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Transfer of resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) from *Sinapis alba* L. (white mustard) to the *Brassica napus* L. gene pool by means of sexual and somatic hybridization

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Summary. Sexual and somatic hybrid plants have been produced between *Sinapis alba* L. (white mustard) and *Brassica napus* L. (oil-seed rape), with the aim to transfer resistance to the beet cyst nematode *Heterodera schachtii* Schm. (BCN) from white mustard into the oil-seed rape gene pool. Only crosses between diploid accessions of *S. alba* ($2n = 24$, $S_{al}S_{al}$) as the pistillate parent and several *B. napus* accessions ($2n = 38$, AACC) yielded hybrid plants with 31 chromosomes. Crosses between tetraploid accessions of *S. alba* ($2n = 48$, $S_{al}S_{al}S_{al}S_{al}$) and *B. napus* were unsuccessful. Somatic hybrid plants were also obtained between a diploid accession of *S. alba* and *B. napus*. These hybrids were mitotically unstable, the number of chromosomes ranging from 56 to more than 90. Analysis of total DNA using a pea rDNA probe confirmed the hybrid nature of the sexual hybrids, whereas for the somatic hybrids a pattern identical to that of *B. napus* was obtained. Using chloroplast (cp) and mitochondrial (mt) DNA sequences, we found that all of the sexual F_1 hybrids and somatic hybrids contained cpDNA and mtDNA of the *S. alba* parent. No recombinant mtDNA or cpDNA pattern was observed. Three BC_1 plants were obtained when sexual hybrids were backcrossed with *B. napus*. Backcrossing of somatic hybrids with *B. napus* was not successful. Three sexual hybrids and one BC_1 plant, the latter obtained from a cross between a sexual hybrid and *B. napus*, were found to show a high level of BCN resistance. The level of BCN resistance of the somatic hybrids was in general high, but varied between cuttings from the same plant. Results from cytological studies of chromosome association at meiotic metaphase I in the sexual hybrids suggest partial homology between chromosomes of the AC and S_{al} genomes and thus their potential for gene exchange.

Key words: Intergeneric crosses – Somatic hybridization – *Sinapis alba* – *Brassica napus* – *Heterodera schachtii* – Nematode resistance

Introduction

In the *Cruciferae* family disease resistance, earliness, quality components and other agronomically important traits have been transferred between species belonging to the same or to different genera by means of sexual (Agnihotri et al. 1990; Batra et al. 1990; Chiang et al. 1977; Hossain et al. 1990; Rouxel et al. 1990) or somatic hybridization (Glimelius et al. 1989; Sikdar et al. 1990; Toriyama et al. 1987).

Sinapis alba L. (white mustard) is a source of resistance to *Heterodera schachtii* Schm., the beet cyst nematode (BCN) (Toxopeus and Lubberts 1979). Although *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk. (oil-seed rape) as a crop is not affected by BCN, it is a good host for the multiplication of this nematode, which causes considerable damage to a sugar beet crop. BCN resistance, therefore, would be an important trait for *B. napus* in Northwest Europe, since only resistant cultivars can be cultivated in a crop rotation system with sugar beet as one of the major crops. No

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B. napus genotypes resistant to the beet cyst nematode are known.

Several unsuccessful attempts have been made to sexually hybridize *B. napus* with *S. alba* by means of reciprocal crosses, polyploid genotypes or embryo rescue techniques (Turesson and Nordenskiöld 1943; Zenkteler 1990). Very recently, successful sexual hybridization has been reported between *S. alba* and *B. napus* (Ripley and Arnison 1990; Mathias 1991). The occurrence of chromosome association at meiotic metaphase I indicated the possibility of an exchange of traits between *S. alba* and *B. napus* (Ripley and Arnison 1990). Somatic hybrids between *S. alba* and *B. napus* have also been reported (Primard et al. 1988). These somatic and sexual hybrids, however, were not made to transfer BCN resistance but resistance to *Alternaria brassicae* or cytoplasmic traits.

This paper describes the production of BCN-resistant sexual and somatic hybrids between *S. alba* and *B. napus* and presents preliminary data on the resistance of the BC_1 generation derived from the sexual hybrids and results from morphological and cytological studies and DNA analyses of the hybrids.

