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SMALL-SCALE ISOLATION OF MEIOCYTES FROM *ARABIDOPSIS THALIANA* ANTHERS FOR CYTOLOGICAL ANALYSES

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Abstract

In flowering plants, the meiocytes develop within nourishing tissues – the anthers or the ovaries. Although most plants bear one or more anthers in each bisexual or male flower, the size of anthers that undergo meiosis is relatively small, and each anther contains only a small fraction of male meiocytes. As a consequence, the isolation of plant meiocytes is very challenging due to their relative inaccessibility. Efficient manual dissection of meiocytes from young *Arabidopsis* anthers for cytological and cytogenetic analyses is technically challenging. Indirect detection of cellular or nuclear phenotypes is impaired by poor penetration of the reagents, and cell type-specific imaging is deminished by the lack of clarity in whole-mount tissues. Here, we describe a method that allows the efficient isolation of male meiocytes of *Arabidopsis*. Anthers undergoing meiosis were dissected and collected into appropriate isolation/labeling buffer, depending on the downstream application. Meiocytes were released by crushing with a cover slip on a glass slide. The isolated meiocytes could be analyzed using a microscope. The technical skills required are simple, and the procedure does not require any special equipment. Isolated meiocytes are suitable for a variety of dowstream applications, such as cell morphological investigation, reporter gene assays, chromosome analyses, and callose staining.

Key words: *Arabidopsis thaliana*, Callose, Chromosomes, Pollen mother cells, Meiosis, Reporter assays**.**

Introduction

Meiosis is an essential process in all sexually reproductive eukaryotes during which homologous chromosome pairing, synapsis, and recombination are achieved (Yanowitz, 2010). It is of basic interest to the geneticist and cell biologist to understand this complex process. With the advances of genetics and molecular biology, significant progress in meiosis research has been achieved in the study of flowering plants, particularly in *Arabidopsis thaliana* (Osman *et al.*, 2011). Through mutagenesis and homologous study, a number of meiotic genes have been identified (Mercier and Grelon, 2008; Osman *et al.*, 2011; Wijeratne & Ma, 2007). This, combined with the plant's amenability to cytogenetic analysis, has allowed the study of *Arabidopsis* meiosis to continue to make an important contribution to our understanding of this fundamental process.

Male meiocytes of *Arabidopsis* occupy only a small fraction of the anther tissue and are surrounded by somatic anther lobes, which include four layers of supporting cells: tapetum, middle layer, endothecium, and epidermis (Fig. 1), making the isolation and analysis of *Arabidopsis* challenging. Since anthers undergoing meiosis are relatively easily accessible, they were used to study plant meiosis (Wijeratne *et al.*, 2007); however, this will inevitably generate background by nonmeiotic cells of the anthers. An effective meiocyte collection method was recently established (Chen *et al.*, 2010; Dukowic-Schulze *et al.*, 2014). In this method, meiocytes were released into isolation buffer by squeezing the anther with forceps in a droplet of buffer placed on a glass slide on an inverted light microscope. A mouth- or micromanipulator-controlled glass capillary tube was then used to collect meiocytes from the droplet. This large-scale meiocyte isolation method has been successfully applied in transcriptome studies (Chen *et al.*, 2010; Dukowic-Schulze *et al.*, 2014; Yang *et al.*, 2011).

Meiosis research is based on cytological studies, particularly on the studies of meiotic chromosome behavior. Chromosome spreading (Ross *et al.*, 1996), which is usually performed in conjunction with 4',6-diamidino-2-phenylindole (DAPI) staining, is often used for visualization of meiotic chromosomes, and it is also a preparative procedure for fluorescence *in situ* hybridization (FISH) analysis. Typical procedures for this technique involve the fixation of flower buds, enzymatic digestion, flower dissection, chromosome spreading, and staining with DAPI. This procedure is a tedious and time-consuming task, not only during slide preparation, but also during microscopic investigation, since only a small portion of cells in the microscopic preparations are meiocytes. Thus, the method does not allow for quick acquisition of data. Furthermore, researchers sometimes encountered chromosome sample loss due to the long experimental operation and/or slide contamination; this is especially discouraging for beginners.

