Chapter 17

Imaging and Analysis of the Content of Callose, Pectin, and Cellulose in the Cell Wall of Arabidopsis Pollen Tubes Grown In Vitro

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Abstract

To achieve fertilization, pollen tubes have to protect and properly deliver sperm cells through the pistil to the ovules. Pollen tube growth is a representative example of polarized growth where new components of the cell wall and plasma membrane are continuously deposited at the tip of the growing cell. The integrity of the cell wall is of fundamental importance to maintain apical growth. For this reason, pollen tube growth has become an excellent model to study the role of polysaccharides and structural cell wall proteins involved in polar cell expansion. However, quantification of structural polysaccharides at the pollen tube cell wall has been challenging due to technical complexity and the difficulty of finding specific dyes. Here, we propose simple methods for imaging and quantification of callose, pectin, and cellulose using specific dyes such as Aniline Blue, Propidium Iodide, and Pontamine Fast Scarlet 4B.

Key words Pollen tubes, Imaging, Callose, Cellulose, Pectin, Cell wall

1 Introduction

Spatial distribution of different polysaccharides and the role of structural proteins such as hydroxyproline-rich glycoproteins (HRGPs) are crucial to maintain the integrity of the pollen tube cell wall. Generally, in angiosperms, the apical region of pollen tubes is composed of a single, flexible, and dynamic wall layer which is constantly remodeling at the tip. In distal regions where expansion has ceased, the cell wall gets stiffened through enzymatic action promoting polymer linkages, for example the gelation of pectin, and through deposition of stiffer materials such as cellulose and callose [1]. This stiffening locks in place the width of the tube and maintains the constant diameter of the tubular shank of the cell [2]. In contrast to other plant cells, the cell wall of the pollen tube

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contains a large amount of callose and pectin and only relatively low amounts of cellulose.

Callose is a (1 → 3)-β-D-glucan synthetized at the plasma membrane by callose synthase complexes. It is deposited along the pollen tube under the fibrous pectic layer in the cylindrical portion of the tube and is absent at the tip [3]. The first reports describing the detection of callose with Aniline Blue staining date back to 1949 (reported in Currier [4]). Aniline Blue has been widely used to stain callose in pollen tubes both in vitro and in vivo, and to detect callose associated to plasmodesmata between neighboring cells and phloem sieve elements, and plant cell responses to pathogen attack [4, 5].

Pectin is a complex polymer mainly composed of homogalacturonan (HG) and rhamnogalacturonan I and II (RGI and RGII) [6] that is deposited at the apical region of the pollen tube in a highly methylesterified state. The degree of pectin esterification decreases toward the distal regions of the pollen tube where the enzyme pectin methylesterase remains active [3]. In distal regions, free pectin carboxyl groups interact with Ca^{2+} ions, forming a gel that stiffens the region preventing any further lateral expansion [3]. Propidium iodide (phenanthridinium, 3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenyl-,diiodide) is a red-fluorescent dye which competes with Ca^{2+} for binding to carboxyl residues of HGs and has been used as an indicator of the degree of crosslinking of pectic HGs in pollen tube cell walls [7].

Cellulose, a polysaccharide with a lower relative abundance in pollen than in most other plant cells, is a (1 → 4)-β-D-glucan. Cellulose is synthetized at the plasma membrane by multimeric cellulose synthase (CESA) complexes [8]. In pollen tube cell walls, cellulose is organized as crystalline microfibrils that form a tight network with pectin and callose [3]. Calcofluor white has been used for cellulose staining, although this dye is not specific since it has also affinity for callose and for (1 → 4)-β-*N*-acetyl-D-glucosamine (known as chitin). Alternatively, Pontamine Fast Scarlet 4B (S4B) (also known as Direct Red 23 and Levacell Scarlet E-3B) has been shown to be selective for polysaccharides containing β-D-(1 → 4) bonds and fluoresces more intensely in the presence of cellulose than with any other plant polysaccharide [9]. S4B has been used to stain cellulose microfibrils in the primary cell wall of *Arabidopsis thaliana* roots [9] and recently, in pollen tubes [10], and cotyledons [11].

The synthesis and assembly of the plant cell wall components in *Arabidopsis thaliana* are controlled by a myriad of proteins with different expression patterns, degrees of functional redundancy, and contribution to the overall polymer production. A typical approach to study the role of genes involved in plant cell wall synthesis and assembly implies obtaining and characterizing single and multiple mutants for the genes of interest. While the

quantification of gene expression in different mutant backgrounds is relatively straightforward, determining the final effect on the cell wall secretion and assembly can be challenging. For example, localized production and deposition of polymers (as in the growing pollen tube tip), requires sensitive and unambiguous detection and quantification methods that allow direct comparison and statistical analysis. Here, we present protocols for the detection, quantification, and statistical analysis of the abundance of callose, pectin, and cellulose in growing pollen tubes of Arabidopsis.

