Cytogenetic Analyses of Arabidopsis

1. Introduction

The complete genomic sequence of *Arabidopsis thaliana* ***(1,2)***, the public availability of a BAC tiling path for each chromosome, and the steadily growing collections of mutants and transgenic lines make this species a model of still growing importance for plant research. The very small genome (~157 Mb) ***(3)*** is represented by five chromosome pairs with most of the repetitive sequences (~15% of the genome) ***(4)*** clustered within the pericentromeric heterochromatin and within the distal nucleolus organizers (NORs) on the short arms of chromosomes 2 and 4. These heterochromatic regions form conspicuous chromocenters in interphase nuclei (at maximum 14: 10 pericentromeres and 4 NORs; but because of fusion tendency, rarely more than 10), distinguishable by high chromatin density ***(5)*** and strong DNA fluorescence intensity after 4,6-diamidino-2-phenylindole (DAPI) staining. The *Arabidopsis* mitotic chromosomes are rather small (~1.5 μm) and therefore hardly accessible to detailed cytogenetic analysis. Nevertheless, chromosome C-banding ***(6)*** and localization of repetitive sequences by fluorescence *in situ* hybridization (FISH) ***(7,8)*** have been performed early. Chromosome counts in aneuploids and polyploids were conducted by conventional staining ***(***e.g., ***9,10)*** and by flow-cytometric analysis ("flow-karyotyping") ***(11)***. Since the late 1990s, preparation of meiotic (pachytene) chromosomes became feasible ***(12,13)*** and allowed high-resolution physical mapping of DNA sequences by FISH (***14,15***; for review *see* ref. ***16***). The improvement was based on the advantage that *Arabidopsis* chromosomes are 25-fold longer during pachytene than during mitotic metaphase. Meiotic chromosome synapsis and recombination can be studied by FISH and immunostaining ***(17)***. A further improvement of the mapping resolution was accomplished by the FISH on extended chromatin fibers, which has been adapted to plants using *Arabidopsis* nuclei ***(18)***. Recently, cytogenetic analysis of chromatin alterations in interphase nuclei became possible due to the availability of antisera against chromatin epitopes and the application of nuclei isolated from different organs. This approach allowed to trace the dynamics of epigenetic modifications, such as DNA methylation, methylation, and acetylation of histones, within distinct chromatin domains (addressed by FISH) along cell cycle and developmental stages ***(19–21)***. With transgenically expressed and fluorescently (e.g., by green fluorescent protein [GFP]) labeled fusion proteins, the position and movement of such proteins could be monitored *in situ* and in vivo ***(22,23)***. Furthermore, the low DNA content and the relatively simple organization of the *Arabidopsis* genome enabled for the first time to paint all chromosomes of a euploid plant species individually using pools of BAC contigs as chromosome-specific probes for FISH ***(24,25)***. By chromosome painting the interphase organization of chromosome territories and their potential dynamics can be studied and chromosome rearrangements can be visualized. In addition, painting probes specifically designed for *Arabidopsis* chromosomes proved to be suitable to detect homologous chromosome regions within the pachytene karyotypes of several other Brassicaceae species and to reconstruct the evolutionary history of chromosome complements for differently related species of this family ***(25)***. Labeling of the entire chromosome complement by genomic *in situ* hybridization (GISH) is difficult with small genomes because usually these show only heterochromatic blocks labeled after GISH. However, under appropriate conditions GISH turns out to be applicable in interspecific hybrids of *Arabidopsis* ***(26)***. To study genotoxic impacts cytologically, the "comet-assay" with isolated nuclei has been adopted for *Arabidopsis* ***(27)***. For detection of somatic recombination via sister chromatid exchanges, *Arabidopsis* chromosomes are too small. However, the involvement in rearrangements of NOR-bearing chromosomes could be demonstrated by FISH in anaphase nuclei of pistil cells from telomerase-deficient mutants ***(28)***. Applying multicolor painting of all chromosomes simultaneously, reciprocal translocation between any chromosomes should become identifiable. Thus, almost the entire spectrum of cytogenetic approaches is now applicable to this model species.

