-drained soils with moderate watering; adding sand to the potting mixture will help to improve drainage. After bolting, most of the secondary inflorescences should be trimmed, and no more than four floral stems should be allowed to rise per plant. To maintain the strength of the floral meristem all tertiary branching and older flowers should be removed. This will keep the plant floral meristem active longer and reduces the wasteful allocation of nutrients to nonessential organs. An additional benefit is that it will make the flower harvest task swifter and less confusing. Also, the flower quality from secondary to tertiary branching drops exponentially. The main apical meristem and secondary meristems should provide each an average of four flowers (Fig. 1a); therefore, a strong healthy plant treated in this manner should provide about 50 flowers daily for 3 weeks. A single pot with five plants will thus generate up to 250 flowers. The flowers prepared by this method will look bigger than usual, produce bigger anthers, and thus produce more pollen.

2.3 Reagents

1. Pollen culture medium: 10% Saccharose, 5 mM KCl, 5 mM CaCl₂, 1 mM MgSO₄, 0.01% boric acid, pH 7.5 (Table 1).
RNA extraction:
2. Purezol Bio-Rad.
3. Chloroform.

Fig. 1 Plants and materials. Good quality pollen is the key for success in this protocol (a). The flowers should be harvested freshly open (blue arrows). Pollen from older flowers (red arrows) and young closed flowers (yellow arrows) germinates poorly. The essential material for this protocol are a pair of fine tip tweezers, centrifuge tubes, and flat bottom glass flasks (b). Nylon filters and funnel are only required to filter out the pollen tubes for extraction of RNA, protein, or cell wall. A dark incubation box is useful to help maintain a stable temperature

Table 1
Pollen culture medium guide

Pollen tube culture medium composition					
	Concentration	Molar mass (g/mol)	Direct weighing	Stock solutions	
			For 200 mL of culture medium	Stock fold	For 50 mL stock
KCl	5 mM	74.55	0.076 g	100×	1.86 g
CaCl	5 mM	110.99	0.11 g	100×	2.78 g
MgSO ₄	1 mM	120.4	0.024 g	100×	0.60 g
Boric acid	0.01 %(w/v)	n.a.	0.02 g	10×	0.05 g
Sucrose	10 %(w/v)	n.a.	20 g Add ddH ₂ O to 200 mL	n.a.	n.a.
Adjust to pH 7.5					

Pollen tube culture medium should be prepared fresh. For preparing large volumes use direct weighing. To prepare small volume use stock solutions

4. Isopropanol.
Cell wall extraction:
5. Absolute and 70% ethanol.
6. 1:1 Methanol-Chloroform.
7. Myo-Inositol.

8. 2:1 Methanol-HCl.
9. HMDS-TMCS.
10. Cyclohexane.

Light microscopy:

11. Methanol-free formaldehyde.
12. Glutaraldehyde.
13. Ethanol 10%, 30%, 50%, 70%, 90%, 96%, 100%.
14. LR-White medium grade epoxy resin.
15. Size 1 gelatin capsules.
16. *Protein extraction buffer*: 65 mM Tris-HCl pH 6.8, 1% SDS, 5% glycerol, 2.5% β -mercaptoethanol.

3 Methods

3.1 Pollen Tube Culture

This general protocol deals with the harvest of *Arabidopsis thaliana* pollen and initiation of the in vitro pollen tube culture in liquid medium (Table 1). Duration of culture and timing of pollen tube harvest method depend on the objective (Table 2).

In this method, pollen are harvested by dipping flowers in a liquid culture medium. After discarding undesired plant material, the pollen is pelleted by mild centrifugation and cultured at 22 °C in a 69 mm² flat bottom flask (*see Note 1*).

