Theor Appl Genet (2000) 100:665–669

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Detection of 5S and 25S rRNA genes in *Sinapis alba, Raphanus sativus* and *Brassica napus* by double fluorescence in situ hybridization

Received: 20 July 1999 / Accepted: 8 October 1999

Abstract Different ribosomal RNA (5S and 25S) genes were investigated simultaneously by fluorescence in situ hybridization (FISH) in *Sinapis alba, Raphanus sativus* and *Brassica napus.* The chromosomes of *S. alba* carried four 5S and six 25S gene sites, and those of *R. sativus* four sites of each gene, respectively. These two species have one chromosome pair with both rDNA genes; the two are closely located on a short arm of *S. alba*, while in *R. sativus* one is distal on the short arm (5S) and the other more proximal on the long arm (25S). In *B. napus* we have confirmed 12 sites of 25S rDNA. The detection of 5S rDNA genes revealed 14 signals on 12 chromosomes. Of these, six chromosomes had signals for both rDNA genes. The FISH with 5S rDNA probes detected two sites closely adjacent in four chromosomes of *B napus*. These results are discussed in relation to a probable homoeologous chromosome pair in *B. oleracea*.

Key words *Brassica napus* · *Sinapis alba* · *Raphanus sativus* · FISH · 5S rDNA genes · 25S rDNA genes

Introduction

One of the most extensively studied genetic units in the plant genome are the ribosomal RNA genes (18S-5.8S-25S and 5S rDNA) and their chromosomal localization via fluorescence in situ hybridization (FISH). Because of the numerous copies (hundreds to many thousands), in one or more clusters, of these highly conserved families of repeated ribosomal gene sequences, their gene sites can be easily visualized by fluorescence in situ hybridization (FISH). The simultaneous detection of 5S and 18S-5.8S-25S rDNA genes after double FISH with heterologous ribosomal probes allows the physical localizations of both rDNA genes in a single metaphase (Castilho and Heslop-Harrison 1995; Linares et al. 1996; Schrader et al. 1997; Fransz et al. 1998). These genes are excellent cytological markers for karyotype analysis especially in species with many small and equal chromosomes, as in the *Brassicaceae*. For the 18S-5.8S-25S rDNA 12 loci were described by Maluszynska and Heslop-Harrison (1993) and Snowdon et al. (1997) in *Brassica napus*. Investigations using double FISH of both rDNA genes have not yet been reported in the species *Raphanus sativus, Sinapis alba* and *B. napus.* In the present paper we identify the physical localization of gene clusters of 5S and 25S rDNA in these three species by double FISH.

Communicated by K. Glimelius

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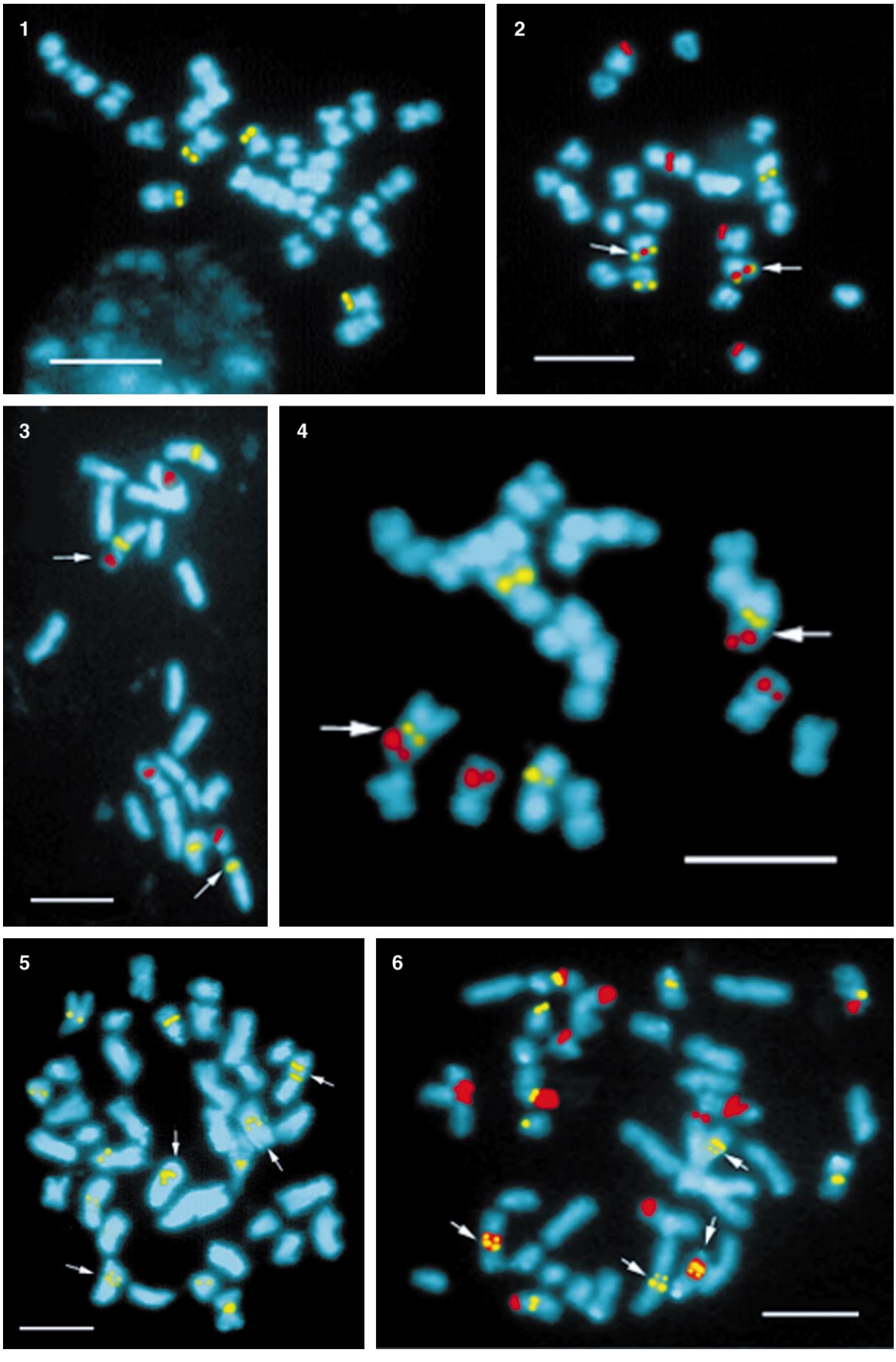
Material and methods

