Cytogenetic analysis of F_1, F_2 and BC_1 plants from intergeneric sexual hybridization between *Sinapis alba* and *Brassica oleracea* by genomic *in situ* hybridization

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Abstract

By intergeneric sexual hybridization between *Sinapis alba* and *Brassica oleracea* F_1, F_2 and BC_1 progeny plants were produced. *S. alba* plants (genome SS, 2n=24) were pollinated with *B. oleracea* (genome CC, 2n=18), and the fertile F_1 plants were pollinated with *B. oleracea* to obtain BC_1 plants. GISH analysis showed that 10 of 12 F_1 plants had 12 *S. alba* chromosomes (one full S chromosome set) and 9 *B. oleracea* chromosomes (one C chromosome set), representing the expected hybrids. However, two F_1 plants had 12 S chromosomes and 18 C chromosomes (two C chromosome set), indicating unexpected hybrids. A maximum of 3 trivalents between C and S chromosomes were identified at metaphase I of semi-fertile F_1 pollen mother cells (PMCs), which indicates homology and chromosome pairing between these two genomes. The C genome had obviously been doubled in two F_2 plants from selfed semi-fertile F_1 plants. BC_1 plants consisted of 18 C chromosomes and variable number of 1, 5 and 6 additional S chromosomes, respectively. Monosomic alien addition lines developed in the present study can be used for *B. oleracea* breeding and *Sinapis alba* gene mapping.

Key words: *Sinapis alba*, *Brassica* oleracea, intergeneric sexual hybridization, F_1, F_2 and BC1 hybrid progenies, genomic in situ hybridization (GISH)

Sinapis alba L. (genome SS, 2n=24), a member of the *Brassica*ceae (Cruciferae) family, possesses important agronomic traits. For example, it is highly resistant to many diseases and insect pests of crucifers (Salisbury 1987, Muehlchen and Parke 1990, Golinowski and Magnusson 1991, Hansen and Earle 1997, Brown et al. 2004), and it is also tolerant to high temperatures and drought stress (Brown et al. 1997). Therefore, it is a valuable germplasm resource for Cruciferae crop breeding. By intergeneric sexual hybridization between *S. alba* and *B. rapa* L. (genome AA, 2n=20), alternaria leaf spot resistance has been transferred to Chinese cabbage (Gong et al. 1994). Successful sexual hybridization between *B. napus* L. (genome AACC, 2n=38) and *S. alba* has also been reported by some researchers (Chevre et al. 1994, Brown et al. 1997). Somatic hybridizations between *S. alba* and *B. napus* have been performed by Wang et al. (2005a, 2005b), between *S. alba* and *B. juncea* L. (genome AABB, 2n=36) by Gaikwad et al. (1996), between *S. alba* and *B. oleracea* L. (genome CC, 2n=18) by Hansen and Earle (1997). The purpose of the hybridizations mentioned above was to transfer valuable characteristics from *S. alba* to other Cruciferae crop plants.

B. oleracea is one of the most important vegetable crops in China. But, the crop is often susceptible to aphids, frost, mildew, black spot and black rot diseases, resulting in yield reduction of. Therefore, the interspecific transfer of positive traits of *S. alba* to *B. oleracea* is desirable to expand the genetic variability of *B. oleracea*. In the present study, intergeneric sexual crosses between *S. alba* and *B. oleracea* were performed successfully by using sources of insect pest and disease resistance of *S. alba* for the first time with the aim of improving *B. oleracea*. In order to create a full set of monosomic alien addition lines of *S. alba* in a *B. oleracea* genome background, backcrosses with *B. oleracea* as a recurrent parent were subsequently carried out.

Genomic *in situ* hybridization (GISH) enables the distinction of the parental chromosomes in interspecific and intergeneric hybrids. In addition, GISH also allows an analysis of genomic constitution and chromosome behavior (Kamstra et al. 1999, Ji et al. 2004, Wang et al. 2005b). To identify *Brassica* intergeneric hybrids, GISH has been applied effectively by others, e.g. Benabdelmouna et al. (2003). GISH can also be successful used to detect intergenomic recombination in meiotic preparations (Wang et al. 2004, 2005b), although its application has turned out to be difficult for detecting small *Brassica* genome segments (Snowdon et al. 1997).

