Theor Appl Genet (2002) 104:533–538

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Identifying the chromosomes of the A- and C-genome diploid *Brassica* species *B. rapa* (syn. *campestris*) and *B. oleracea* in their amphidiploid *B. napus*

Received: 13 July 2001 / Accepted: 23 August 2001

Abstract Oilseed rape (*Brassica napus* L.) is an amphidiploid species that originated from a spontaneous hybridisation of *Brassica rapa* L. (syn. *campestris*) and *Brassica oleracea* L., and contains the complete diploid chromosome sets of both parental genomes. The metaphase chromosomes of the highly homoeologous A genome of *B. rapa* and the C genome of *B. oleracea* cannot be reliably distinguished in *B. napus* because of their morphological similarity. Fluorescence in situ hybridisation (FISH) with 5S and 25S ribosomal DNA probes to prometaphase chromosomes, in combination with DAPI staining, allows more dependable identification of *Brassica* chromosomes. By comparing rDNA hybridisation and DAPI staining patterns from *B. rapa* and *B. oleracea* prometaphase chromosomes with those from *B. napus*, we were able to identify the putative homologues of *B. napus* chromosomes in the diploid chromosome sets of *B. rapa* and *B. oleracea*, respectively. In some cases, differences were observed between the rDNA hybridisation patterns of chromosomes in the diploid species and their putative homologue in *B. napus*, indicating locus losses or alterations in rDNA copy number. The ability to reliably identify A and C genome chromosomes in *B. napus* is discussed with respect to evolutionary and breeding aspects.

Keywords *Brassica* · Chromosome · Karyotype · rDNA · Fluorescence in situ hybridisation

Introduction

The genetics of oilseed rape (*Brassica napus* L. 2n=38, genome AACC), now one of the most important oilseed crops worldwide, are complex because it is an amphidiploid composed of two related genomes which themselves are thought to represent ancestral hexaploids (Lagercrantz and Lydiate 1996; Lagercrantz et al. 1996; see also Truco et al. 1996; Quiros 1999). *B. napus* originated from a spontaneous hybridisation of *Brassica rapa* L. (syn. *campestris*; AA, 2n=20) and *Brassica oleracea* L. (CC, 2n=18) and contains the entire diploid chromosome sets of both parental genomes. The chromosomes of the highly homoeologous A genome of *B. rapa* and the C genome of *B. oleracea*, which diverged relatively recently from a common ancestor (Lagercrantz and Lydiate 1996), are difficult to reliably distinguish in *B. napus* because of their small size and morphological similarity (Röbbelen 1960; Olin-Fatih and Heneen 1992). Fluorescence in situ hybridisation (FISH) with ribosomal DNA probes, however, considerably improves the identification of *Brassica* chromosomes, particularly in prometaphase. FISH with 25S ribosomal DNA probes has been used to assist chromosome identification in the three diploid *Brassica* species *B. rapa*, *Brassica nigra* (BB, 2n=16) and *B. oleracea* (Fukui et al. 1998), and to speculate on the evolution of rDNA loci in modern-day *Brassica* amphidiploids (Maluszynska and Heslop-Harrison 1993; Snowdon et al. 1997a; Hasterok and Maluszynska 2000). Moreover, multicolour FISH with 25S and 5S rDNA probes has been applied to reliably identify a number of chromosomes in *B. oleracea* (Armstrong et al. 1998) and *B. napus* (Kamisugi et al. 1998; Schrader et al. 2000; Snowdon et al. 2000). Despite this, the association of individual *B. napus* chromosomes with their homologues in *B. rapa* and *B. oleracea* remains problematic to-date.