1. Collect 40 freshly opened flowers (Fig. 1a) per each 1.5 mL Eppendorf tubes (*see Note 2*).
2. Add 1 mL of pollen tube growth medium per tube (Fig. 2a).
3. Gently shake the tubes for 5 min. The solution in the tubes will turn cloudy and slightly yellow.
4. Remove the flowers with forceps and check the solution for contaminants, like petals, sepals, stamens, or other flower residues, under a stereoscope (*see Note 3*) (Fig. 2b, d).
5. Centrifuge the Eppendorf tubes at $3200 \times g$ for 6 min and remove the supernatant with a pipette (Fig. 2c).
6. Add 200 μ L of fresh culture medium to each tube and suspend the pollen pellets.
7. Group the pollen extracts of three tubes (120 flowers) in a 69 mm² flat bottom flask (Fig. 1e).
8. Gather any remaining pollen by rinsing the empty tubes with 150 μ L of culture medium, and transferring to the culture flask (final culture volume of 750 μ L) (*see Note 4*). Seal the tubes with parafilm to avoid evaporation or contamination during culture (Fig. 1f).

Table 2
Culture time for different objectives

Pollen tube culture time	
	Recommended
Pollen germination	5 h
Pollen tube elongation	5 h
mRNA extraction	6 h
Protein extraction	6 h
Cell wall extraction	Over 12 h

The time of culture will depend on the experiment objectives. For germination rate essays, the culture time should be between 4 and 6 h. Under 4 h some less responsive pollen grains may still germinate and over 6 h the percentage of pollen tubes bursting tends to increase. The same applies to the pollen tube cultures for gene expression studies, and the culture should be kept under 6 h to avoid any overcrowding, oxygen- or nutrient-related stress effects on gene expression. For cell wall harvest, the culture time can be extended beyond 12 h to maximize yields. If trying to combine and compare results from both essays, then stop the two cultures at the shortest culture time

Fig. 2 Pollen harvest and pollen tube culture. This process will require fine tip tweezers, pollen tube germination media, centrifuge tubes, and flat bottom glass flasks (a). After transferring the harvested flowers to the centrifuge tubes and shaking, the solution will turn yellowish and the flowers are removed (b). Some flower residues (red arrows) may remain and will need to be removed using tweezers (c). Greenish yellow pollen pellets will form by the end of the mild centrifugation (d). These pellets are suspended in fresh culture media, and transferred to flat bottom glass flasks (e). The flasks are sealed and transferred to a dark culture chamber at 22 °C (f)

9. Incubate the culture in the dark at 22 °C, culture time will depend on the culture objective (Table 2).
10. Check the germination rate for each batch to ensure consistency (*see* **Note 5**).

3.2 Pollen Tube Culture Sub-protocols

The following inter-combinable protocols present simple ways to simultaneously analyze multiple important parameters for the study of pollen tube elongation.

3.2.1 Germination and Elongation Rate

Germination and elongation rates are the two simplest parameters to evaluate pollen fitness and culture conditions. In addition, they provide important support data for publication.

1. Stop cultures between 4 and 6 h.
2. Gently stir the culture flasks to homogenize the solution.
3. With a clipped 1000 µL pipette tips, transfer the solution to a 2-mL centrifuge tube.
4. Gently centrifuge at $3200 \times g$ for 6 min, discard the supernatant, and suspend in 500 µL of fresh culture medium (*see* **Note 6**).
5. If a Neubauer counting chamber is available, make estimates for at least five samples.
6. If a Neubauer counting chamber is not available, transfer 10 µL drops to a microscope slide.
7. Count germinated pollen under a microscope at low magnification (*see* **Note 6**).
8. Pollen tube elongation essays will require an imaging system and measuring software (*see* **Note 7**).

3.2.2 Embedding for Immunohistochemistry

The study of the pollen tube cell wall by immunocytochemistry is an excellent complement to any biochemical essay (*see* Table 3 for useful antibodies). The following protocol describes a simple method for embedding of pollen tubes. Briefly, the pollen tube culture will be fixed in formaldehyde and pelleted by centrifugation. The resulting pellets will be trapped in an agarose matrix and then embedded in LR-white resin for light microscopy.

