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Chapter 9

Immunolocalization of Meiotic Proteins in *Brassicaceae*: Method 1

Liudmila A. Chelysheva, Laurie Grandont, and Mathilde Grelon

Abstract

Plant meiosis studies have enjoyed a fantastic boom in recent years with the use of *Arabidopsis thaliana* as an important model species for developmental studies because of its small genome, short life cycle, and large mutant collections. Unlike other eukaryotic models, plant meiosis does not display strict checkpoints and rarely commits to apoptotic processes, which makes it possible to investigate the whole meiotic process (spanning from premeiotic interphase to spore formation) in knockout mutants. In this chapter we describe a protocol for immunolabelling *Arabidopsis* and *Brassica* meiotic proteins on robustly spread chromosomes. This protocol allows the detection of a large range of proteins on well-preserved chromosomes and throughout the entire meiotic process.

Keywords *Arabidopsis*, *Brassica napus*, Cytogenetics, Chromosome, Meiosis, Immunolabelling

1 Introduction

Significant progress in the study of *Arabidopsis* meiosis has been made these last years. With the development of cytogenetic techniques (1–3) chromosome organization and behavior could be analyzed during meiotic progression in wild type and various meiotic mutants. These first protocols were based on an acetic acid cell-spreading of flower buds fixed in Carnoy's fixative. Then, Armstrong and colleagues (4) developed techniques for immunolabelling meiotic proteins, which allowed the detailed analysis of spatial and temporal expression of different proteins throughout prophase of meiosis I. This commonly used protocol requires fresh material on which lypsol or Triton X-100 spreading and paraformaldehyde fixation are applied (see Chapter 10). Unfortunately this method of immunolabelling has limitations: often poor resolution because of mild spreading efficiency of lypsol, loss of stages beyond pachytene, and poorly preserved chromosome organization. To overcome these limitations, we developed a technique that allows

immunolocalization of proteins using Carnoy's fixative and acetic acid chromosome spreads (5). This method combines a strong fixation to preserve chromosome structure, acetic acid spreading to remove the cytoplasm, and microwave treatment to increase the accessibility of protein to antibodies.

2 Materials

2.1 Plants

1. *Arabidopsis thaliana* plants grown in greenhouse, photoperiod 16 h day and 8 h night; temperature 20°C day and night; humidity 70% (see Note 1).
2. *Brassica napus* plants grown in greenhouse, photoperiod 16 h per day and 8 h per night; temperature 22°C day and 18°C night; humidity 65% (see Note 1).

2.2 Chromosome Preparation

1. Carnoy's fixative: mix absolute ethanol (3 volumes) and glacial acetic acid (1 volume).
2. Citrate buffer pH 4.5: 10 mM sodium citrate/citric acid, pH 4.5. Weigh 2.94 g of tri-sodium citrate and transfer to a graduated glass beaker. Add distilled water up to 800 ml. Prepare 100 ml of 100 mM citric acid (2.1 g in 100 ml of distilled water). Progressively add citric acid solution to the 800 ml of tri-sodium citrate solution until pH 4.5. Top up to 1 l with distilled water. Sterilize and make 40 ml aliquots. Store at -20°C.
3. Citrate buffer pH 6.0: 10 mM sodium citrate/citric acid, pH 6.0. Prepare 0.1 M citric acid (21.01 g in 1 l of distilled water). Prepare 0.1 M tri-sodium citrate (29.41 g in 1 l of distilled water). Add 9 ml of 0.1 M citric acid solution to 41 ml of 0.1 M tri-sodium citrate solution. Top up to 500 ml with distilled water. Store at 4°C (see Note 2).
4. Digestion enzyme mixture: 0.3% (w/v) cellulase Onozuka R-10 (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.3% (w/v) pectolyase Y-23 (MP Biomedicals, Santa Ana, CA, USA), 0.3% (w/v) cytohellicase (Sigma Aldrich, St. Louis, MO, USA) in 10 mM citrate buffer pH 4.5. Weigh 1.5 g of each enzyme and dissolve in 500 ml of sterilized 10 mM citrate buffer pH 4.5. Aliquot by 40 ml and store at -20°C.
5. 60% acetic acid.
6. Embryo dishes.
7. Stereomicroscope equipped with eyepiece micrometer disk.
8. Standard microscope slides (Knittel Glaser, Braunschweig, Germany) cleaned with 70% ethanol.
9. Dissection needles, a hook (curved dissection needle), and a fine forceps.

