Annals of Botany **86**: 201–204, 2000
doi:10.1006/anbo.2000.1156, available online at http://www.idealibrary.com on IDEAL®

SHORT COMMUNICATION

Molecular Cytogenetic Localization and Characterization of 5S and 25S rDNA Loci for Chromosome Identification in Oilseed Rape (*Brassica napus* L.)

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Received: 4 January 2000 Returned for revision: 15 February 2000 Accepted: 16 March 2000

Chromosome identification in oilseed rape (*Brassica napus* L.) is extremely difficult using conventional cytogenetic techniques because amphidiploid *Brassica* species possess numerous very small chromosomes with few cytogenetic landmarks. In combination with methods for improved chromosome preparations, we used a simplified fluorescence *in situ* hybridization (FISH) technique to localize simultaneously the gene families coding for 5S and 25S rDNA in *B. napus*. The resulting hybridization patterns enabled ten of the 19 oilseed rape chromosome pairs to be unequivocally identified.

Key words: *Brassica napus*, oilseed rape, rDNA, molecular cytogenetics, FISH, chromosome identification.

INTRODUCTION

Oilseed rape (*Brassica napus* L., genome AACC, $2n = 38$) is one of the most important industrial and food oil crops worldwide, with high production in Europe, North America and Asia being complemented by rapidly increasing cultivation in Australasia. By using modern biotechnological methods like protoplast fusion or embryo rescue to overcome incompatibility barriers, it is now possible to generate interspecific and intergeneric hybrids between *B. napus* and a wide range of related crop and wild species (see Friedt and Lühs, 1998; Glimelius, 1999). In this way, the available genetic pool for breeding of potential new rapeseed varieties has been considerably extended. However, hybrid breeding strategies can be considerably simplified by the ability to screen offspring cytologically, enabling selection of plants containing the trait of interest in a minimal donor-genome background. For other important crop plants, particularly cereals, detailed karyological information is available which allows a ready identification and physical characterization of addition, substitution and introgression lines (e.g. Cabrera *et al*., 1995; Friebe *et al*., 1996; Linde-Laursen *et al*., 1997). On the other hand, the very small and uniform *Brassica* chromosomes are unable to be reliably distinguished by conventional cytogenetic techniques. Moreover, the chromosomes are often highly condensed in standard squash preparations, heterochromatin is restricted almost exclusively to centromeric regions, and banding techniques (e.g. C-banding, see Olin-Fatih and Heneen, 1992) therefore give only very limited information for chromosome identification. Thus, hybrids and chromosome addition lines could previously generally only be accurately characterized by complex molecular marker studies.

The molecular cytogenetic technique of fluorescence *in situ* hybridization (FISH) provides one potential solution to this problem. Hybridization of repetitive DNA probes, like the DNA coding for ribosomal RNA gene families, has, in recent years, been applied to assist karyological and genome analysis in a large number of different plant species (see Jiang and Gill, 1996; Kubis *et al*., 1998; Schmidt and Heslop-Harrison, 1998). The localization of 25S rDNA probes by FISH in *Brassica* species (e.g. Maluszynska and Heslop-Harrison, 1993; Snowdon *et al*., 1997) allowed genomic comparisons of rDNA-carrying chromosomes in amphidiploid species with their diploid progenitors. By combining this technique with computer-based imaging methods, Fukui *et al*. (1998) published 'quantitative karyotypes' for diploid *Brassica* species based on computer-densitometry measurements of chromosome lengths and centromere positions. However, a simple method for reliable identification of chromosomes has not previously been available. Here we describe the simultaneous hybridization of 5S and 25S rDNA probes which, in combination with a simplified method for generation of high-quality *Brassica* chromosome preparations, enables the accurate and reliable visual identification of more than half of the *B. napus* chromosome pairs.

