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Behaviour of *Sinapis alba* chromosomes in a *Brassica napus* background revealed by genomic *in-situ* hybridization

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Abstract

Genomic *in-situ* hybridization (GISH) was applied to study the behaviour of addition chromosomes in first and second backcross (BC) progenies of hybrids between *Brassica napus* sp. napus L. (AACC, 2n = 38) and *Sinapis alba* L. (SS, 2n = 24) produced by electrofusion. With GISH using genomic DNA of *S. alba* as a probe it was possible to clearly distinguish both of the parental genomes and effectively monitor the fate of *S. alba* chromosomes in the BC_1 and BC_2 progenies. GISH analysis confirmed the sesquidiploid genome composition (AACCS) of the BC_1 progenies, which contained 38 chromosomes from *B. napus* and 12 chromosomes from *S. alba*. Genome painting in the pollen mother cells (PMCs) of the BC_1 plants revealed intergenomic association between *B. napus* and *S. alba* chromosomes, whereby a maximum of 4 trivalents between AC and S chromosomes were identified at metaphase I. In the BC_2 progenies, aneuploids with different numbers of additional chromosomes from *S. alba*, ranging from 1 to 7, were confirmed. Three putative monosomic alien addition lines were characterized, and the results are discussed with respect to the potential for intergenomic chromosome recombination.

Introduction

Sinapis alba L. (genome SS, 2n = 24) is phylogenetically close to *Brassica* species and possesses desirable agronomic characteristics such as yellow seed colour, reduced pod shattering and resistance to various diseases including black spot (*Alternaria brassicae* Berk., Hansen & Earle 1997), beet cyst nematode (*Heterodera schachtii* Schm.) and clubroot (*Plasmodiophora brassicae* Wor., Lelivelt *et al.* 1993), as well as tolerance to flea beetles (Bodnaryk & Lamb 1991, Brown *et al.* 2004). It is also tolerant to high temperatures and drought stress (Brown *et al.* 1997), therefore *S. alba* has considerable promise as an alternative cruciferous oilseed crop in areas with short, dry growing seasons such as those found in the semi-arid regions of Western Canada and Australia (Rakow *et al.* 2000). On the other hand, the seed and oil yield are relatively low in comparison to oilseed rape/canola (*Brassica napus* ssp. *napus*, AACC, 2n = 38), therefore the interspecific transfer of these positive traits to *B. napus* is desirable to expand the genetic variability for these traits in the more important crop species. Successful sexual hybridization between *B. napus* and *S. alba* has been reported through embryo rescue or ovary culture (Ripley &

Arnison 1990, Mathias 1991, Lelivelt *et al*. 1993, Chevre *et al*. 1994, Brown *et al*. 1997) and by protoplast fusion (Primard *et al*. 1988, Lelivelt *et al*. 1993, Wang *et al*. 2005b). In our previous work, somatic hybridization was performed between *B*. *napus* and *S*. *alba* via electrofusion, and seven somatic hybrids were produced (Wang *et al*. 2005b) with the aim of enriching sources of disease resistance of *B*. *napus* and introduction of the yellow seed trait from *S*. *alba*. In order to create a full set of monosomic alien addition lines of *S*. *alba* in a *Brassica napus* genomic background, and to eliminate undesirable traits, backcrosses with *B*. *napus* were subsequently carried out.