Parkin et al. (1995) and Sharpe et al. (1995) demonstrated that the molecular linkage groups of *B. rapa* and *B. oleracea* could be identified in *B. napus* with only relatively minor reorganisation. Most linkage groups were more or less intact, indicating that recombination between A- and C-genome chromosomes has not been extensive in the evolution of modern *B. napus* (Parkin and Lydiate 1997). In a similar study, Axelsson et al. (2000) by com-

Communicated by C. Möllers

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parative mapping of re-synthesised and natural *Brassica juncea* demonstrated that very little recombination has occurred between A- and B-genome chromosomes during the evolution of the AABB-genome *Brassica* amphidiploid. This could also be effectively demonstrated by the fact that genomic in situ hybridisation (GISH) allowed the respective A- and B-genome chromosome sets to be clearly distinguished in *B. juncea* (Snowdon et al. 1997b). The same was true for the B and C genomes of *Brassica carinata*, but the A and C genomes of *B. napus*, on the other hand, were too highly homoeologous to label specifically by GISH (Snowdon et al. 1997b). Chromatin condensation patterns have been shown to differ in *B. rapa* and *B. oleracea* prometaphase chromosomes (Cheng et al. 1995; Chen et al. 1997), and using image-analysis equipment Kamisugi et al. (1998) were able to divide *B. napus* chromosomes into those with *rapa*-type and those with *oleracea*-type chromatin condensation. A direct association of *B. rapa* and *B. oleracea* chromosomes with their homologues in *B. napus* was however not made.

Besides providing information on chromosome and genome evolution, the ability to reliably identify A- and C-genome chromosomes is important for the genomic location of transgene inserts in genetically transformed oilseed rape. The well-documented ability of *B. napus* to outcross with *B. rapa* poses a risk for transgene spread from transgenic oilseed rape to wild *B. rapa* populations (see Mikkelsen et al. 1996a). On the other hand, it has long been known that sexual crosses between *B. napus* and *B. oleracea* are extremely difficult to generate with *B. napus* as the pollen donor (Roemer 1935); hence the probability of transgene spread from *B. napus* to the *B. oleracea* genome is by comparison very low. On this basis it has been suggested that transgenes introgressed into the C genome of *B. napus* would be considerably less likely to be introgressed into wild *Brassica* populations than those present on A-genome chromosomes, because *B. napus*×*B. oleracea* hybrids are extremely rare and transgene-carrying C-genome chromosomes will be eliminated from wild *B. napus*×*B. rapa* backcross offspring due to the absence of homologues (Mikkelsen 1996b; Metz et al. 1997; but see also Tomiuk et al. 2000). In combination with the methods described here, localisation of introgressed transgenes by FISH (see Moscone et al. 1996; Ten Hoopen et al. 1999; Snowdon et al. 2001) will assist in the selection of *B. napus* plants with transgene inserts on C-genome chromosomes.

In this study we compared 5S/25S rDNA hybridisation and DAPI staining patterns of *B. rapa* and *B. oleracea* chromosomes with those from *B. napus*, in order to match the A- and C-genome chromosomes of the amphidiploid *B. napus* with their putative homologues in the diploid species.

Materials and methods

Plant material

Seeds from *B. napus* L. subsp. *oleifera* (winter oilseed rape cv "Lirajet"), *B. rapa* L. subsp. *trilocularis* (yellow sarson, accession number YSPb-24) and *B. oleracea* L. var. *capitata* (white cabbage, cv "Braunschweiger") were germinated on moist filter paper until the primary roots were 2–3 cm long. Whole seedlings were treated for 2 h at room temperature and 2 h at 4°C in 2 mM of 8-hydroxyquinoline to accumulate metaphases, then fixed in ethanol-acetic acid fixative (3:1). Root tips were excised and chromosome spreads were made from protoplast suspensions using a method described previously (Snowdon et al. 2000, adapted from Schwarzacher et al. 1994).

Fluorescence in situ hybridisation (FISH)

The 25S rDNA subclone pAt27 from *Arabidopsis thaliana* and a 5S rDNA subunit from *Beta vulgaris* were directly labelled by nick translation with the fluorochromes Cy3 and fluorescein-isothiocyanate (FITC), respectively. FISH methods followed Snowdon et al. (2000). After hybridisation, slides were washed at 42°C for 5 min in 2×SSC and 10 min in 0.2×SSC. Chromosomes were counter-stained with DAPI and fluorescence was visualised using a Leica DM-R microscope. At least five to ten well-spread prometaphases were observed for each species and images were obtained using a Cohu 4912 uncooled CCD camera and Leica QFISH software. Individual chromosomes were dissected from the FISH images and karyotypes were constructed using the Adobe Photoshop.

