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Pollen Grain Counting Using a Cell Counter

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Cover page

Chapter Title

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Pollen grain counting using a cell counter

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Chapter Title

Pollen Grain Counting Using a Cell Counter

Authors

Hiroyuki Kakui, Misako Yamazaki, Naoto-Benjamin Hamaya, Kentaro K. Shimizu

Abstract

The number of pollen grains is a critical part of the reproductive strategies in plants and varies greatly between and within species. In agriculture, pollen viability is important for crop breeding. It is a laborious work to count pollen tubes using a counting chamber under a microscope. Here, we present a method of counting the number of pollen grains using a cell counter. In this method, the counting step is shortened to 3 minutes per flower, which, in our setting, is more than five times faster than the counting chamber method. This technique is applicable to species with a lower and higher number of pollen grains, as it can count particles in a wide range, from 0 to 20,000 particles, in one measurement. The cell counter also estimates the size of the particles together with the number. Because aborted pollen shows abnormal membrane characteristics and/or a distorted or smaller shape, a cell counter can quantify the number of normal and aborted pollen separately. We explain how to count the number of pollen grains and measure pollen size in *Arabidopsis thaliana*, *Arabidopsis kamchatica* and wheat (*Triticum aestivum*).

Keywords: Pollen number, Pollen size, Pollen viability, Cell counter (CASY Cell Counter), *Arabidopsis thaliana*, *Arabidopsis kamchatica*, Wheat (*Triticum aestivum*)

1. Introduction

Pollen grain number has long been studied from agricultural and evolutionary viewpoints

[1-~~43~~]. For example, the number of pollen grains was shown to have decreased during rice domestication [~~54~~]. The number of viable pollen grains is critical for breeding because decreased yield due to pollen abortion can be induced by diverse abiotic stresses, such as low/high temperature, salt stress, and osmotic shock [~~65~~, ~~67~~]. To control pollen mass is a major challenge to increase the efficiency of hybrid breeding in wheat [7]. In an evolutionary context, shifts from outcrossing to selfing are one of the most frequent evolutionary transitions in angiosperms [~~4~~, 8]. Selfing plants tend to show a set of traits termed selfing syndrome, such as smaller flower size, ~~less-reduced~~ scent and nectar, and a reduced pollen grain number (or low pollen–ovule ratio). The selfing syndrome is regarded as adaptive evolution because selfing plants do not need an excess amount of pollen to assure mating success and, instead of producing pollen, use that resource for other purposes.

Recently, we identified a pollen-grain-number controlling gene in *Arabidopsis thaliana* by a genome-wide association study. We named this gene *REDUCED POLLEN NUMBER 1 (RDPI, [9])*. We found that *rdp1* null mutants produced about half the number of pollen grains per flower compared with wild type. In our study, we also found that pollen grain number per flower varied greatly between accessions (an average of 2,000-8,000 pollen grains from 144 accessions) and within samples from the same individual (1,000-5,000 pollen grains in Col-0). Because of this large variation, many samples (~50 samples per genotype) had to be counted to obtain a reliable estimate of pollen grain number from an accession and a genotype. Traditionally, pollen grain number is counted under a microscope using a counting chamber (hemocytometer) [10-12], but this method is time-consuming and laborious. A more recent approach is based

Kommentar [KKS1]: Original reference 4 was not appropriate in this context. Another relevant reference was added together with a sentence.

on automated image analysis. Researchers can apply this method to the images obtained using counting chambers or conventional glass slides. Its advantage is that the analytical software is available for free and saves time compared with manual counting [13].

However, this method has difficulties with handling samples that contain aborted pollen and/or debris, because these can adversely affect the identification of the normal pollen.

Another drawback of the counting chamber method arises when measuring pollen size. A small difference in microscope handling (i.e., focus, light intensity, position in the optical field, etc.) may lead to a different region being recognized as a pollen and may cause a large difference in size calculation.

