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ORIGINAL PAPER

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Molecular cytogenetic analysis of *Brassica rapa-Brassica oleracea* var. *alboglabra* monosomic addition lines

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Abstract Interspecific alien chromosome addition lines can be very useful for gene mapping and studying chromosome homoeology between closely related species. In this study we demonstrate a simple but robust manner of identifying individual C-genome chromosomes (C5, C8 and C9) in the A-genome background through the simultaneous use of 5S and 25S ribosomal probes on mitotic and meiotic chromosomes of three different *Brassica rapa-B. oleracea* var. *alboglabra* monosomic addition lines. Sequential silver staining and fluorescence in situ hybridisation indicated that 18S-5.8S-25S rRNA genes on the additional chromosome C9 are expressed in the A-genome background. Meiotic behaviour of the additional chromosomes was studied in pollen mother cells at diakinesis and metaphase I. In all of the addition lines the alien chromosome was most frequently observed as a univalent. The alien chromosome C5, which carries an intercalary 5S rDNA locus, occasionally formed trivalents that involved either rDNA- or non rDNA-carrying chromosomes from the A genome. In the case of chromosomes C8 and C9, the most frequently observed intergenomic associations involved the regions occupied by 18S-5.8S-25S ribosomal RNA genes. It is possible that not all such associations represent true pairing but are remnants of nucleolar associations from the preceding interphase. Variations in the numbers and distribution of 5S and 25S rDNA sites between cultivars of *B. oleracea*, *B. oleracea* var. *alboglabra* and *B. rapa* are discussed.

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Introduction

Six closely related species of the genus *Brassica* [*B. nigra* (L.) Koch., genome BB, $2n = 2x = 16$; *B. oleracea* L., genome CC, $2n = 2x = 18$; *B. rapa* L., genome AA, $2n = 2x = 20$; *B. carinata* A. Br., genomes BBCC, $2n = 4x = 34$; *B. juncea* (L.) Czern., genomes AABB, $2n = 4x = 36$; *B. napus* L., genomes AACC, $2n = 4x = 38$] (U 1935) are of worldwide agronomical importance. Not surprisingly, therefore, these species have received considerable attention within the framework of crop improvement since the early twentieth century (Prakash and Hinata 1980). Recently, *Brassica* species have been the subject of extensive cytogenetic and molecular analyses (Lagercrantz 1998; Jackson et al. 2000; Lan et al. 2000; Howell et al. 2002; Parkin et al. 2002; Wang et al. 2002; Ziolkowski and Sadowski 2002; Lukens et al. 2003; Osborn et al. 2003; Maluszynska and Hasterok 2005). The main goal of these studies was to understand better, at both the chromosomal and molecular levels, the evolutionary origin and the intergenomic relationships within the genus, as well as between this genus and its close allies, with special emphasis on *Arabidopsis thaliana*. Such an understanding is especially important when assessing the potential of intergenomic gene transfer, which can be exploited both by conventional plant breeding and through biotechnology.

Most of the above approaches often rely upon accurate chromosome identification and karyotypical analyses. However, the chromosomes of *Brassica* are inordinately small and morphologically uniform both within and between genomes, making their identification

and subsequent karyotype construction difficult by conventional cytological means, such as chromosome length, arm ratio and simple cytological methods of chromatin staining (Olin-Fatih and Heneen 1992; Cheng et al. 1995a). Under such circumstances, fluorescence in situ hybridisation (FISH) is the method of choice for the Brassicas, as it offers a wide range of possibilities for chromosome and genome analyses and identification. For example, to date, FISH has been successfully used in this genus to analyse the origin of some centromeric repeat families (Harrison and Heslop-Harrison 1995; Koo et al. 2004; Schelfhout et al. 2004). The probes based on total genomic DNA (genomic in situ hybridisation, GISH) have been used to study both the chromosomal distribution of repetitive DNA sequences and to identify and determine the origin of the chromosomes belonging to the constituent genomes of the allopolyploid species of *Brassica* (Snowdon et al. 1997a). Recent FISH experiments that have exploited BAC (bacterial artificial chromosome) clones on low-resolution substrates (i.e. somatic metaphase chromosomes) and high-resolution substrates (such as meiotic pachytene spreads and extended DNA fibres), have resulted in significant progress being made with respect to the identification of individual *Brassica* chromosomes, the integration of genetic linkage and physical maps and comparative physical mapping of different *Brassica* species and *A. thaliana* (Jackson et al. 2000; Howell et al. 2002; Ziolkowski and Sadowski 2002). The less condensed pachytene chromosomes enabled an accurate determination of the number and position of the FISH signals (Armstrong et al. 1998; Howell et al. 2002; Ziolkowski and Sadowski 2002; Koo et al. 2004).