Results

Based on 5S and 25S rDNA hybridisation patterns with DAPI counterstaining, the prometaphase chromosomes of *B. rapa*, *B. oleracea* and *B. napus*, respectively, could be identified and paired. Figure 1 shows the chromosome sets of the three species, aligned according to their FISH and DAPI staining patterns, chromosome size and morphology. The chromosomes of *B. rapa* and *B. oleracea* were numbered according to Fukui et al. (1998), whose chromosome assignment for *B. oleracea* matches that of Armstrong et al. (1998) with the exception of chromosomes 2 and 4 which are exchanged. In accordance with Armstrong (1998) the chromosome with the larger 25S rDNA locus was designated chromosome 7. Chromosome-arm length ratios were not calculated here, because the intense DAPI staining at *Brassica* centromeres only permitted the approximate localisation of the centromere.

In *B. rapa*, four 25S rDNA loci were observed near the centromeres of the metacentric chromosomes 1, 4, 5 and 7. Chromosome 1 could be easily differentiated from chromosomes 4 and 5 by size, whereas the large 25S locus on chromosome 4 was readily distinguishable from the small locus on chromosome 5. The NOR-bearing chromosome 2 contains the fifth largest 25S rDNA locus, which extended over the NOR and chromosome satellite. The large 25S locus located interstitially on chromosome 7 co-localised with a large 5S rDNA locus, giving a strong double-hybridisation signal that allowed this chromosome to be readily identified. Two further 5S loci were localised on the short arms of chromosomes 2 and 10, the largest and the smallest acrocentric *B. rapa* chromosomes, respectively.

As described previously by Armstrong et al. (1998), the *B. oleracea* 5S rRNA genes were located in two