We have produced a large number of F_1, F_2, and BC_1 plants from intergeneric sexual hybridization between *S. alba* and *B. oleracea* assisted by ovary culture. To identify the genomic constitutions of these plants and their genetic backgrounds is necessary for their use in breeding and genetic improvement of *Brassica* crop plants.

Materials and Methods

Plant materials: Female parent *S. alba* (2n=24, SS genome; white mustard var. 'Asta') plants were pollinated by the male parent *B. oleracea* (2n=18, CC genome; var. 'Hong-Kong Zhonghua Jielan'). Post-inseminated ovaries of *S. alba* were

cultured *in vitro* on MS medium added with 500mg/l hydrolyzed casein and without any hormones. A total of 38 young seedlings were recovered and transferred to the field, where 12 F_1 plants survived. Two F_2 plants were obtained from self-pollination of fertile F_1 plants. From hybridization between fertile maternal F_1 and paternal *B. oleracea* a total of 68 BC_1 plants could be recovered (Figure 1). Flower buds of the parental F_1, F_2, and BC_1 plants were collected during meiosis for mitotic or meiotic chromosome preparations.

Chromosome preparation: Chromosome preparation method is developed using the technique described by Wei et al. (2005) with some modifications. Briefly, flower buds were fixed in a mixture of ethanol : acetic acid glacial (3 : 1) at 4 °C over night. They were washed 3-5 times with distilled water, then digested in 1% (W/V) cellulase "Onozuka" R-10 (Yakult Honsha Co., Ltd) and 1% (W/V) pectolyase Y-23 (Yakult Honsha Co., Ltd) dissolved in distilled water at 28 °C for 2.5-3 h. Then they were subjected to a hypotonic treatment in distilled water for 30 min before preparing spreads by the flame drying method.

Genomic DNA extraction: The extraction of *S. alba* and *B. oleracea* genomic DNAs was performed using the procedure described by Doyle and Doyle (1988).

Probes labeling and GISH: The procedures described by Wei et al. (2003, 2005) were used. In brief, *S. alba* genomic DNA was labeled with Biotin-Nick Translation Mix (Roche, Cat. No. 11745824910) and *B. oleracea* genomic DNA was labeled with Dig-Nick Translation Mix (Roche, Cat. No. 11745816910) as probes according to the procedures of the kits, respectively. The labeling results were evaluated by means of dot blots. Chromosome preparations were pretreated with 100 μg/ml RNase (in 2×SSC) at 37 °C for 1 h, rinsed briefly in 2×SSC. Chromosomal DNA was then denatured by immersing the slide in 70% deionized formamide at 70 °C for 3 min. After dehydration of the preparation in an ice-cold 70%, 95% and 100% ethanol series and air drying, 40 μl of denatured probe cocktail (5 ng/μl labeled probe DNA each, 0.5 μg/μl sheared salmon sperm DNA, 10% dextran sulphate, 50% deionized formamide, 0.1% SDS, 2×SSC) was added to the slide and hybridization was carried out at 37 °C overnight. Post-hybridization washes included a stringent wash in 20% formamide, a wash in 2×SSC and a wash in 0.1×SSC at 42 °C for 10 min, respectively, to remove weakly bound probe. Signals were firstly detected with Streptavidin-Cy3 (Amersham, Cat. No. PA43001), washed in PBS for 10 min. An immediate sequential detection was performed with Anti-Digoxigenin-Fluorescein (Roche, Cat. No. 1207741), washed in PBS for 10 min again. Slides were counterstained with 2 μg/ml DAPI (4',6-diamidino-2-phenylindole) and examined under a Leica DM IRB fluorescence microscope assembled with DFC300 CCD and FW4000 software.

Pollen viability determination: Newly opened flowers were sampled at 9-10 a.m.. Anthers were squashed and pollen grains were stained with 1% aceto-carmine and observed under microscopy. Pollen viability rate was calculated as the number of stained pollen grains/total pollen grains×100. Three views per flower were observed with nearly 500-1000 pollen grains.