Recently, cell counters (e.g., CASY cell counter (OLS OMNI Life Science [14]) or Ampha Z32 (Amphasys [15])) provided convenient and reliable methods for the counting of pollen grain number, the measurement of the size of pollen grains and examination of pollen viability. Here, we describe a pollen grain counting method that uses a CASY cell counter. Cell counters measure the number and the size of cells based on electrical impulses. For example, the CASY Model TT uses an established method called “Electrical Sensing Zone” paired with “Pulse Area Analysis” [14]. Pollen is suspended in an electrolyte (CASYton) that is aspirated through a precision measuring pore. The electrolyte then moves through the pore, which has a defined electrical resistance, at a constant flow speed. Each pollen grain that passes through the measuring pore creates a change in voltage and is recorded as a cell count. The amplitude of the signal corresponds to the volume of each pollen grain, as it displaces as much electrolyte as its own volume. Moreover, the shape and time course of the electrical impulse are recorded. From that, the cell counter can translate the change in voltage into biologically interpretable values, such as cell diameter, because it calculates the integral of the electrical impulse. The sampling rate of the cell counter is 1 MHz, which means that the measuring pore performs 1

million measurements per second. Furthermore, it is possible to distinguish between viable and dead cells. Given that viable cells are electrical isolators because of their cell membrane, their volume can be determined precisely. Conversely, the cell membrane of dead cells may not be intact, leading to the detection of a reduced signal compared with viable cells [14].

Figure 1 shows a comparative overview of the two counting methods, one (A) using a counting chamber and the other (B) using a cell counter. Both methods follow the same procedures for flower collection (in Methods 3.1), incubation, and sonication (in Methods 3.2). The next step in the counting chamber method requires image capturing and image analysis (in Methods 3.3-A), which are time-consuming (in total, 17 min per sample in our setting). The cell counter method needs sample preparation before measurement (in Methods 3.3-B); including this preparation time, measurement requires in total 3 min per sample, meaning that the cell counter method is more than five times faster than the counting chamber method.

[Fig. 1 near here]

2. Materials

2.1 Preparation of plant materials

1. Plants with flower buds (i.e. *A. thaliana*, *A. kamchatica* or wheat (*Triticum aestivum*); see Note 1).
2. Fine forceps.
3. 0.5 ml tube.
4. Needle (18G size).
5. 60°C incubator.

2.2 Sonication

1. Sonicator (Bioruptor Plus, Diagenode, Belgium).
2. 5% (w/w) Tween-20 (SIGMA, St. Louis, MO, USA).

2.3 Pollen counting

1. Counting chamber (Neubauer, Paul Marienfeld, Germany).
2. Light microscope.
3. Fiji [16].
4. Cell counter (model: CASY TT, OLS OMNI Life Science, Germany).
5. Isotonic buffer for CASY (CASYton, OLS OMNI Life Science).
6. Measuring vessel for CASY (CASYcups, OLS OMNI Life Science).
7. Software for CASY (CASYworX 1.26, OLS OMNI Life Science).

2.4 Data analysis

R software [17].

3. Methods

3.1 Flower collection

1. Choose closed anthers with fully matured pollen. Mature anthers are typically yellow, whereas immature anthers are green. Select *A. thaliana* or *A. kamchatica* flowers from late flower stage 12 to early stage 13 [18, 19]. Flowers from the main stem tend to produce more pollen grains compared with those from the side stems and side branches of the main inflorescence [9]. Because of this characteristic and the limitation of the number of flowers on the apical tip of one branch, take samples from the side stems or side branches of the main inflorescence, to collect more flowers on the same day. Avoid the first and second flowers on each branch because they tend to have an abnormal flower shape and pollen number (for wheat, see Note 2).

2. Open a flower with fine forceps and place it in a 0.5 ml tube. Close the tube, make a hole in it using a needle, and incubate it overnight at 60°C, to dry and open the anthers (see Note 3).