10. Moist chamber for enzyme digestion. A hermetic plastic box 180 × 120 × 75 mm³ with a wet paper on bottom.
11. Glass Pasteur pipette.
12. Heating block (45°C).
13. Incubator (37°C).
14. Glass and plastic Hellendahl jars (VWR, Radnor, PA, USA).
15. Microwave oven.

2.3 Immunolabelling

1. 1× PBS: 10 mM sodium phosphate, pH 7.0, 143 mM NaCl.
2. PBST: 0.1% Triton-X 100 in 1× PBS.
3. PBST-BSA: 1% BSA in 1× PBST.
4. Secondary antibodies conjugated to Alexa (Invitrogen, Carlsbad, CA, USA) or DyLight (Thermo Fisher Scientific, Waltham, Massachusetts, USA).
5. DAPI solution: 2 µg/mL DAPI (4',6-diamidino-2-phenylindole) in Vectashield (Vector Labs, Burlingame, CA, USA).
6. Laboratory film Parafilm "M" (Sigma Aldrich, St. Louis, MO, USA).
7. Coverslips 24 × 32 mm.
8. Moist chamber: a hermetic container (e.g., square Petri dish) with wet paper on the bottom and two rails in order to keep microscopic slides horizontally.
9. Incubator (37°C).
10. Fluorescence microscope equipped with a high resolution CCD camera.

3 Methods

3.1 Chromosome Preparation

1. Fix the inflorescences in freshly prepared Carnoy's fixative for 3 × 60 min and keep them in fixative for at least 3 days at 4°C (see Note 3). Fixed material can be stored in fixative at 4°C or -20°C for several months.
2. Rinse the fixed inflorescences once with distilled water in an embryo dish.
3. Replace the water with citrate buffer pH 4.5 and wash 2 × 5 min.
4. Replace the citrate buffer with the digestion enzyme mixture. Incubate for 3 h (3.5 h for *Brassica* inflorescences) in a moist chamber at 37°C. Ensure that all material is submerged in the digestion mixture.
5. Replace the digestion mix with water and from now on keep material on ice. Digested inflorescences can be stored at 4°C overnight.

6. Take a single inflorescence in a drop of distilled water on a slide and select three flower buds of appropriate stage (see Notes 4 and 5). Extract anthers and place them in 4 μl of water on an ethanol-cleaned slide. For *Brassica* samples, prepare each slide with a single anther.
7. Crush anthers with a hook until a fine suspension has formed (see Note 6).
8. Add 10 μl of 60% acetic acid to the suspension on the slide. Place the slide on the heating block for 2 min at 45°C. Add another 10 μl of 60% acetic acid after the first minute of heating. For *Brassica*, add 20 μl of 60% acetic acid on the slide and place it for 5 min at 45°C. Add 10 μl of 60% acetic acid after 2 min of heating.
9. During step 8, stir the drop with the hook without stopping (see Note 7) in order to remove as much cytoplasm as possible.
10. Fix the cells on slides first by pipetting cold Carnoy's fixative around the drop of the cleared cell suspension. Then, at the end, add the remaining Carnoy's by pipetting directly onto the top of the suspension. Hold the slide vertically to get rid of the liquid. Air-dry the preparation.
11. Put up to eight slides in 10 mM citrate buffer pH 6 in a plastic Hellendahl jar and microwave them for 45 s at 850 W.
12. Transfer the slides immediately to PBS-T in a glass Hellendahl jar. Incubate for 5 min at RT.

3.2 Immunolabelling

1. Dilute the primary antibodies to the appropriate concentration in PBST-BSA and centrifuge them 3 min at 10,000 $\times g$.
2. Use 50 μl of the antibody working solution per slide, cover with 24 \times 40 mm pieces of parafilm.
3. Incubate at 4°C for 24–48 h in a moist chamber.
4. Rinse the slides in PBST, 3 \times 15 min at RT.
5. Mix the secondary antibodies in PBST-BSA at a dilution of 1:100 to 1:2,000 (see Note 8).
6. Use 50 μl of solution per slide, cover with 24 \times 40 mm pieces of parafilm and incubate at 37°C for 1 h in a dark moist chamber.
7. Rinse the slides in PBST, 3 \times 10 min at RT.
8. Put 15 μl of DAPI solution per slide and cover with a 24 \times 32 coverslip.
9. Observe the slides under a fluorescent microscope using the appropriate filters (see Note 9) (Figs. 1, 2, and 3).