Materials and methods

Plant materials

Sexual hybridization. The BCN-resistant *S. alba* L. material used consisted of the diploid cvs 'Emergo' and 'Maxi', the tetraploid cv 'Condor' and the tetraploid accession Oscar (Limagrain Genetics, Scheemda, The Netherlands). The following 14 cultivars of *B. napus* L. (oil-seed rape and fodder rape), all susceptible to *H. schachtii* Schm., were used: 'Akela', 'Blako', 'Bridger', 'Cascade', 'Darmor', 'Emerald', 'Jet Neuf', 'Mansholts Hamburger', 'Petranova', 'Rapekale', 'Rapol', 'Tantal', 'Tower', and 'Zephyr'.

Somatic hybridization. For the somatic hybridization experiments the cvs 'Emergo' and 'Maxi' of *S. alba* L. and the cvs 'Barrapo', 'Jet Neuf', 'Tower', 'Tantal' and the accession K1 (Van der Have B. V., Rilland, The Netherlands) of *B. napus* L. were used.

Sexual hybridization

Plants were grown in pots in a greenhouse without temperature and humidity control during the spring and summer and in a heated greenhouse during the autumn and winter. Reciprocal crosses were made between diploid and tetraploid accessions of *S. alba* on the one hand and *B. napus* on the other. The buds were emasculated by hand and bagged 1–2 days before anthesis. Pollination was performed 2–3 days after emasculation using freshly collected pollen. A similar procedure was followed for backcrosses.

Embryo rescue. Crosses followed by embryo rescue were performed between the *B. napus* cvs 'Jet Neuf' and 'Tantal' and the four *S. alba* accessions described above. Between 2 and 14 days after pollination developing siliquae were collected, surface sterilized and cultured according to Lelivelt and Krens (1992) with the following modifications: MS (Murashige and Skoog 1962)-based media were supplemented either with 1 mg/l indole-3-acetic acid (IAA) + 0.2 mg/l kinetin (kin) or with 2 mg/l IAA + 0.5 mg/l kin (instead of 1 mg/l IAA + 0.5 mg/l kin). After 3–4 weeks ovules were excised from the ovaries and cultured on the same medium. Emerging embryos were transferred to Medium 1 (Lelivelt and Krens 1992). After rooting plantlets were transferred to soil and grown in a greenhouse. Putative sexual hybrids hereafter are referred to as $H_{sex}1$, $H_{sex}2$, etc.

Somatic hybridization

Protoplast isolation, culture and regeneration. Protoplasts were isolated from leaves of in vitro-grown plants or from hypocotyls of in vitro-germinated seeds according to Pelletier et al. (1983) with minor modifications. The enzyme mixture used for hypocotyl segments contained 0.1% (w/v) Pectolyase Y23 (Seishin Pharmaceutical, Japan) and 1.0% (w/v) Cellulase 'Onozuka' R-10 (Yakult, Honsha Co, Japan), while an additional washing step with W5 medium (Menczel and Wolfe 1984) was performed prior to protoplast plating. Isolated protoplasts were plated at a density of 5×10^4 protoplasts/ml culture medium. Two procedures for protoplast culture were followed: the first as described by Pelletier et al. (1983) and the other according to Glimelius (1984). Calli, when 4–5 weeks old, were transferred to proliferation medium MS11 (Lelivelt and Krens 1992) before transfer to regeneration media E (Pelletier et al. 1983) or K3R (Glimelius 1984). Regenerants were placed on Medium 1 (Lelivelt and Krens 1992), and after the rooting plants were transferred to a greenhouse.

Protoplast fusion. PEG fusion, rinsing and plating of protoplasts were carried out as described earlier (Lelivelt and Krens 1992). Hypocotyl protoplasts were stained with fluorescein diacetate (FDA) by adding 30–40 µl FDA solution (5 mg FDA/ml acetone) to 20 ml enzyme solution at the beginning of protoplast isolation. Heterofusion frequency was determined by counting the number of fusion products per 500 cells. Heterokaryons could be identified by the emission of a combination of red autofluorescence by the mesophyll protoplasts and yellow fluorescein fluorescence by the hypocotyl protoplasts under UV light. After 2 days fusion products were collected by means of a micromanipulator. They were either transferred to Cuprak dishes or to Falcon dishes at a density of approximately 150 cells/0.25 ml culture medium, or alternatively 10–15 cells were cultured in 10–15 µl drops of culture medium (5–7 drops/dish) under mineral oil (Sigma) in a Falcon dish. Fusion products were cultured as described above for protoplasts. Putative somatic hybrids hereafter are described as $H_{som}1$, $H_{som}2$, etc.

