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FISH AND GISH ANALYSIS OF *BRASSICA* GENOMES

ROBERT HASTEROK*, TOMASZ KSIAZCZYK, ELZBIETA WOLNY, AND JOLANTA MALUSZYNSKA

Department of Plant Anatomy and Cytology, University of Silesia, Jagiellonska 28, 40–032 Katowice, Poland

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Fluorescence and genomic in situ hybridization (FISH and GISH) methods were used for discrimination of *Brassica* genomes. The three diploid and three allotetraploid species of *Brassica*, known as the "U-triangle," represent an attractive model for molecular and cytological analysis of genome changes during phylogeny in the genus *Brassica*. The use of genomic DNA probes enabled unambiguous discrimination of the ancestral genomes in *B. juncea* and *B. carinata*, and was only partially successful in *B. napus*. GISH signals in all genomes were localized predominantly in pericentromeric regions of chromosomes. Simultaneous application of genomic and ribosomal DNA probes in multicolor GISH and FISH allowed identification of a significant number of chromosomes in the *B. juncea* complement. The study also revealed that species of *Brassica* possess *Arabidopsis*-type telomeric repeats which in all genomes occupied exclusively terminal, that is, telomeric, locations of chromosomes.

Key words: 5S rDNA, 25S rDNA, *Brassica*, FISH, GISH, telomeres.

INTRODUCTION

The genus *Brassica* comprises many species. Six of them are economically important, widely cultivated, and of interest for basic research. The phylogenetic relationships between the six cultivated *Brassica* species, presented for the first time by U (1935) in the form of a triangle, were based on crossability, chromosome pairing and fertility studies on the interspecific hybrids. This "U-triangle" consists of three elementary diploid species, namely *B. campestris*, *B. oleracea* and *B. nigra*, and three allotetraploid species, which originated from crosses between pairs of the three diploid species. The allotetraploids are *B. carinata*, *B. juncea* and *B napus*. These species present a very attractive model for analysis of the origin and genetic relationships of the genus *Brassica*. Not surprisingly, in recent years they have been investigated extensively at the molecular and cytological levels. Genetic analyses have demonstrated that genome duplication occurred very early in the phylogeny of *Brassica* diploid species, and that the duplicated chromosomal segments are highly rearranged and dispersed. These findings supported the hypothesis that modern diploid species of *Brassica* actually are paleopolyploids (Quiros, 1995).

Chromosomes of *Brassica* species are relatively small and poorly differentiated in their morphology, so they are difficult to study by cytogenetic analysis. Comparative genome analysis has disclosed important information on the genome structure of *Brassica* species (Lagercrantz and Lydiate, 1996). The application of fluorescent staining and FISH has delivered markers for identification of some chromosomes in the complement of diploid (Hasterok and Maluszynska, 2000a) as well as allotetraploid species (Hasterok and Maluszynska, 2000b; Hasterok et al., 2001). GISH is also widely applied to distinguish genomes of the ancestral species both in natural allopolyploids and in plant hybrids (Benabdelmouna et al., 2001; Hasterok et al., 2004; Marasek et al., 2004), and to identify alien chromatin (Iqbal et al., 2000; Shi and Endo, 2000; Morgan et al., 2001; Pasakinskiene and Jones, 2005). Total nuclear DNA, comprising all sequences of a genome, can be used as a probe for GISH to show the locations of these sequences.

The aim of this study was to compare the distributions of genomic DNA probe signals and to visualize the chromosomal localization of repetitive DNA in the diploid species of *Brassica*, as well as to discriminate the ancestral genomes in the allopolyploids. We also determined the type and localization of telomeric repeats in *Brassica* genomes. Simultaneous probing of chromosomes of the allotetraploid species with different DNA sequences in a multicolor GISH and FISH experiment demonstrated the usefulness of this technique for chromosome identification in *Brassica* genomes.

*e-mail: hasterok@us.edu.pl

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MATERIALS AND METHODS

PLANT MATERIAL

Seeds of the following diploid and allotetraploid species were obtained from botanical gardens, plant breeding stations and commercial sources: *B. nigra* cv. 1858 (genome BB; 2n = 2x = 16), *B. oleracea* var. *capitata* cv. Kamienna Glowa and var. *botrytis* (genome CC; 2n = 2x = 18), *B. campestris* var. *rapifera* cv. Goldball, var. *trilocularis* cv. K–151 and var. *pekinensis* (genome AA; 2n = 2x = 20), *B. carinata* cv. Yellow Dodolla (genome BBCC; 2n = 4x = 34), *B. juncea* cv. Malopolska and cv. Bpl 4–1 (rapid cycling) (genome AABB; 2n = 4x = 36), as well as *B. napus* var. *napus* cv. Licosmos 00 and Kana (genome AACC; 2n = 4x = 38). No differences were observed between the varieties we used in respect to the number and chromosomal distribution of 25 rDNA sites, and the appearance of GISH signals.