Plant materials

Seeds of *S. alba* (cv Kastor, 2n=24, SS), *R. sativus* (oilradish, cv Pegletta, 2n=18, RR), and *B. napus* (cv Madora, 2n=38, AACC) were sown on moist filter paper in Petri dishes and germinated at 24°C. To enhance (up to five times) the number of metaphases in root-tip meristems, the cell cycle was synchronized by the treatment of 0.5–1-cm-long roots of seedlings with 1.25 mM hydroxyurea (HU) for 17 h (Pan et al. 1993). In a modification to the protocol of these authors the time of DNA synthesis after HU treatment was 9 h for *S. alba* and *R. sativus,* and 9 h and 10 min for *B. napus*, at 24°C. Seedlings were pre-treated with 8-hydroxyquinoline (2 mM aqueous solution, applied at 24°C for 2 h and 40 min), fixed in a freshly prepared mixture of absolute ethanol/glacial acetic acid (3:1) for 24 h and stored in 70% ethanol at –20°C.

Chromosome preparation

Fixed root tips were washed in distilled water for 30 min and then digested with an enzyme mixture of 4% cellulase ‘Onozuka R-10’ (Serva) and 1% pectlyase Y-23 (Seishin Pharmaceutical) in 75 mM KCl and 7.5 mM Na-EDTA, pH 4.0 (Kakeda et al. 1991), at 37°C for 40 min in *S. alba* and for 30 min in *R. sativus* and *B. napus*. After a short rinse in the same buffer the macerated root tips were softened in 45% acetic acid for 1–2 min and squashed.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The slides were frozen at –84°C and the coverslips were removed with a razor blade. The specimens were dried overnight at 24°C and stored at –20°C.

Preparation of DNA probes and hybridization mixtures

For the detection of 18S-5.8S-25S rDNA loci a gene-specific probe of the 25S-repeat (220 bp) was labelled with biotin-16-dUTP (Boehringer Mannheim) during polymerase chain reaction (PCR)-amplification of the genomic DNA of *Allium ampeloprasum* with primers designed according to the sequence published by Yokata et al. (1989). For the localization of 5S rDNA genes, a 117-bp fragment obtained after PCR-amplication from the same genomic DNA with specific primers coding for this region (Gottlob-McHugh et al. 1990) was used. The labelling of this amplified probe was performed with digoxigenin-11-dUTP (Boehringer Mannheim). The hybridization mixture contained, per slide, 80 ng of each DNA probe (5S- and 25S-rDNA) and 10 µg of salmon-sperm DNA in 20 µl of hybridization buffer (50% de-ioinized formamide, 10% dextran sulphate, 2×SSC).