DNA analyses

Total DNA was extracted from greenhouse-grown plants according to Dellaporta et al. (1983). Digestion of plant DNA (5–10 µg) with the restriction enzymes *Hin*dIII, *Bam*HI, *Eco*RI, *Eco*RV, and *Dra*I, Southern blotting transfer of the DNA onto nylon membranes (Hybond N, Amersham) and crosslinking of the DNA were carried out according to Kreike et al. (1990). A pea ribosomal DNA (rDNA), chloroplast DNA (cpDNA) and several mitochondrial DNA (mtDNA) clones were used as probes in the hybridization experiments. The rDNA probe (a 4.0 kb *Eco*RI subclone in pACyc1184 from a partial genomic library of *Pisum sativum* cv 'Rondo') and the chloroplast probe pPhcPS1 (rbc-L) were obtained from Dr. P. Zabel (Agricultural University Wageningen, The Netherlands). The petunia cpDNA probe pPCY64 has been described by de Haas et al. (1986). Maize cytochrome C oxidase subunit I (*cox*I), cytochrome C oxidase subunit 2 (*cox*II) and the alpha subunit of ATPase (*atp*a)

were provided by the University of Edinburgh, UK (Prof. C. Leaver). Maize ATPase subunits 6 (*atp6*) and 9 (*atp9*) were supplied by Dr. C. S. Levings III (North Carolina State University, USA) and cytochrome C oxidase subunit 3 (*cox*III) by Dr A. Brennicke (Institute for Gene Biological Research, Berlin, FRG). The pHH22 probe (a 10 kb *Pst*I fragment of *Spirodela oligorhiza*) has been described by de Heij et al. (1985). Probe DNA was labelled non-radioactively with digoxigenin-dUTP, hybridized to the target DNA and visualized by chemiluminescence according to Kreike et al. (1990).

Cytological observations and pollen viability

Treatment of the plant material used for cytological observations and estimation of pollen viability were carried out as described by Lelivelt and Krens (1992).

Beet cyst nematode resistance tests

Cuttings, propagated in vitro, were tested for resistance to the beet cyst nematode as described by Lelivelt and Krens (1992). Cuttings were transplanted into 96-ml PVC tubes, and after 2 weeks each was inoculated with a suspension of approximately 500 pre-hatched L2 larvae of *Heterodera schachtii* Schm. in 2 ml tap water using a veterinary syringe. Four weeks after inoculation the root system was washed free from sand and the number of mature females, hereafter referred to as cysts, was counted. A *t*-test was applied for statistical analysis of the results of the BCN resistance tests.

Results

Sexual hybridization

Out of approximately 7000 crosses that were carried out without embryo rescue and with *B. napus* as the female parent, some 2000 seeds were obtained. From these seeds only *B. napus*-type plants could be recovered; there were no putative hybrids. The reciprocal crosses with *S. alba* as the female parent, also without the application of embryo rescue techniques, were found to produce only a few seeds, from which *S. alba*-type plants but no putative hybrid plants could be obtained.

When embryo rescue was carried out the majority of the cultured ovaries contained either no seeds at all or shrivelled ovules without an embryo; some only produced embryos up to the globular stage of development, which did not result in plant formation. From the crosses with *B. napus* as female parent only 11 plants were recovered after embryo rescue, but these were not hybrids but *B. napus*-type plants. Six well-developed putative hybrid embryos and plants were obtained from crosses with the diploid accessions of *S. alba* as the pistillate parent (Table 1). $H_{sex}1$ and $H_{sex}2$ were derived from crosses between *S. alba* cv 'Emergo' and the winter oil-seed rape cv 'Jet Neuf'. $H_{sex}3$ and $H_{sex}4$ were obtained from crosses between *S. alba* cv 'Maxi' and spring oil-seed rape cv 'Tantal' and $H_{sex}5$ and $H_{sex}6$ were from *S. alba* cv 'Emergo' and *B. napus* cv 'Tantal'.