SLIDE PREPARATION FOR CHROMOSOME ANALYSIS

Root tip meristematic cells were used as a source of mitoses. All seeds were germinated on filter paper moistened with tap water at 20–22˚C in the dark until the roots were 1.5–2 cm long. Whole seedlings were then treated with 2 mM 8-hydroxyquinoline for 1–4 h at room temperature, fixed in a 3:1 (v/v) mixture of methanol and glacial acetic acid, and stored at -20˚C until required. Excised roots were washed in 0.01 M citric acid-sodium citrate buffer (pH ~ 4.8) for 20 min and digested enzymatically for 1–1.5 h at 37˚C in a mixture of 1% (w/v) cellulase (Calbiochem), 1% (w/v) Onozuka R–10 cellulase (Serva) and 20% (v/v) pectinase (Sigma). After separation from the non-meristematic parts, root tips were squashed in a drop of 45% acetic acid and the preparations were frozen. Cover slips were removed, and the preparations were postfixed in chilled 3:1 (v/v) ethanol:glacial acetic acid, followed by dehydration in absolute ethanol and air-drying.

DNA PROBES

The following probes were used:

(1) For genomic in situ hybridization, total nuclear DNA from *B. nigra*, *B. oleracea* and *B. campestris* was extracted from young plants using a standard procedure (Murray and Thompson, 1980). In single-color GISH experiments, sheared (boiled for 7 min at 100˚C) genomic DNA was labelled by nick translation with tetramethyl-rhodamine-dUTP; for dual-color GISH, tetramethyl-rhodamine-dUTP and digoxigenin-dUTP were used simultaneously.

(2) The 25S rDNA probe was generated by nick translation with digoxigenin-dUTP of a 2.3 kb *Cla*I subclone of the 25S rDNA coding region of *Arabidopsis thaliana* (Unfried and Gruendler, 1990). This probe was used to detect the loci of 18S–5.8S–25S rRNA genes.

(3) A 5S rDNA-specific probe was amplified and labelled by PCR with tetramethyl-rhodamine-dUTP from the wheat clone pTa794 (Gerlach and Dyer, 1980) using universal M13 forward (5'-CAG GGT TTT CCC AGT CAC GA–3') and reverse (5'-CGG ATA ACA ATT TCA CAC AGG A–3') sequencing primers. The labelling conditions were as follows: 94˚C for 1 min, 43 cycles of 94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 90 sec, and 1 cycle of 72˚C for 5 min.

(4) The telomeric probe (HT100.3) consisted of arrays of *Arabidopsis*-type telomeric repeats $(TTTAGGG)_n$ (Hajdera et al., 2003). A clone containing approximately 30 copies of the repeat was used as the template for subsequent PCR labelling reaction. Primers, the label and conditions of PCR labelling were the same as for the 5S rDNA probe.

FISH PROCEDURE

The slides were pretreated with RNase (100 μg/ml) in 2 × SSC at 37˚C for 1 h, washed in 2 × SSC and dehydrated in ethanol. For the cloned probes, the hybridization mixture consisted of 50% deionized formamide, 10% dextran sulphate, 2 × SSC, 0.5% SDS, salmon sperm blocking DNA (50–100 times the excess of labelled probe), and ~3 ng/μl (100–150 ng/slide) of each probe DNA. To reduce cross-hybridization of the genomic probes, sheared and unlabelled total nuclear DNA of the complementary genome (70 times the excess of the labelled genomic probe) was added as blocking DNA. The hybridization mixture was pre-denatured at 75˚C for 10 min and applied to the chromosome preparations. Slides and DNA probes were then denatured together at 75˚C for 5 min in an in situ thermal cycler (Hybaid) and then allowed to hybridize overnight in a humid chamber at 37˚C. After stringent washes (10–20% formamide in 0.1 × SSC at 42˚C for 10 min), immunodetection of digoxigenated probes was carried out with FITC-conjugated anti-digoxigenin antibodies (Roche). Counterstaining and mounting of dehydrated preparations was done using 2.5 g/ml DAPI in Vectashield antifade buffer.