Genomic *in-situ* hybridization (GISH) enables not only the distinction of the parental chromosomes in a large number of interspecific and intergeneric hybrids, but also the detection of genomic constitution and chromosome rearrangements (Schwarzacher *et al*. 1989, Anamthawat-Jonsson *et al*. 1990, Jacobsen *et al*. 1995, Kamstra *et al*. 1999, Takahashi *et al*. 1997, Stevenson *et al*. 1998, Karlov *et al*. 1999, Ji *et al*. 2004). GISH has also been applied successfully for identification of *Brassica* intergeneric hybrids (Fahleson *et al*. 1997, Skarzhinskaya *et al*. 1998, Snowdon *et al*. 2000, Benabdelmouna *et al*. 2003, Wang *et al*. 2004); however, it is difficult to detect intergenomic rearrangements in *Brassica* chromosomes. Also, due to a concentration of heterochromatin around the centromeric regions, and the extensive intergenomic homoeology among the Brassicaceae, genomic probes often do not hybridize uniformly across the entire length of the chromosomes (Snowdon *et al*. 1997). On the other hand, we have shown that GISH on meiotic preparations can allow the detection of intergenomic recombination between *B*. *napus* and *Crambe abyssinica* (Wang *et al*. 2004).

The transfer of genes and chromosomes from alien species and genera has contributed a great deal to the improvement of numerous crops in the past (Lim *et al*. 2000), and interspecific hybrids can also be used to generate novel *B*. *napus* genotypes with genetically diverse polyploidy genome components (Li *et al*. 2004). GISH has proved invaluable in monitoring the fate of alien chromatin through subsequent generations of wide hybrids (Raina & Rani 2001). In order to establish the number of *B*. *napus* and *S*. *alba* chromosomes, and to determine whether homoeologous recombination has occurred in the BC_1 and BC_2 plants, mitotic and meiotic GISH was performed in plants of the BC_1 and BC_2 progenies from hybrids between *B*. *napus* and *S*. *alba*.

Materials and methods

Plant materials

Somatic hybrids obtained by electrofusion of *Brassica napus* L. cv. 'Maplus' (AACC, 2n = 38) protoplasts with *Sinapis alba* L. protoplasts have been described previously (Wang *et al*. 2005b). Sexual progeny of the hybrid was obtained by backcrossing twice with *B*. *napus* cv. 'Maplus'. Root tips from seeds harvested from BC_1 and BC_2 plants were used for mitotic GISH analysis, whereas flower buds of the BC_1 and BC_2 plants were used as experimental material for meiotic GISH. The crossing scheme for the development of hybrid progeny is shown in Figure 1. All plants were grown in the greenhouse.

Chromosome preparation

For study of mitotic metaphase complements, the root tips of young seedlings from BC_1 and BC_2 seeds were collected, and the whole seedlings were pretreated in 2 mM 8-hydroxyquinoline for 2 h at 25°C followed by 2 h at 10°C. Material was fixed in Farmer's solution (acetic acid: ethanol = 1:3) and stored at −20°C until use. Flower buds from BC_1 and BC_2 plants were fixed directly in Farmer's solution. Both root tips and anthers were incubated in an enzyme mixture containing 2% (v/w) cellulase and 20% (v/v) pectinase in 4 mmol/L citrate buffer (pH 4.8) for about 1.5–2 h at 37°C and subjected to a 45 min treatment in 75 mmol/L KCl. Subsequently

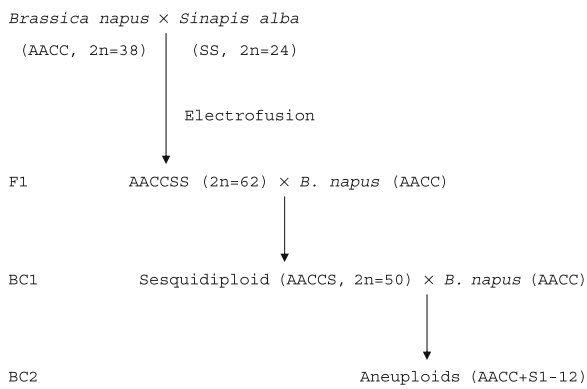

Figure 1. Crossing scheme for the development of hybrid progeny.

each tip or anther was transferred to chilled slides directly using a pipette, and 60% acetic acid was added to clear the cytoplasm followed by washing with ice-cold Farmer's solution to spread the cells on the slides. Slides were air-dried before further use.