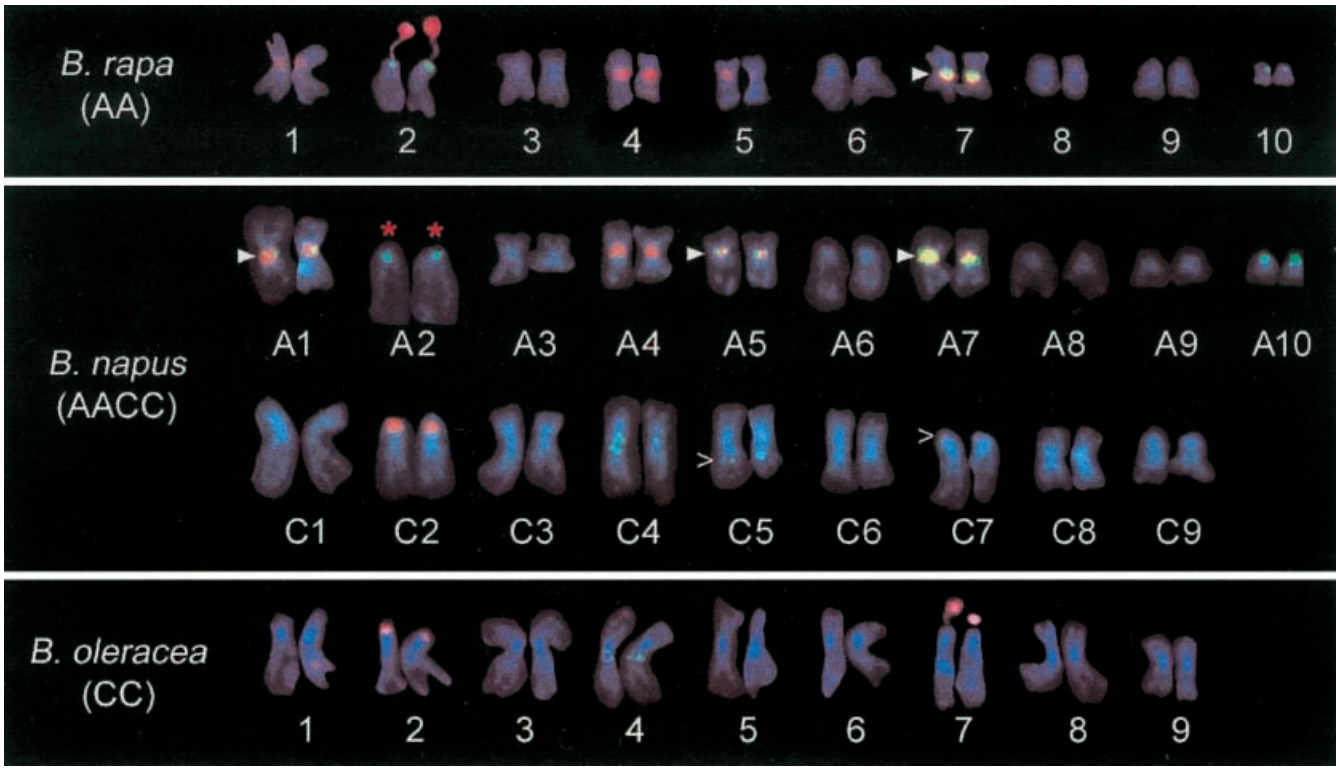

Fig. 1 Karyotypes based on fluorescence in situ hybridisation patterns with 5S (*green*) and 25S (*red*) rDNA probes and DAPI staining (*blue*), for *B. rapa* L., *B. oleracea* L. and their amphidiploid *B. napus* L. Closed arrowheads indicate co-localisation of 5S and 25S loci, whereas *open arrowheads* show a weak 5S locus and a weak 25S locus on *B. napus* chromosomes C5 and C7, respectively. The *red asterisks* represent the position of a large 25S rDNA locus located on the satellite of *B. napus* chromosome A2, which in this spread was lost during chromosome preparation. The *B. napus* karyotype is divided into two sets of chromosomes with differing chromatin condensation patterns resembling, respectively, those of *B. rapa* (A) and *B. oleracea* (C). Each *B. napus* chromosome is aligned and numbered in accordance with its putative homologue in the *B. rapa* or *B. oleracea* genome

closely adjacent loci on the long arm of a single large submetacentric chromosome, designated here and by Fukui (1998) as chromosome 4. The two acrocentric, satellite-carrying *B. oleracea* chromosomes (2 and 7) both had 25S loci at the terminal ends of their short arms and extending over the satellite.

The chromosomal positions of rDNA loci in *B. napus* have been described previously (see Kamisugi et al. 1998; Snowdon et al. 2000) and the *B. napus* karyotype in Fig. 1 derives from the chromosome spread shown by Snowdon et al. (2000). In the present study, however, the large 25S locus covering the chromosome satellite of *B. rapa* chromosome 2 led us to investigate the number of 25S loci in *B. napus* in more detail, because it appeared that this locus was absent from the corresponding chromosome in *B. napus*. After observation of a large number of metaphases it was revealed that chromosome A2 in fact also possesses a chromosome satellite with a large 25S locus adjacent to the 5S locus, corresponding to *B. rapa* chromosome 2. In prometaphase, however, this satellite can be highly distended (Maluszynska and Heslop-Harrison 1993) or alternatively completely lost during chromosome preparation. This was the case for *B. napus* chromosome A2 in the karyotype shown, hence the location of this 25S locus has been indicated by asterisks in Fig. 1.

When the chromosome sets from the diploid species *B. rapa* (A genome) and *B. oleracea* (C genome) were compared to those from *B. napus*, the AACC amphidiploid, chromosomes could be identified in *B. napus* which closely resembled all rDNA-carrying chromosomes from the two diploid species. Chromosomes not containing rDNA loci could also be matched to their putative homologues due to similarities in size, centromere position and DAPI staining pattern. In prometaphase, different chromatin condensation patterns allow *B. rapa* chromosomes to be distinguished from *B. oleracea* chromosomes (Cheng et al. 1995; Chen et al. 1997). Hence, the *B. napus* karyotype could be clearly divided into ten A-genome (*B. rapa*) and nine C-genome (*B. oleracea*) chromosomes, as described previously by Kamisugi et al. (1998). Moreover, based on rDNA hybridisation and DAPI staining patterns, the chromosomes of *B. napus* could be aligned with their putative homologues in the respective diploid species (Fig. 1), and were designated A1–A10 and C1–C9 to reflect their putative origin from either *B. rapa* or *B. oleracea*, respectively.