IMAGE CAPTURING AND PROCESSING

All images were acquired using either a Hamamatsu C5810 CCD camera attached to an Olympus Provis AX epifluorescence microscope or an Olympus Camedia C-4040Z digital camera attached to a Leica DMRB epifluorescence microscope. Image processing and superimposition were done using Micrografx (Corel) Picture Publisher software.

RESULTS

DIPLOID SPECIES OF *BRASSICA*

Total nuclear DNA of *B. nigra* (B genome) hybridizes with pericentromeric regions of all chromosomes of this species (Fig. 1a, pale red fluorescence). Additionally, six chromosomes in the complement usually display a characteristic, intensively red fluorescence in the distal parts of their short arms (Fig. 1a, arrows). The use of the 25S rDNA probe (Fig. 1b, green fluorescence) suggests that these are probably the nucleolar organizer regions (NOR) of the three pairs of chromosomes in the B genome that contain clusters of 18S–5.8S–25S ribosomal RNA genes. Intensively red signals of the total genomic DNA probe can also be tracked down in *B. nigra* interphase nuclei (Fig. 1c, arrows). As would be expected, such signals reveal some topographical connections with the nucleolus/nucleoli in most cases.

Figure 1d shows the results of a similar experiment with the total nuclear DNA of the C genome hybridizing to pericentromeric regions (red fluorescence) of all chromosomes in the somatic complement of *B. oleracea*. Likewise in *B. nigra*, the genomic probe gives strong signals in the regions of *B. oleracea* chromosomes that probably carry rRNA gene sites (for comparison see: Fig. 1e, green fluorescence), although in this species the phenomenon is not so prominent in interphase nuclei (only two out of four sites unambiguously defined; Fig. 1f).

In the metaphase chromosomes of the third diploid species, *B. campestris* (Fig. 1g, red fluorescence), the hybridization pattern of the probe based on the total nuclear DNA of the A genome more resembles the pattern observed in *B. oleracea* than in *B. nigra*. Apart from rather distinct signals in the pericentromeric regions of most chromosomes in the complement, two intensively red signals of hybridization (arrows) most likely mark the satellites of the only pair of NOR-chromosomes in *B. campetris*. FISH with the 25S rDNA probe suggests that the two very intensive signals revealed by GISH indeed correspond to the only non-intercalary loci of genes encoding for 18S–5.8S–25S rRNA in the *B. campestris* chromosome complement (Fig. 1h, green fluorescence, arrows). The remaining eight sites of rDNA are distributed at pericentromeric locations of chromosomes, which may explain why they cannot be clearly discriminated from the heterochromatin by GISH signals in mitotic chromosomes (Fig. 1g) and interphase nuclei (Fig. 1i).

ALLOTETRAPLOID SPECIES OF *BRASSICA*

Brassica carinata metaphase chromosomes were subjected to genomic in situ hybridization with total nuclear DNA of the B genome (Fig. 1j) and C genome (Fig. 1k). Regardless which DNA is used as the probe for GISH, in *B. carinata* it allows fairly unambiguous discrimination of 16 chromosomes belonging to one putative ancestral species and 18 of the other parental species, although cross-hybridization of genomic probes in the regions occupied by the ribosomal genes may be disadvantageous for efficient genome discrimination. In *B. napus*, a similar situation is observed after GISH with the A genome DNA as the probe (Fig. 2a). In contrast, when the chromosomes of *B. napus* were probed with total nuclear DNA of the C genome, a significant level of probe cross-hybridization thwarted attempts at effective and reliable genome discrimination (Fig. 2b).

In *B. juncea*, as in *B. carinata*, the efficiency of genome discrimination was similar, regardless of which ancestral species total nuclear DNA was used as a probe. Because the signals differ in size, in single-target GISH we found the nuclear DNA of the A genome (Fig. 2c) slightly less effective for this purpose than B genome DNA (Fig. 2d). However, when the two total nuclear DNAs were simultaneously applied in dual-color GISH (Fig. 2e), discrimination of all 16 chromosomes of the *B. nigra* complement (red fluorescence) from the 20 chromosomes originating from the *B. campestris* complement (green fluorescence) was less ambiguous than in single-color GISH. Figure 2f shows a further step towards multicolor and multiprobe in situ hybridization experiments in brassicas. Three different probes were simultaneously applied in a combined GISH and FISH experiment on *B. juncea*. Genomic DNA of *B. nigra* (pale pink fluorescence) allowed discrimination of chromosomes belonging to the B genome from those of the A genome, while 5S (red fluorescence) and 25S (green fluorescence) ribosomal DNA probes provided landmarks for 20 out of the 36 chromosomes in the complement.

