CHAPTER 2

Nucleolar activity in allotetraploid *Brassica napus* and its putative ancestral diploid species

1. Introduction to the topic

1.1. Why are we using *Brassica* species as a model?

B. napus (2n = 4x = 38; genomes AA CC) is an allotetraploid species which originated by interspecific hybridisation between *B. oleracea* (2n = 2x = 18; genome CC) and *B. campestris* (2n = 2x = 20; genome AA). *B. napus* has two different genomes, derived from both ancestral species. Results of some analyses suggest that these genomes may differ from the genomes of extant putative ancestral diploid species.

1.2. 45S ribosomal RNA genes (= rDNA) — their number and localisation

Fluorescent *in situ* hybridisation applied to root-tip meristematic cells of *Brassica* species shows that *B. oleracea* has two pairs, *B. campestris* — five pairs, and *B. napus* — six pairs of rDNA loci (Fig. 2.1). These loci are distributed in different chromosomal locations, i.e. at known positions of

nucleolus organiser regions (NORs), and in pericentric and terminal regions of some chromosomes. It is significant that the number of loci in allotetraploid species is lower than the sum of these loci in both diploid species that presumably are the donors of the genomes for *B. napus*. This suggests that some intragenomic rearrangements including translocations and/or deletion of 45S rRNA genes may have occurred during speciation within the genus *Brassica*.

1.3. What is known about nucleolar activity in *Brassicas?*

45S rRNA genes belong to the category of so-called "housekeeping genes". This means that in all cells they have to have at least basal activity, which ensures sufficient synthesis of ribosome subunits. Many species possess in their diploid somatic cells only one pair of 45S rRNA gene loci. In such cases usually both loci show rRNA gene expression. Genomes of *Brassica* species have at least two pairs of rDNA loci distributed over different chromosomes. Then nucleolar activity of a particular locus can vary.

1.4. Connection between DNA methylation and gene activity

DNA methylation is one of several epigenetic mechanisms, which can significantly modulate the expression of genes in eukaryotic cells. It is well documented that an increase in the level of methylated cytosine in promoter regions of genes inhibits their expression. 5-azacytidine (5-aza-C), a synthetic analog of cytosine, is known as a strong hypomethylation agent. Its application prevents DNA from maintaining its methylation status and may, in many cases, increase the level of activity of numerous genes. It would be interesting to see, if the expression of 45S rRNA genes depends on DNA methylation.

1.5. Why are we applying silver staining?

The silver staining technique is a simple, reliable cytogenetic method for demonstration of 45S ribosomal gene activity. It enables the detection of