This chapter describes (1) the preparation of mitotic and meiotic chromosomes, (2) FISH on meiotic chromosomes and interphase nuclei (3) painting of *Arabidopsis* chromosomes and comparative chromosome painting in other Brassicaceae species using BAC contigs specific for *Arabidopsis* chromosomes as probes.

2. Materials

2.1. Chromosome Preparation by Spreading

1. Freshly prepared ice-cold Carnoy's fixative (ethanol:glacial acetic acid, 3:1) (*see* **Note 1**).
2. 70% ethanol.
3. Citrate buffer: 10 m*M* sodium citrate/citric acid in distilled water, pH 4.5.

4. Pectolytic enzyme mixture: 0.3% (w/v) cellulase, 0.3% (w/v) pectolyase, 0.3% (w/v) cytohelicase (all from Sigma) in citrate buffer (*see* **Note 2**).
5. 60% acetic acid.
6. Stereo microscope.
7. Light microscope with phase contrast.
8. Microscopic slides.
9. Dissection needles and fine forceps.
10. Small Petri dishes.
11. Moist chamber for enzyme digestion.
12. Glass capillary tubes.
13. Heating block.
14. Incubator (37°C).

2.2. Probe Labeling

2.2.1. Probe labeling using nick translation mix

1. Nick Translation Mix (Roche).
2. Nucleotides: dATP, dCTP, dGTP, dTTP and either biotin-dUTP or digoxigenin-dUTP (all from Roche), DNP-dUTP (PerkinElmer), Cy3-dUTP (Amersham), or DEAC-dUTP (Perkin Elmer).
3. 3 *M* sodium acetate, pH 5.2.
4. 70% and 96% ice-cold ethanol.
5. 0.5 *M* EDTA, pH 8.0.
6. Eppendorf tubes.
7. Water baths (15°C, 65°C).
8. Centrifuge for Eppendorf tubes.

2.2.2. Alternative single-component approach for nick translation

1. All materials from **Subheading 2.1.2.1.** except **1**.
2. 10X NT buffer: 0.5 *M* Tris-HCl, pH 7.5, 50 m*M* $MgCl_2$, 0.05% bovine serum albumin (BSA). Store in aliquots at –20°C.
3. 0.1 *M* β-mercaptoethanol. Store in aliquots at –20°C.
4. DNase I (Roche). Use a 1:250 dilution of a 1 mg/mL DNase stock in 0.15 *M* NaCl, 50% glycerol (*see* **Note 3**).
5. DNA polymerase I (10 U/μL, MBI Fermentas).
6. Electrophoresis system.
7. 1% agarose gel.
8. 100-bp ladder DNA marker.

2.3. In situ Hybridization

1. Labeled probe(s).
2. Hybridization buffer (HB50): 50% deionized formamide, 2X SSC (1X SSC = 0.15 *M* NaCl/ 0.015 *M* Na_3-citrate), 50 m*M* sodium phosphate, pH 7.0.
3. 20% dextran sulfate in HB50.
4. 3 *M* sodium acetate, pH 5.2.
5. 2X SSC.
6. 100 μg/mL RNase (Roche) in 2X SSC.
7. 1X phosphate-buffered saline (PBS) (10 m*M* sodium phosphate, pH 7.0, 143 m*M* NaCl) (conveniently from 10X stock).
8. 4% formaldehyde in 1X PBS (*see* **Note 4**).
9. Ethanol series (70%, 90%, 100%).
10. Moist chamber.
11. Eppendorf tubes.
12. Cover slips 24 × 50 and 22 × 22 or 32 × 24 mm.
13. Heating block (80°C).
14. Incubators (60°C, 37°C).
15. Centrifuge.
16. Vacuum centrifuge.