Probe preparation and in-situ *hybridization*

Total genomic DNA was extracted from young leaves of *B. napus* and *S. alba* plants using the DNeasy Plant Maxi Kit (Qiagen, Germany). Genomic DNA of *S. alba* was labelled with fluorescein-12-dUTP using a nick-translation kit (Catalogue No. 976776, Roche, Germany) according to the manufacturer's instructions. To prevent non-specific intergenomic cross-hybridization, a 30-fold excess of sheared genomic DNA from *B. napus* was added to the hybridization solution. The DNA was sheared by autoclaving (5 min, 1 bar), yielding fragments of around 300–500 bp in size. Labelled probe and chromosomes were denatured simultaneously on cleaned microscope slides at 80°C for 4 min and hybridized overnight at 37°C. After hybridization the slides were washed at 42°C for 5 min each in 2 × SSC and 0.4 × SSC, respectively. Chromosomes were counterstained with propidium iodide (PI) and fluorescence was visualized using an Olympus BX51 microscope. At least 5 cells were observed for each preparation. Photographs were taken using a computer-assisted cooled charge-coupled device (CCD) camera and images were merged with Image-Pro Plus Version 5.0 software.

Results

GISH analysis of BC_1 plants

Using genomic DNA of *S. alba* as a probe, the *B. napus* and *S. alba* genomes were clearly distinguished in the hybrid progeny. As expected, the GISH studies showed that the chromosome constitution in the seeds harvested from F_1 hybrids via backcrosses with *B. napus* (BC_1) was 2n = 50 (AACCS, sesquidiploid), i.e., 38 chromosomes of *B. napus* origin were strongly painted in red, while 12 chromosomes of *S. alba* origin fluoresced in yellow (Figure 2a). We examined the possibility of meiotic pairing between *B. napus* (AC) and *S. alba* (S) chromosomes in the BC_1 plants. At diakinesis I stage of the pollen mother cells (PMCs), *B. napus* chromosomes formed 19 normal bivalents, while the 12 *S. alba* chromosomes formed univalents and no intragenomic pairing was observed. However, chromatin association between AC and S chromosomes was detected by GISH to meiotic preparations. At metaphase I, AC chromosomes from *B. napus* aligned on the equatorial plate and paired preferentially, whereby between one and four trivalents between AC and S chromosomes were also formed (Table 1). The example shown in Figure 2b shows eight S chromosomes present as univalents and distributed around the exterior of the PMC, while four S chromosomes form trivalents with AC genome chromosomes and were typically found to be oriented more centrally. At anaphase I the 12 chromosomes from *S. alba* were randomly distributed to both poles. Among 65 PMCs scored, 4.6%, 7.7%, 6.2%, 18.5%, 35.4% and 27.7%, respectively, were counted at the ratios of 1:11, 2:10, 3:9, 4:8, 5:7 and 6:6 (Table 1). The example given in Figure 2c shows separation in a ratio of 5:7, with two chromosomes from *S. alba* exhibiting association with *B. napus* chromosomes (arrow). Figure 2d shows a PMC at telophase I with seven chromosomes from *S. alba*, one of which is combined with an AACC chromosome (arrow).

GISH analysis of BC_2 plants

Seeds harvested from BC_1 plants were also examined by GISH on mitotic root tip preparations. Among 36 seeds tested, all had more than 38 chromosomes, ranging from 39 to 45, and were confirmed as aneuploids. Thirty-eight chromosomes from *B. napus* and 1, 3, 4, 5, 6 and 7 additional chromosomes from *S. alba*, respectively, were observed (Table 2, Figure 2e, 2g). Of the progeny investigated, three were

Table 1. Chromosome behaviour of BC_1 plants at diakinesis I or metaphase I and anaphase I analyzed by GISH.