1. Stop the culture between 4 and 6 h by pipetting out the culture medium and replacing it with 1.5 mL of fixative solution (2% formaldehyde, 2.5% glutaraldehyde in pollen culture medium). Keep for 2 h at room temperature with mild agitation, followed by overnight at 4 °C.
2. Gently swirl the culture to suspend and transfer the pollen culture to a 2 mL centrifuge tube with a clipped 1000 µL pipette tip.
3. Centrifuge at $5000 \times g$ for 5 min and discard supernatant.

Table 3
Useful cell wall-related monoclonal antibodies (MAbs)

Name	Affinity	Available at
LM2	Arabinogalactan protein	L
LM5	Pectins	L
LM6	Neoglycoprotein	L
LM7	Homogalacturonan	L
LM8	Xylogalacturonan	L
LM16	Processed Arabinan—RG-I	L
LM19	Homogalacturonan	L
LM20	Homogalacturonan	L/G
JIM5	Low methyl-esterified homogalacturonan	L/G
JIM7	Highly methyl-esterified homogalacturonan	L/G
JIM4	Arabinogalactan protein	G
JIM8	Arabinogalactan protein	L
JIM13	Arabinogalactan protein	L
JIM20	Extensin	G
MAC207	Arabinogalactan protein	G

All of these MAbs are obtained in rat and are unlabeled. For a comprehensive protocol for immunocytochemistry, *see* Coimbra et al. [29]

G CarboSource Georgia; L plant probes Leeds

4. Prepare a 2% low-melt agarose gel in ddH_2O .
5. Pour the hot low-melt agarose gel into a small petri dish. After the temperature has cooled to 40 °C transfer the pollen tubes pellets to the agarose, apply a mild swirl and let set.
6. Once the agarose has set, with sharp scalpel cut blocks of gel containing pollen tube pellet of no more than 5 mm size, and transfer to glass vials.
7. Dehydrate the samples in an ethanol series (25%, 35%, 50%, 70%, 80%, 90%, and 100%), 15 min at each step, followed by two additional incubations in ethanol 100% for 10 min each.
8. Gradually replace ethanol by LR-white resin (3:1, 2:1, 1:1, 1:2, 1:3, ethanol:resin), incubating each step for 12 h.
9. Leave an extra 6 h in fresh LR-white resin and transfer the embedded blocks to size 1 gelatin capsules, fill with resin and hermetically close with the caps. Let polymerize at 55 °C for 48 h.
10. Make semi-thin sections with an ultramicrotome (*see* Note 8).

3.2.3 Preparation for RNA Extraction

Gene expression analysis is an important tool to understand cell mechanisms. Pollen tubes must be harvested early, as they tend to burst at later times (*see* Table 2). In this protocol, a variation of the phenol/chloroform method to isolate total RNA is used, after filtering out the pollen tubes from the culture by passing the culture through a nylon mesh filter.

1. Culture pollen tubes for 4–5 h.
2. Filter the cultures through a 60- μ m mesh nylon filter (Fig. 3a), to discard ungerminated pollen (*see* Note 9). Rinse the filter with culture medium.
3. Pollen tubes will gather at middle of the nylon filter (Fig. 3b). With a pair of sharp forceps transfer them to an RNase free tube (Fig. 3c).
4. Freeze de samples in liquid nitrogen and store until a significant quantity of pollen tubes is collected (*see* Note 10).
5. Grind the frozen pollen tubes to a fine powder in liquid nitrogen, add 1 mL of Purezol (Bio-Rad), homogenize, and let stand for 5 min at RT.
6. Add 200 μ L of chloroform and mix vigorously for 15 s and let stand for 5 min at RT.
7. Centrifuge at $12,000 \times g$ for 15 min at 4 °C.
8. Transfer the upper liquid phase to a new tube and mix in 500 μ L of ice cold 2-propanol.
9. Let rest for 5 min on ice and centrifuge for 10 min at $12,000 \times g$ at 4 °C.
10. Discard supernatant and wash the pellet with 1 mL of 75% cold ethanol by inverting the tube several times. Centrifuge for 5 min at $7500 \times g$ at 4 °C.
11. Let the pellet air dry and dissolve in 50 μ L of RNase free water (*see* Note 11). Check for quality and DNA contaminants.