Another kind of repetitive sequence probed with the chromosomes of *Brassica* was *Arabidopsis*-type telomeric repeats (TRS). We determined their presence in all six "U-triangle" species of *Brassica*. The signals of hybridization were small, which may suggest a low copy number of TRS in *Brassica*, found exclusively at terminal locations of most arms of almost all chromosomes in the different complements. Figure 2g shows an example of the distribution of these repeats in metaphase chromosomes of *B. juncea* (red fluorescence).

DISCUSSION

EVOLUTIONARY AND TECHNICAL ASPECTS OF PROBING *BRASSICA* CHROMOSOMES WITH GENOMIC DNA

A noticeable feature of GISH in *Brassica* species is the peculiar way total nuclear DNA probes hybridize to metaphase or prometaphase chromosomes, with the signals limited almost exclusively to pericentromeric regions. This is strongly connected with the small size

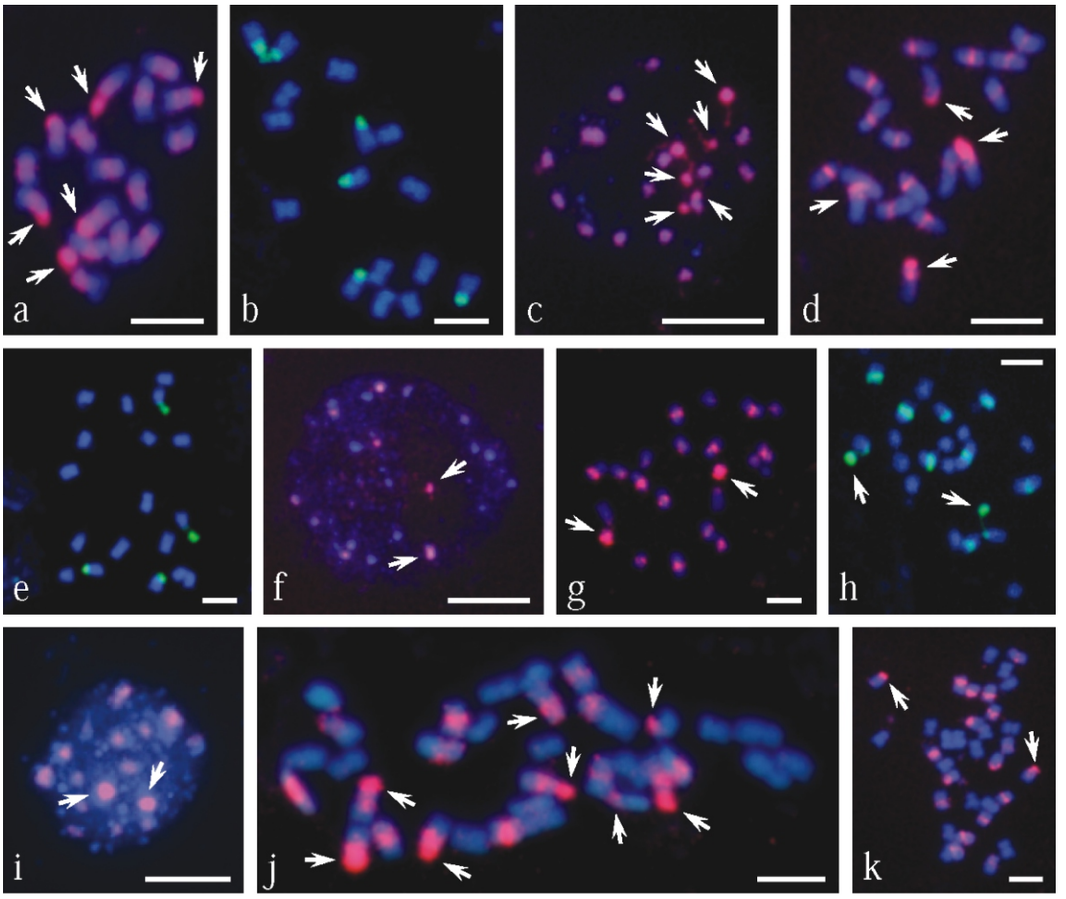

Fig. 1. (**a,c,d,f,g,i–k**) Single-target genomic in situ hybridization to somatic metaphase chromosomes (**a,d,g,j,k**) and interphase nuclei (**c,f,i**) in various species of *Brassica* (red fluorescence). **(a,c)** Genomic DNA of the B genome hybridizing to *B. nigra*, (**d,f**) Genomic DNA of the C genome probed to *B. oleracea*, (**g,i**) *B. campestris* material subjected to GISH with A genome total nuclear DNA, (**j,k**) *B. carinata* chromosomes subjected to GISH with genomic DNA of the B genome (**j**) and C genome (**k**). Arrows indicate putative localization of ribosomal DNA sites. (**b,e,h**) Single-target FISH of 25S rDNA probe (green fluorescence) to chromosomes of *B. nigra* (**b**), *B. oleracea* (**e**) and *B. campestris* (**h**). Bar = 5 µm.