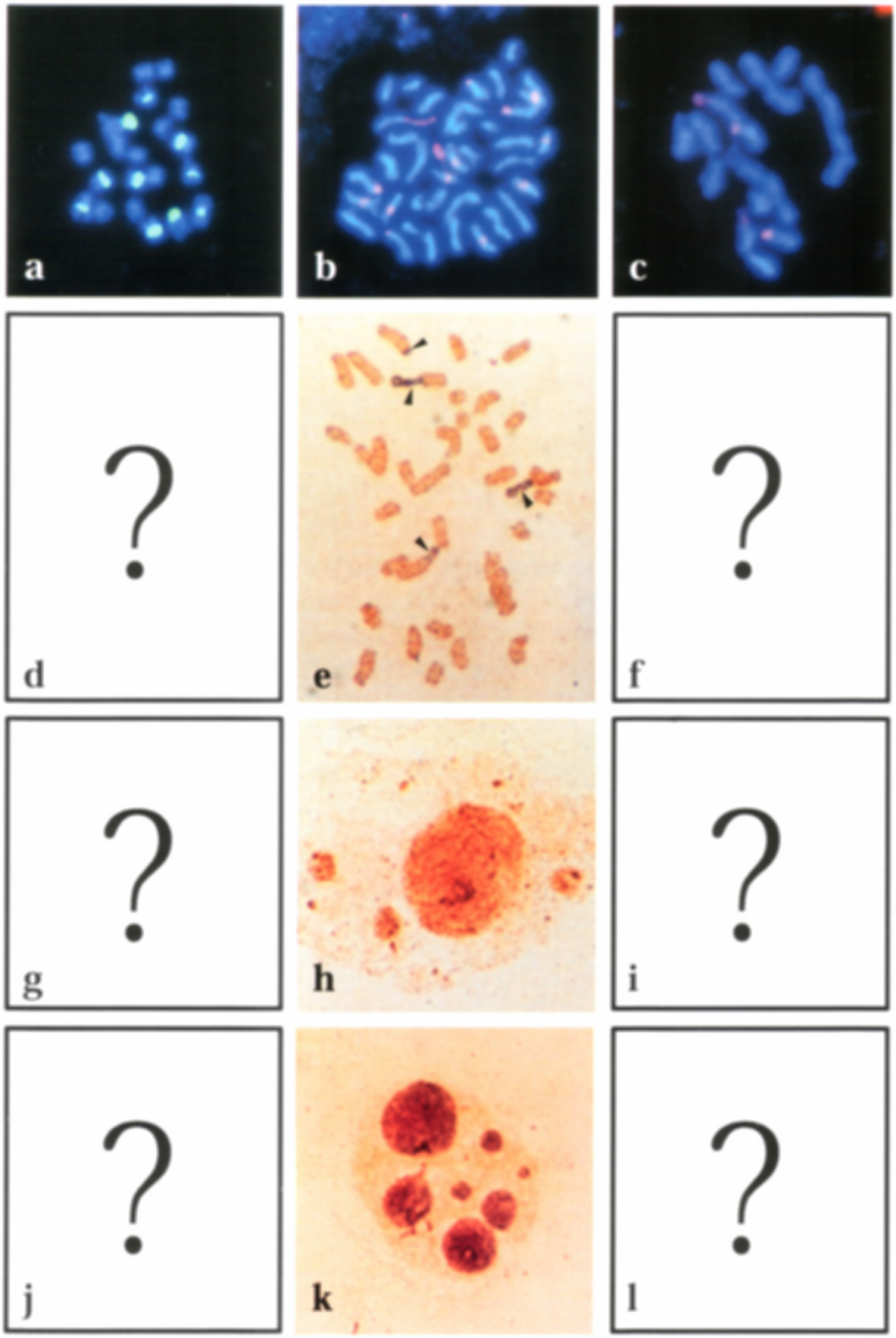

Fig. 2.1. (a, d, g, j) *Brassica campestris* (2n = 2x = 20; genome AA), (b, e, h, k) *Brassica napus* (2n = 4x = 38; genomes AACC), (c, f, i, l) *Brassica oleracea* (2n = 2x = 18; genome CC). (a, b, c) *in situ* hybridisation with 25S rDNA; five (*B. campestris*), seven (*B. napus*) and two (*B. oleracea*) pairs of 25S rDNA loci respectively. (e) silver staining; two pairs of Ag-NORs on metaphase chromosomes, (h) max. four nucleoli in interphase, (k) up to seven nucleoli can be seen after 5-azaC treatment. Empty fields (d, g, j) for *B. campestris* and (f, i, l) for *B. oleracea* respectively should be supplemented with your own results

nucleoli in interphase nuclei and NORs on the chromosomes in standard cytogenetic preparations. Available cytochemical data suggest that the silver staining reaction stains some nucleolar-specific proteins during interphase. These proteins can remain around the NOR even during the division of the cell. Hence, positive silver staining of NORs (AgNORs) is evidence for transcriptional activity of its rRNA genes in the previous interphase.

2. Scientific problem

The scientific aim in this part of the course is the determination of the number of active 45S ribosomal loci both in the diploid parental species and in the allotetraploid species. Inactivity of some rDNA loci may be due to DNA methylation. If methylation is involved in regulation of rRNA genes activity, 5-aza-C treatment should restore the expression of inactive rDNA loci.

3. The research skills you will acquire

In conducting this experiment you will acquire research skills in the following:

- Germination of the seeds in the presence (experimental group) and absence (control group) of 5-azacytidine
- Preparing of preparations of mitotic chromosomes and somatic nuclei for silver staining
- Silver staining reaction
- Ordinary light microscopy and photography
- Analysis, interpretation and presentation
- Descriptive statistics.

4. Scientific goals and questions you should be able to answer

- On which chromosomes in these species are the FISH signals of the 45S rRNA genes and which of the NORs of these loci are active?
- Why can we infer from the NOR silver staining pattern of metaphase chromosomes the activity of the ribosomal genes?
- Is the number of nucleoli in interphase nuclei equal to the number of active rDNA loci observed on chromosomes (AgNORs)?
- Do you observe nucleolar dominance in *B. napus*?
- Is DNA methylation involved in regulation of rRNA gene activity?

5. Report

You will be expected to conduct the experiment alone or in a small group of participants. You will present your results in the form of an illustrated protocol. In addition you might be selected to present your result in the form of the poster presentation. You are also expected to complete the chart below and empty fields in Fig. 2.1 and to include it with the final protocol.

	B. campestris	*B. napus*	*B. oleracea*
chromosome number			
45S rRNA FISH signals			
AgNORs (untreated)			
AgNORs after 5-aza-C treatment			

6. Methods

6.1. 5-azacytidine treatment

(1) Divide the seeds of all three species into two groups. You should have at least 100 seeds in each group.

(2) Germinate the seeds at 18—22°C in a dark incubator on filter paper moistened with tap water for control group and with a 0.1 mM solution of 5-aza-C (Appendix-1) for experimental group in 14 cm Petri dishes until roots are 1.5—2.0 cm long. Because of the instability of 5-aza-C it is necessary to change the solution each day.

6.2. Chromosome and slide preparation

(1) Treatment for metaphase accumulation and condensation of chromosomes: seedlings with 1.5—2.0 cm root tips are immersed immediately in 2 mM 8-hydroxyquinoline (Appendix-2) for 2 h at room temperature and then for 2 h in a refrigerator.