2 Materials

2.1 Plant Material

Arabidopsis thaliana var. Col-0 (wild type) seeds are obtained from the Arabidopsis Biological Resource Center (ABRC) or the Nottingham Arabidopsis Stock Centre (NASC). To perform the assays, select one-day open flowers (Fig. 1); at this stage, flowers are fully opened and mature pollen grains are released from the anthers (*see* **Note 1**).

2.2 Pollen Germination Media

Two alternative pollen germination media (PGM) can be used: *semi*-solid and *semi*-liquid. Prepare stock solutions for PGM according to Boavida and McCormick (2007) [12]. Final concentrations are 0.01% H_3BO_3, 5 mM KCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, and 10% sucrose. Set the pH to 7.5–8 with 0.1 M KOH.

- To prepare *semi*-solid medium, reach the final volume with MilliQ H_2O, add low-melting agarose to a final concentration of 1% and melt it at 95 °C for 5 min using a block heater (*see* **Note 2**). This medium makes a gel resistant to compression.
- To prepare *semi*-liquid medium, reach the final volume using 0.15% agarose pre-melted solution prepared in MilliQ H_2O (*see* **Note 3**). Pollen tubes grow on the solution surface and do not sink.

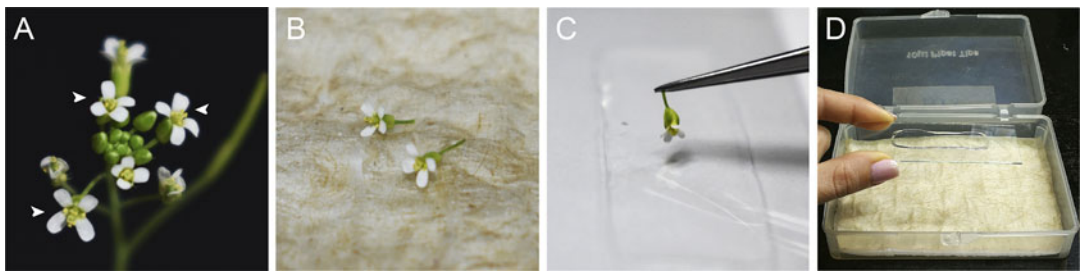

Fig. 1 Step-by-step pollen germination method on *semi*-solid medium. One-day open *Arabidopsis thaliana* flowers (**a**, white arrowheads) are positioned over wet paper towels (**b**) and incubated for 30 min at room temperature in a humidity chamber (similar to **d**). Pollen grains from pre-incubated flowers are transferred to the medium using forceps (**c**). Finally, a coverslip is placed on top of the medium and slides are incubated in a humidity chamber at 22 °C for 2–3 h (**d**)

2.3 Dyes

All dye stock solutions must be prepared in MilliQ H_2O and stored at 4 °C protected from light. To avoid osmotic shock, use liquid PGM (reach the final volume by using MiliQ H_2O without agarose addition) to prepare fresh working solutions from all dye stocks before performing the assays.

1. Propidium iodide (PI) stock solution: 1 mg/ml. To prepare a PI working solution, make a fresh 1/5 dilution.
2. Aniline blue (AB) stock solution: 0.1% (w/v) in 108 mM K_3PO_4 pH ~11 [13]. Store the solution overnight at 4 °C and then filter it. Finally, add glycerol to a final concentration of 2% (v/v). To prepare an AB working solution, make a fresh 1/2 dilution.
3. Pontamine Fast Scarlet 4B (S4B) stock solution: 1% (w/v). To prepare a S4B working solution, make a fresh 1/100 dilution.

2.4 Equipment

1. Pollen incubation chamber: A tip rack can be used to build a humidity chamber, placing a wet paper towel at the bottom of the rack (Fig. 1).
2. Block heater.
3. pH meter.
4. Confocal microscope.
5. ImageJ/Fiji Software.

3 Methods

3.1 Pollen Germination

Collect at least two one-day open flowers and pre-incubate them directly on wet paper towels for 30 min at room temperature in a pollen incubation chamber (Fig. 1).