2.4. Fluorescent Detection of Hybridized Probes (see **Note 5**)

1. 2X SSC.
2. SF50 buffer: 50% formamide in 2X SSC, pH 7.0.
3. Water bath of 42°C (in fume hood for incubation in SF50 buffer).
4. Coplin jars.
5. 4T buffer: 4X SSC, pH 7.0, 0.05% (v/v) Tween-20.
6. Blocking solution: 5% BSA, 0.2% (v/v) Tween-20 in 4X SSC.
7. TNT buffer: 100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, with 0.05% (v/v) Tween-20.
8. TNB buffer: 100 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 0.5% Boehringer blocking reagent.
9. Avidin~Texas Red (Vector Laboratories).
10. Goat anti-avidin~biotin (Vector Laboratories).
11. Mouse anti-digoxigenin (Jackson Laboratories).
12. Rabbit anti-DNP (Sigma).
13. Goat anti-mouse~Alexa 488 (Molecular Probes).
14. Goat anti-rabbit~Cy5 (Jackson Laboratories).
15. Vectashield antifade mounting medium (Vector Laboratories) with 2 μg/mL DAPI.
16. Ethanol series (70%, 90%, 100%).
17. Cover slips (24 × 50 mm).
18. Moist chamber.
19. Incubator (37°C).
20. Fluorescence microscope equipped with optical filters for DAPI, FITC/Alexa 488, Texas Red, Cy5, DEAC and Cy3 fluorochromes (all filters from AHF Analysentechnik).
21. Digital charge-coupled device (CCD) camera.
22. Image acquisition software (e.g., MetaVue, Spot).

3. Methods

3.1. Chromosome Preparation by Spreading

The most crucial step in the procedure is the preparation of high-quality chromosome spreads. Because the anthers containing pollen mother cells at pachytene are hardly visible, even with a standard binocular, entire flower buds are used for preparation of pachytene chromosome spreads. A major advantage of using immature flower buds is the presence of meiotic and mitotic cells as well. The following protocol is applicable also for leaf or root tissue and for chromosome preparation from other Brassicaceae taxa as well.

1. Fix young inflorescences in freshly prepared ice-cold Carnoy's fixative (*see* **Note 1**) for at least 3 × 30 min. Fixed material can be stored in fixative or in 70% ethanol at 4°C or –20°C for several months.
2. Wash fixed inflorescences with distilled water 2 × 10 min in a small Petri dish.
3. Replace the water by citrate buffer and wash 3 × 5 min; remove unwanted parts of the inflorescences under a stereomicroscope.
4. Incubate three inflorescences in ~1 mL of pectolytic enzyme mixture for 3 h in a moist chamber at 37°C.
5. Take an inflorescence and select a flower bud. Usually, the large yellowish flower buds and the first white flower bud contain anthers with pollen and microspores or tetrad stages, respectively. The second white bud has pollen mother cells (PMCs) in meiosis II. PMCs at prophase I, including diakinesis and pachytene, can be found in the third or fourth white flower bud. Put the flower bud on a clean microscopic slide.
6. Remove excess of fluid (by capillary forces of a fine forceps or a glass capillary tube). Keep ~4 μL of liquid.
7. Tap the flower bud with a dissection needle until a fine suspension has formed. If there are still large tissue fragments, then extend the enzyme incubation time for further digestion. If a fine suspension has been obtained, replace the enzyme mixture in the Petri dish by citrate buffer and keep on ice or at 4°C.
8. Add about 15 μL of 60% acetic acid to the suspension on slide to clear the fine suspension and to make cells adhere to the slide. Place the slide on a heating block at 45 to 50°C, add twice ~15 μL of 60% acetic acid, and spread the drop by careful stirring for 15 s (*see* **Note 6**).
9. Precipitate the cells by careful pipetting ~100 μL of Carnoy's fixative around the drop with cleared cell suspension and wait until the fixative covers the whole slide. Discard excessive solution by tilting the slide.
10. Air-dry the preparation (*see* **Note 7**).
11. Examine the preparations for specific meiotic stages in the phase contrast microscope. (*see* ref. ***12*** for a detailed microscopic atlas of meiosis in *Arabidopsis*). Check that cells are spherical or oval, without remnants of cell wall material. Mitotic metaphase chromosomes should appear as dark gray or black structures (not white). Nuclei look dark gray with