3.2 Sonication

Add 30 µl of 5% Tween-20 and centrifuge at 100 x g for 1 min (for wheat, see Note 4). Place the tubes into the sonicator and start sonication at 4°C for five cycles of sonication-ON for 30 sec and sonication-OFF for 30 sec, to release the pollen grains from the anthers (the majority of pollen grains are released into the Tween-20 (Fig. 2; see Note 5)).

[Fig. 2 near here]

3.3-A Pollen counting using a counting chamber

1. Apply a cover slip to the counting chamber and Add-add 10 µl of well-mixed sample solution which is prepared in 3.2-to the counting chamber and apply a cover slip. Capture nine images per sample using a light microscope to obtain robust data (Fig. 3; see Note 6).

[Fig. 3 near here]

2. The number of pollen grains per image is counted using the particle counting implemented in Fiji. The total number of pollen grains per flower is estimated based on the image size and the total volume.

Calculated total pollen number = counted pollen number x sample volume (30 µl) / (image size-area (µm²) x height of the chamber (100 µm))

Kommentar [KKS2]: The parentheses are necessary

3.3-B Pollen counting using a cell counter (CASY cell counter)

1. Set the parameters on the CASY cell counter for *Arabidopsis thaliana* as described in Table 1 (see Note 7 for *A. kamchatica* and Note 8 for wheat).

[Table 1 near here]

2. Fill the CASYcup with 10 ml of CASYton. Take 0.3 ml of CASYton from the CASYcup and mix the solution in a 0.5 ml tube after sonication. Transfer the entire liquid to the CASYcup (without the flower sample). Put a cap on the CASYcup and mix gently. Measure the sample immediately using the CASY cell counter (see Note 9).
3. A result screen appears after the measurement. A few examples are displayed in Fig. 4. The measurement data are transferred automatically to CASYworX.

[Fig. 4 A-D near here]

3.4 Data analysis

Import pollen grain number (or size) data from CASYworX to R, to generate a ~~box~~-violin plot (Fig. 5). In *A. thaliana*, the pollen grain number is highly variable, even between flowers taken from the same individual (e.g., around 2,000-6,000 pollen grains from *A. thaliana*; Fig. 5A). Therefore, to obtain a robust result, the collection of more than 50 samples per genotype is recommended. *A. kamchatica* produced a great quantity of pollen (Figs. 4C and 5A), and wheat produced larger pollen grains (Figs. 4D and 5B) compared with the other two species.

[Fig. 5 near here]

4. Notes

1. For *A. thaliana*, plants flower typically 10 days after bolting. For wheat, choose mature ears with flowers in some spikelets. To obtain reproducible results, it is important to determine the anther stage.
2. For wheat anther sampling, open spikelets and identify closed (yellow) anthers (stage 13-14 [20]). Hold the ear in your hand and wait until the anther filament elongates (a few minutes). Collect elongated anthers before they release pollen.
3. Flower samples can be stored in the 60°C incubator for about one week.
4. Add 5% Tween-20 to the sample and cut both tips of the anther using a syringe to help release the pollen after sonication (Fig. 6). To obtain a reliable result, it is essential to confirm the setup to release almost all pollen grains from the anther. The observation of several anthers after sonication using Alexander staining is recommended [21].

[Fig. 6 near here]

5. At the sonication step, use a blank tube if your sample has an empty slot in the sonication holder, to sonicate each sample homogeneously.
6. At the image analyzing step, pollen grains at the edge of the image should not be counted. (Fig. 3C bottom).

7.

[Table 2 near here]

8.

[Table 3 near here]

9. At the counting step, if error messages appear, clean the capillary three times using CASYton (blank sample) by pushing the “clean” button.

Acknowledgements

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Figure Captions

Fig. 1 Overview of pollen grain counting using a counting chamber (A) or a cell counter (B). The numbers in red indicate the estimated time required for the counting of a single sample in our setting.