Fluorescence *in situ* hybridization

The FISH procedure was performed according to Fuchs and Schubert (1995) with the following modifications. Prior to hybridization, slides were incubated in 50 ng/µl of DNase-free RNase in 2×SSC for 1 h at 37°C, washed three times in 2×SSC for 5 min and treated with 0.5 ng/µl of proteinase K for 10 min at 37°C, followed by washing in 2×SSC for 15 min. The slides were then post-fixed in 4% paraformaldehyde for 10 min, washed in 2×SSC for 15 min, dehydrated in a graded ethanol series (70, 80 and 96%) at –20°C, and air-dried. The hybridization mixture (probe) was denaturated in a 75°C water bath for 7 min, incubated on ice (about 5 min), dropped onto slides, covered with coverslips, and sealed with rubber cement. Probes and chromosomes were denaturated together on a Zytotherm (Schutron) for 7 min at 80°C. The slides were then incubated overnight at 37°C in a humidity chamber. After hybridization, the coverslips were removed and the slides were washed in 50% formamid in 2×SSC at 37°C three times for 5 min each, followed by three 5-min washes in 0.5×SSC at 60°C and then blocked for 30 min at 37°C with a solution of 4×SSC, 3% BSA and 0.1% Tween 20. The biotinylated probe was detected with 12 ng/µl of streptavidin-Cy3 (Dianova) and amplified with two steps of 11 ng/µl of biotinylated anti-streptavidin (Vector) and 10 ng/µl strepavidin-Cy3. Together with the first amplification step of the biotin-labelled probe the detection of the digoxigenin-labelled probe with 9 ng/µl of anti-digoxigenin-fluorescein (Boehringer Mannheim) was done and then amplified with 8 ng/µl anti-sheep-fluorescein (Dianova). Chromosomes were counterstained with 1.5 ng/µl of 4´,6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 5 min at 37°C, rinsed in distilled water, and mounted in VECTASHIELD antifade solution (Vector Laboratories).

Figs. 1–6 Location of 5S and 25S rDNA gene loci by the FISH technique. **1 and 5** FISH using the digoxigenin-labelled 5S rDNA probe with detection by anti-digoxigenin-FITC (*yellow*) and counterstaining by DAPI. **1** *S. alba*, **5** *B. napus*: four chromosomes with closely adjacent double signals (*arrows*). **2, 3–4 and 6** double FISH of 5S and 25S rDNA gene loci. The digoxigenin-labelled 5S rDNA probe was detected by anti-digoxigenin-FITC (*yellow*), the biotinylated 25S rDNA probe by streptavidin-Cy3 (*red*), and the chromosomes were counterstained with DAPI. **2** *S. alba*: *arrows* mark the locations of both rDNA genes in a chromosome pair. **3–4** Prometaphase and metaphase of *R. sativus* with *arrow-marked* locations of the 5S and 25S rDNA genes in one chromosome. **6** *B. napus* with two sites of 5S rDNA genes closely adjacent in each of four chromosomes (*arrows*). The scale bars indicate 5 µm

Microscopy and image analysis

Images were captured for each fluorescence dye separately with a cooled CCD camera system (S/L Microtest, Jena) on a Zeiss Axioskop with the following filter combinations: 02 (DAPI), 10 (FITC) and 20 (Cy3). Pseudocoloration and mergence of images were done with Adobe Photoshop 5.0.

Results

FISH of *S. alba* chromosomes with the 5S rDNA specific probe resulted in four equally strong signals located distally on the short arms of two submetacentric chromosome pairs differing in their total length (Fig. 1). In the same species the double-target in situ hybridization with digoxigenin-labelled 5S and biotin-labelled 25S rDNA probes revealed, besides the four 5S rDNA loci, six 25S rDNA gene loci on the short arms of six chromosomes (Fig. 2). Both rDNA genes were closely linked on the short arm of one chromosome pair. In *R. sativus* simultaneous FISH of 5S and 25S rDNA gene loci showed four signals of each (Figs. 3 and 4). The localization of both rDNA genes was detectable in one of the longest chromosome pairs, distally on the short arm (25S) and near the centromere on the long arm (5S). The other 5S or 25S rDNA genes were separately located on the long arms of two chromosome pairs. Figure 5 shows the hybridization pattern of 12 5S rDNA signals in *B. napus*, at which two closely adjacent sites were visible in two long submetacentric chromosome pairs. Double FISH of the 5S and 25S rDNA genes in this species revealed 12 signals for both 5S and 25S rDNA (Fig. 6). In six chromosomes both rDNA genes were localized together, of which two pairs were closely linked.