3.2.4 Preparation for Cell Wall Extraction

This protocol is designed to obtain a maximum yield of cell wall mass for GC-MS cell wall composition analysis. Pollen tubes will be cultured for over 16 h to maximize growth, thus increasing the yield of cell wall mass per batch.

1. Stop cultures by adding three volumes of absolute ethanol to each culture vial (Fig. 3d) and swirl slowly. Pollen tubes will tend to cluster forming a loose hank (Fig. 3e).
2. Filter the culture through a 60 μ m mesh nylon filter.
3. Wash the filter by passing 70% ethanol through it to discard any remaining ungerminated pollen grains, salts, and sucrose (Fig. 3f).

Fig. 3 Pollen tube isolation. The pollen tube culture may be used for various applications. Minor changes must be made to the protocol to maximize pollen tube recovery depending on the subsequent experiment. For RNA and protein extraction: the pollen tube cultures are filtered through a 60-μm nylon filter (a), and rinsed with culture media. The pollen tubes must be gently gathered with tweezers (b) and transferred to the respective extraction buffer (c). For cell wall extraction (d): 3 volumes of 100% ethanol is added directly to the culture. The pollen tubes will gather forming a loose hank (e). This solution is filtered through a 60-μm nylon filter (f), and the pollen tubes are rinsed with 70% ethanol. Pollen tubes will gather at the bottom of the filter (g). Pollen tubes can be stocked in 70% ethanol until the needed quantity is collected (h)

4. With a pair of forceps, slightly scrape the nylon filter to gather the pollen tubes (Fig. 3g).
5. Transfer the pollen tube bundles to a vial containing 70% ethanol and store at 4 °C until gathering sufficient material (12 mg per replicate) (Fig. 3h).
6. Grind samples in 500 µL of 70% ethanol. Incubate at 70 °C for 15 min. Centrifuge at $5000 \times g$ for 10 min, remove supernatant, and mix in 1 mL of 70% ethanol. Repeat once.
7. Add 1 mL of methanol-chloroform (1:1 v/v), mix and centrifuge at $5000 \times g$ for 10 min. Discard supernatant and let air dry. Add 2.5 µL (4.3^{-3} M Myo-Inositol) and freeze dry.
8. Add 250 µL of 2:1 methanol-HCl, transfer to a GC-vial and incubate for 16 h at 80 °C.
9. Mix in 200 µL of methanol and air dry. Repeat twice.
10. Add 200-µL HMDS-TMCS and incubate at 110 °C for 20 min.
11. Add 200-µL cyclohexane and let evaporate. Repeat twice.
12. Finally dissolve sample in cyclohexane 100 µL and analyze by GC-MS (*see* **Note 12**).

3.2.5 Preparation for Protein Extraction

For an integral study of pollen tube development, proteome analysis is an important tool. This protocol provides a simple method for a basic study of cytoplasmic proteins in a polyacrylamide gel.

1. Filter the pollen tube culture through a 60 µm mesh filter (Fig. 3a).
2. Wash out the pollen grains with culture medium.
3. Pollen tubes will collect at the bottom of the filter (Fig. 3b).
4. With a pair of sharp tweezers, transfer the samples to a sterile 1.5 mL centrifuge tube containing 250 µL protein extraction buffer (Fig. 3c).
5. Grind with a 1.5 mL centrifuge tube compatible pestle for 3 min, the solution should turn yellowish.
6. Centrifuge at $14,000 \times g$ for 30 min.
7. Transfer the supernatant into a new centrifuge tube, add 250 µL of protein extraction buffer and repeat **step 6**.
8. The protein extract can be stored at 4 °C until use.
9. Load samples in a 30% acrylamide gel and stain with Coomassie blue (*see* **Note 13**).