Minor differences were observed between the rDNA hybridisation patterns of some of the presumed homologues, with respect to signal strengths (indicating differences in copy number) or absence of loci. Details of these discrepancies are listed in Table 1.

Table 1 Differences observed in 5S and 25S rDNA occurrence and signal strength between chromosomes of *B. napus* (genome AACC) and their putative homologues in *B. rapa* (AA) and *B. oleracea* (CC)

rDNA locus	*B. napus* (genome AACC)		Diploid genome homologue	
	Chromosome	FISH signal strength	*B. rapa* (AA)	*B. oleracea* (CC)
5S	A1	Weak	Absent	
5S	A5	Low	Absent	
5S	A10	Strong	Weak	
5S	C5	Low		Absent
25S	A1	Strong	Weak	
25S	C7	Weak		Strong

Discussion

Metaphase chromosomes from *Brassica* species are extremely difficult to reliably identify in standard cytological preparations because they are very small and normally highly condensed. They can be more-readily identified in prometaphase, but because heterochromatin is located almost solely in the pericentromeric regions of *Brassica* chromosomes few useful cytogenetic landmarks are available using classical banding techniques (Olin-Fatih and Heneen 1992). Because of this, the ability to recognise chromosomes of the highly homoeologous diploid genomes of *B. rapa* and *B. oleracea* in their amphidiploid *B. napus* has remained elusive to-date.

Various comparative mapping studies (e.g. Parkin et al. 1995; Sharpe et al. 1995; Parkin and Lydiate 1997) have demonstrated that no more than minor recombination has occurred between the A- and C-genome linkage groups since the spontaneous origin of *B. napus*. This implies that the diploid karyotypes should be largely intact in the amphidiploid, as has been found by GISH to be the case in *B. juncea* and *B. carinata*, the amphidiploids that contain the *Brassica* B genome (Snowdon et al. 1997b). The high level of homoeology between the A- and C-genomes prevents their differentiation by GISH, however (Snowdon et al. 1997b), and might be expected to promote intergenomic recombination. Here we provide molecular cytogenetic evidence that the physical karyotypes of *B. rapa* and *B. oleracea* are largely intact in *B. napus*.

In this study we were able to identify and distinguish the A- and C-genome chromosomes in *B. napus* by multicolor FISH with rDNA probes in combination with DAPI staining. The resulting karyotype for *B. napus* closely resembles that produced by Kamisugi et al. (1998), whereby the inclusion of 5S rDNA loci aided considerably in comparing *B. napus* chromosomes to those of *B. rapa* and *B. oleracea*. Schrader et al. (2000) note that such a comparison should preferably be performed in meiotic prophase or mitotic prometaphase, where a higher resolution of FISH signals is possible than in *Brassica* mitotic metaphase. This was confirmed in the present study, where mitotic prometaphase chromosomes gave clear, reproducible hybridisation signals that could be used effectively for chromosome identification and interspecies comparisons. All rDNA-carrying chromosomes in the diploid species had a characteristic 5S and/or 25S rDNA hybridisation pattern closely resembling a putative homologue with a similar centromere position and chromosome size in the amphidiploid. The remaining chromosomes could be identified by the DAPI staining pattern, chromosome size and morphology, and could be allocated to either the A or C genome due to differences in DAPI staining patterns caused by differential chromatin condensation (Cheng et al. 1995; Chen et al. 1997). Most chromosomes not containing rDNA could also be matched to a putative homologue in *B. napus* with a similar DAPI staining pattern and chromosome morphology.