Results

Chromosome constitution of the parents, F_1, F_2 and BC_1 plants

GISH results confirmed that the parents used for intergeneric hybridization had normal chromosome sets: *B. oleracea* (var. 'Hong-Kong Zhonghua Jielan') 2n=18 (Figure 2a), *S. alba* (var. 'Asta') 2n=24 (Figure 2c). Spreads of 10 F_1 hybrid plants all showed 21 chromosomes, consisting of 12 S genome chromosomes and 9 C genome chromosomes (Figure 2d and 2e), as expected. Two further F_1 hybrid plants identically showed 30 chromosomes, consisting of 12 S genome chromosomes and 18 C genome chromosomes (Figure 2f and 2g), therefore containing two set of paternal genome, This phenomenon was probably due to the formation of unreduced male gametes. Two F_2 plants showed 10 S genome chromosomes and more than 18 C genome chromosomes (Figure 2h and 2i), which indicated that unusual F_1 gametes had also been produced. In addition, BC_1 plants with 18 C genome chromosomes and 1, 5, and 6 S genome chromosomes, respectively, have been identified (Figure 2j-l), these results are summarized in Table 1.

Chromosome behavior of semi-fertile F_1 and BC_1 plants

A meiotic spread at metaphase I of a semi-fertile F_1 plant with 18 C chromosomes from *B. oleracea* aligned on the equatorial plate and paired preferentially is shown in Figure 3a: 9 S chromosomes from *S. alba* are present as univalents, other 3 S chromosomes are associated with C chromosomes, forming 3 trivalents (arrow). This indicates that homology between the C and S genomes is sufficient for chromosome pairing and the possible transfer of genetic information between these two species. The corresponding frequencies of 1-3 trivalents in F_1 PMCs are presented in Table 2. As expected chromosome segregation in anaphase/telophase I was not always regular: Figure 3b shows an S chromosome separation ratio of 6:6 in F_1 PMCs, among 62 F_1 PMCs scored, ratios of 1:11, 2:10, 3:9, 4:8, 5:7 and 6:6 were counted at frequencies of 3.2%, 11.3%, 16.1%, 17.7%, 27.4% and 24.2%, respectively (Table 2). Such, deviating segregations can result in alien addition lines in BC_1. Figure 3c shows a meiotic spread of F_1 PMCs in anaphase I, showing 4 chromosomes from *S. alba*.

A mitotic spread of BC_1-3 consisting of 18 C genome chromosomes and 6 S genome chromosomes is presented in Figure 3d, where one of the C chromosomes appears to undergo chromatin exchange with an S chromosome (arrow). Figures 3e-g indicate chromosome behavior of BC_1-3 where PMCs with six additional chromosomes of *S. alba*, two of which (arrow) are possibly associated with C chromosomes (Figures 3f). Figure 3h shows a PMC of BC_1-2 with five additional *S. alba* chromosomes, probably a pentavalent consisting of a C chromosome pair and three S chromosomes (arrow). Figures 3i-k present the chromosome behavior of BC_1-1 PMCs with one additional chromosome of *S. alba*; at diplotene, associations between C and an additional monosomic S chromosome were observed (Figures 3i).

Agronomic characters of the MAAL plants

One putative monosomic alien addition line (MAAL-1) (Fig 4c) looked much similar in morphology to the male parent *B. oleracea* (Fig 4b), and showed no aspects which is characteristic of female parent *S. alba* (Fig 4a), such as lyrate, pinnatifid, pinnatisect leaf or leaf trichomes. At the same time, MAAL-1 plant showed numerous branches like *S. alba* and smaller flower bud than *B. oleracea*, bright green sepals, varied number of petals (apetalous or deficient), reduced pollen fertility with 77.4% for MAAL-1 in contrast to 97.6% for *B. oleracea*. MAAL-1 plant was also different from *B. oleracea* by showing slender and oblanceolate leaves, basal lobes on each side of midvein, basal cauline leaves petiolate and auriculate at base.