4 Notes

1. If using 69 mm² flat bottom flasks (9.4 mm diameter), each batch of pollen tube culture will require 120 flowers. If using a different diameter culture vessel a ratio of 1.74 flower/mm² is desired.

This proportion will ensure an average of 520 pollen grains/mm², which is close to the optimal pollen density for *Arabidopsis* pollen tube culture [16, 25, 26]. Flowers are collected in groups to keep the average concentration of pollen in culture stable.

It is always a good idea to try working in batches. For example, we propose the use of 120 flowers batches distributed over three tubes with 40 flowers each. Always check if the number of flowers is sufficient before starting a new batch to avoid wasting time and flowers.

2. The collected flowers will tend to wither even in the centrifuge tubes. Withered flowers tend to shed less pollen. Therefore, proceed to the next step as quickly as possible. After addition of the culture medium, the pollen will start to hydrate almost immediately. Shake the tubes gently and continuously. The gentle movement will help improve the release of the pollen to the medium; by the end of the 5 min, the solution will look cloudy (Fig. 2b). Speed is important; the harvesting window should not extend over the 30-min mark in order to obtain a synchronous germination. It is also important to harvest a maximum of top quality pollen from freshly opened flowers (Fig. 1a). Young flowers in which anthesis has not occurred will not contribute any pollen. If the young anthers burst, the pollen will be immature and compete for resources with the mature pollen. The same applies to pollen collected from older flowers already pollinated, the little that remains usually germinates poorly affecting the overall pollen tube yields, in a similar manner as the immature pollen. Flowers that are freshly opened will both give maximum yields of pollen grains and the best pollen tubes.
3. The contamination of the culture with flower residues (Fig. 2c) alters the conditions of culture, not only through competition for oxygen and nutrients but also by introducing additional contaminants, thus greatly affecting the pollen tube culture yields. The foreign tissues may also affect the purity of the pollen tube RNA, protein and cell wall extracts.
4. Pollen tends to stick to the plastic wall of the tubes. The final wash by sequentially passing the 150 µL of culture medium through the three tubes will help to gather some of the remaining pollen. The pollen grains will hydrate and tend to sediment at the bottom of the culture flask. By gently swirling the culture flask, a cloud of pollen should be visible. If not, it may be

possible that the harvest has been ineffective. Mix the culture and check the concentration of pollen grains by pipetting 10 μ L of the suspension to a Neubauer counting chamber. Alternatively, pipette 5 μ L onto a slide and count all the pollen grains. The expected concentration for Colombia-0 wild type is of 50 ± 10 grains/ μ L. If below 50% of this value repeat the extraction process. After **step 6** the liquid in the centrifuge tube should be yellowish and cloudy. If this is not the case verify that a pellet is formed after centrifugation in **step 5**. If a pellet is not formed after **step 5** then, check **Note 2**.

5. To check germination rates gently swirl culture flasks to homogenize the culture. Pipette 10 μ L samples of the culture to a slide and observe. Count the total number of pollen tubes and divide by the total number of objects (pollen tubes and ungerminated pollen grains) in five samples. The average germination rate of Colombia-0 wild-type pollen should normally be above 80%. Low germination rate, or pollen tube burst above 10% for cultures of up to 5 h, are indicative of a problem with the culture medium or culture parameter. Reduced germination and/or elongation rates are a trait of certain mutant lines. Checking that the germination rate is consistent between cultures and batches of the same lines may help to identify phenotypes. It is unusual but from time to time batches with significant divergent germination rates may occur. If they deviate significantly (over 20% variation) from the average germination rate for that specific line, discard it as it may generate artifacts in downstream applications.

If the germination rate varies substantially in systematic manner, the culture media stock solutions should be checked for contaminants. Verify that the pH is perfectly adjusted to 7.5 with HCl. Make sure that the culture chamber temperature is stable at 22 °C.

Glassware is a common source of contamination. Wash the glassware with a mild detergent, and rinse several times with deionized water, 100% ethanol and then deionized water again before letting it dry bottom side up. This should insure that no detergent or salts make it into the culture.