Table 1. Seed set and plant recovery from reciprocal crosses between diploid (2x, 2n=24) and tetraploid (4x, 2n=48) accessions of *S. alba* and *B. napus*, followed by embryo rescue

Cross	Ovaries cultured	Plants	Hybrids
B. napus × *S. alba* 2x	890	8	0
B. napus × *S. alba* 4x	388	3	0
S. alba 2x × *B. napus*	412	6	6
S. alba 4x × *B. napus*	322	0	0

Somatic hybridization

Protoplast regeneration. The culture of mesophyll protoplasts of *S. alba* cv 'Maxi' and 'Emergo' was unsuccessful: all of the cells died after two to three divisions. Hypocotyl protoplasts of *S. alba* cv 'Emergo' were observed to be dividing after 1 day in culture; the hypocotyl protoplasts of *S. alba* cv 'Maxi' and the mesophyll protoplasts of all of the *B. napus* cultivars used were observed to undergo cell division only after a much longer culture period. Irrespective of the two culture media used, up to 50% of the hypocotyl protoplasts of *S. alba* cv 'Emergo' began cell division, whereas only 3–5% of the protoplasts of *S. alba* cv 'Maxi' were found to divide. After 4 weeks the frequency of microcalli, when using the culture media according to Glimelius (1984), was found to be 0.5% and less than 0.01% for *S. alba* cv 'Emergo' and *S. alba* cv 'Maxi', respectively. For callus growth and regeneration, hypocotyl protoplasts were found to do better in the media of Glimelius (1984), while *B. napus* mesophyll protoplasts performed better in the media of Pelletier et al. (1983). In *B. napus* average cell division and callus formation frequencies ranged from approximately 15% and 0.2% for cv 'Jet Neuf' to 50% and 1.5% for cvs 'Barrapo' and 'Tower', respectively. Shoot regeneration from calli of *S. alba* cv 'Emergo' was observed on both regeneration media, with the highest frequency (5%) occurring on medium K3R (Glimelius 1984). Calli of *B. napus* normally produced 1 to 2 shoots on media E (Pelletier et al. 1983) or K3R. The frequency of regeneration on medium E was found to be less than 2% for *B. napus* cvs 'Jet Neuf' and 'Barrapo' and up to 22% for accession K1. Calli of *S. alba* cv 'Emergo' were found to produce up to 40 shoots per callus on medium K3R. Shoot regeneration from calli of *S. alba* or *B. napus* was observed within 1 month after transfer to regeneration medium. A study of the ploidy level of 31 regenerants of *S. alba* cv 'Emergo' revealed that 25 were diploid and 6 were tetraploid or diploid/tetraploid chimerae. Ten regenerants of *B. napus* accession K1 were all found to have the parental number of chromosomes.

Protoplast fusion. Since hypocotyl protoplasts of *S. alba* cv 'Emergo' showed better regeneration than those of cv 'Maxi', the majority of fusion experiments was carried out with protoplasts of the former cultivar and mesophyll protoplasts of the five *B. napus* accessions. PEG-induced fusion yielded 8–10% heterokaryons. Cell division and callus growth of the heterokaryons were much better in media of Glimelius (1984) than in media of Pelletier et al. (1983). Fusion experiments performed with the five *B. napus* accessions and *S. alba* cv 'Emergo' resulted in callus formation for all combination, with the highest frequencies (22–25%) being obtained when *B. napus* cv 'Tantal' or cv 'Tower' was used. Most of the putative hybrid calli produced only roots and leaf structures that lacked an apical meristem. The growth of putative hybrid calli and shoot regeneration from these calli occurred at a slower rate than on calli of the parental species *B. napus* and *S. alba*; most shoots on the putative hybrid calli were observed after 4–6 weeks of culture on medium K3R. Some of the regenerants died before transfer to Medium 1. Eight shoots from 4 putative hybrid calli were analyzed for their hybrid nature. $H_{som}1$ and $H_{som}2$-1–$H_{som}2$-5 were derived from fusion between *S. alba* cv 'Emergo' and *B. napus* accession K1. $H_{som}2$-1–$H_{som}2$-5 were obtained from the same callus containing five shoot-inducing regions, each region resulting in a large number of regenerants after subculture on Medium 1. $H_{som}3$ and $H_{som}4$ were obtained from fusion between *S. alba* cv 'Emergo' and *B. napus* cv 'Tower'.