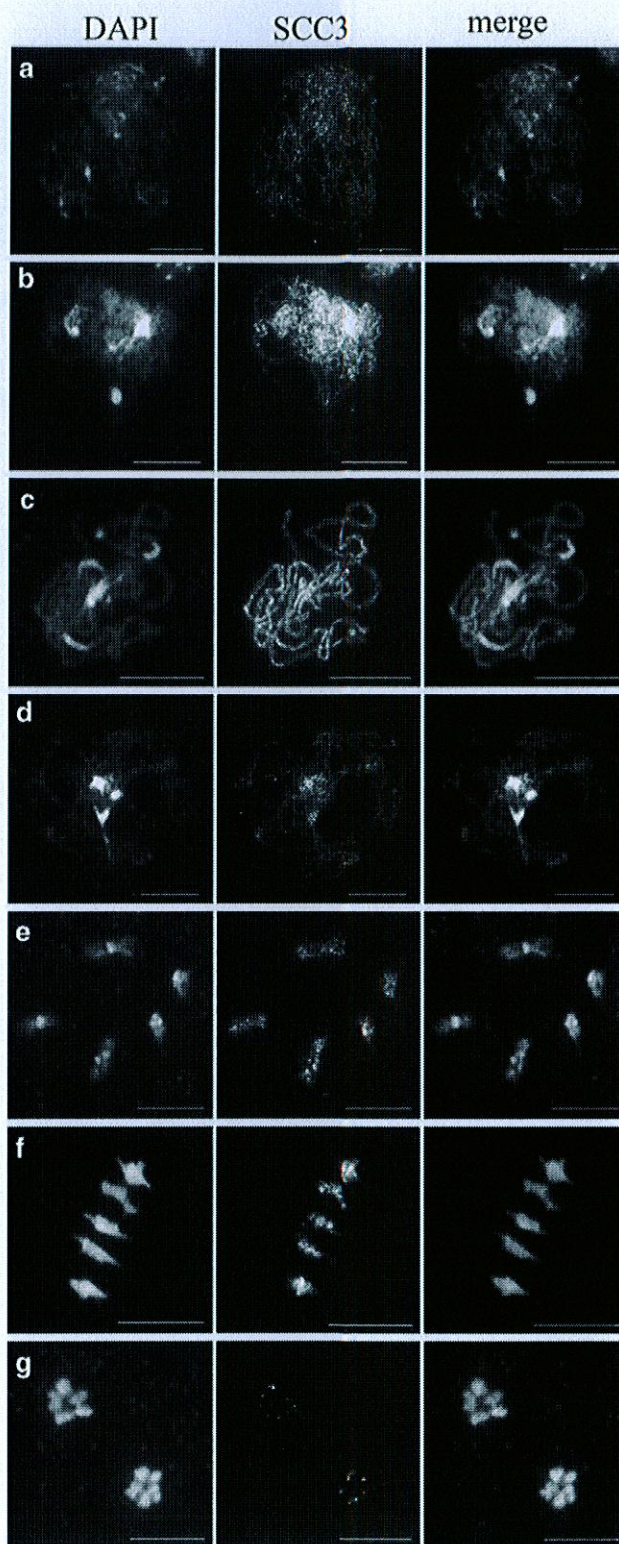


Fig. 1 Immunolocalization of AtSCC3 on *Arabidopsis* pollen mother cells. The cohesin AtSCC3 (6) was immunolocalized on *Arabidopsis* pollen mother cells chromosomes after acetic acid spreading. For each cell DAPI, AtSCC3 and merged signals are shown. (a) Leptotene. (b) Zygotene. (c) Pachytene. (d) Diplotene. (e) Diakinesis. (f) Metaphase I. (g) Telophase I. Bar: 10 μ m