Plastic ware, especially centrifuge tubes, is another common source of chemical contamination. Sadly, a simple wash won't solve the problem. If after checking culture parameter, medium and glassware, low Col-0 germination rates persist, consider using a different brand of centrifuge tubes.

Yet another sign for problems are ruptured pollen grains. If appearing in great numbers accompanied by a low germination rate it may be a sign that centrifugation force was excessive. Observe a sample of pollen after centrifugation in **step 5** of Subheading 3.1. If damaged pollen appears, reduce the

centrifugation speed. If pollen burst is only observed after some culture time has elapsed, then most probably it is a hydration issue, and the culture medium is hypotonic. Retry with a fresh batch of culture medium.

6. Pollen is considered to have germinated when the emerging pollen tube is longer than $1.5 \times$ length of the pollen grain. Pollen grain burst may occur and is very different from pollen tube emergence. Slides with reaction chambers can be used to make this task slightly easier. Calculate the germination rate dividing the germinated pollen average by the total number of pollen grains.
7. There are several free tools with the capacity of recording measurements; ImageJ is one of those versatile tools for microscopy (<https://imagej.nih.gov/ij/download.html>). Always use the same magnification and imaging resolution. Make measurements on unaltered images. Changing image resolution or sizing might cause the measuring tool to give inaccurate results.
8. LR-white resin is compatible with a wide range of water soluble stains. Also, it has low auto-fluorescence, making it perfect for fluorescence immunocytochemistry. A simple and efficient protocol for immunolocalization on LR-White sections of Arabidopsis tissues can be found in Coimbra et al. [29].
9. A minimum of four cultures should be pooled, as there are some losses during the filtering process. Some pollen tubes will stick to the filter mesh and will be unrecoverable, others will pass through the mesh.
10. Up to 10 ng can be purified from a single culture (120 flowers). For most microarray essays, replicates of 150 ng of RNA will be required (about 14 cultures, or 1680 Flowers per replicate). qPCR essays (*see* Table 4 for a list of tested qPCR primers) and RNASeq can be performed with as little as 1 ng. Consider these numbers before starting an experiment.
11. DEPC treatment is the most common solution to make RNase free water. However, DEPC is toxic and some handling training is required. Other safer and easier solutions may be considered, as commercial RNase free water may be purchased at low cost.
12. Culture medium carry over sucrose contamination is a frequent problem with this protocol, and will be manifested by a peak of glucose in chromatography. Make sure to rinse the pollen tubes thoroughly with 70% ethanol. Sugars from the pollen grain exine and intine may reduce the resolution of the test, especially if the pollen tubes are short. The pollen grain will (for the most part) remain attached to the pollen tube. It is therefore inevitable to see its influence in the final analysis. To create a