The two types of rRNA genes—45S rDNA encoding for 18S-5.8S-25S ribosomal RNAs and 5S rDNA—possess common features, such as high copy number and tandem repeat arrangement at one or several chromosomal loci, which make them very useful as chromosome markers in FISH experiments. Such studies have already been performed on diverse plant species (Maluszynska 2002). Since the early 1990s, the 45S ribosomal probe has been exploited intensively to study *Brassica* chromosomes, providing a chromosomal landmark with various degrees of reliability (Maluszynska and Heslop-Harrison 1993; Snowdon et al. 1997b; Fukui et al. 1998; Hasterok and Maluszynska 2000a, b, c). However, the application of a combination of both the 25S/45S and 5S ribosomal probes to somatic metaphase chromosomes of the six "U-Triangle" species of *Brassica* (U 1935) yielded an unexpectedly high number of rDNA loci, thereby providing new chromosomal landmarks for a substantial number of *Brassica* chromosomes (Snowdon et al. 2000) and enabling the discrimination of eight morphological types of rDNA-bearing chromosomes (Hasterok et al. 2001). Further studies, which combined the above either with a morphometric analysis of mitotic and pachytene chromosomes (Kulak et al. 2002; Koo et al. 2004) or with the simultaneous multicolour use of both rDNA and genomic probes (FISH and GISH) (Maluszynska and Hasterok 2005) have provided many additional chromosome- and/or genome specific landmarks.

In the study reported here, we demonstrate the potential of double target FISH with the ribosomal probes for the detection of alien chromosomes at mitosis and the analysis of their behaviour during meiosis in three different *B. rapa-B. oleracea* var. *alboglabra* monosomic addition lines (MALs). Eight of the possible nine MALs have been developed (Heneen and Jørgensen 2001). The MALs and their parental species have been useful in studies on isozyme, molecular and cytological characterisation of genomes and chromosomes, intergenomic chromosome homoeology and introgression and the genetic control of seed and flower colour (Chen et al. 1989, 1992, 1997a, b; Cheng et al. 1994a, b, 1995a, b; Jørgensen et al. 1996; Heneen et al. 1995; Heneen and Brismar 2001; Heneen and Jørgensen 2001). Of relevance in this context is the study of Nozaki et al. (2000) on the transmission of *B. oleracea* var. *alboglabra* synteny groups in the genetic background of *B. rapa*. Addition lines in other materials as well have proved to be very useful as models for studying sequence homoeology using DNA markers (Qi et al. 1997; Serizawa et al. 2001) and for identifying linkage groups in order to facilitate the transfer of agronomically important traits between species (Gao et al. 2001; Ji and Chetelat 2003).

Materials and methods

Plant material

The *Brassica rapa-B. oleracea* var. *alboglabra* MALs ($2n = 2x = 20 + 1$; AA + 1C) were developed from a resynthesised *B. napus* L. accession, No. 7406, obtained from a cross between black-seeded and white-flowered *B. oleracea* var. *alboglabra* (Bail.) Sun No. 4003 and yellow-seeded and yellow-flowered *B. rapa* L. K-151 following backcrossing to the *B. rapa* parent (Chen et al. 1988; Heneen and Jørgensen 2001). The MALs used in this study were carriers of *B. oleracea* var. *alboglabra* chromosomes C5, C8 and C9, according to the nomenclature of Cheng et al. (1995a). These three lines were chosen following analysis of the eight available lines using FISH with 5S and 25S rDNA probes (R. Hasterok, unpublished data) and the finding or verification that they contain ribosomal DNA sites on the alien chromosome. The MAL 5 was developed (W. K. Heneen, unpublished data) from the *B. rapa–B. oleracea* var. *alboglabra* aneuploid material studied by Heneen and Jørgensen (2001), in which this alien chromosome was referred to as chromosome G. The MALs 8 and 9 were developed by Chen et al. (1997a). That C8 and C9 contain rDNA sites has been also shown in an earlier study (Cheng et al. 1995b).

Root meristem and anther cell preparations

Seeds of the parental species and the three MALs were germinated on filter paper moistened with tap water at 20–22°C in the dark for 3–5 days. Whole seedlings with 1- to 2-cm-long roots were immersed in 2 mM 8-hydroxyquinoline for 1–2 h at room temperature, fixed in 3:1 (v/v) methanol:glacial acetic acid and stored at −20°C. After several washes in 0.01 M citric acid-sodium citrate buffer (pH 4.6–4.8), the excised roots were subjected to enzymatic digestion in a mixture comprising 20% (v/v) pectinase (Sigma, St. Louis, Mo.), 1% (w/v) cellulase (Calbiochem, San Diego, Calif.) and 1% cellulase 'Onozuka R-10' (Serva, Heidelberg) for 90–120 min at 37°C. Meristems were dissected out from root tips and then squashed in a drop of 45% acetic acid. After freezing, coverslips were removed, preparations were postfixed in 3:1 ethanol:glacial acetic acid and finally dehydrated in absolute ethanol and air dried.