Morphological observations and mitosis

Sexual hybrids. All of the putative hybrids showed intermediate morphological characteristics, e.g. beak length of the pods, but also characteristics typical for one of the parents. In petal colour, petal length and morphology of buds, for example, the hybrids closely resembled the *B. napus* parent; the morphology of the first leaves was more similar to that of the *S. alba* parent. All plants grew vigorously, but sometimes the leaves and stems showed growth abnormalities. Plants had flowers with six stamens and pollen production was, except for $H_{sex}3$, normal and similar to that of *B. napus* and *S. alba*. All plants had 31 chromosomes. On the basis of the results of the cytological and morphological analyses these plants are assumed to be hybrids.

Somatic hybrids. The putative somatic hybrids $H_{som}1$ and $H_{som}2$-1–$H_{som}2$-5 showed irregular growth of the leaves and flowers. The leaves were dark green and in many cases wrinkled and thick; the flowers were yellow, contained six stamens and were smaller than those of the sexual hybrids. Flower petal and sepal shape, anther and stigma morphology and beak length varied between flowers and pods on the same hybrid plant. Bud shape and morphology also varied and resembled those of either *B. napus*, *S. alba* or were intermediate. Not all of the flowers from these plants produced pollen, and in general pollen production was low. The plants did not grow as vigorously as the sexual hybrids. The numbers of chromosomes in the root-tip cells varied between and within the plants, in total ranging from 56 to more than 90. Morphological and cytological analyses strongly indicated the hybrid nature of $H_{som}1$ and $H_{som}2$. $H_{som}3$ and $H_{som}4$, however, closely resembled the *B. napus* parent and, based on morphology and cytological analyses, are assumed not to be hybrids but *B. napus* regenerants. Therefore, only data from the analyses of $H_{som}1$ and $H_{som}2$ will be given in the results described in the following sections.

DNA analyses

Nuclear DNA. Digestion of total plant DNA with *Bam*HI and hybridization with the rDNA probe were used to discriminate between the parental species from the sexual and somatic hybrids. For *B. napus* six main bands were visible at 6.0, 5.2, 5.0, 3.5 (vague), 2.5 and 2.2 kb, and for *S. alba* three main bands appeared at 5.2, 3.8 and 3.6 kb. The *B. napus* accessions each had a similar DNA pattern when hybridized with the rDNA probe, and no polymorphism was found between the two *S. alba* accessions when using this probe/enzyme combination. Hybridization of DNA from $H_{sex}1$–$H_{sex}6$ resulted in a pattern with bands from both parental species, a result that thus confirmed their hybrid nature. For hybrids $H_{som}1$ and $H_{som}2$ patterns equal to that of the *B. napus* parent were found, meaning that the hybrid nature of the somatic hybrids could not be proven with this probe/enzyme combination. Other rDNA/enzyme combinations that were tested did not show any polymorphisms.

Organelle DNA. Hybridization of total plant DNA isolated from the sexual hybrids $H_{sex}1$–$H_{sex}6$, digested with *Hin*dIII and probed with the chloroplast probe pPhcPS1(rbc-L) showed a pattern identical to that of the *S. alba* parent. No polymorphism between the parental species from the somatic hybrids was found with this probe/enzyme combination. Hybridization of *Bam*HI-digested DNA with the cpDNA sequence pPCY64, however, resulted in polymorphism between the parental species used for protoplast fusion. The somatic hybrids $H_{som}1$ and $H_{som}2$-1–$H_{som}2$-5 were found to have a cpDNA pattern equal to that of *S. alba* cv 'Emergo'.

Table 2 presents results of the analysis of mtDNA. Combinations that resulted in polymorphisms between the parental species showed that the sexual hybrids

Table 2. Southern blot analyses of total plant DNA derived from two diploid *S. alba* cultivars, three *B. napus* accessions, the sexual hybrids $H_{sex}1$–$H_{sex}6$ (*S. alba* × *B. napus*) and somatic hybrids $H_{som}1$ and $H_{som}2$ (*B. napus* (+) *S. alba*) digested with *Bam*HI, *Hin*dIII, *Eco*RI and *Eco*RV and probed with the mtDNA sequences *atp*a, *atp*6, *atp*9, *cox*I and *cox*II. For each restriction enzyme/probe combination the estimated molecular weight in kb of the polymorphic DNA is given