black conspicuous spots (i.e., heterochromatic chromocenters); cytoplasm is almost invisible; pachytene chromosomes should be free of cytoplasm. If chromosomes and nuclei appear to be covered by cytoplasm, preparations aimed for FISH should be treated with pepsin (**Note 8**).
12. Store selected and dried slides in a dust-free box at 4°C. Let stored slides adapt to room temperature before further use.

3.2. Probe Labeling

Molecular biology laboratory manuals contain detailed protocols for isolation of DNA from living plant tissues, bacteria, and yeast. For DNA isolation from BAC clones we recommend to use the plasmid isolation protocol of Sambrook et al. ***(29)*** and to scale up the volumes to 50 mL of bacterial culture (*see* **Note 9**).

3.2.1. Probe labeling using nick translation mix

1. Combine in an Eppendorf tube:
a. 1 μg DNA in 12 μL sterile distilled water.
b. 4 μL nucleotide mix (250 μ*M* dATP, dCTP, dGTP; 170 μ*M* dTTP and 80 μ*M* of either biotin-dUTP or digoxigenin dUTP, DNP-dUTP, Cy3-dUTP or DEAC-dUTP).
c. 4 μL Nick Translation Mix.
2. Mix carefully and centrifuge briefly.
3. Incubate for 90 min at 15°C.
4. Stop the reaction by adding 1 μL 0.5 *M* EDTA, pH 8.0, and heating to 65°C for 10 min.

3.1.2.2. Alternative single-component approach for nick translation (*see* **Note 10**)

1. Combine in an Eppendorf tube:
a. 1–2 μg DNA.
b. 5 μL nucleotide mix (2 m*M* dATP, dCTP, dGTP, 400 μ*M* dTTP).
c. 5 μL 10X NT-buffer.
d. 1–4 μL 1 m*M* X-dUTP (in which X is either biotin, digoxigenin, DNP, Cy3, or DEAC).
e. 5 μL 0.1 *M* β-mercaptoethanol.
f. Fill up with distilled water to 47 μL.
g. 2 μL DNase I.
h. 1 μL DNA polymerase I, mix gently, and centrifuge briefly.
2. Incubate for 90 min at 15°C.
3. Transfer the tubes to ice and load 1/10 of the reaction volume on a 1% agarose gel together with a 100-bp ladder DNA marker. When the bulk of labeled fragments is ~400 to 500 bp long, stop the reaction by adding 1 μL 0.5 *M* EDTA, pH 8.0, and heat the probe to 65°C for 10 min (*see* **Note 11**).
4. For purification it is recommended to precipitate the probe by adding 1/10 vol of 3 *M* sodium acetate, pH 5.2, and 2.5 vol ice-cold 96% ethanol and keep at –70°C for 30 min or at –20°C overnight. Centrifuge the precipitate with 13,000*g* for 30 min at 4°C, wash pellet with 70% ethanol, centrifuge again for 15 min, air-dry, and resuspend the pellet in 50 μL of sterile distilled water.