of *Brassica* genomes, which contain relatively little of the moderately and repetitive DNA families, the fractions that are major if not predominant components of any total genomic DNA probe (Harrison and Heslop-Harrison, 1995; Schwarzacher and Heslop-Harrison, 2000; Shibata and Hizume, 2002). Such delimited hybridization of genomic probes has been observed not only in brassicas but also in other plant species with comparably small genomes, for example in rice (Li et al., 2001) and in another grass species, *Brachypodium distachyon* (Hasterok et al., 2004). The characteristic lack of chromosome painting along entire chromosome arms, which is so typical for GISH performed on species with larger genomes, narrows the possible application of this technique in analysis of *Brassica* chromosomes. For example, it is impracticable to use genomic probes to study tiny introgressions from one genome into another in allotetraploid brassicas, although in similar studies carried out in cereals and forage grasses, for example, GISH can be considered the method of choice (Iqbal et al., 2000; Morgan et al., 2001). Interestingly, in some plant species, for example in the genera *Lycopersicon*, *Solanum* (Gavrilenko et al., 2001) and *Musa* (D'Hont et al., 2000), despite their relatively small genome size, total nuclear DNA-based probes hybridize to entire chromosome arms. An explanation for this may be that in these species, unlike in brassicas, repetitive DNA families are distributed more evenly along the chromosomes.

A detailed comparison of different *Brassica* genomes revealed that the hybridization pattern of the B genome nuclear DNA differs from that of the