Discussion

FISH of diploid species

Knowledge of the relative physical locations and the number of multicopy rDNA gene loci is important and useful both for the construction of physical maps of chromosomes and for phylogenetic studies. In the *Brassicaceae*, *S. alba* has not previously been investigated molecular-cytologically. It possess very short chromosomes, 1–2 µm length in mitotic metaphases, with only slight karyotypic differentiation following homogeneous staining and also after C-banding and staining with several fluorochromes (Geber and Schweizer 1988). These latter authors detected three active NORs after silver-staining. The six locations of 25S rDNA genes in our experiment showed that, in addition to the three active NORs, three further possibly inactive loci were detectable via FISH. But six active NORs were shown in the results of Cheng and Heneen (1995) in the lower chromosome number species *Sinapis arvensis* (2n=18). Also six 25S rDNA gene loci were detected via FISH in two additional diploid species *Brassica nigra* (Fukui et al.

1998) and *Brassica oleracea* (Maluszynska and Heslop-Harrison 1993; Snowdon et al. 1997; Armstrong et al. 1998). Taken together these results support the conclusion that the number of three chromosome pairs carrying the 25S rDNA genes is basic for the *Brassicaceae*. Several authors had reported that the diploid species of *Brassicaceae* have evolved from a common ancestor but with different basic chromosome numbers of *x*=6 (Röbbelen 1960) and *x*=3 (Hussein and Abobakr 1976; Cheng and Heneen 1991, 1995). The detection of three chromosome pairs with 25S rDNA sites or active NORs in the above mentioned species of *Brassica* and *Sinapis* supports the hypothesis of a basic chromosome number of *x*=3 (with one active NOR-chromosome) in these genera.

In contrast to *S. alba* the results in *R. sativus* with four sites of 5S und 25S rDNA respectively (Figs. 3 and 4) demonstrated that the number in the latter species was decreased. In confirmation with this finding Mukherjee (1979) also described maximum of four chromosomes with a secondary constriction in karyotypes of several strains of *R. sativus*. On the basis of restriction maps of the molecular length of the 18S and 25S rDNA repeats and their intergenic spacer regions, Delseny et al. (1990) showed marked similarities between *R. sativus* and diploid *Brassica* species, which confirms the high conservation of this genetic region. The presence of both 5S and 25S rDNA genes in one chromosome pair was common to both *S. alba* and *R. sativus*. But the different position and arm-location of 5S compared to the 25S rDNA suggest distinct evolutionary pathways for this chromosome pair, in both species, from a common ancestor.

FISH of *B. napus*

The FISH of 5S rDNA indicates a high number of 16 sites in six chromosome pairs in *B. napus* two of which had closely linked double signals near to the centromeres (Figs. 5 and 6). In pachytene chromosome preparations of *B. oleracea*, Armstrong et al. (1998) had earlier demonstrated only one location of 5S rDNA genes at two closely adjacent chromosome sites near to the centromere. Our results demonstrating 5S rDNA gene localizations with two double-signal chromosome pairs suggest an origin of one of the two from the C-genome progenitor of *B. napus*. The 12 localized 25S rDNA sites were consistent with the findings of Maluszynska and Heslop-Harrison (1993) and Snowdon (1997) in *B. napus*. However, the investigation of 5S and 25S rDNA genes by double FISH revealed that six of the 12 25S marked chromosomes had both rDNA genes closely linked proximal to the centromere in two of the submetacentrics. Additionally, these two submetacentric chromosomes had the above-discussed two closely adjacent sites of 5S rDNA clusters. Therefore they coincide with the pattern of 5S and 25S rDNA genes in chromosome number 2 of the *B. oleracea* karyotype (Armstrong et al. 1998). The other four double-marked chromosomes were probably descended from the A-genome progenitor.

The detected number of 12 chromosomes which carry 5S rDNA gene clusters does not confirm the postulation of Long and Dawid (1980) that this family member of tandemly repeated genes is organized at a small number of chromosomal sites. The high number of 5S rDNA sites can probably be related to the A-genome progenitor. The A-genome had also been partly subject to nonreciprocal translocations (Sharpe et al. 1995; Parkin et al. 1995) that could potentially enhance the number of rDNA sites. Additionally, the sites of rDNA may have changed their position as a result of chromosome fission (Hall and Parker 1995) and a jumping of the nucleolus organizing regions (Schubert and Wobus 1985).

Further work must be done with a higher resolution of rDNA sites, using with FISH and double FISH at meiotic prophase and mitotic pro-metaphase, in order to map these genes quantitatively in the karyotype of *B. napus* and to compare it with members of the A-genome (*Brassica rapa and Brassica campestris*).

Acknowledgements We are greatful to K. Maier and E. Koblitz for their exellent technical assistance and to Dr. H. Peterka for critical reading of the manuscript.

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