3.3. In Situ Hybridization

1. To apply one to three labeled probe(s) for FISH, add 1 to 3 μL of the probe(s) to 2 μL distilled water in an Eppendorf tube and centrifuge in a vacuum centrifuge at medium speed for 12 to 15 min. To the vacuum-dried probe add 10 μL HB50 and 10 μL 20% dextran sulfate in HB50 (*see* **Note 12**). If more BAC probes must be applied simultaneously, e.g., for chromosome painting, it is recommended to label individual BAC clones separately and pool them for hybridization. Pipet 3 to 5 μL of each labeled probe into an Eppendorf tube. Precipitate the DNA with 1/10 volume of 3 *M* sodium acetate, pH 5.2, and 2.5 vol of ice-cold 96% ethanol. Mix well and keep on ice or at for –20°C at least 30 min. Spin down at 13,000*g* for 30 min at 4°C. Discard the supernatant, dry the pellet, dissolve it in 10 μL HB50 at 37–42°C, and add 10 μL 20% dextran sulfate in HB50 (*see* **Note 12**).
2. Bake the slides at 60°C for 30 min.
3. Pipet 100 μL RNase solution on the slides and cover with a 24 × 50 mm cover slip and incubate at 37°C for 60 min.
4. Rinse in 2X SSC at RT for 2 × 5 min. All washing steps are performed in Coplin jars (*see* **Note 8**).
5. Rinse in 1X PBS for 5 min.
6. Post-fix in 4% formaldehyde in 1X PBS (*see* **Note 4**) at room temperature for 10 min.
7. Rinse in 1X PBS for 2 × 5 min.

8. Dehydrate the slides through an ethanol series (70%, 90%, 100%), each step 1 to 3 min.
9. Air-dry the slides.
10. Add 20 μL probe and cover with a 22 × 22 or 32 × 24 mm cover slip.
11. Denature the probe DNA and chromosomal DNA together on the slides at 80°C for 2 min on a heating block.
12. Put the slides in a moist chamber and incubate overnight at 37°C (*see* **Note 13**).

3.4. Fluorescent Detection of Hybridized Probes

Signal detection and amplification are exemplified for hapten-labeled probes (biotin-, digoxigenin-, DNP-dUTP) visualized by indirect immunofluorescence via Texas Red, Alexa 488, and Cy5. (If the first detection step for hapten-labeled probes yields a sufficiently strong signal, amplification can be omitted.) This protocol can be combined with the application of probes containing directly fluorochrome-labeled nucleotides (e.g., Cy3- or DEAC-dUTP) that require only posthybridization washing prior to microscopic evaluation. FISH can be performed with single probes labeled according to one of the above options or with various combinations of differently labeled probes. Avoid drying of slides during the entire procedure.
1. Wash slides in a Coplin jar with 2X SSC at 42°C for 2 min. Keep the slides in the dark as much as possible when fluorochrome-labeled probes are used (valid for all following steps).
2. Wash slides in SF50 at 42°C 3 × 5 min (*see* **Note 14**).
3. Wash in 2X SSC at 42°C for 2 min. If only fluorochrome-labeled probes are used, rinse the slides in 2X SSC at RT and proceed with **step 14**.
4. Rinse the slides briefly in 4T at 42°C.
5. Pipet 100 μL blocking solution onto slide, put cover slip on slide, and incubate in a moist chamber at 37°C for 30 min.
6. Rinse in 4T at 42°C for 2 × 5 min.
7. Rinse in TNT at 42°C for 5 min.
8. For detection of biotin-labeled probes pipet 100 μL avidin~Texas Red in TNB (1:1000) onto slide, put cover slip on slide and incubate in a moist chamber at 37°C for 30 min. Keep the slides in the dark as much as possible during the following steps.
9. Rinse in TNT at 42°C for 3 × 5 min.
10. Mix goat anti-avidin~biotin, mouse anti-digoxigenin, and rabbit anti-DNP in TNB that the final concentration is 1:200, 1:250, and 1:400, respectively, and pipet 100 μL onto slide, put cover slip on slide and incubate in a moist chamber at 37°C for 30 min.
11. Rinse in TNT at 42°C for 3 × 5 min.
12. Mix avidin~Texas Red, goat anti-mouse~Alexa 488, and goat anti-rabbit~Cy5 in TNB (final concentrations: 1:1000, 1:200, and 1:100), pipet 100 μL onto slide, put cover slip on slide, and incubate in a moist chamber at 37°C for 30 min.
13. Rinse in TNT at 42°C for 3 × 5 min.
14. Dehydrate in an ethanol series of 70%, 90%, and 100%, 1 to 3 min each, and air-dry.
15. Mount in Vectashield with DAPI.
16. Check the slide under a fluorescence microscope using the appropriate filters. In particular the fluorescence signals of far-red fluorochromes (e.g., Cy5) require a CCD camera and image acquisition software for visualization.