1. If *semi*-solid medium is used, melt the agarose and quickly place a thin layer of medium with a pipette on a glass slide. Once PGM has gelled, use forceps to gently brush the pre-incubated flowers on the medium to spread pollen grains. Cover the medium with a coverslip (*see* **Note 4**) by applying some pressure and incubate in the pollen incubation chamber at 22 °C for 2–3 h in continuous light (Fig. 1).
2. If *semi*-liquid medium is used, place a drop (~20 μl) of medium with a pipette on a glass slide and use forceps to gently brush the pre-incubated flowers on the medium to spread pollen grains. Cover the medium with a coverslip and incubate in the pollen incubation chamber at 22 °C for 2–3 h under continuous light (Fig. 1).

3.2 Pectin Staining

1. Carefully lift the coverslip, add 5 μl of the PI working solution in the *semi*-solid or *semi*-liquid PGM and cover it again.

2. Incubate 5 min before imaging (*see* **Note 5**). PI has an excitation maximum at 535 nm and an emission peak at 617 nm. PI can be alternatively excited with a 488 or a 532 nm laser line.

3.3 Callose Staining

1. Carefully lift the coverslip, add 5 μl of the AB working solution in the *semi*-solid or *semi*-liquid PGM and cover it again.
2. Proceed with the imaging. AB has an excitation maximum at 320–380 nm and an emission peak at 450 nm.

3.4 Cellulose Staining

1. Carefully lift the coverslip, add 5 μl of S4B working solution in the *semi*-solid or *semi*-liquid PGM and cover it again.
2. Proceed with the imaging. S4B has an excitation maximum at 405 nm and an emission peak at 570 nm.

4 Image Acquisition

Confocal scanning settings must be optimized according to the microscope in use and its hardware configuration (*see* **Note 6**). The images shown here were obtained with a Leica SPE confocal laser scanning microscope, using a 1024 × 1024 format, bidirectional scanning at a speed of 600 Hz, an APO/ACS 63×/1.30 oil objective and a 3× digital zoom (Fig. 2). Pinhole was set to 1 Airy unit. The transmitted light was used to focus and analyze pollen tube tips (*see* **Note 7**). Laser lines were selected depending on the excitation/emission spectra of the fluorophores.

4.1 ImageJ Analysis

To quantify the fluorescence intensity at the pollen tube margins, make measurements along the perimeter of the pollen tubes, including their apical and subapical regions (Fig. 3).

1. Open the ImageJ/Fiji application. In "File" go to "Open" and find the location of the file.
2. Click the "Line tool" from the toolbar (fifth option from the left), set "Line Width" in 15 by double clicking and activate

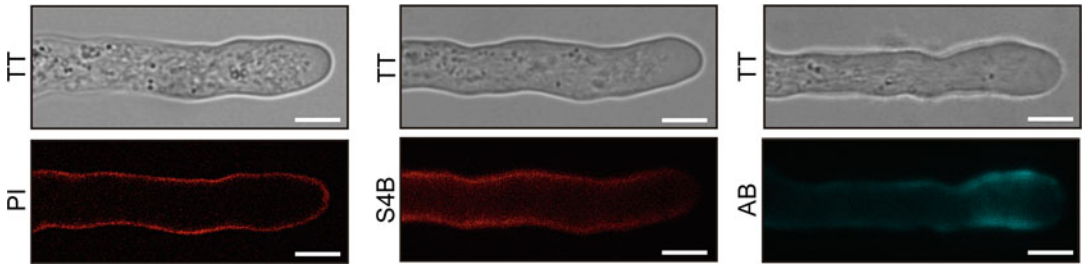

Fig. 2 Representative confocal images of Col-0 pollen tubes stained with propidium iodide (PI) for pectin (left panel), Pontamine Fast Scarlet 4B (S4B) for cellulose (middle panel), and aniline blue (AB) for callose (right panel). *TT* transmitted light. Scale bar = 5 μm

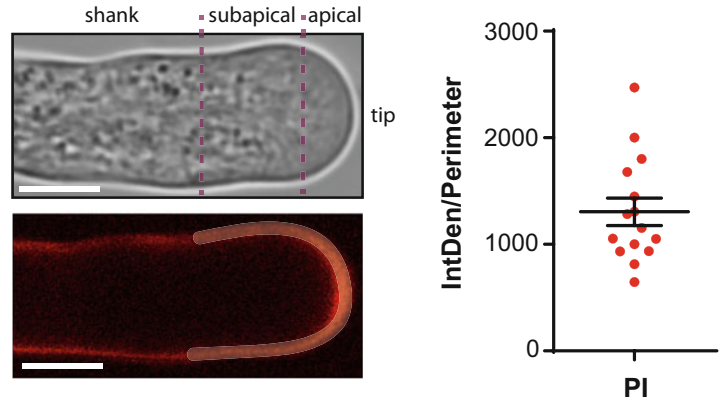

Fig. 3 Pollen tube spatial organization: tip, apical region, subapical region, and shank are indicated (left, upper panel). Representative image of a Col-0 pollen tube stained with PI (left, bottom panel). Measurement of PI fluorescence intensity at the margins of Col-0 pollen tubes; the fluorescent signal was normalized to the perimeter of the measured region. Recorded fluorescence of $n = 15$ pollen tubes from one independent experiment is shown. Scale bar = 5 μm