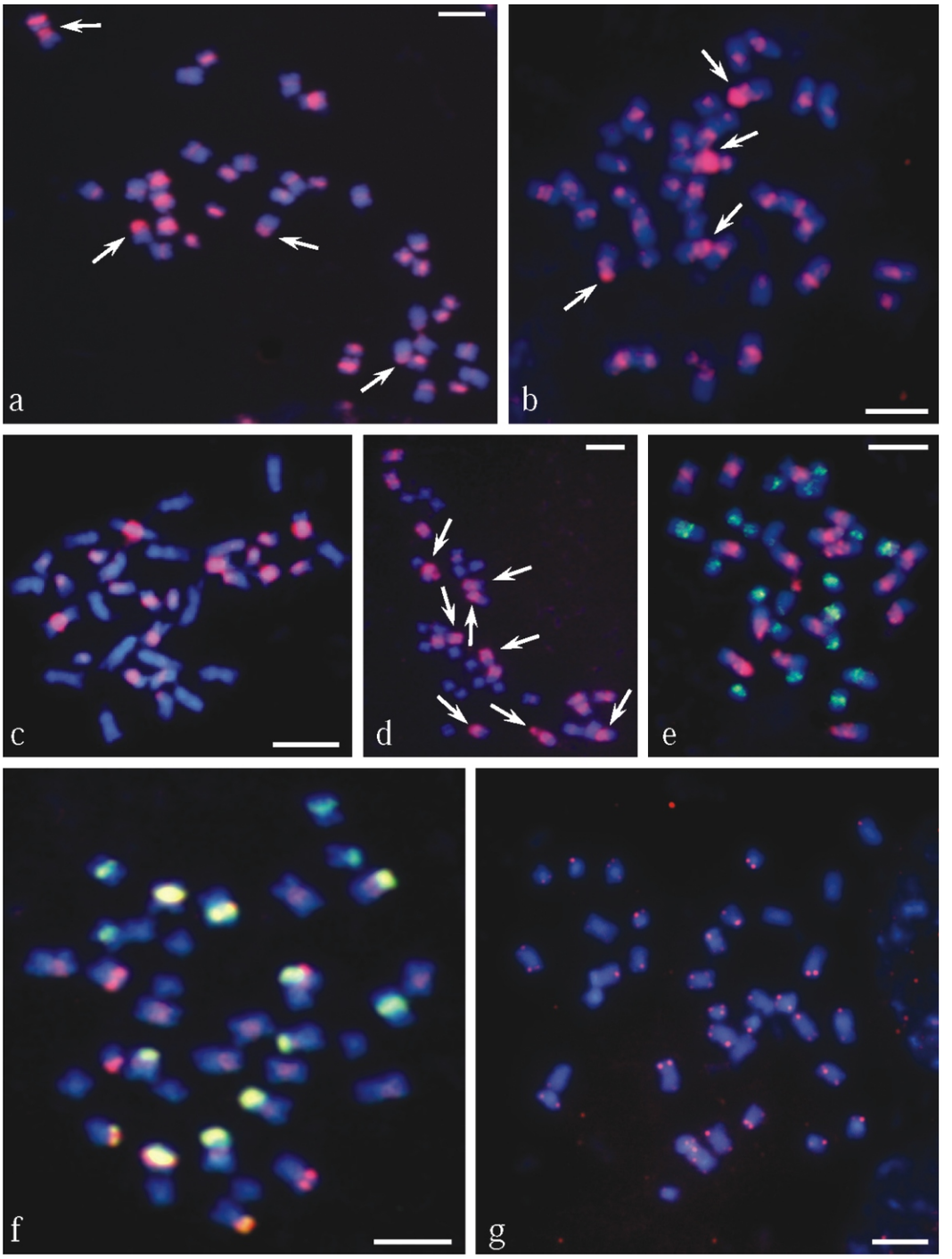

Fig. 2. (**a,b**) Genomic DNA of the A genome (**a**) and C genome (**b**) hybridizing to *B. napus* chromosomes, (**c,d**) *B. juncea* chromosomes probed with labelled nuclear DNA of the A genome (**c**) and B genome (**d**). Arrows indicate putative localization of ribosomal DNA sites. (**e**) Chromosomes of *B. juncea* probed simultaneously with nuclear DNA of the A genome (green fluorescence) and B genome (red fluorescence), (**f**) Combined multicolor GISH and FISH with B genome nuclear DNA (pale red fluorescence), 25S rDNA (green fluorescence) and 5S rDNA (red fluorescence) hybridizing to *B. juncea* chromosomes, (**g**) FISH of the *Arabidopsis*-type $(TTTAGGG)_n$ telomeric repeat sequence to *B. juncea* chromosomes. Bar = 5 µm.

A and C genomes. In the chromosomes of the B genome of *B. nigra* (Fig. 1a) and the two allotetraploids (Fig. 1j, 2d,e, red fluorescence), although the GISH signals are localized predominantly in the pericentromeric regions, clear extensions of the signals towards the intercalary parts of the arms can be seen. In contrast, in the A and C genomes (Fig. 1d,g,k, 2a–c,e, green fluorescence), the signals are restricted almost exclusively to the pericentromeric regions; this may suggest some differences in the distribution of repetitive DNA between the B genome and the A/C genomes. The results of most other cytogenetic (Harrison and Heslop-Harrison, 1995) and molecular (Song et al., 1988; Warwick and Black, 1993) studies clearly suggest that these differences are not only quantitative but also qualitative. This supports the hypothesis that the B genome was the first to diverge from the common ancestral *Brassica* genome (Quiros, 1995).

The above-mentioned phylogenetic relationships may to some extent be reflected in the technical difficulties faced when applying GISH to these species. In spite of some evolutionary divergence, the general profile of repetitive DNA is similar enough to allow the sequences to cross-hybridize. Both in our study and in some previous reports (Snowdon et al., 1997), cross-hybridization was most prevalent in *B. napus*, the species that contains the two genomes most closely related evolutionarily (A and C). In order to diminish cross-reactions, strict control of hybridization conditions, such as stringency and the application of specific blocking DNA, is required, making GISH experiments technically demanding in *Brassica* allopolyploids in general and in *B. napus* in particular. The reverse is observed in, for example, allotetraploid species of *Brachypodium* (Hasterok et al., 2004) and also some other monocotyledonous allopolyploids and interspecific hybrids, where such strict control of GISH conditions is not needed.