(2) Fix immediately in fresh ethanol: acetic acid mixture (Appendix-3) for 3 h at room temperature and store at −20°C until use.

(3) Wash material for 10 min in citrate buffer (Appendix-4). Cut off about 1 cm long sections of roots containing the root-tips and put them into another change of citrate buffer for the next 10 min to remove the remaining fixative.

(4) Put material into enzyme solution (1 ml; Appendix-5) and incubate for 30—60 min at 37°C.

(5) Remove carefully the enzyme and wash the material for 30 min in two changes of citrate buffer.

(6) Cut off the root-tips from the rest of the root.

(7) Using a glass-pipette take carefully three root-tips and place them in the dish with 45% acetic acid. If possible remove the cap and extrude the meristem under a dissecting microscope. When meristems become transparent using a micropipette transfer them with small volume (3 µl) of 45% acetic acid in the centre of a clean slide (Appendix-6).

(8) Using thin needles macerate the material and apply carefully a 22 × 22 mm coverslip, tap gently with a matchstick and squash between folded paper.

(9) Scan under ×20 and ×40 phase contrast to check well-spread metaphases.

(10) Freeze preparation in deep freeze, flick off coverslip with blade, and air dry immediately at RT.

(11) Slides may be stored at room temperature or in a refrigerator for several days.

6.3. Silver staining reaction

(1) Wash the slides in borate buffer (Appendix-7) at room temperature for 15—30 min. Dry slides for several minutes.

(2) Place 50 µl of 50% aqueous solution of silver nitrate (Appendix-8) on each slide and cover with a piece of polyamide cloth (Appendix-9).

(3) Place the slides immediately in a sealed box and incubate at 45°C for 40—60 min. Monitor the progress of staining under a microscope.

(4) When the polyamide cloth and the chromosome preparation are deep yellow rinse the slides in 4—5 changes of distilled water.

(5) Air-dry slides and (optionally) mount in DPX.

Correctly stained preparations appear as follows:

structure	colour
cytoplasm	pale yellow outer circle
nucleus	deep yellow inner circle
nucleolus	brown or black stained large dot inside the inner circle
NOR	dark stained small two dots or band on the chromosome, in most cases located at the secondary constrictions of the satellited chromosomes
background	white or almost white without precipitates

6.4. Some questions regarding the method

(1) How would you optimise the concentration of 5-azacytidine?

(2) What determines the time of the silver staining reaction?

(3) What is the advantage of sequential silver staining and FISH to 45S rRNA genes compared to the application of these two techniques in separate experiments?

7. Appendix

(1) 0.1 mM aqueous solution of 5-azacytidine (Sigma — A-2385)

0.0024 g 5-azacytidine/100 ml distilled water

The solution has to be prepared directly prior to use.
The solution is unstable so must be prepared fresh each day.

(2) 8-hydroxyquinoline — 2 mM

0.28 g 8-hydroxyquinoline/1000 ml distilled water at 60°C

Using a magnetic stirrer dissolve 8-hydroxyquinoline in preheated (60°C) distilled water. Store in refrigerator.

(3) alcohol-acetic acid fixative

3 volumes of pure ethanol or methanol
1 volume of glacial acetic acid

Mix well. Prepare prior to use.

(4) 10 mM citrate buffer (pH 4.8)

A. 2.1 g citric acid monohydrate ($C_6H_8O_7 \times H_2O$)/100 ml distilled water (0.1 M)
B. 2.94 g trisodium citrate dihydrate ($C_6H_5O_7Na_3 \times 2H_2O$)/100 ml distilled water (0.1 M)

Mix 60 ml A and 90 ml B. Dilute 10×.
TECHNICALLY THE DEGREE OF HYDRATION WILL EFFECT THE WEIGHT YOU USE.

(5) enzyme solution

1% w/v cellulase (Calbiochem 21947)
1% w/v cellulase (Onozuka RS)
20% v/v pectinase (Sigma P4802)

Dissolve enzymes in 10 mM citrate buffer, divide into 1.5 ml aliquots and store at −20°C.

(6) microscope slides

Place new microscope slides and coverslips into 70% ethanol. Remove and dry with tissue immediately prior to use.

(7) 10 mM borate buffer (pH 9.2)

1.9 g $Na_2B_4O_7 \times 10\ H_2O$/500 ml distilled water

Adjust to a pH of 9.2. Keep frozen or in refrigerator.
Ready to use solution (Merck, Art. 1645) also is available.

(8) 50% aqueous solution of silver nitrate

0.25 g $AgNO_3$ (Merck, Art. 1.01512)/0.5 ml deionised water (volume for 10 preparations)

It is recommended that the solution is prepared immediately prior to use.

(9) polyamide cloth

Prepare a number of pieces (2.5 × 2.5 cm) of polyamide mesh (Silk & Progress, 50—150 μm mesh size) equal to the number of preparations.

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Contents

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A PRACTICAL COURSE MANUAL

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