Both the MALs and plants of the parental species were grown in a glasshouse. For meiotic analysis, immature inflorescences (flower buds) of different size, collected from three (MAL 5 and MAL 8) or two (MAL 9) individual plants, were fixed in 3:1 ethanol:glacial acetic acid and stored at −20°C until required. For FISH analysis, individual anthers were dissected from the buds and washed in 0.01 M citric acid-sodium citrate buffer (pH 4.6–4.8) for 20 min before enzymatic digestion for 2.5–3 h in a mixture comprising 10% (v/v) pectinase (Sigma), 0.65% (w/v) cellulase 'Onozuka R-10' (Serva), 0.5% (w/v) cellulase (Calbiochem), 0.15% (w/v) cytohelicase (Sigma) and 0.15% (w/v) pectolyase (Sigma) in 10 mM citric buffer (pH 4.6–4.8) followed by washes of anthers in citric buffer. The anthers were then transferred onto a slide and gently homogenised in a drop of 45% acetic acid. The subsequent stages of the procedure were the same as for somatic chromosome preparations. In the FISH experiments, chromosome pairing was observed in pollen mother cells (PMCs) at diakinesis or metaphase I, which were the most suitable phases for chromosome behaviour analysis, both in the parental species and in the MALs. Three preparations of each individual plant were subjected to FISH. The total number of PMCs analysed was approximately 800 for MAL 5 and MAL 8, and 400 for MAL 9.

DNA probes and FISH

Two probes were used in this study:

1. The 5S rDNA probe, wheat clone pTa794 (Gerlach and Dyer 1980), was amplified and labelled with tetramethyl-rhodamine-5-dUTP (Roche, Indianapolis, Ind.) using PCR.
2. The 25S rDNA was prepared by nick translation of a 2.3-kb *Cla*I sub-clone of the 25S rDNA coding region of *A. thaliana* (Unfried and Gruendler 1990) and labelled with digoxigenin-11-dUTP (Roche). This probe was used to detect the loci of 18S-5.8S-25S rRNA genes (45S rDNA).

The general conditions of the FISH procedure were as follows. The hybridisation mixture consisted of 50% deionised formamide, 2× SSC and salmon sperm blocking DNA in 50–100× excess of the labelled probes. Each probe DNA was mixed to a concentration of 2.5–3.0 ng/μl. Chromosome preparations and the denatured (80°C for 10 min) hybridisation mixture were denatured together (70°C for 4.5 min) and allowed to hybridise overnight in a humid chamber at 37°C. The post-hybridisation washes were carried out for 10 min in 20% deionised formamide in 0.1× SSC at 42°C (which is an equivalent of 85% of stringency). Immunodetection of the digoxygenated probe was done according to standard protocol using FITC-conjugated anti-digoxigenin antibodies (Roche). Both the above DNA labelling and FISH procedures have been described in detail in Hasterok et al. (2002).

Silver staining

Silver staining was carried out on somatic chromosome preparations of MAL 9 that were made the same way as those for FISH. The staining was done according to the method of Hizume et al. (1980) with some modifications (Hasterok and Maluszynska 2000a). Prior to use for FISH, the silver-stained slides were de-stained using the method described in Hasterok and Maluszynska (2000d).

Image capturing and processing

All fluorescent and silver staining images were acquired either using a Hamamatsu C5810 CCD camera attached to an Olympus Provis AX microscope or with an Olympus Camedia C-4040Z digital camera attached to a Leica DMRB microscope, and then processed uniformly using Micrographx (Corel) Picture Publisher software.

Results

The parental species

As expected on the basis of our previous studies, the ribosomal probes provided chromosome- and/or genome-specific markers for *B. oleracea* var. *alboglabra* and *B. rapa*. These markers comprise different chromosomal types (Fig. 1a) that have been introduced and described in detail in Hasterok et al. (2001). The 18 somatic chromosomes of *B. oleracea* var. *alboglabra* No. 4003 are shown in Fig. 1b. One pair presumably bears the secondary constriction with a large 25S rDNA locus in