"Spline fit" option (*see* **Note 8**). Then, by pressing the right button of the mouse, select the "Segmented line" option.

3. Draw the perimeter of the pollen tube, including the apical and subapical region, and finish the drawing by double-clicking on the last traced point.
4. To record the fluorescence, go to "Analyze" and press "Measure" (or Ctrl+M). A new window will appear with the data.
5. Copy and paste the data into the appropriate data analysis software for further analysis.

To quantify the fluorescence intensity of the cytoplasm and the margins of the pollen tube, trace a longitudinal or a transversal line (Fig. 4a, b):

1. Open the Image J/Fiji application. In "File" go to "Open" and find the location of the file.
2. Click the "Line tool" from the toolbar (fifth option from the left) and set "Line width" in 30 by double clicking (*see* **Note 9**). Then select the "Straight Line" option.
3. Trace the longitudinal or the transversal line, go to "Analyze" and press "Plot profile" (or Ctrl+K). A two-dimensional graph with the intensity recorded along the line drawn will appear in a new window.
4. To export the data, select "More" at the bottom of the window, then press "Copy All Data" and paste it into the appropriate data analysis software for further analysis.

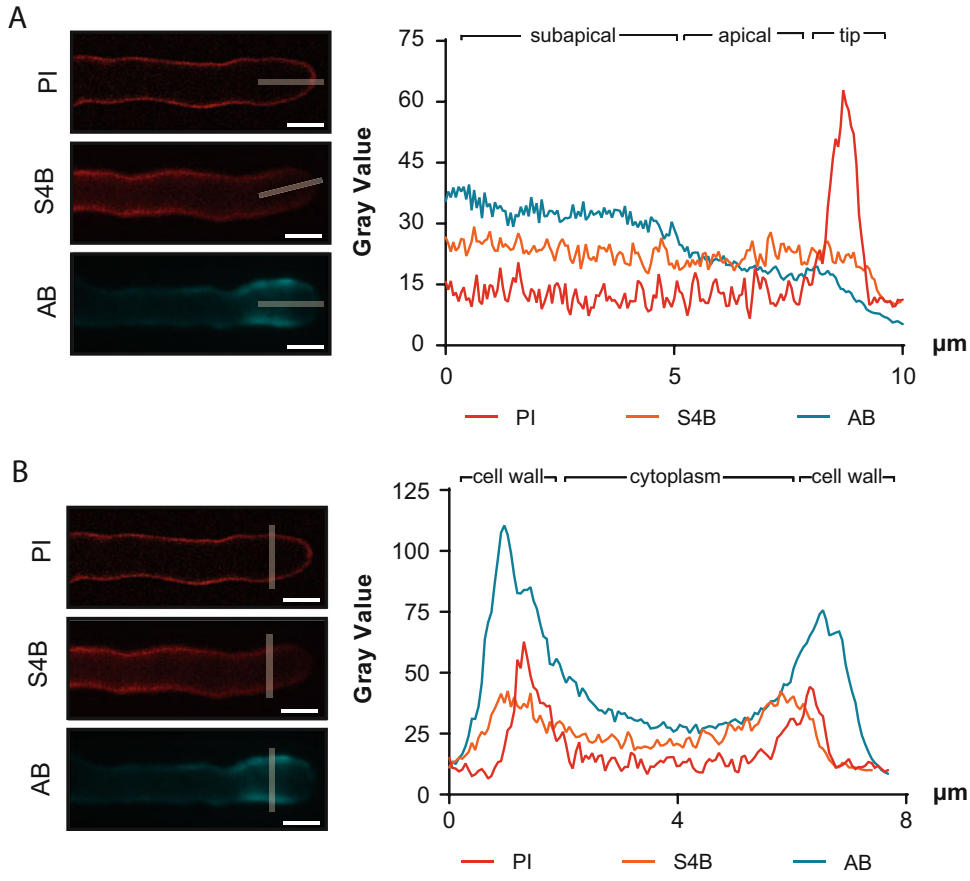

Fig. 4 Representative images and intensity measurement of cell wall components of Col-0 pollen tubes stained with propidium iodide (PI), Pontamine Fast Scarlet 4B (S4B), and aniline blue (AB). (**a**) Longitudinal fluorescence measurement (upper panel). (**b**) Transversal fluorescence measurement (bottom panel). Scale bar = 5 µm