Another plant of the putative monosomic alien addition line (MAAL-2) was similar to MAAL-1 in morphology. But the flower of MAAL-2 often showed three petals (Fig 4d) with pollen fertility being 68.5%, lower than MAAL-1.

Discussion

Intergeneric sexual F_1 hybrids between *S. alba* and *B. oleracea* were synthesized artificially. The somatic number of chromosomes of F_1 hybrid should be 21, or 42 if doubled. Unexpectedly GISH-dcFISH results showed two chromosome numbers. One was 21 as expected, consisting of a C genome from *B. oleraea* and a S genome from *S. alba*, and another was unexpectedly 30 chromosomes, consisting of two C genomes and one S genome. We suggest an occasional generation of those unexpected F_1 plants by unreduced gametes of paternal *B. oleracea*. This phenomenon was also discovered by others (Inomata 2002). Unreduced male and female gametes are generally rare but they do occur in Brassiceae (Heyn 1977, Ayotte et al. 1987). This was also found in two F_2 plants in this experiment because the C chromosome number was over eighteen as shown in Fig. 2. It is very important to obtain the unexpected F_1 hybrid plants for the production of monosomic alien addition lines. We were fortunate to have obtained aneuploids, even monosomic alien addition lines, in BC_1 plants while this had been impossible in previous experiments of distant hybridization (Gaikwad et al. 1996, Peterka et al. 2004, Wang et al. 2005b).

In F_1 and BC_1 plants, *B. oleracea* chromosomes were shown to pair preferentially and part of *S. alba* chromosomes formed trivalents with *B. oleracea* chromosomes, suggesting that intergenomic exchange might occur in the nuclei of F_1 and BC_1 plants. This was in agreement with other reports in somatic hybrids between *Brassica juncea* and *S. alba* (Gaikwad et al. 1996), and between *B. napus* and *S. alba* (Wang et al. 2005b). Therefore, the introgression of alien chromosome segments or genes from *S. alba* to *B. oleracea* can be achieved through this intergenomic recombination in backcross progenies.

We detected two BC_1 plants added with a single alien chromosome from *S. alba* in a *B. oleracea* background. A full set of monosomic alien addition lines is expected by addition of each of the twelve *S. alba* chromosomes. In the present study, the petals of MAAL-1 is apetalous or deficient, and MAAL-2 often show three petals**.** It is considered that the additional chromosomes carry genes for petal development. Monosomic alien addition lines are then powerful tool to localize genes controlling relevant agronomic traits. During diakinesis, the univalent *S. alba* chromosome looked smaller when compared to the bivalents of *B. oleracea* chromosomes (Figure 3j) and is easy to identify. Through microdissecting the univalent chromosome, some new chromosome-specific markers can be developed as described in Li et al. (2005) and Fominaya et al. (2005). In order to obtain stable disomic alien addition lines, doubled haploids (DHs) are produced underway through microspore culture and colchicine treatment. This DH lines will be useful in constructing high density genetic map and cloning objective genes, e.g. the genes controlling petal development, in our future work. In addition, these monosomic alien addition lines or DH disomic addition lines will be of interest as an intermediate material to transfer genes from *S. alba* to *B. napus* through resynthesis of *B. napus* with *B. oleracea*-*S. alba* alien addition lines and *B. rapa* (Li et al. 2006).

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Table 1: Chromosome constitutions of the parents, F_1, F_2, and BC_1 plants analyzed by GISH

Genotype	Chromosome number	Genomic constitution	Chromosomes from *B. oleracea*	Chromosomes from *S. alba*
B. oleracea	18	CC	18	
S. alba	24	SS		24
F_1	21	CS	9	12(10)[a]
(*S. alba* ♀ × *B. oleracea* ♂)	30	CCS	18	12(2)
F_2 (F_1 selfing)	>30	CC+10S+?C	>18	10(2)
BC_1		CCS(1-12)		
(Fertile F_1 ♀ × *B. oleracea* ♂)				
BC_1-1			18	1(2)
BC_1-2			18	5(4)
BC_1-3			18	6(3)