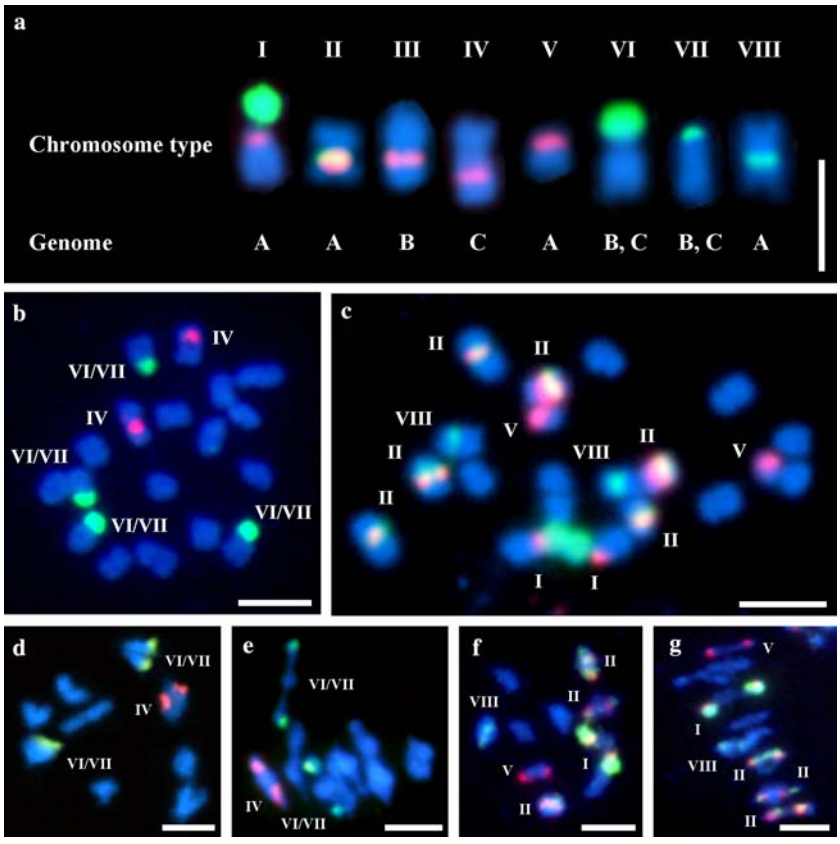

Fig. 1 (**a**) The eight types of mitotic chromosomes bearing 5S rDNA (*red*) and 25S rDNA (*green*) of the three genomes present in the six "U-Triangle" species of *Brassica* originally presented in Hasterok et al. (2001). The chromosomes were extracted from Fig. 2a–c, except for type III, which was extracted from a *Brassica nigra* chromosome complement. (**b–g**) Double-target FISH of 5S rDNA (*red*) and 25S rDNA (*green*) probes to somatic metaphase chromosomes (**b,c**) and diakinesis and metaphase I chromosomes in PMCs **(d–g)**. **(b,d,e)** *B. oleracea* var. *alboglabra* No. 4003, (**c,f,g**) *B. rapa* K-151. The nomenclature of rDNA-bearing chromosome types (*Roman numerals*) in all microphotographs is according to Hasterok et al. (2001). *Bar*: 5 μm

the short arm and the signal usually encompasses the whole arm (type VI). Another pair (type VII), of a similar morphology, also has a locus in the short arm, but the signal is usually less pronounced than in type VI. These two pairs of loci are often undistinguishable from one another when the secondary constriction in the chromosome type VI is not distended. One pair of chromosomes exhibits a signal of 5S rDNA in the proximal region of the long arm. This chromosome is designated type IV, in spite of the fact that it exhibits only one instead of two adjacent signals that have been recorded in other *B. oleracea* materials (Armstrong et al. 1998; Hasterok et al. 2001; Snowdon et al. 2002). Thus, it is similar to chromosome type III found in the B genome.

The somatic complement of chromosomes of *B. rapa* K-151 has ten loci of 5S rDNA and ten loci of 25S rDNA (Fig. 1c). In contrast to the *B. oleracea* var. *alboglabra* genome (Fig. 1b), the number of both kinds of ribosomal RNA gene loci in *B. rapa* K-151 is much higher. However, due to co-localisation of rRNA genes in some chromosomes, the two rDNA probes provide landmarks only for a total of 12 chromosomes. These landmarks distinguish four different, A-genome-specific types of rDNA-bearing chromosomes (I, II, V, VIII), of which all except type II are chromosome-specific. Chromosome type I contains the nucleolus organiser region (NOR) and usually has a distended secondary constriction in its short arm. It has two adjacent sites of 5S rDNA and 25S rDNA in its NOR. Three pairs of type-II chromosomes also have the two kinds of ribosomal RNA genes closely linked or co-localised but, unlike the previous type, occupy pericentromeric regions. A single pericentromeric locus of 25S rDNA is characteristic for the pair of type-VIII chromosomes, while a short arm terminal locus of 5S rDNA is diagnostic for the pair of type-V chromosomes (Fig. 1c).

At diakinesis and metaphase I, nine bivalents in *B. oleracea* var. *alboglabra* (Fig. 1d,e) and ten in *B. rapa* (Fig. 1f,g) are regularly observed. At these stages and in both species, all of the rDNA-bearing chromosome types described for the somatic chromosomes are clearly distinguishable (Fig. 1d–g).

Somatic chromosomes of the MALs

The additional chromosomes C5, C8 and C9 in their respective *B. rapa*-*B. oleracea* var. *alboglabra* MALs were carriers of either 5S rDNA or 25S rDNA loci (Fig. 2a–c). Chromosome C5 represents chromosome type IV (Fig. 2a), C8 represents type VII (Fig. 2b) and C9 represents type VI (Fig. 2c). As none of these types exists in the A genome, double-target FISH with ribosomal probes provides the unique genome- and chromosome-specific landmarks for these three alien chromosomes of *B. oleracea* var. *alboglabra*, which in turn enables their unambiguous discrimination in the background of the *B. rapa* chromosomes.