In this report, we describe a protocol for small-scale isolation of male meiocytes in *Arabidopsis*, and, if necessary, the subsequent labeling of meiotic chromosomes. The labeling step was combined with the isolation step; thus, the protocol is simple, efficient, and does not require any special equipment other than normal cytogenetic equipment.

Materials and Methods

Plant growth: For reporter gene assays, confirmed transgenic lines harboring any transgene of interest (e.g., the green fluorescent protein [GFP], as described by Li *et al.* (2012), and wild-type lines are required. Growth of healthy plants is important for success in the investigation of meiotic phenotype. Seeds were sown on half peat and half vermiculite and imbibed at 4°C for 3 days in the dark. Plants were then grown for 3 to 4 weeks in long-day conditions (16 h light/8 h dark) at 22°C, 40%–60% relative humidity, and 63 $mE \cdot s^{-1} \cdot m^{-2}$ light intensity.

Anther collection: Anthers undergoing meiosis were dissected and collected into appropriate isolation/ labeling buffer, depending on the downstream application. DAPI (Life Technologies, USA) 1 μg/mL for nuclear staining, 5 μM SYTOX Green (Life Technologies) for chromosome labeling, and 0.005% aniline blue (Sigma-Aldrich, USA) for callose staining were all diluted using phosphate-buffered saline (PBS) (1×) and were used as isolation/labeling buffers. Two to three separate drops (around 2.5 μl/each drop) of isolation/labeling buffer were placed on a glass slide set on a dissecting microscope stage. The anthers were dissected from the flower buds using a pair of 1 mL disposable syringes with needle, and 4–6 anthers that appeared transparent were collected and transferred to a drop of buffer. *Arabidopsis* flower buds must be at stage 9, as was determined by Smyth *et al.* (1990). In our growing conditions, the average number of flower buds in a healthy wild-type (*Columbia* [Col-0] ecotype) inflorescence undergoing meiosis is 5.1 (Fig. 2a); anthers undergoing meiosis should appear transparent (Fig. 2b). Flower debris and anthers that did not look transparent was removed from the glass slide before proceeding to the next step.

Meiocyte isolation, staining, and detection: A cover-slip was placed over the sample and was gentle pressed to squeeze the anthers and release the meiocytes. The anthers situated in separate isolation/labeling buffer drops released their meiocytes at distinct sites on the slide. Meiocyte staining, if required, was achieved in this same step. The integrity of the isolated meiocytes was inspected before proceeding with each experiment to ensure that use of valuable materials was reserved for high-quality samples only. Success in this stage was contingent on practice to apply the appropriate strength required for meiocyte release. The collected meiocytes were examined under the appropriate microscope and conditions to visualize the meiotic cell morphology, the reporter gene, or fluorescence labeling under bright field or appropriate fluorescence. The DAPI filter was used for DAPI stain and aniline blue, and the GFP filter was used for GFP reporter label and SYTOX Green. Images in this study were taken using a Zeiss Axioskop 40 microscope (Carl Zeiss, Jena, Germany).

Results

An efficient, large-scale meiocyte isolation method has been recently developed (Chen *et al.*, 2010; Dukowic-Schulze *et al.*, 2014; Yang *et al.*, 2011); however, this system is most appropriate for the isolation and analyses of meiocytes in bulk and is thus not amenable for rapid investigation of multiple samples. Based on this need and the general utility of a small-scale isolation protocol for cytological studies, we developed a robust, small-scale isolation/ labeling method for meiocytes.

Representative data showing isolated and stained meiocytes obtained using our small-scale protocol is shown in Figure 3. Under the bright-field light microscope, isolated meiocytes could be easily distinguished from somatic cells by their characteristic worm-like cell clusters in early stages (Fig. 3a) and a transparent callose-containing cell wall in later stages (Fig. 3b).