Table 4
qPCR primers

	AGI	Gene	Forward primer	Reverse primer
Reference genes	At4g27960	UBC9	GTTTCACCACCCCTTTCTTC	AAATCCCACGATCAAATTC
	At4g05320	UBQ10	GCTCCGACACCACTTGACAAC	ACGCAGGACCAAGTGAAGAG
	At5g59370	ACT4	CACCAAACGAAGACGGAAGT	CATTGGGAGTGAAATGTGT
Cell wall-related genes	At1g71380	CEL3	TCTCAAAGTCATTTGCTCTTCTTCC	CGTTGTCTCCTGCGTCATAGTAC
	At3g60570	EXPB5	GCAAACGGTGATGGGAACTTCG	GGACAACGGCGGAGGTAAGC
	At1g71380	CEL3	TCTCAAAGTCATTTGCTCTTCTTCC	CGTTGTCTCCTGCGTCATAGTAC
	At3g60570	EXPB5	GCAAACGGTGATGGGAACTTCG	GGACAACGGCGGAGGTAAGC
	At5g42260	BGLU13	CCATTGGGACACACCCGCAAGC	ACACCTTCTCTGGAGCCATTACACC
	At5g14380	AGP6	TTCCTAAGTTAAGTCGTCCAC	GACATTAGGTTTATATTACTCC
	At3g01700	AGP11	CCACGTAATGTCAAGC	CAACAGGGGATGATGCTTTC
	At3g57690	AGP23	AATGGAGATGAAGAAGATTG	TGCAAGTAGTAGCTGAAG
	At5g40730	AGP24	CGGTTACAGATGGAGGAGGCACTC	AGGACACCAACACGTAGCCACAATC
	At3g20865	AGP40	GCGGCTACAATGGAGTCTC	CAAGAAAGCGGAGAGTGATG
	AT4G35010	BGAL11	GGAATGGGAAAAGGGTTGAT	GCTCAGGCTTCACATTAGGC
	AT2G16730	BGAL13	AAACAAGGCGGTTTGAACAC	GGAAGTCCTCCATGAGTCCA
	AT3G01040	GAUT13	GAATTTTGTGGTTGTCTGT	ACAACATCACTCCCTACGC
	AT1G06780	GAUT6	CGGCTTAAGAGCATCACTCC	CCATCACTTTTACGACAGACGA
	At4g33240	PI4K	TCCTCCAGCCACACGCAGAATTG	GAAGCATAGCGGAAGCAAGCAACC
	At2g39890	PROT1	ATGGCGAGAGCGGGGTAC	GTGGTTGGCTAGATGAATGTGAG
	AT1G69940	PM1E1	ATGCCCTACACCGAGATGACC	AGTTTACAAAGCGGGTGGTG
	AT2G47040	VGD1	ACTGGCCCAAGTCATTCAAC	CCACCGAGACCACTTTT

List of qPCR primers previously tested for useful cell-wall-related genes in pollen tube [24]

base line a pollen cell wall extract can be used. Gather pollen by the method described in this chapter, using ddwater instead of culture media. At the end of **step 5** group all the pollen pellets and proceed as described in the cell wall extraction sub protocol starting at **step 6**.

13. A single culture of 120 flowers will yield an average of 80 µg of protein.

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References

1. Lopes AL, Moreira D, Ferreira MJ, Pereira AM, Coimbra S (2019) Insights into secrets along the pollen tube pathway in need to be discovered. *J Exp Bot* 70:2979–2992
2. Malhó R, Serrazina S, Saavedra L, Dias FV, Ul-Rehman R (2015) Ion and lipid signaling in apical growth – a dynamic machinery responding to extracellular cues. *Front Plant Sci* 6:816
3. Cheung A, Wu HM (2008) Structural and signaling networks for the polar cell growth machinery in pollen tubes. *Annu Rev Plant Biol* 59:547–572
4. Gomes TM, Spitzer NC (1999) In vivo regulation of axon extension and pathfinding by growth-cone calcium transients. *Nature* 397:350–355
5. Galway ME, Heckman JW Jr, Schiefelbein JW (1997) Growth and ultrastructure of arabidopsis root hairs: the *rhd3* mutation alters vacuole enlargement and tip growth. *Planta* 201:209–218
6. Wymer CL, Bibikova TN, Gilroy S (1997) Cytoplasmic free calcium distribution during development of root hairs of *Arabidopsis thaliana*. *Plant J* 12:427–439
7. Pierson ES, Miller DD, Callahan DA, van Aken J, Hackett G, Hepler PK (1996) Tip-located calcium entry fluctuates during pollen tube growth. *Dev Biol* 174:160–173
8. Johnson M, Harper J, Palanivelu R (2019) A fruitful journey: Pollen tube navigation from germination to fertilization. *Annu Rev Plant Biol* 70:809–837
9. Chebli Y, Kaneda M, Zerzour R, Geitmann A (2012) The cell wall of the arabidopsis pollen tube—spatial distribution, recycling, and network formation of polysaccharides. *Plant Physiol* 160:1940–1955
10. Campanoni P, Blatt MR (2007) Membrane trafficking and polar growth in root hairs and pollen tubes. *J Exp Bot* 58:65–74
11. Grebnev G, Ntefidou M, Kost B (2017) Secretion and endocytosis in pollen tubes: models of tip growth in the spot light. *Front Plant Sci* 8:154. <https://doi.org/10.3389/s.2017.00154>
12. Malhó R, Liu Q, Monteiro D, Rato C, Camacho L, Dinis A (2006) Signalling pathways in pollen germination and tube growth. *Protoplasma* 228:21–30
13. Onelli E, Moscatelli A (2013) Endocytic pathways and recycling in growing pollen tubes. *Plan Theory* 3:211–229
14. Sekereš J, Pleskot R, Pejchar P, Žárský V, Potocký M (2015) The song of lipids and proteins: dynamic lipid–protein interfaces in the regulation of plant cell polarity at different scales. *J Exp Bot* 66:1587–1598
15. Pina C, Pinto F, Feijó JA, Becker JD (2005) Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene