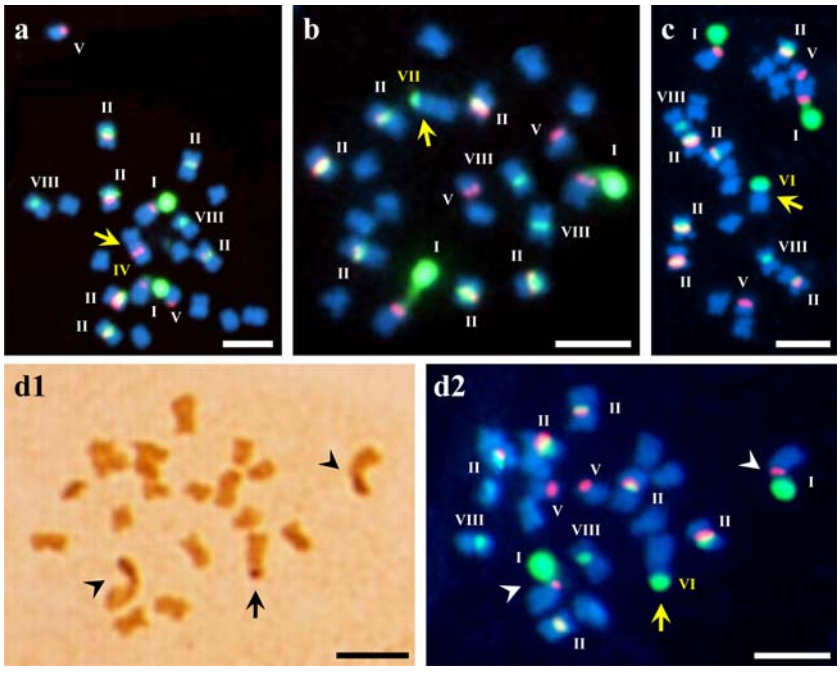

Fig. 2 Somatic metaphase chromosomes of *B. rapa*-*B. oleracea* var. *alboglabra* MAL 5 (**a**), 8 (**b**), and 9 (**c**). FISH with 5S rDNA (*red*) and 25S rDNA (*green*) probes. Sequential silver staining (**d1**) and FISH with the same set of probes (**d2**) to somatic metaphase chromosomes of MAL 9. Silver-stained regions and FISH signals are indicated by *arrows* (additional chromosome from the C genome) and *arrowheads* (chromosomes from the A genome). Additional chromosomes are indicated by *yellow numerals* and *arrows*. *Bar*: 5 μm

Figure 2d1 and d2 show the results of silver staining (Fig. 2d1) and FISH with the ribosomal probes (Fig. 2d2) when applied sequentially to the same somatic chromosomes of MAL 9. The results of silver staining, which is considered to be a marker of transcriptional activity of the 18S-5.8S-25S rRNA genes, reveal that expressed loci occupy the secondary constrictions of the pair of *B. rapa* type-I chromosomes (Fig. 2d1, *arrowheads*) and the locus in the NOR of the alien chromosome C9 (*arrow*).

Chromosome pairing at meiosis of the MALs

The frequencies of various meiotic configurations at diakinesis and metaphase I were calculated from the total number of PMCs analysed in all preparations made from anthers of three individual plants in each of MAL 5 and MAL 8 and two plants of MAL 9. No significant differences between individuals were observed with respect to alien chromosome behaviour.

In the *B. rapa* K-151 background of MAL 5, the alien chromosome was most frequently observed as a univalent (65.8% of the PMCs analysed) (Fig. 3a). Trivalent formation involving the alien chromosome and two *B. rapa* type-II chromosomes was observed in 7% of the PMCs (Fig. 3b). In 15.8% of the PMCs, the alien chromosome paired with two chromosomes of the A genome that did not possess any rDNA landmark (Fig. 3c). In the remaining 11.4% of the PMCs it was difficult to describe precisely the mode of pairing of the alien chromosome C5.

The alien chromosome C8 was also most often seen as a univalent (49.3% of the PMCs) (Fig. 3d). When it paired, this type-VII rDNA-bearing chromosome was most frequently involved (23% of the PMCs) in trivalent formation with the nucleolar chromosomes of the A genome (Fig. 3e,f). Such meiotic behaviour almost always involved the short arms bearing 25S rRNA genes of both the alien chromosome and the NOR chromosomes of the A genome (Fig. 3e), while the long arm associations were observed only sporadically (Fig. 3f). In 18.5% of the PMCs, the alien chromosome was associated with two chromosomes of type II in a way that involved the 25S rDNA loci of these chromosomes, often leading to the formation of characteristic frying pan-like configurations (Fig. 3g). In 3.8% of the PMCs, chromosome C8 was involved in a possible association with chromosomes of type I and type II (data not shown), while in 5.4% of the PMCs it was difficult to interpret the chromosome pairing.

Also in the MAL 9, univalency of the nucleolar alien chromosome (type VI) was the predominant disposition (60.9% of the PMCs) (Fig. 3h). Trivalent formation between its rDNA locus-bearing short arm and the NOR of the type-I chromosomes of the A genome was observed in 20.8% of the PMCs (Fig. 3I), while probable associations between the alien chromosome C9 and a pair of type-I and type-II chromosomes of the A genome occurred in 7.5% of the PMCs (data not shown). In the remaining 10.8% of the PMCs, the behaviour of the alien chromosome C9 was unclear.