MATERIALS AND METHODS

Plant material

Seedlings from winter oilseed rape (*B. napus* L.) were germinated in the dark for 3–4 d on moist filter paper. When the primary roots were 4–5 cm long, the terminal 1–2 cm was excised and incubated for 2 h at room

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0305-7364/00/070201 + 04 $35.00/00

temperature, followed by a further 2 h at 4°C, in 2 mM 8-hydroxyquinoline. The material was then transferred to ethanol-acetic acid fixative (3:1) and stored at −20°C for a minimum of 24 h.

Cell suspensions

Chromosomes were generated from air-dried cell suspensions using a method adapted from Schwarzacher *et al.* (1994). Initially, the fixed root material was washed briefly in enzyme buffer (40 mM citric acid, 60 mM tri-sodium citrate; pH 4·8). Root tips were then removed and digested in 2 % (w/v) cellulase (Calbiochem) and 20 % (v/v) pectinase (from *Aspergillus niger*, Sigma) in enzyme buffer for 90–100 min at 37°C. The enzyme solution was removed by washing twice in enzyme buffer. The still-intact root tips were then treated for 35 min in 75 mM KCl. Subsequently, cytoplasm was cleared by washing in repeated changes of 60 % glacial acetic acid, for 20–25 min, until the root tips were almost invisible. Finally, cells were dispersed in a small quantity of fixative solution by passing the material four or five times gently through a fine pipette tip.

Chromosome preparations

Ten µl of cell suspension was placed on a clean microscope slide which had been pre-chilled to −20°C. The best chromosome spreads were obtained when evaporation of the cells was slowed by adding further drops of fresh fixative over a period of 2–3 min. Slides were subsequently air-dried and either used immediately for FISH or stored at room temperature for up to 4 weeks.

FISH

A 25S rDNA clone from *Arabidopsis thaliana* and the 5S rDNA subunit from *Beta vulgaris* were directly labelled by nick translation with the fluorochromes Cy3 and fluorescein, respectively. The labelled probes were resuspended with a 50-times excess of sheared salmon sperm DNA in a hybridization mixture comprised of 50 % formamide, 2 × SSC and 5 % dextran sulphate. Chromosomal DNA was stabilized by washing slides for 10 min in 1 × PBS and 10 min in 1 × PBS/50 mM $MgCl_2$/1 % formaldehyde, followed by dehydration in a graded ethanol series at −20°C. Fifteen µl of probe solution was added to each slide and, after simultaneous denaturation of probe and chromosomal DNA on a hotplate for 5 min at 80°C, the slides were transferred to a humid chamber for overnight hybridization at 37°C.

Slides were washed at 42°C for 5 min in 2 × SSC followed by 10 min in 0·2 × SSC. Chromosomes were then counter-stained with DAPI-antifade (Quantum-Appligene) and fluorescence was visualized using a Leica DM-R microscope and Leica QFISH software. Signals were optimized by adjustment only of contrast and brightness settings affecting the complete image.

RESULTS AND DISCUSSION

Cytological preparations

A good number of well-spread metaphase and prometaphase chromosomes was consistently achieved using the cell suspension method described. Chromosomes were free of cytoplasm and less condensed than in standard squash preparations, making them extremely suitable for FISH. Although a hypotonic treatment might not be expected to have any effect on fixed cells, exclusion of the 0·75 mM KCl step from the chromosome preparation protocol tended to result in poorer chromosome spreading. We hypothesize that the root tip cells were sufficiently re-hydrolysed by the prior enzymatic treatment to enable at least a limited osmotic action.

Non-specific hybridization after FISH was negligible, even though the stringent washes were performed in 0·2 × SSC, with the advantage of reduced exposure to harmful formamide fumes. Moreover, the use of directly-labelled probes resulted in stronger hybridization signals than with indirect labelling (results not shown) and also removed the need for time-consuming detection steps. Equally good results were obtained using this procedure for chromosome preparations from other *Brassica* species and further plants with small chromosomes, including *Raphanus* and *Helianthus* species.

rDNA localization and chromosome identification

Hybridization patterns from simultaneous FISH with the two rDNA probes are shown in Fig. 1. Six pairs of 25S rDNA loci could be localized to chromosome sub-arm level in concurrence with previously published results (see Snowdon *et al.*, 1997; Fukui *et al.*, 1998). Eight 5S rDNA loci were observed on seven chromosome pairs. Three 5S loci co-localized with centromeric 25S rDNA, whereby the resulting double hybridization patterns enabled these three respective chromosome pairs to be distinguished from one another (see Fig. 1B). Two metacentric chromosome pairs which could not be identified based on their 25S rDNA loci alone possessed a distinguishable large and small 5S locus, respectively.