4. Notes

1. Alternatively, 6:3:1 fixative (ethanol:chloroform:glacial acetic acid, 6:3:1) can be used.
2. A stock solution containing 1% of each enzyme in citrate buffer is stored at –20°C.
3. Dilution should be prepared freshly and the stock can be stored at –20°C.
4. For postfixation on slides, either 4% formaldehyde prepared from commercially available 37% formaldehyde or 4% paraformaldehyde prepared from solid substance by stirring for ~30 min on a heating plate (~60ₒC) under a fume hood and subsequent filtration through filter paper is suitable. Formaldehyde is hygroscopic; therefore, once opened, the concentration will be, after longer storage, less than 37% in the original vessels.
5. When using differently hapten-labeled probes, select antibodies for detection/amplification of FISH signals in a way that will prevent unintentional cross-reaction.
6. In this step and the following one the acid-soluble proteins and various cytoplasmic components will dissolve in the acetic acid and clear up the spread preparation. Omitting this step will result in chromosome spreads that are still surrounded by cytoplasm. Too little acetic acid or too high a density of cells will result in an undesirable layer of cytoplasmic debris. Monitor the result under a phase contrast microscope. If needed add either more acetic acid or extend exposure time to acetic acid. The cells move in the droplet and settle down at the periphery of the drop let, forming a ring

of cells. To prevent disposition of too many cells at the same ring position, the droplet should be stirred now and then with a needle without touching the glass surface.
7. For unknown reasons the standard spreading protocol sometimes may yield faint chromosome/ nuclei preparations. This phenomenon becomes visible on slides after denaturation at high temperature. It can be prevented by formaldehyde postfixation immediately after spreading and precipitation in Carnoy's fixative. To this, after **step 9** rinse the slide in water and postfix the material in 4% freshly prepared formaldehyde in 1X PBS (10 m*M* sodium phosphate, pH 7.0, 143 m*M* NaCl) or in distilled water for 10 min and air-dry.
8. If chromosomes/nuclei appear to be covered by cytoplasm after checking of dry slides in a phase contrast microscope, pipet 100 µL pepsin (100 µg/mL in 0.01 *N* HCl) on the slide, cover with a 24 × 50 mm cover slip, and incubate at 37°C until cytoplasm is no longer microscopically detectable. Too long a treatment with pepsin may degrade chromatin structures.
9. Occasional milky appearance of the DNA solution after isolation, indicating contamination with proteins and polysaccharides, does not impair the hybridization efficiency of the probe.
10. The single-component approach for nick translation allows adjusting the desired probe length.
11. If the fragments are clearly longer than 500 bp, extend the incubation at 15°C for another 30 min. Additional DNase can be added as well. If the probe is too short, DNase can be reduced in a repeated experiment. The suitable incubation time and DNase concentration should be determined empirically. The conditions may slightly differ from probe to probe.
12. Insufficiently dissolved DNA may cause a high background.
13. Hybridization of *Arabidopsis* probes to chromosomes of other Brassicaceae species requires longer hybridisation times (48–72 h) to ensure proper hybridisation to the homeologous target sequences. Sealing of cover slips by rubber cement (e.g., Fixogum) avoids drying out of the probe and/or its dilution by condensing water during long hybridization times.
14. If applying *Arabidopsis* probes to chromosomes of other Brassicaceae species, wash under less stringent conditions (20% formamide in 2X SSC, pH 7.0) at 42°C for 3 × 5 min.

5. References

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