- expression regulation. *Plant Physiol* 138:744–756
16. Costa M, Nobre M, Becker J, Masiero S, Amorim M, Pereira LG, Coimbra S (2013) Expression-based and co-localization detection of arabinogalactan protein 6 and arabinogalactan protein 11 interactors in *Arabidopsis* pollen and pollen tubes. *BMC Plant Biol* 13:7
17. Liepman AH, Wightman R, Geshi N, Turner SR, Scheller HV (2010) *Arabidopsis*—a powerful model system for plant cell wall research. *Plant J* 61:1107–1121
18. The *Arabidopsis* Genome initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408:796–815
19. Taylor LP, Hepler PK (1997) Pollen germination and tube growth. *Annu Rev Plant Physiol Plant Mol Biol* 48:461–491
20. Fan LM, Wang YF, Wang H, Wu WH (2001) In vitro *arabidopsis* pollen germination and characterization of the inward potassium currents in *arabidopsis* pollen grain protoplasts. *J Exp Bot* 52:1603–1614
21. LiH LY, Heath RM, Zhu MX, Yang Z (1999) Control of pollen tube tip growth by a Rop GTPase-dependent pathway that leads to tip-localized calcium influx. *Plant Cell* 11:1731–1742
22. Boavida LC (2005). Genetic dissection of cell-to-cell interactions in sexual reproduction in *Arabidopsis thaliana*. s.l. : Ph.D. thesis, Departamento de Biologia Vegetal, Faculdade de Ciencias da Universidade de Lisboa, Lisbon
23. Johnson-Brousseau SA, McCormick S (2004) A compendium of methods useful for characterizing *arabidopsis* pollen mutants and gametophytically-expressed genes. *Plant J* 39:761–775
24. Costa M, Pereira LG, Coimbra S (2013) Growth media induces variation in cell wall associated gene expression in *Arabidopsis thaliana* pollen tube. *Plan Theory* 2:429–440
25. Boavida L, McCormick S (2007) Technical advance: temperature as a determinant factor for increased and reproducible in vitro pollen germination in *Arabidopsis thaliana*. *Plant J* 52:570–582
26. Dardelle F, Lehner A, Ramdani Y, Bardor M, Lerouge P, Driouich A, Mollet JC (2010) Biochemical and immunocytological characterizations of *arabidopsis* pollen tube cell wall. *Plant Physiol* 153:153–176
27. Shivanna KR, Linskens HF, Cresti M (1991) Pollen viability and pollen vigor. *Theor Appl Genet* 81:38–42
28. Hashida SN, Takahashi H, Kawai-Yamada M, Uchimiya H (2007) *Arabidopsis thaliana* nicotinate/nicotinamide mononucleotide adenylyltransferase (AtNMNAT) is required for pollen tube growth. *Plant J* 49:694–703
29. Coimbra S, Almeida J, Junqueira V, Costa ML, Pereira LG (2007) Arabinogalactan proteins as molecular markers in *Arabidopsis thaliana* sexual reproduction. *J Exp Bot* 58:4027–4035