In three cases *B. napus* possessed a 5S rDNA locus that was not present on the putative homologues in the diploid species, and strong variation in signal strength was apparent for a further 5S locus and two 25S loci (see Table 1). Losses or additions of rDNA loci and alterations in copy number are quite common in polyploid plant genomes (e.g. Dubcovsky and Dvorak 1995; Osuji et al. 1998; Taketa et al. 1999), and have been reported previously in *Brassica* amphidiploids (Maluszynska and Heslop-Harrison 1993; Snowdon et al. 1997a). In *Brassica*, differences are often observed between the number of 25S rDNA loci in different subspecies or varieties of the same species. For example, a third, interstitial 25 S locus is present in *B. oleracea* var. *alboglabra* (Armstrong et al. 1998) and various studies report the presence of either six (Malusynska and Heslop-Harrison 1993; Schrader et al. 2000) or seven (e.g. Kamisugi et al. 1998) loci in *B. napus*. The latter may arise from differences in hybridisation conditions in different laboratories, which can affect the resolution of small loci in particular. In any case, the *B. rapa* and *B. oleracea* varieties investigated in the present study do not represent the original parental genotypes of modern oilseed rape, hence minor discrepancies in rDNA locus and copy number are not unexpected. On the other hand, it was shown that on the whole the *B. rapa* and *B. oleracea* chromosome sets are largely conserved in *B. napus*, indicating that the overall karyotype structures of the respective A and C genomes have not changed excessively during the course of *B. napus* evolution.

Comparative analysis of the *Brassica* A, B and C genomes has unveiled a remarkably high degree of intergenomic similarity and intragenome duplication, sug-

gesting that the three genomes each possess full but rearranged copies of what is now thought to be a hexaploid ancestral genome (Lagercrantz and Lydiate 1996). Through chromosome fusions and rearrangements, the genome of the *Brassica* predecessor has been reorganised into 8, 9 and 10 chromosomes in *B. nigra*, *B. oleracea* and *B. rapa*, respectively. The results presented here, and by Parkin et al. (1995), suggest that, despite their high homoeology, the evolutionary reorganisation of the respective diploid genomes has been sufficiently extensive and divergent to result in physically distinct chromosome sets that have kept intergenomic recombination to a minimum, not only in *B. carinata* (Snowdon et al. 1997b) and *B. juncea* (Snowdon et al. 1997b; Axelsson et al. 2000) but also between the A and C genomes in *B. napus*. The distinct A- and C-genome chromosomes *in B. napus* mean that the narrow genetic variability present in *B. napus* breeding material can be expanded by interspecific crosses with *B. rapa* and *B. oleracea*, which can be valuable sources for resistance (e.g. Crouch et al. 1994; Scholze and Hammer 1998) or to increase heterotic potential (Seyis et al. 2001).

Although the presence of rDNA loci as physical markers provided convincing evidence for matching chromosome homologues between species, the identification of homologues in the absence of hybridisation signals still needs to be viewed with some caution. For example, *B. rapa* chromosomes 8 and 9 were distinguished by the presence of a DAPI-stained region at the distal end of the long arm of chromosome 8. Chromosomes A8 and A9 in *B. napus*, on the other hand, show extremely similar DAPI staining and could be distinguished only by the larger long arm of A8. Therefore, further markers will be necessary before A8 and A9 can be confirmed as the homologues of *B. rapa* chromosomes 8 and 9, respectively. Similarly, the absence of markers on six *B. oleracea* chromosomes means that the identification of their putative homologues in the *B. napus* C genome can presently only be based on similarities in chromosome morphology. In order to generate physical markers to positively identify all *B. oleracea* chromosomes, Armstrong et al. (2001) are developing FISH probes derived from anchored BAC clones with which the physical karyotype of *B. oleracea* can be aligned with its molecular-marker linkage groups. Such markers should also enable confirmation of *B. oleracea* chromosome homologues in *B. napus*.

As further cytogenetic markers become available, the accuracy of chromosome identification will increase and it should also become possible to physically identify regions of interest on specific chromosomes, delivering much more detailed information about A- and C-genome chromosome structure. This will enable the accurate designation of transgene introgressions to A- or C-genome chromosomes or specific chromosomal regions, and will also bring benefits for studies of *Brassica* genome evolution and for physical mapping.

Acknowledgements The authors acknowledge the helpful comments on this manuscript from two anonymous reviewers. The work presented here was funded by a grant from the Deutsche Forschungsgemeinschaft.

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