[a]Figures in parentheses = frequency

Table 2: Chromosome behavior of semi-fertile F_1 plants at diakinesis I (or metaphase I) and anaphase I analyzed by GISH

Meiosis	No. of trivalents per PMC at diakinesis I (or metaphase I)			Separation ratio of *S. alba* chromosomes at anaphase I					
	1	2	3	1:11	2:10	3:9	4:8	5:7	6:6
No. of PMCs scored	27	35	9	2	7	10	11	17	15
Frequency(%)	38.0	49.3	12.7	3.2	11.3	16.1	17.7	27.4	24.2

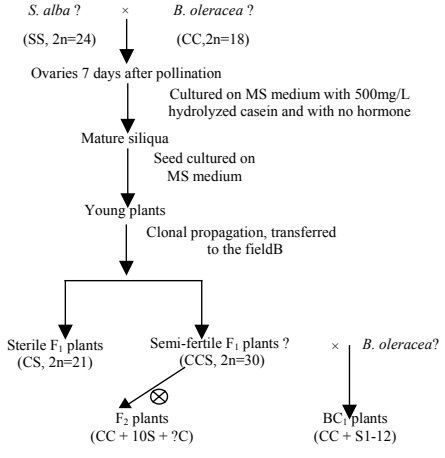

Fig. 1: The origin of F_1, F_2 and BC_1 plants

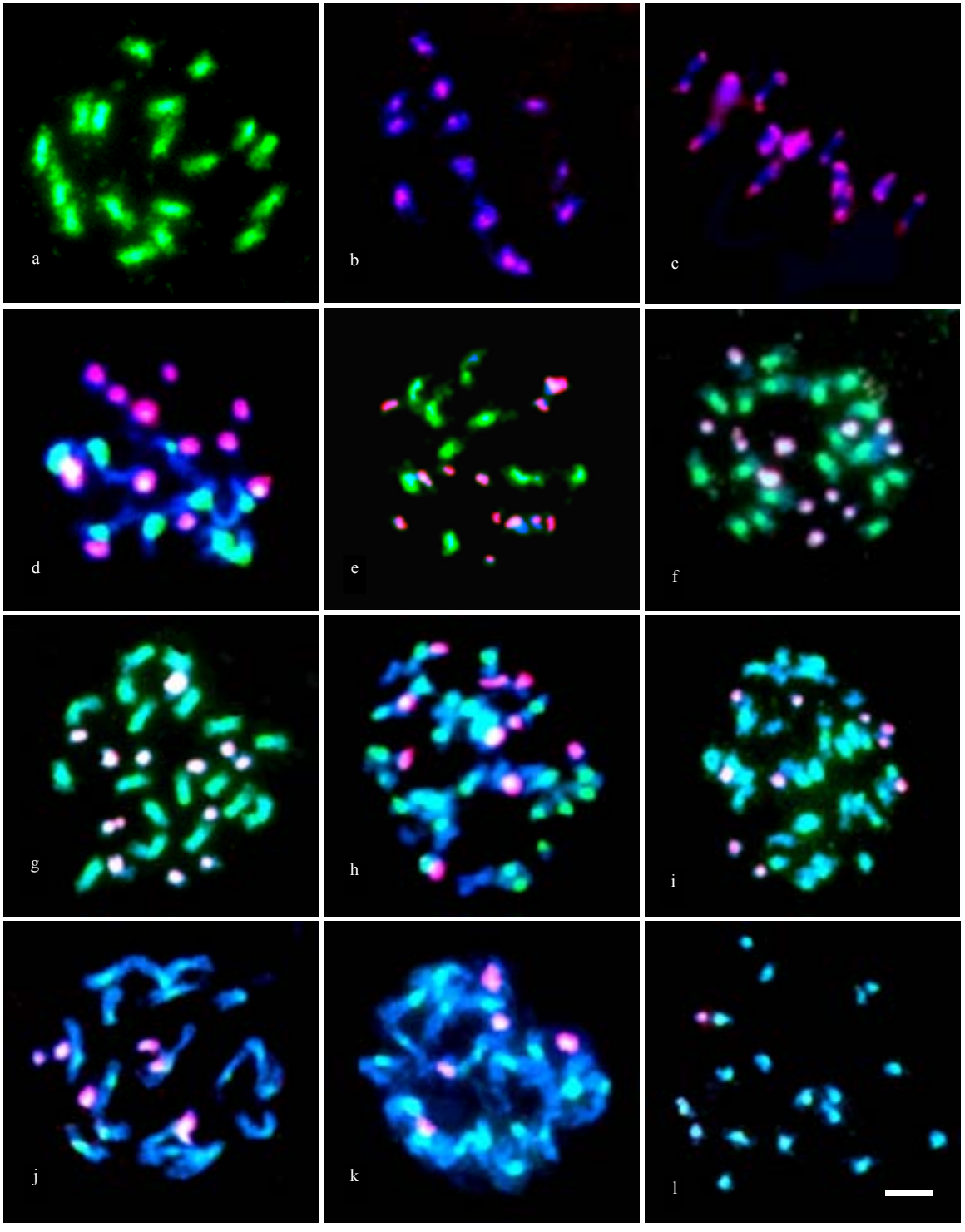

Fig. 2: Identification of chromosome constitutions in parents, F_1, F_2 and BC_1 plants by GISH. Chromatin of *S. alba* is labeled red or pale red with Cy3, while *B. oleracea* chromatin is labeled green or blue-green with Fluorescein. (a-c) Parent plants: (a) mitotic spread of *B. oleracea* with 18 chromosomes; (b) meiotic telophase I of *S. alba* with 12 chromosomes; (c) metaphase I spread of *S. alba* with 12 divalents. (d-g) F_1 plants: (d and e) mitotic spreads of sterile F_1 plants with 9 *B. oleracea* chromosomes and 12 *S. alba* chromosomes; (f and g) mitotic spreads of semi-fertile F_1 plants with 18 *B. oleracea* chromosomes and 12 *S. alba* chromosomes. (h and i) F_2 plants: mitotic spreads with 10 *S. alba* chromosomes and over 18 *B. oleracea* chromosomes. (j-l) BC_1 plants: (j) mitotic spread with 18 *B. oleracea* chromosomes and 6 additional chromosomes from *S. alba*; (k) mitotic spread with 18 *B. oleracea* chromosomes and 5 additional chromosomes from *S. alba*; (l) mitotic spread with 18 *B. oleracea* chromosomes and 1 additional chromosome from *S. alba*. Scale bar represents 5 μm.