	No. of trivalents per PMC at diakinesis I or metaphase I				Separation ratio of *S. alba* chromosomes at anaphase I					
Meiosis	1	2	3	4	1:11	2:10	3:9	4:8	5:7	6:6
No. of PMCs scored	12	18	24	11	3	5	4	12	23	18
Frequency (%)	18.5	27.7	36.9	16.9	4.6	7.7	6.2	18.5	35.4	27.7

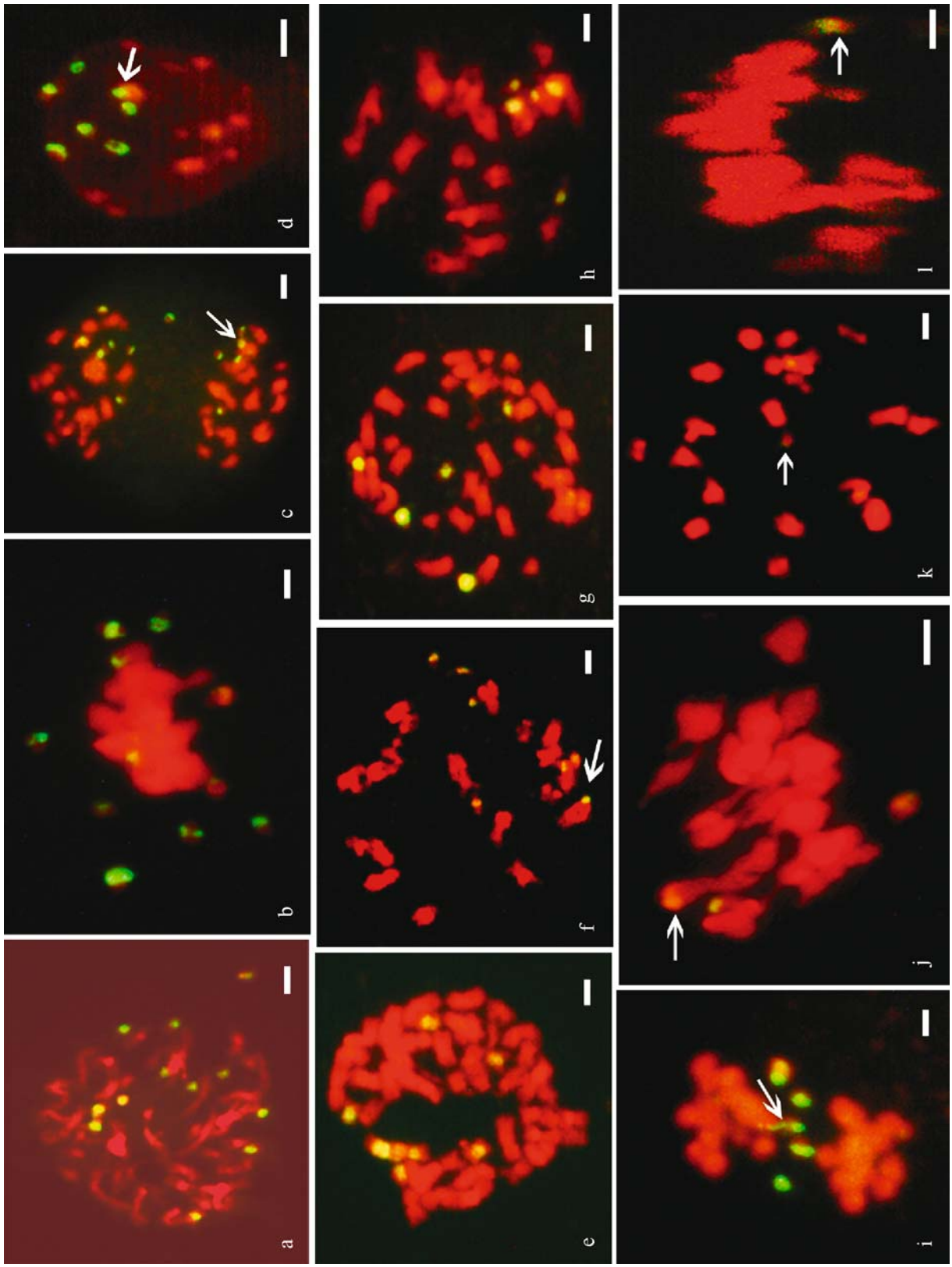
a
b
c
d
e
f
g
h
i
j
k
l

Table 2. Chromosome constitution of somatic hybrids and the BC_1 and BC_2 progenies analysed by GISH.