The four chromosomes with only 5S rDNA loci could be discriminated based on hybridization pattern and chromosome size or structure. One large metacentric chromosome pair exhibited a pair of 5S loci flanking the centromere, whereas a similar chromosome had only a single locus located near the centromere on the long arm. Two acrocentric chromosome pairs with centromeric 5S loci were readily distinguished by chromosome size, with the long arms of one pair being considerably longer. Three further chromosomes exhibited only 25S rDNA loci. One locus was centromeric on a small metacentric chromosome, while a further large locus localized to the NOR of the satellite-carrying chromosome and was thus easy to distinguish from the last, very small telomeric locus on a large, submetacentric chromosome.

To our knowledge this is the first description of *in situ* hybridization of 5S rDNA in *B. napus* and consequently the first simultaneous localization of 5S and 25S rDNA in this

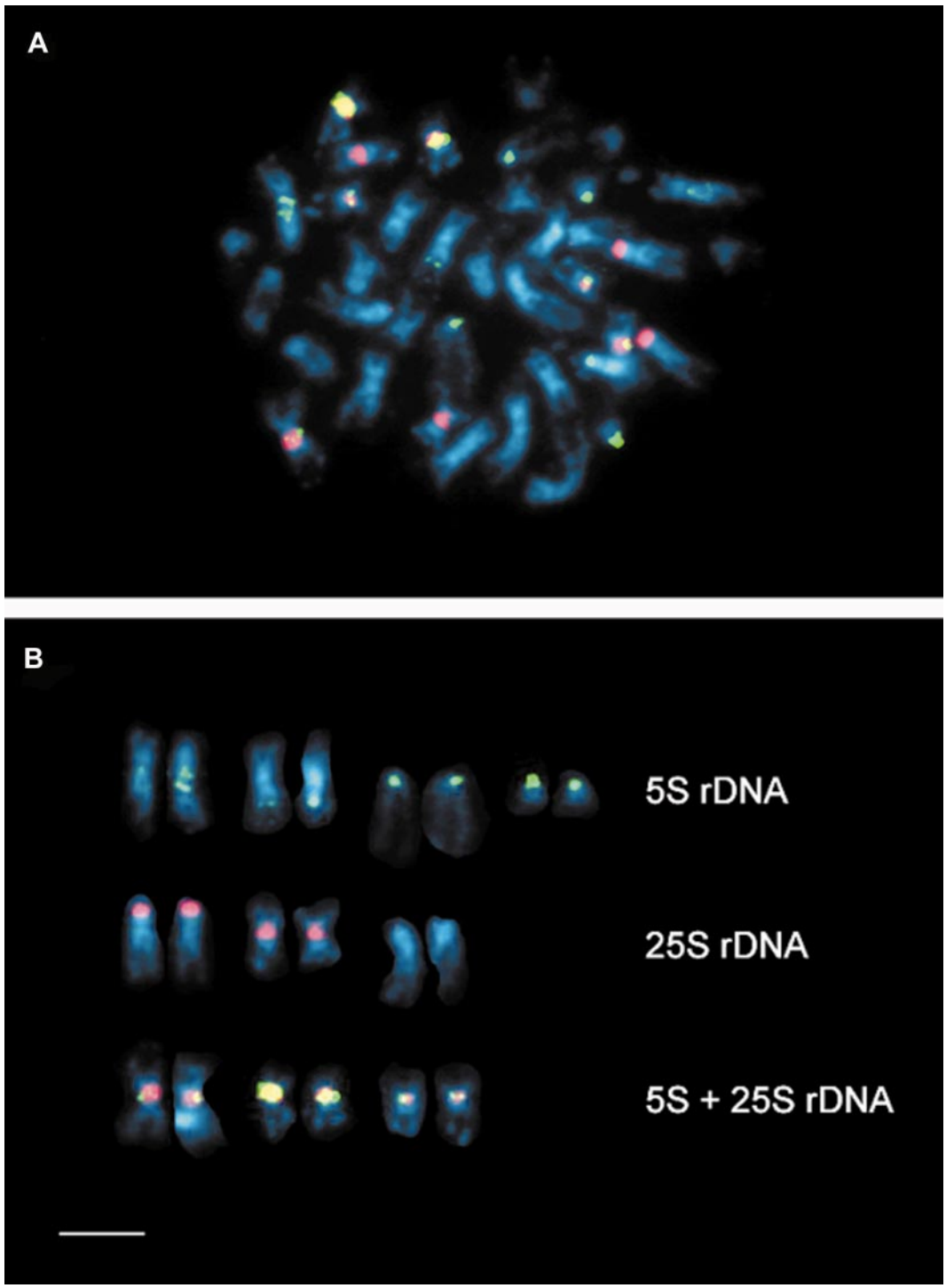

FIG. 1. A. Localization of 5S and 25S rDNA loci on prometaphase chromosomes of *Brassica napus* by fluorescence *in situ* hybridization. Chromosomes are stained blue with DAPI, 5S rDNA are detected with FITC (green) and 25S rDNA sites are labelled with Cy3 (red). Bright yellow signals indicate co-hybridization of 25S and 5S loci, at centromeric sites on three chromosome pairs. B. Identification of ten *B. napus* chromosome pairs based on their 5S and/or 25S rDNA hybridization patterns. Bar = 10 μm.

species. Because *B. napus* is an amphidiploid originating from the hybridization of the two diploid species *B. rapa* (A genome) and *B. oleracea* (C genome), it is interesting to compare the rDNA loci of *B. napus* with those in its diploid progenitors. Intergenomic recombination between the highly homologous A and C genomes in modern *B. napus* (reviewed by Quiros, 1999) means it is difficult to compare *B. napus* chromosomes directly with those of its diploid