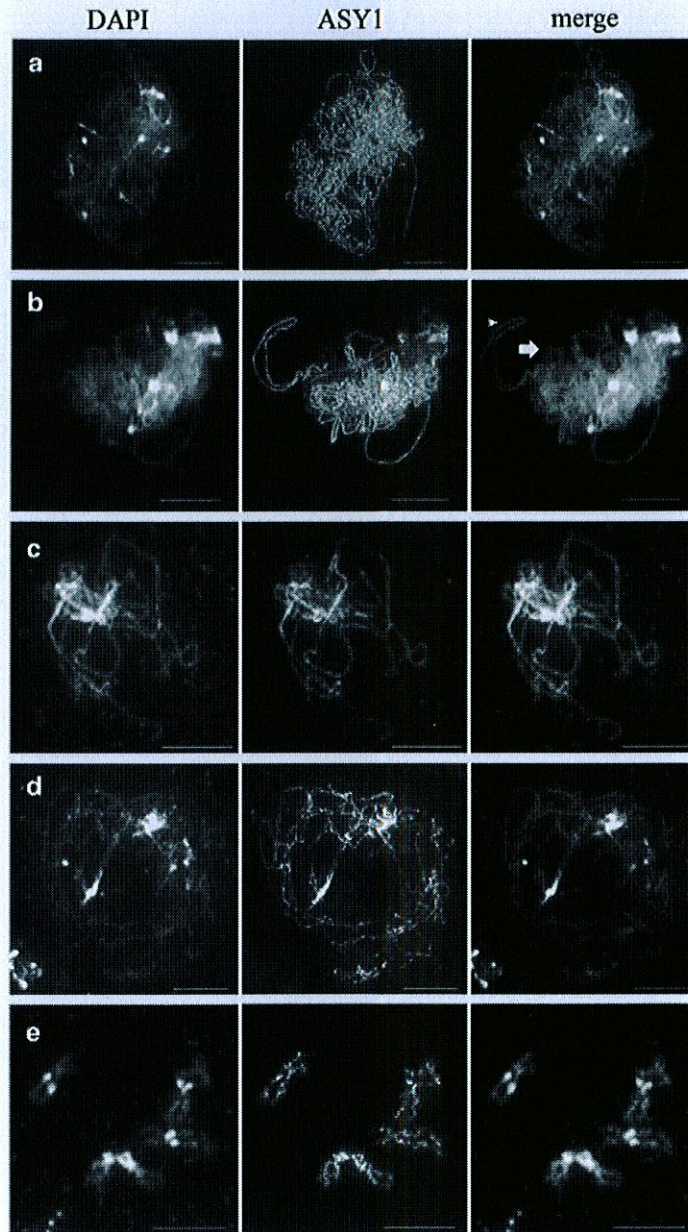


Fig. 2 Immunolocalization of ASY1 on *Arabidopsis* pollen mother cells. The axis-associated protein ASY1 was immunolocalized on *Arabidopsis* pollen mother cells chromosomes after acetic acid spreading (4). For each cell DAPI, ASY1 and merged signals are shown. (a) A leptotene cell showing numerous dotted lines corresponding to the ASY1 signal. (b) A zygotene cell showing continuous ASY1 signals along chromosomal axes; the *arrowhead* indicates a bright ASY1-labelled unsynapsed region, while the *arrow* indicates a synapsed region, with faint ASY1 signal. (c) Pachytene. (d) Diplotene. (e) Diakinesis. Bar, 10 μ m