We have successfully used this method in analyzing the GFP reporter in male meiocytes (Li *et al.*, 2012) (Fig. 3c), and our method is particularly efficient in the investigation of multiple transgenic lines. Meiotic chromosomes incubated with our modified chromosome labeling buffer showed a bright green fluorescence, and the stages of meiosis could be determined at the single-cell level (Fig. 3d and f). We tested another nuclei acid stain, DAPI, with our cell preparations, as is shown in Figure 3e and g, and the staining of meiotic chromosomes was barely visible. Thus, DAPI is not suitable for the direct labeling of meiotic chromosomes in those unfixed tissues, but it can be used as an inexpensive nuclear indicator. Furthermore, the process of callose deposition during meiosis can be monitored after staining with aniline blue (Fig. 3h and i) (Xie *et al.*, 2010)

Discussion

Our protocol is valuable in several applications regarding meiotic phenotype investigation, including the analysis of cell morphology, reporter gene, chromosome, and callose (Fig. 3). In particular, we proposed a rapid, one-step staining protocol for the visualization of meiotic chromosomes (Fig. 3d and f) using a combination of meiocyte release through squeezing and immediate staining.

The proposed method is quick, allowing for timely analysis of meiotic progress in plants, compared to the general method, which includes time-consuming fixation, enzymatic digestion, and DAPI staining. Compared with the ferrous acetocarmine method (Belling, 1926), the method described here is easy for beginners, especially considering that the ferrous coloring step requires some training and use of an alcohol lamp for heat. The use of SYTOX Green make this quick protocol feasible (Li *et al.*, 2014) due to its ability to penetrate membranes of our cell preparations and/or its bright fluorescence after excitation. Previously, SYTOX Green was commonly used as an indicator of dead cells (Truernit and Haseloff, 2008). In our study, the commonly used nucleic acid dye DAPI was not as efficient as SYTOX Green (Fig. 3e and g).

In flowering plants, the synchrony among anthers within a single flower has been observed in many species (Armstrong *et al.*, 2003; Bennett, 1971; Li *et al.*, 2015; Nonomura *et al.*, 2011). The presented protocol allows for the rapid determination of the specific meiotic stage of a flower by investigating one anther, with other anthers left intact. Thus, this method could be a substantial aid for identifying, monitoring, and enriching meiocytes at a particular stage, which is useful for some experiments, e.g., FISH assay. Our described methods may also provide a reference for cytogenetic analyses of meiotic chromosomes in other species.

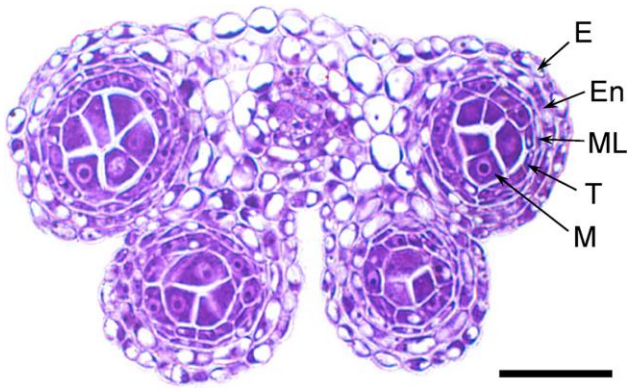

Fig. 1. Cross sectional view of an *Arabidopsis thaliana* anther. E, epidermis; En, endothecium; ML, middle layer; T, tapetum; M, meiocyte. Bar = 25 μm.

Fig. 2. Photographs of flowers and anthers undergoing meiosis suitable for meiocyte isolation. a, healthy *Arabidopsis* inflorescence is used to isolate meiocytes. Young floral buds were cut from the white frame. Bar = 1 mm. b, anthers dissected from the floral buds using a pair of disposable syringes with needle. Transparent ones (right) are retained, and those that do not look transparent (left) are discarded. Bar = 0.1 mm.