progenitors, particularly since modern *Brassica* varieties are the product of intensive breeding. Comparisons of respective locus numbers for repetitive DNA sequences, however, can give interesting information about genome evolution. Numerous authors have described the distribution of 45S rDNA in the species representing the *Brassica* A and C genomes (e.g. Maluszynska and Heslop-Harrison, 1993; Snowdon *et al*., 1997; Fukui *et al*., 1998). On the other hand, little work has been published regarding 5S rDNA in *Brassica*, and the high number of 5S rDNA loci we observed in *B. napus* was particularly interesting. Armstrong *et al*. (1998) reported only a single chromosome with 5S rDNA (two loci) in *B. oleracea*, suggesting that as many as six of the *B. napus* 5S loci derive from the A genome. In terms of genome evolution, the implication is that despite the high homology of the A and C genomes, the 5S RNA genes have been amplified to a far greater extent in *B. rapa* than in *B. oleracea* during their evolution from a common ancestral genome.

In total, ten *B. napus* chromosome pairs could be unequivocally identified by the presence of either 5S or 25S rDNA or both (Fig. 1B). Hybridization of further repetitive FISH probes using the methods described should provide further molecular cytogenetic markers for the efficient identification of all oilseed rape chromosomes. This will not only create the opportunity for much more effective generation and characterization of addition and substitution lines for use in breeding, but also represents a first step towards establishing the physical association between *B. napus* chromosomes and their respective molecular marker linkage groups. Furthermore, a greater understanding of the physical distribution of repeat sequences, which represent a substantial proportion of *Brassica* and other crop plant genomes, should also provide important information for molecular mapping and genomic studies.

ACKNOWLEDGEMENTS

This work was supported by a grant from the German Research Association (DFG grant KO 701/18-1).

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