Discussion

The parental species

The number and chromosomal locations of 5S and 25S rDNA sites observed in *B. oleracea* var. *alboglabra* No. 4003 in the present investigation are in agreement with previous findings in other *B. oleracea* cultivars and

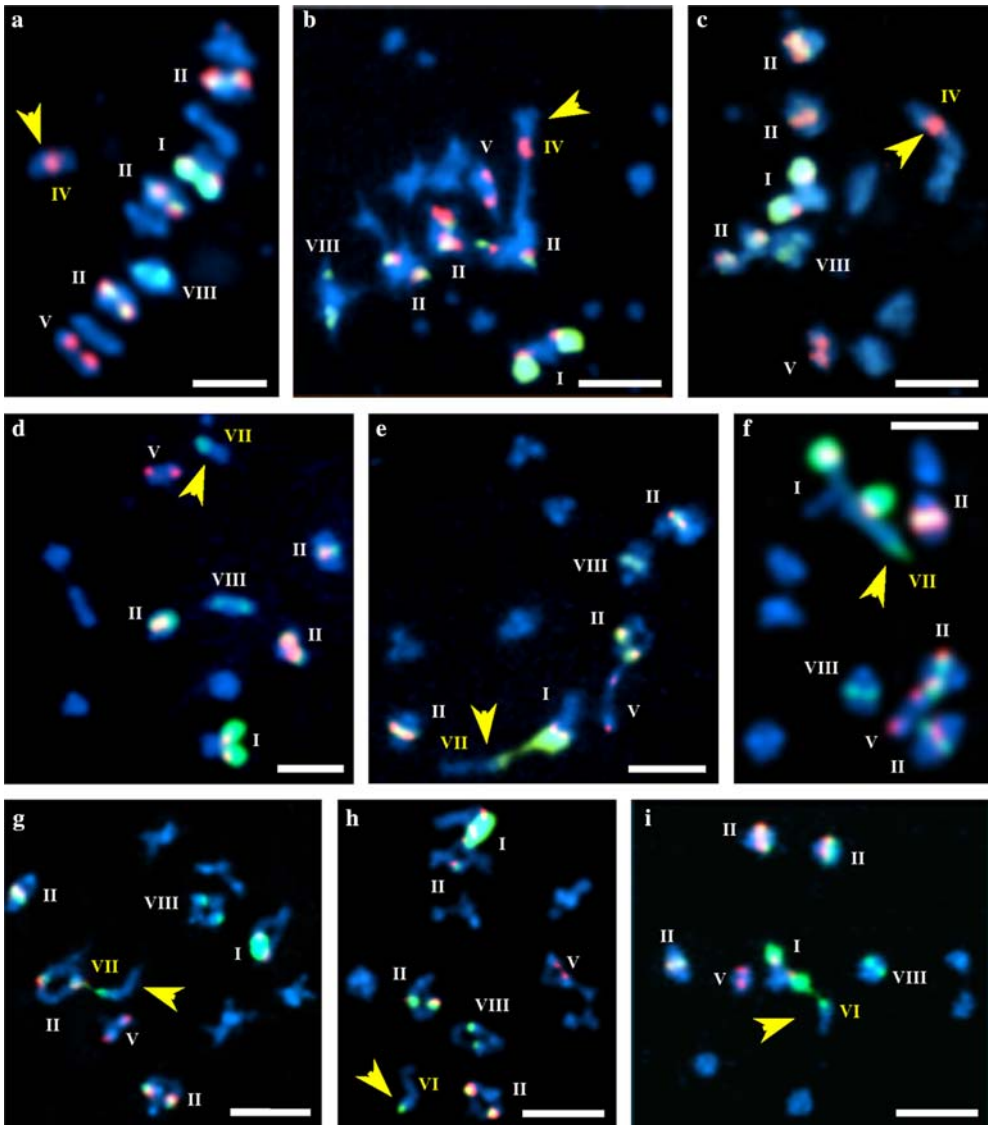

Fig. 3 Double-target FISH of 5S rDNA (*red*) and 25S rDNA (*green*) probes to diakinesis and metaphase-I chromosomes in PMCs of *B. rapa*-*B. oleracea* var. *alboglabra* MAL 5 (**a–c**), 8 (**d–g**) and 9 (**h–l**). Additional chromosomes are indicated by *yellow numerals* and *arrows*. (**a,d,h**) 10 II + 1 I. The respective alien chromosomes stay as univalents. (**b**) 1 III + 9 II. Trivalent formation between the alien chromosome type IV and a pair of chromosomes type II of the A genome. (**c**) 1 III + 9 II. Trivalent formation between the alien chromosome type IV and a pair with no rDNA from the A genome. (**e**) 1 III + 9 II. Trivalent formation by short-arm association of the alien chromosome type VII with the short arm of the nucleolar pair (type I). (**f**) 1 III + 9 II. The alien chromosome type VII pairs by its long arm with the long arm of the nucleolar pair (type I). (**g**) A "frying pan" formation between the alien chromosome type VII and a pair of chromosomes type II of the A genome. (**i**) 1 III + 9 II. The short arm of the alien chromosome type VI associating with the short arm of the nucleolar pair (type I). *Bar*: 5 μm