Genotype	Chromosome number (2n)	Genomic constitution	Genome constitution Chromosomes from	
			B. napus	*S. alba*
B. napus	38	AACC	38	
S. alba	24	SS		24
Somatic hybrids (F_1)[a]	62	AACCSS	38	24
BC1 (F1 × *B. napus*)	50	AACCS	38	12
BC2 (BC × *B. napus*)		AACCS(1–12)		
BC2-1			38	1 (3)[b]
BC2-2			38	3 (8)
BC2-3			38	4 (9)
BC2-4			38	5 (7)
BC2-5			38	6 (4)
BC2-6			38	7 (5)

[a] For details see Wang *et al*. (2006).
[b] Figures in parentheses = frequency.

confirmed to contain 39 chromosomes comprising 38 from *B. napus* and one monosomic addition from *S. alba*. Selected BC_2 plants were used for further meiotic GISH analysis. Figure 2f shows a PMC with seven additional chromosomes from *S. alba*, two of which were associated with *B. napus* chromosomes at diakinesis I (arrows). Figures 2h and 2i indicate chromosome behaviour of PMCs with five additional chromosomes of *S. alba*, three of which are possibly associated with AC chromosomes (Figure 2h). Five laggards of *S. alba* were clearly displayed at anaphase I, one of them with chromatin bridge between *S. alba* and *B. napus* (Figure 2i, arrow). Figure 2j shows a PMC with three additional *S. alba* chromosomes, one of which appears to be undergoing chromatin recombination with *B. napus* chromatin (arrow). Potential *B. napus* + *S. alba* monosomic alien addition lines with one *S. alba* chromosome were identified in the BC_2 populations. At diakinesis I, 19 bivalents derived from *B. napus* and 1 univalent derived from *S. alba* were clearly detected (Figure 2k). At metaphase I, one univalent of alien *S. alba* chromosome was observed (Figure 2l).

Morphological characterization of the monosomic alien addition lines

Considerable morphological variation was observed among the BC_1 and BC_2 progenies. Three putative monosomic alien addition lines (MAALs) detected by GISH grew vigorously, were taller than *B. napus* and were differentiated morphologically from each other. Plant BC2-1-1 had very dark green leaves without wax, while plant BC2-1-2 had a thick stem with numerous trichomes and plant BC2-1-3 had a compact shape resembling *S. alba*. The MAALs had a higher pollen fertility, ranging from 82% to 90%. The average seed set of the MAALs by self-

Figure 2. Genomic *in situ* hybridization (GISH) to mitotic and meiotic chromosomes of BC_1 and BC_2 progeny from somatic hybrids between *Brassica napus* ssp. *napus* (AACC, 2n = 38) and *Sinapis alba* L. (SS, 2n = 24). Chromatin of *S. alba* is labelled yellow with FITC, while *B. napus* chromatin is counterstained red with PI. (a–d) BC_1 plants: (**a**) mitotic chromosomes of sesquidiploid BC_1 plants with 38 *B. napus* chromosomes and 12 *S. alba* chromosomes; (**b**) metaphase I in the sesquidiploid hybrid. In this example the *B. napus* chromosomes are oriented on the equatorial plate and eight *S. alba* chromosomes separate to form univalents, while four *S. alba* chromosomes are involved in putative trivalents with *B. napus* chromosomes; (**c**) example of anaphase I showing a 5:7 separation of *S. alba* chromosomes and association of AC and S chromosomes (arrow); (**d**) example of telophase I showing seven chromosomes from *S. alba*, one of which shows association with a *B. napus* chromosome (arrow). (**e**–**l**) BC_2 plants: (**e**, **f**) plants with seven additional chromosomes from *S. alba*; (**e**) mitotic cell with 38 *B. napus* chromosomes and seven *S. alba* chromosomes (yellow); (**f**) example of diakinesis showing a PMC with 19II from *B. napus*, five *S. alba* univalents and two putative AC-S trivalents (arrow); (**g**–**i**) plants with five additional chromosomes from *S. alba*; (**g**) mitotic cell with 38 *B. napus* chromosomes and five *S. alba* chromosomes; (**h**) example of diakinesis in a PMC with 19II *B. napus*, two *S. alba* univalents and three putative AC-S trivalents (arrows); (**i**) example of anaphase I showing five *S. alba* laggards, one of which exhibits a chromatin bridge with a *B. napus* chromosome (arrow); (**j**) metaphase I in a PMC with three additional *S. alba* chromosomes, one of which shows an intergenomic recombination with a *B. napus* chromosome (arrow); (**k**, **l**) monosomic addition line carrying a single *S. alba* chromosome at diakinesis I (**k**) and metaphase I (**l**) in the form of a univalent. Scale bar represents 2 μm.