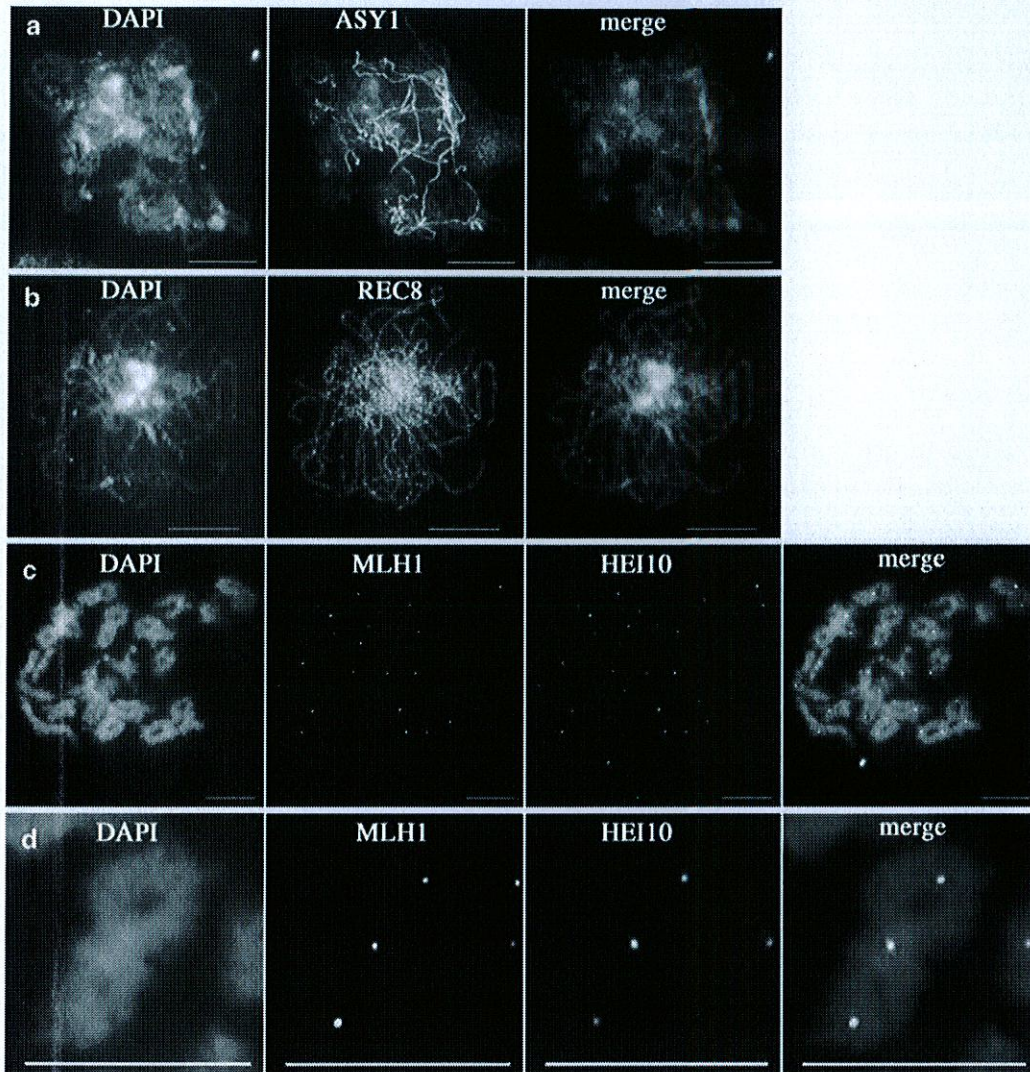


Fig. 3 Immunolocalization of AtASY1, AtREC8, AtMLH1, and AtHEI10 on *Brassica napus* pollen mother cells. (a) Immunolocalization of the axis protein ASY1 on *Brassica napus* pollen mother cells chromosomes at zygotene using an antibody directed against the *Arabidopsis thaliana* ASY1 protein (4). DAPI, ASY1 and merged signals are shown. (b) Immunolocalization of the meiotic cohesin REC8 on *Brassica napus* pollen mother cells chromosomes at pachytene using an antibody directed against the *Arabidopsis thaliana* REC8 protein (7). DAPI, REC8 and merged signals are shown. (c) Co-immunolocalization of MLH1 and HEI10 proteins on *Brassica napus* pollen mother cells chromosomes at diakinesis. Anti-MLH1 and anti-HEI10 sera were developed against the *Arabidopsis thaliana* proteins (5). (d) A zoom on one bivalent from (c). For (c) and (d), DAPI, MLH1, HEI10, and merged signals are shown. Bar, 10 μm

4 Notes

1. Good and reproducible growth conditions are particularly important because inappropriate growth conditions can considerably affect the dynamics of meiotic progression. Furthermore, it can be difficult to recover enough material from unhealthy plants.
2. We found that the utilization of commercial citrate buffer pH 6.0 (Diapath S.p.A., Martinengo, Italy) gives very good results.
3. A fixation shorter than 3 days can be insufficient to make all proteins available to immunodetection. In consequence, meiocytes extracted from partially fixed buds will show only partial chromosome immunostaining.
4. In *Arabidopsis*, pollen mother cells develop synchronously, which make it possible to relate the meiotic progression to the bud size. Nevertheless, this correspondence is dependent on the genotype (variability among the ecotypes or between wild type and mutants) and growth conditions. Therefore, it is very important to define it for your own material. Roughly, 0.4 mm buds contain prophase I pollen mother cells, 0.5 mm buds contain metaphase I meiocytes, 0.6 mm buds—metaphase II to telophase II, and 0.75 mm buds tetrads and microspores in Columbia-0.
5. In *Brassica*, bud size is poorly correlated with pollen mother cell development stages but development stages are homogeneous among the various anthers of a single bud. Therefore, it is possible to select appropriate buds after acetocarmine staining of one anther per bud. To do it, extract one anther from a fixed bud and place it on a slide. Add 30 μ l of acetocarmine and crush the anther. Cover with a coverslip 24 \times 32 mm. Place the slide on the heating block for 8 min at 45°C. Observe the slides under a bright field microscope.
6. It is very important to make a fine cell suspension in order to treat all cells efficiently with acetic acid and to get rid of most of the cytoplasm. However, it is also very important to prevent drop evaporation that would make meiocytes stick to the slide and dry, rendering them unavailable to the subsequent acetic acid treatment. In this case, many faintly stained meiocytes will be observed, surrounded by cytoplasm.
7. To amplify acetic acid treatment, the droplet with cells should be stirred vigorously with a hook without touching the glass surface.
8. The appropriate dilution for secondary antibodies should be determined experimentally and can sometimes be more diluted than suggested by the supplier.
9. The fluorescence signal of far-red fluorochromes (e.g., Alexa-647, DyLight-640) can be visualized only by image acquisition.

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