accessions (Hasterok et al. 2001; Snowdon et al. 2002). An additional 45S rDNA site on the chromosome with 5S rDNA was observed in *B. oleracea* var. *alboglabra* (Armstrong et al. 1998), while an additional 5S rDNA site on the same chromosome was observed in three other varieties of *B. oleracea* (Ziolkowski and Sadowski 2002). The karyotype of *B. rapa* is not only morphometrically the most asymmetric among the three diploid "U-Triangle" species of *Brassica* (U 1935), but it also contains the highest number of 5S and 25S rDNA loci at different chromosomal locations (Hasterok et al. 2001; Snowdon et al. 2002). The above features make the distinction of a significant number of chromosomes at mitosis, or bivalents at meiosis, relatively easy in the A genome compared with the two other genomes (B and C). In contrast to previous results that were obtained for other cultivars, we observed as many as ten loci of 5S rDNA loci on five chromosome pairs in *B. rapa* K-151 instead of only six (Hasterok et al. 2001; Snowdon et al. 2002) or eight (Koo et al. 2004) on three chromosome pairs. All of the extra loci in *B. rapa* K-151 were found near the centromere of two pairs of chromosomes, leading to over-representation of type-II chromosomes in which 5S rDNA was closely linked or co-localised with the site of 25S rDNA. In the cultivars that do not contain the extra loci of 5S rDNA, these chromosomes would appear to be type VIII. Comparing these results with those that were obtained earlier from different cultivars of *B. juncea* and *B. napus* (Hasterok et al. 2001; Kulak et al. 2002; Snowdon et al. 2002) suggests that the observed polymorphism in the number of 5S rRNA genes loci in the A genome is restricted to the types-II and -VIII chromosomes, either of which may increase or decrease in number, depending upon the presence or absence of the given polymorphic locus of 5S rDNA.

Thus, variations in the numbers of 5S and 45S rDNA sites occur in the limited materials of *B. rapa* and *B. oleracea* studied (Armstrong et al. 1998; Hasterok et al. 2001; Snowdon et al. 2002; Ziolkowski and Sadowski 2002; Koo et al. 2004). Similar variations were also re-

ported in some other plant species regarding 5S rDNA sites (Cuadrado and Jouve 1997) and more frequently in the case of 18S-5.8S-25S ribosomal gene loci (Hanson et al. 1996; Moscone et al. 1999; Shishido et al. 2000). Different mechanisms may be invoked to explain this phenomenon, such as unequal crossing over, chromosome rearrangements, gene conversion or even transpositional events involving ribosomal genes (Leitch and Heslop-Harrison 1993; Hall and Parker 1995; Shishido et al. 2000). It also cannot be ruled out that intervarietal and interindividual polymorphism in number of rDNA sites can be caused by differences in copy number of ribosomal cistrons, which may make some loci undetectable by FISH. A higher resolution of the number and sites of rDNA loci was achieved when the less condensed pachytene chromosomes were studied (Armstrong et al. 1998; Howell et al. 2002; Ziolkowski and Sadowski 2002). One labelled site on condensed mitotic chromosomes, or meiotic diakinesis or metaphase-I chromosomes, might appear as labelled neighbouring loci in pachytene chromosomes (Koo et al. 2004). However, resolution of all pachytene chromosomes in a complement is difficult due to frequent clustering of the pericentromeric heterochromatic regions.

The MALs

In mitotic metaphases as well as at diakinesis and metaphase I of meiosis the two ribosomal probes enabled the unambiguous identification of alien chromosomes in the three MALs studied due to the presence on these chromosomes of either 5S rDNA or 25S rDNA markers, whose localisation is unique and diagnostic for the C genome. The presence of such landmarks was the main reason why we chose these particular three MALs out of the eight available for the detailed FISH-based analyses.

Equally important is the distribution of exceptionally numerous and specific 5S rDNA and/or 25S rDNA sites on the A-genome chromosomes. This makes it possible to determine which chromosome, or at least which chromosome type, of the *B. rapa* parent is involved in possible associations with the alien chromosome. Such chromosome-specific markers that are present both on the alien and host chromosomes make it considerably easier to make more detailed observations of possible homoeologous interactions at meiosis than was possible in earlier studies that were based on conventional chromosome staining—for example, with carmine (Cheng et al. 1994b). On the other hand, carmine-stained preparations provide a better definition of chromosome morphology and enable preferential staining of the heterochromatin. Therefore, to achieve the best effects it may be advisable to use carmine staining and FISH with the ribosomal probes sequentially on the same chromosome preparations.

FISH analysis of meiosis in the *B. rapa*-*B. oleracea* var. *alboglabra* MALs revealed that in all three cases univalency of the alien chromosome was most frequently observed. The additional chromosome C5 is the only one that carries proximally located 5S rRNA genes. Regardless of its association with two labelled or unlabelled chromosomes of the A genome, it seems that such intergenomic associations represent proper chiasma-based trivalents.