Another interesting aspect of genomic in situ hybridization in brassicas is the prevalence of cross-hybridization of total nuclear DNA to the ribosomal DNA loci. This phenomenon is clearly explainable by the high level of evolutionary conservation of these genes. Of course, this effect is much more evident in these species, where the genomic probes do not tend to paint whole chromosome arms evenly. In analysis of *Brassica* allotetraploid species, such cross-hybridization may be unwanted sometimes, especially if it concerns the pericentromeric rDNA loci in the A genome, because it may seriously hamper proper discrimination of the genomic origin of chromosomes. Technically, however, this effect may be difficult to eliminate because of the very high level of ribosomal DNA sequence homology. The use of sheared, unlabelled ribosomal DNA sequences as a block may at least partially solve this problem.

DISTRIBUTION OF TELOMERIC ARRAYS

The *Arabidopsis*-type telomeric repeat sequence $(TTTAGGG)_n$ motif is known to be present in many though not all species, both dicots and monocots, for example in *Secale cereale* (Schwarzacher and Heslop-Harrison, 1991), *Crepis capillaris* (Maluszynska et al., 2003) and *Lupinus* species (Hajdera et al., 2003). While in most species the telomeric repeat sequence (TRS) is distributed at the usual chromosomal locations, that is, their termini, in some species such as *Vicia faba* (Schubert, 1992) and *Arabidopsis thaliana* (Richards et al., 1992) the TRS has also been observed in intercalary chromosomal locations.

In all "U-triangle" species of *Brassica*, weak signals of TRS were present exclusively at the termini of the vast majority of chromosomes. The apparent lack of signals at the ends of some arms may indicate that the copy number at such sites was so low that the signals were below the detection level. No intercalary signals were detected, seriously limiting the use of this sequence as a marker for tracing translocations and identifying chromosomes. The latter application may be feasible to some extent, considering that not all chromosomes have detectable TRS signals. Also, as in *S. cereale* (Schwarzacher and Heslop-Harrison, 1991), the size of telomeric signals on different chromosomes seems to vary, perhaps the result of differences in the number of repeats at the telomere of each arm. However, the observed differences are too inconsistent to be safely used as reliable landmarks for chromosome identification.

TOWARDS MULTICOLOR FISH EXPERIMENTS

As stated above, the chromosomes in *Brassica* genomes are small, numerous, and both inter- and intragenomically uniform in their morphology. This makes their identification and determination of genome origin difficult, especially in the allotetraploids. There are two ways to overcome these difficulties. One is to utilize more effectively the already existing chromosome landmarks provided by genomic and ribosomal DNA probes, by combining them in multicolor FISH. Because the two classes of ribosomal genes are present in *Brassica* genomes at numerous loci and at very different chromosomal locations, even a relatively simple dual color combination of 5S rDNA and 25S rDNA probes yields many more landmarks for chromosome identification than in separate single target experiments (Hasterok and Maluszynska, 2000c; Hasterok et al., 2001). The same applies to GISH, where simultaneous use of two differentially labelled genomic probes significantly improves the quality and ease of genome origin determination (Fig. 2e). A combination of genomic and ribosomal probes in one experiment (Fig. 2f) is even more effective, extending the above by

the possibility of further classification of the chromosomes with different rDNA types. In the case of some chromosomes, this may even allow unambiguous identification of specific pairs. There are several reasons why combined GISH and FISH experiments work most effectively in *B. juncea* (Maluszynska and Hasterok, 2005), but currently we are trying to apply them to the two other allotetraploids, particularly *B. napus*.

The second way to improve *Brassica* chromosome identification is to introduce new, preferably fully chromosome-specific markers. The landmarks that can prove useful for this task are the libraries of clones based on bacterial artificial chromosomes (BACs). BAC-FISH was recently used for fully discriminative chromosome painting in *A. thaliana* (Lysak et al., 2003; Pecinka et al., 2004). Also, Howell et al. (2002) utilized BAC clones for identification of individual chromosomes and integration of cytogenetic and genetic linkage maps of *B. oleracea*, while Schelfhout et al. (2004) demonstrated the utility of a PCR-based marker for reliable discrimination of all B-genome originated chromosomes in *B. juncea*.

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