4.2 Statistical Analysis of Data

Common errors when generating and analyzing pollen tube data are: grouping data from independent experiments (performed on different days), using data from experiments with low pollen germination rates, comparing genotypes that were not analyzed simultaneously, comparing raw data without prior normalization, and using an erroneous N in statistical analysis.

Unlike other species, reproducible germination rates for Arabidopsis pollen are quite difficult to obtain in vitro. Pollen germination after 3 h of incubation can vary from 0% to 60% or more in any given experiment, even when the same PGM is used and the assay is performed in the same growth chamber. In fact, several articles have focused on improving pollen germination and pollen tube growth [12, 14, 15]. In terms of biological variability, pollen tube populations may be different on different days. In terms of sample

representability, we assume that the higher the germination rate, the more adequate the sample is to infer population parameters. Inversely, experiments with low germination rates could produce estimates of biased population parameters because they only consider the most successful pollen tubes grown in suboptimal conditions. For this reason, we suggest that only experiments in which wild-type pollen germination rates are at least 30–40%, after 3 h of incubation, should be considered.

Another source of variation arises from the experiment *per se*. In this case, staining will fluctuate with dye preparations and fluorescence detection will be different between different confocal microscopes or imaging sessions even when identical settings are used. If germination rates are reproducible through experiments and minimal variation is introduced during imaging, sample estimates of population parameters could be obtained and compared. However, if that is not the case, it is necessary to implement normalization strategies to compare data between experiments (*see* **Notes 10** and **11**).

5 Notes

1. For best results, avoid using the first flowers of the primary bolt since most pollen is infertile.
2. H_3BO_3 can be added to the PGM after the agarose melting.
3. Agarose serves as a support and at the same time improves pollen germination and pollen tube growth.
4. Germination rates can be improved when pollen grains are grown on the interface between the coverslip and the PGM.
5. PI is toxic to pollen tubes at high concentrations and after long incubation periods. When the pollen tube is dead, the PI enters the cell and fluorescence is observed throughout the cytoplasm.
6. An optimal microscope configuration must be set for each fluorophore and must be kept constant during imaging sessions and between experiments.
7. Only live pollen tubes should be included for analysis. To ensure that a pollen tube is alive, use transmitted light and verify that the tubes show the typical cytoplasm streaming. The proximity of the big vacuole to the apical region indicates the impending death of the pollen tube.
8. The line width (in pixels) is set according to the image resolution and the zoom chosen. Select a line width thick enough to only encompass the cell wall. Here, the line width for a 1024×1024 image captured with a digital zoom $3\times$ was calculated.

9. In this case, a thicker line width is selected to record and integrate the fluorescence of a larger number of pixels and decrease the measurement error.
10. The first strategy to minimize imaging variations is to subtract the background signal and normalize data. For example, when determining the fluorescence intensity in transects, the background signal (detected in the same image) is subtracted from each value in the series of linear data. Thus, normalization is done by dividing each value to the highest value of the series and data are scaled to the same reference system (between 0 and 1). In each experiment, the observations for each genotype (i.e., Col-0) will be averaged to obtain a sample estimate of the population parameter, in the same way that the sample mean provides an estimate of the population mean. In each case, the number of observations required will be determined (in our experience, 15 observations per genotype per experiment are adequate). Because the purpose of each experiment is to obtain sample estimates for the population parameters, N corresponds to the number of replications per genotype and not to the total number of observations within each replicate or all samples in the experiments. For example, 15 pollen tubes for Col-0 in three independent replicates correspond to an $n = 3$, and not $n = 45$. If data for each genotype are comparable between experiments after this normalization (that is, the standard errors are 10% of the average of the observations), no further normalization is required.
11. A second normalization strategy is to define the variable to be measured as a ratio between the sample estimates of the population parameters for the genotypes or treatments of interest. For example, a mutant versus a wild-type genotype or a treated versus an untreated sample. This strategy is suitable for highly variable experiments. As before, sample estimates can be calculated based on an appropriate number of observations (for example, a number that produces a standard error of 10% of the average). In this case, N corresponds to the number of independent ratios generated.

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