Both of the additional chromosomes C8 and C9 possess 18S-5.8S-25S rRNA genes in a major part of the short arm and exhibit a large, terminal FISH signal, a common feature that may drive their similar behaviour at meiosis. Their ribosomal gene loci associate frequently with type-I and -II chromosomes of the host species. A high affinity between the short arm of these alien chromosomes and the NOR-bearing chromosomes of *B. rapa* as well as the pairing of C8 and C9 with other background chromosomes have been reported by Chen et al. (1997a). Our current analyses have revealed the further prevalence of frying pan-like configurations. Apparently, these are associations between the ribosomal DNA site in the alien chromosome and the ribosomal DNA site in a chromosome of an A-genome bivalent. The apparent achiasmatic association between the rDNA-containing short arm of alien chromosome C8 and the pericentric rDNA of type-II chromosomes urges caution when interpreting putative chiasmatic associations involving rDNA-carrying arms and the lack of silver stain at certain 45S rDNA sites. A possible explanation may be that, at least in some cases, the frequently observed affinity of the rDNA regions is caused by some physiological factors, such as the nucleolar association of 45S ribosomal DNA-bearing chromosomes, a phenomenon described for some plant species (Sato et al. 1981; Dagne and Heneen 1992; Khoudoleeva et al. 2000) and occasionally also observed in somatic metaphase (Fig. 1c) and pachytene chromosomes of *Brassica* (R. Hasterok, unpublished data).

The results of sequential silver staining and FISH with the 25S ribosomal probe on the same somatic chromosome spreads reveal that MAL 9, which carries the alien nucleolar type-VI chromosome, expresses only 3 out of 11 loci of genes encoding for 18S-5.8S-25S rRNA. Transcriptionally active sites were borne on a pair of type-I chromosomes from the A genome, and a locus on the additional chromosome C9 was also expressed. All other 25S rDNA loci that were distributed on type-II and -VIII chromosomes of the A genome did not show activity or their expression was below detectable levels. The lack or undetection of expression of these loci is in agreement with earlier observations (Cheng and Heneen 1995; Hasterok and Maluszynska 2000a, b) in both *B. rapa* and the two A-genome containing allotetraploid species of *Brassica* (*B. juncea*, *B. napus*). Preliminary results of experiments with demethylating agents, such as 5-azacytidine (Hasterok and Maluszynska 1999) suggest that these loci may be permanently inactivated due to some mechanism regulating their expression not necessarily connected with DNA methylation. Such inactivity may also be the result of the

pericentromeric localisation of these loci. It is well known from both C-banding (Olin-Fatih and Heneen 1992) and GISH analyses (Snowdon et al. 1997a; Maluszynska and Hasterok 2005) that the pericentromeric regions in *Brassica* chromosomes are highly heterochromatinised. This or other factors may inactivate the ribosomal genes in such regions, as has been observed in black rat (Stitou et al. 2000), the grasshopper *Eyprepocnemis plorans* (Lopez-Leon et al. 1995), fruit fly and budding yeast (Fourel et al. 2002). As the A genome contains rDNA arrays at many chromosomal locations, it cannot also be ruled out that the ribosomal genes in the proximal regions are no longer functional, presumably due to degeneration and conversion to pseudogenes (Alvarez and Wendel 2003).

The unambiguous expression of the ribosomal site on the additional chromosome C9 in the background of the A-genome chromosomes shows that its 45S rDNA locus does not undergo the nucleolar suppression that has been observed in many allopolyploid species and interspecific hybrids (Pikaard 1999, 2000a, b). This is consistent with the results of Hasterok and Maluszynska (2000a, c) which revealed that a pair of nucleolar chromosomes from both the A- and C-genomes is expressed in root-tip cells of both natural and resynthesised *B. napus*, although molecular analyses of leaf material strongly indicated the prevalence of nucleolar dominance (Chen and Pikaard 1997).

The use of FISH and rDNA probes has shown that this methodology is not only a robust direct marker system for the detecting MALs with ribosomal genes but also for highlighting homoeologous chromosome pairing as well as achiasmatic associations between rDNA sites. Methodology involving the use of molecular DNA markers is complementary to cytology and provides a decisive methodology—in most cases, a prerequisite—for the detection and characterisation of MALs (Jørgensen et al. 1996; Chen et al. 1997a, b; Heneen and Jørgensen 2001). Eight out of the nine possible MALs have been developed (Heneen and Jørgensen 2001), and efforts are currently being made to obtain the missing MAL. These lines are useful for molecular mapping and the designation of available molecular maps to specific chromosomes, the physical localisation of specific DNA sequences to chromosomes and the standardisation of chromosome nomenclature. Chromosome pairing at meiosis elucidates homoeological and phylogenetical relationships between alien and background chromosomes. For applied purposes, the addition lines are suitable for mapping certain agronomic characters such as seed-colour genes (Chen et al. 1997b; Heneen and Brismar 2001; Heneen and Jørgensen 2001) and for marker-assisted breeding for desirable characters in the parental species and their allopolyploid *B. napus*.

It can be concluded that more detailed FISH analyses of mitotic chromosomes and chromosome behaviour at meiosis of *B. rapa*-*B. oleracea* var. *alboglabra* MALs will certainly require going beyond ribosomal landmarks. This could be accomplished through the use of either chromosome-specific or even arm-specific sets of BAC clone-based probes for both *B. rapa* and *B. oleracea* chromosomes (Howell et al. 2002; Ziolkowski and Sadowski 2002; Koo et al. 2004).

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