pollination was 2–3 seeds per pod, in backcrosses with *B. napus* 5–7 seeds per pod were produced. Siliques of all MAALs exhibited a long beak characteristic of *S. alba*. Most of the seeds harvested from the MAALs were larger than that of *B. napus*, and the plant BC2-1-3 produced brown–yellow seed.

Discussion

Genome differentiation by GISH

The results of this study demonstrate the utility of GISH for genome discrimination in hybrid nuclei for an analysis of intergenomic relationships. Furthermore, the transmission and recombination of *S. alba* and *B. napus* chromosomes through meiotic divisions of intergeneric hybrid progeny was also able to be determined by GISH. At meiosis, *B. napus* chromosomes were shown to pair preferentially and some *S. alba* chromosomes formed trivalents with *B. napus* chromosomes, meaning that recombinant chromosomes are present in the nuclei of backcross progeny (Figure 2j). This demonstrates the existence of partial chromosome homology between the genomes of *B. napus* and *S. alba*, as was found previously in somatic hybrids between *B. juncea* and *S. alba* (Gaikwad *et al*. 1996), and therefore the potential for intergenomic recombination. Therefore, the introgression of alien genes from *S. alba* to *B. napus* can be achieved through meiotic cross-over in backcross progenies. This important prerequisite for the integration of agronomically relevant traits from related crucifers into *Brassica* crops is not always fulfilled: In intergeneric hybrids between *B. napus* and the closely related crucifer oil radish (*Raphanus sativus*), for example, radish addition chromosomes were maintained more or less unaltered in the background of the *Brassica* genomes (Peterka *et al*. 2004) and desired resistance traits could not be introgressed from *R. sativus* to the *B. napus* genome despite numerous rounds of backcrossing (Voss *et al*. 2000, Peterka *et al*. 2004).

Chromosome associations in hybrid progeny

The identification of intergeneric or interspecific recombination by GISH in mitotic preparations is questionable in small genomes with a relatively low proportion of medium and highly repetitive DNA families, such as *Brassica* species (Snowdon *et al*. 1997, 2000, Wang *et al*. 2004). In this case, detectable GISH signals are mainly restricted to pericentromeric heterochromatin blocks and sometimes to nucleolus organizers (NORs) where repetitive DNA sequences are clustered. In contrast, our results using GISH to meiotic preparations were able to effectively reveal intergenomic recombination and homoeology. Homoeologous associations between *B. napus* and *S. alba* chromosomes were identified, and in some cases recombinant chromosomes could be clearly detected. The hybrid progenies had a higher seed set when backcrossed with *B. napus*. This result differs from the study of Lelivelt *et al*. (1993), who reported infertility in backcross offspring from somatic hybrids of *B. napus* and *S. alba*. On the other hand, however, GISH can fail to identify very short recombinant segments in interspecific *Brassica* hybrids and the exact size and the position of introgressions can be extremely difficult or impossible to estimate. In such cases, analysis with chromosome-specific markers along the chromosome may assist in more exact chromosomal localization and characterization of the introgressions (Ali *et al*. 2001, Peterka *et al*. 2004).