Fig. 2 Anthers before (A) and after (B) sonication. Only a few pollen grains remained after sonication (B). Anthers from *A. thaliana* were stained by Alexander staining [21]. The arrows indicate pollen grains.

Fig. 3 The principle of the counting chamber. (A) Counting chamber. (B) Graphical illustration of the counting chamber grid, separated into nine fields. One image is captured for each of the nine fields because of the variation of pollen grain number between fields (C, top; $n = 8$ and bottom; $n = 16$).

Fig. 4 Result screen of the CASY cell counter. Plots were created and analyses were performed using the CASYworX 1.26 software (modified). The x-axis indicates the particle size and the y-axis indicates the particle counts. Debris (R1), aborted pollen (R2), and normal pollen (R3) are indicated. “Counts” indicate the measured number of normal pollen grain, “Viable cells/ml” indicate the calculated number of normal pollen grains per sample (calculated by “Counts”, dilution factor, and machine bias), “Total cells/ml” indicate the total number of pollen grains (including aborted pollen), and “Mean diameter” indicates the average size of normal pollen. (A) Wild-type pollen distribution in *A. thaliana*. There is no peak in the region of aborted pollen (R2). (B) Flower with aborted pollen from *A. thaliana*. There is a clear peak in the aborted pollen region (R2). (C) Pollen measurement example from *A. kamchatica*, which produced around four times as many pollen grains as did *A.*

thaliana (1.736×10^4 pollen grains per flower). (D) Pollen measurement in wheat. The size of its pollen is more than twice that of *A. thaliana* (~50 µm in diameter).

Fig. 5 Violin plot for pollen grain number (A) and size (B) from *A. thaliana* (Col-0, $n = 122$), *A. kamchatica* (Hukiinoura [22], $n = 51$) and wheat (ArinaLrFor [23], $n = 60$). Green dots represent samples and red dots indicate medians.

Kommentar [HK3]: We increased sample number from 31 to 51

Fig. 6 Wheat anther. The dashed lines represent cutting lines.

Table Captions

Table 1 Parameter setup used for counting the number of *Arabidopsis thaliana* pollen grains

The number of particles detected between the “Left Norm. Cursor” and “Right Norm. Cursor” represents the total amount of pollen (viable and aborted pollen). The number of particles detected between the “Left Eval. Cursor” and “Right Eval. Cursor” represents viable pollen. The dilution factor is used to calculate the original concentration (“Viable cells/ml” in Fig. 5). If the dilution factor is entered as the same value as the volume (ml) used in the CASYcup, the value of the “Viable cells/ml” can be interpreted as the number of normal pollen grains of the sample.

Table 2 Parameter setup used for counting the number of *Arabidopsis kamchatica* pollen grains

Table3 Parameter setup used for counting the number of wheat pollen grains

Tables

Table 1

Capillary	150 µm
Size Scale	50 µm
Left Norm. Cursor	7.50 µm
Right Norm. Cursor	25.00 µm
Left Eval. Cursor	12.50 µm
Right Eval. Cursor	25.00 µm
Sample volume	3 x 400 µl
Dilution	1.00E+01
Aggregation Corr.	0.000E+00 fl

Table 2

Capillary	150 µm
Size Scale	50 µm
Left Norm. Cursor	7.50 µm
Right Norm. Cursor	30.00 µm
Left Eval. Cursor	15.00 µm
Right Eval. Cursor	30.00 µm
Sample volume	3 x 400 µl
Dilution	1.00E+01
Aggregation Corr.	0.000E+00 fl

Table 3

Capillary	150 µm
Size Scale	80 µm
Left Norm. Cursor	20.00 µm
Right Norm. Cursor	80.00 µm
Left Eval. Cursor	35.00 µm
Right Eval. Cursor	60.00 µm
Sample volume	3 x 400 µl
Dilution	1.00E+01
Aggregation Corr.	0.000E+00 fl