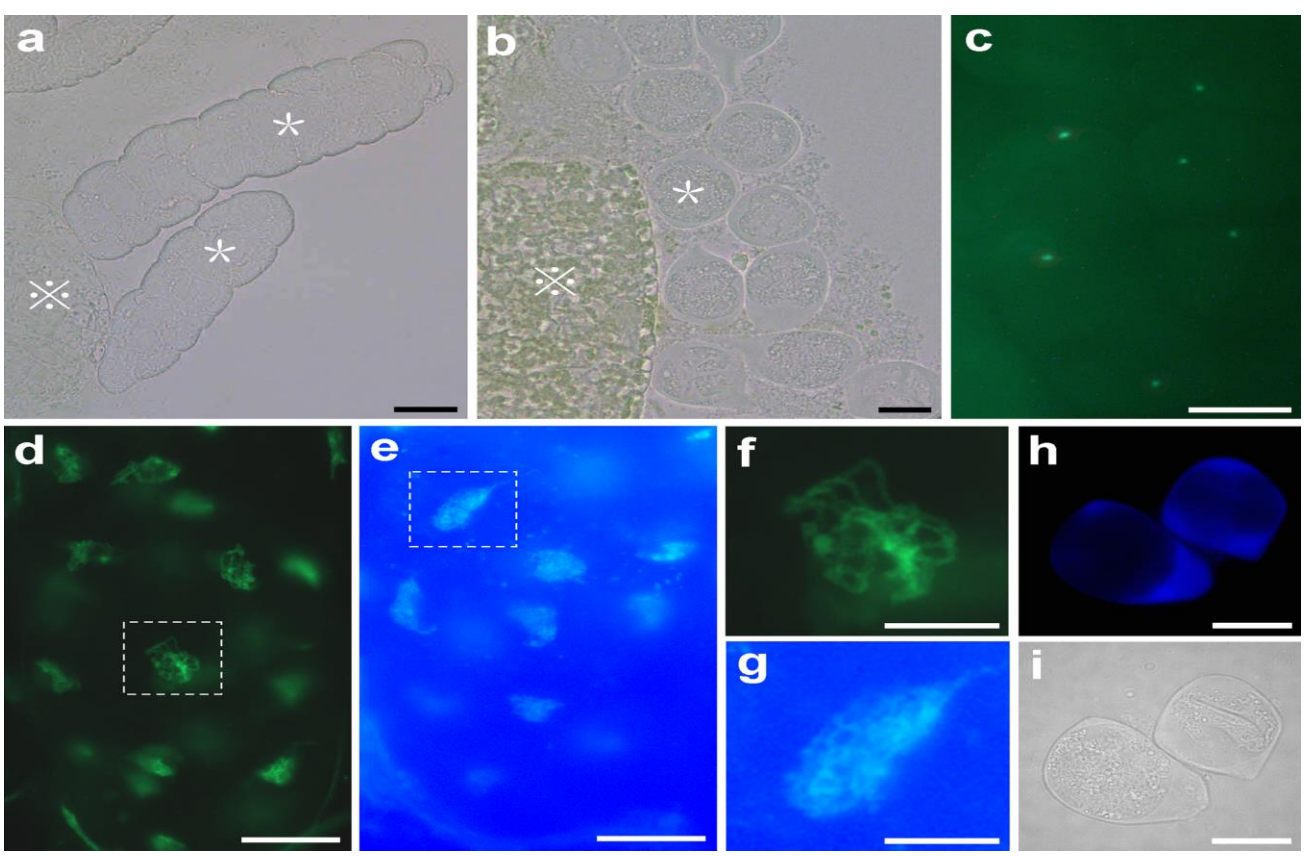

Fig. 3. Isolated *Arabidopsis* meiocytes for cytological analyses. a, isolated worm-like meiosis I meiocytes. Bar = 10 μm. b, isolated meiocytes at later meiosis. Asterisk refers to the meiocytes; ※ refers to the other cells around the meiocytes. Bar = 10 μm. c, observation of the GFP reporter driven by the *Arabidopsis Disrupted Meiotic cDNA 1* (*DMC1*) promoter (Li *et al.*, 2012) in meiocytes. Bar = 10 μm. d and e, nucleic acid stained by SYTOX Green and DAPI, respectively. Bars = 5 μm. f and g, close-up views of the white dash-dotted frames in d and e, respectively. Bars = 2 μm. h, callose wall labeled by aniline blue. Bar = 10 μm. i, the corresponding bright-field image of h. Bar = 10 μm.

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