Utility of the MAALs

S. alba and *B. napus* are closely related species, hence it is possible to transfer individual chromosomes from *S. alba* to *B. napus* to produce monosomic alien addition lines. Obviously, this opens the prospect for establishing a complete set of monosomic *S. alba* alien addition lines within the genomic background of cultivated rapeseed. Our study shows that *S. alba* chromosomes were decreased in subsequent backcrosses with *B. napus*, whereby after only two backcrosses three individuals were detected with only a single *S. alba* chromosome. The identity of individual addition chromosomes can be established in some cases by hybridization with labelled 45S and 5S rDNA probes or other chromosome-specific markers (Schrader *et al*. 2000, Ali *et al*. 2005), or with the help of chromosome-specific PCR markers. This should enable the rapid generation of a complete set of *B. napus* + *S. alba* MAALs as a tool to localize genes of interest controlling relevant agronomic traits in *S. alba*, and to transfer these in a targeted manner into the genome of oilseed rape (Ali *et al*. 2001). MAALs of *B. napus* with alien chromosomes from *S. arvensis* exhibiting resistance

to *Leptosphaeria maculans* were successfully obtained and the resistance was successfully introgressed into *B. napus* by backcrossing and selfing (Snowdon *et al.* 2000). Five different multiple *B. napus* + *R. sativus* (oil radish) addition chromosome lines (a–i) were selected by Peterka *et al.* (2004) and used to identify the oil radish chromosome containing genes for beet cyst nematode resistance. More recently, we produced two MAALs of *B. napus* + *C. abyssinica*, and a fertile, stable doubled haploid disomic addition line was obtained via microspore culture (Wang *et al.* 2006). The evidence presented herein of chromosome recombination and association between *B. napus* and *S. alba* demonstrates in principle the feasibility of gene transfer from *S. alba* to *B. napus*. In previous work (Wang *et al.* 2005b), *fae1* gene introgression from *S. alba* to *B. napus* was confirmed by GISH and cleaved amplified polymorphic sequence (CAPS) analysis of the *fae1* gene in F_1 plants of the hybrid and their progenies (F_2 and BC_1). Our eventual goal is to develop a set of *S. alba* introgression lines or MAALs in a *B. napus* background. Many studies show that loci controlling complex traits are numerous, widespread, and intensively interact. The application of MAALs offers more prospects to dissect these loci and ultimately transform them into simple Mendelian factors via backcrosses, greatly facilitating map-based cloning of the genes in the wild relatives (Fridman *et al.* 2004). A full set of *B. napus* + *S. alba* MAALs containing enhanced traits from the donor species would provide us with a potentially powerful tool to identify and transfer genes of interest from *S. alba* to *B. napus* and ultimately to generate stable rapeseed lines containing disease resistance and other relevant traits, such as yellow seed colour and pod shattering resistance, from the donor genome on small chromosome introgressions with minimal genetic drag.

MAALs are also useful for detecting structural variations in homoeologous chromosomes of related species. By creation of a full set of MAALs from *S. alba* to cultivated rapeseed, genes of interest from *S. alba* could be fine-mapped and tagged with closely linked markers. For meaningful utilization of these valuable materials in further introgression breeding, accurate identification of recombinant chromosomes will be necessary using molecular marker analyses and assessment by fluorescence *in situ* hybridization (FISH) with genomic and chromosome-specific DNA as probes (Wang *et al.* 2005a). Furthermore, the production of substitution lines for recombinant segments is required. Similar studies have been conducted in *Festuca–Lolium* hybrids where some of the agronomic traits have been assigned to specific chromosome segments (King *et al.* 1998). Ultimately, this approach enables exploitation of the *S. alba* gene pool for use in rapeseed breeding.

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