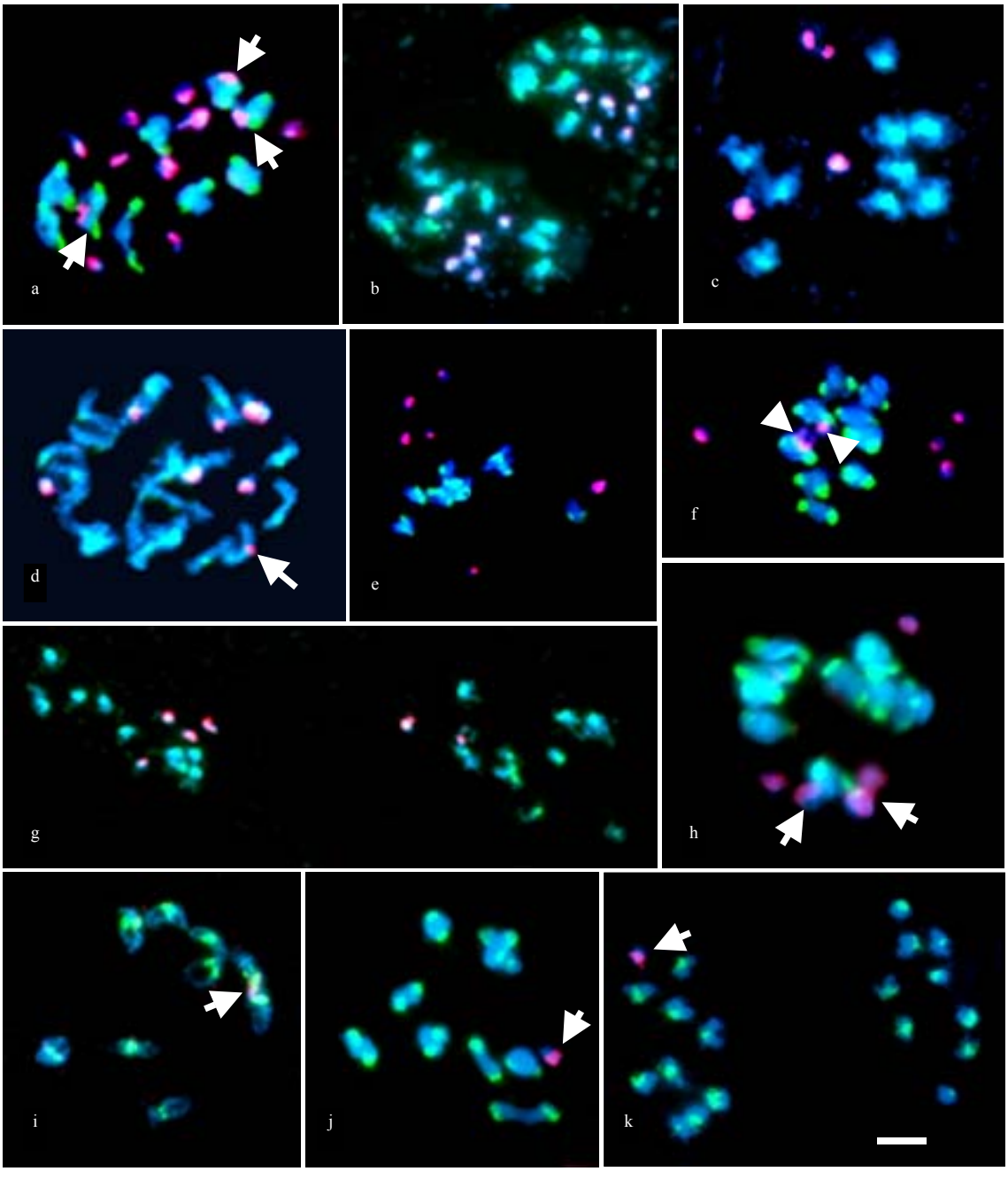

Fig. 3: Chromosome behavior of F_1 and BC_1 plants analysed by GISH. Chromatin of *S. alba* is labeled red or pale red with Cy3, while *B. oleracea* chromatin is labeled green or blue-green with Fluorescein. (a-c) F_1 plants: (a) a meiotic spread at metaphase I of PMCs showing 6 bivalents from *B. oleracea*, 3 trivalents formed by 2 C-chromsomes and one S-chromsome, and 9 univalents from *S. alba*; (b) a meiotic spread at anaphase I of PMCs showing a S chromosome separation ratio of 6:6; (c) anaphase I of a PMC showing one pole of the cell containing 4 S chromosomes. (d-g) BC_1 plants with 18 C chromosomes and 6 S chromosomes: (d) a mitotic spread displaying a S chromosome segments translocated into a C chromosome; (e) diakinesis of a PMC showing 9 II from *B. oleracea* and 6 univalents from *S. alba*; (f) diakinesis of a PMC showing 9 II from *B. oleracea*, 4 *S. alba* univalents and two putative C-S trivalents (arrow); (g) anaphase I showing a 9:9 separation of *B. oleracea* chromosomes and a 4:2 separation of *S. alba* chromosomes. (h) BC_1 plants with 18 *B. oleracea* chromosomes and 5 *S. alba* chromosomes: diakinesis of a PMC showing 8 II from *B. oleracea*, 2 univalents from *S. alba* and 1 putative C-S pentavalent (arrow). (i-k) BC_1 plants with 18 *B. oleracea* chromosomes and 1 *S. alba* chromosome: (i) diplotene of a PMC showing 8 II from *B. oleracea* and 1 putative C-S trivalent; (j) diakinesis of a PMC showing 9 II from *B. oleracea* and 1 univalent from *S. alba*; (k) anaphase I showing a 9:9 separation of *B. oleracea* chromosomes and a 1:0 separation of *S. alba* chromosomes. Scale bar represents 5 μm.

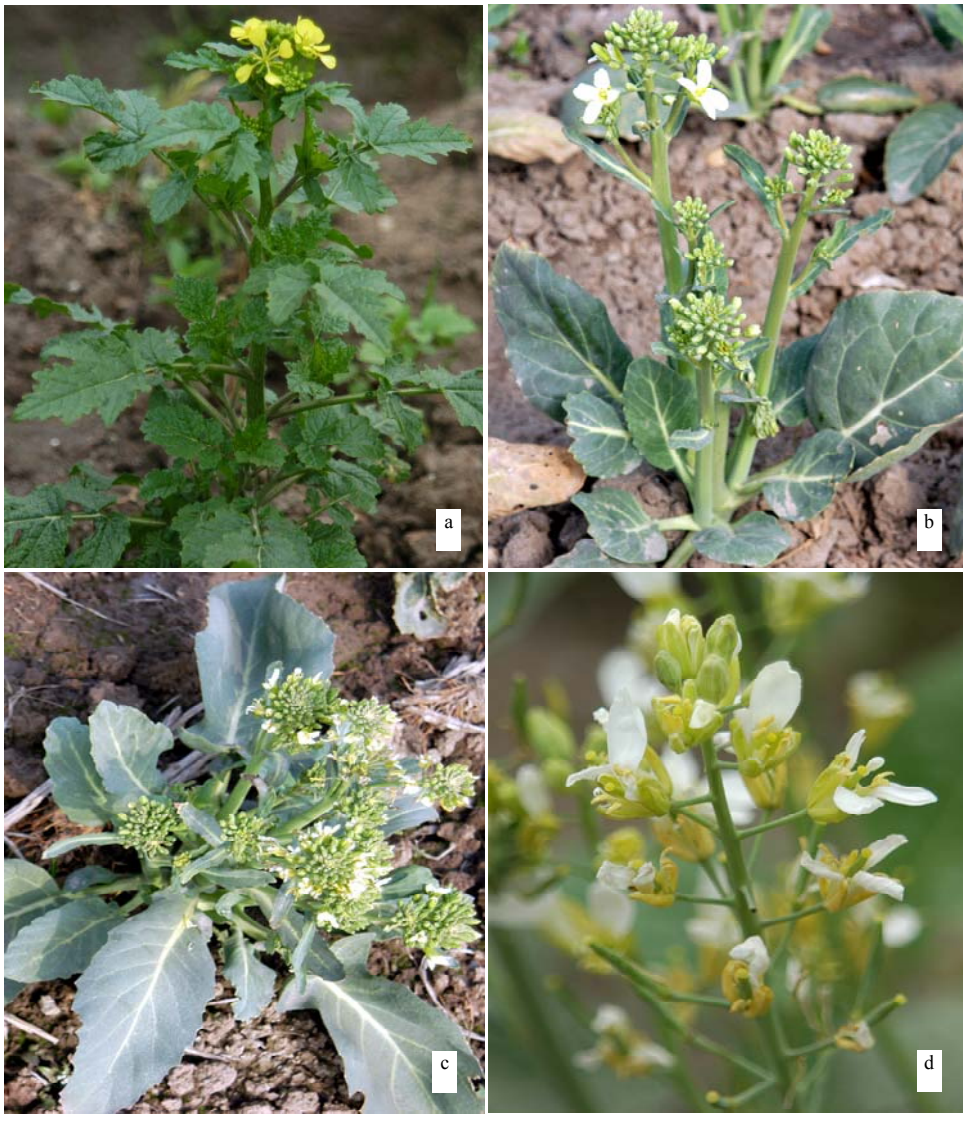

Fig. 4: Morphology of *B. oleracea*-*S. alba* monosomic alien addition lines in comparison with their parents.(a)maternal *S. alba*;(b)paternal *B. oleracea*;(c)Plant of MAAL-1;(d)inflorescence of MAAL-2.