Enzyme	Probe	Object						
		B. napus		*S. alba*		*Hybrids*		
		Jet Neuf Tantal	K1	Emergo	Maxi	$H_{sex}1$, $H_{sex}2$ $H_{sex}5$, $H_{sex}6$	$H_{sex}3$ $H_{sex}4$	$H_{som}1$ $H_{som}2$
*Bam*HI	*atp*a	4.3	19	3.4	3.4	3.4	3.4	3.4
*Hin*dIII	*atp*a	11	5.2/5.3	11	18	11	18	11
*Eco*RI	*atp*a	4.9	10	6	6	6	6	6
*Eco*RV	*atp*a	12	16	16	16	16	16	16
*Hin*dIII	*cox*I	11	13	11	18	11	18	11
*Eco*RI	*cox*I	16	16	18	16	18	16	18
*Eco*RV	*cox*I	7	7	16	7	16	7	16
*Eco*RV	*cox*II	17/19	2.6	2.6	2.6	2.6	2.6	2.6[b]
*Bam*HI	*atp*6	5.3	19	5.3	5.3	5.3	5.3	5.3[b]
*Hin*dIII	*atp*6	3.4	2.3	3.4	3.4	3.4	3.4	3.4[b]
*Eco*RI	*atp*6	6	5.3	6	6	6	6	6[b]
*Eco*RI	*atp*9	2.7/2.8[a]	5.3	5.4	5.4	5.4	5.4	5.4[b]

[a] 2.7/2.8 = two bands visible at 2.7 and 2.8 kb, respectively
[b] Only $H_{som}2$-1 and $H_{som}2$-5 were used

Table 3. Analysis of meiotic metaphase I of pollen mother cells (PMCs) in *S. alba*, *B. napus* and five sexual hybrid plants

Object	Number of PMCs analysed	Frequency (range) of configurations				
		Univalents	Bivalents	Trivalents	Quadrivalent	Hexavalents
S. alba	30	0	12	0	0	0
B. napus	25	0	19	0	0	0
$H_{sex}1$	30	11.07 (7–13)	7.23 (6–10)	0.13 (0–2)	1.20 (0–2)	0.03 (0–1)
$H_{sex}2$	9	11.22 (11–12)	6.00 (6)	0.22 (0–1)	1.78 (1–2)	0
$H_{sex}4$	35	11.80 (10–15)	6.60 (5–9)	0.46 (0–2)	1.14 (0–2)	0
$H_{sex}5$	20	10.95 (9–13)	6.15 (6–9)	0.45 (0–2)	1.60 (0–2)	0
$H_{sex}6$	25	11.36 (11–13)	6.08 (5–9)	0.28 (0–2)	1.60 (0–2)	0.04 (0–1)
Mean of hybrids		11.34	6.53	0.38	1.38	0.02

$H_{sex}1$–$H_{sex}6$ and the somatic hybrids $H_{som}1$ and $H_{som}2$-1–$H_{som}2$-5 have a mtDNA pattern equal to that of the *S. alba* parent (Table 2). No recombinant or new mtDNA restriction fragments were observed in the sexual and somatic hybrids (Table 2).

Meiosis

Sexual hybrids. The association of chromosomes at metaphase I (MI) in hybrid pollen mother cells (PMCs) showed on average 11.3 univalents (I) + 6.5 bivalents (II) + 0.4 trivalents (III) + 1.4 quadrivalents (IV) + 0.02 hexavalents (VI) (Table 3). The most common pairing configuration was 11 I + 6 II + 2 IV, observed in 56% of the cells (Fig. 1A). Multivalent associations were found in 107 cells (90%).

At anaphase I (AI) most cells showed a 15–16 or 14–17 chromosomes distribution. Univalents predominantly moved to the poles, however some laggards were observed. Some univalents probably divided prematurely at AI, as suggested by an increase in the number of chromosomes and their smaller size at AI (Fig. 1C). Predominant chromosome distributions at anaphase II (AII) or metaphase II (MII) were found to be 31–31 or 28–34 in two-poled cells and 17-17-14-14 or 16–16–15–15 in four-poled cells. At the sporad stage dyads, triads, tetrads and pentads were found. Laggards were observed at AI and AII/telophase II, resulting in the formation of microgones at the sporad stage and micropollen. Pollen viability of F_1 hybrids ranged from 0 to 9%.

Somatic hybrids. A study of chromosome pairing behaviour in PMCs of the somatic hybrids was found to be very difficult because of the occurrence of a high number of chromosomes, of mixoploidy and because

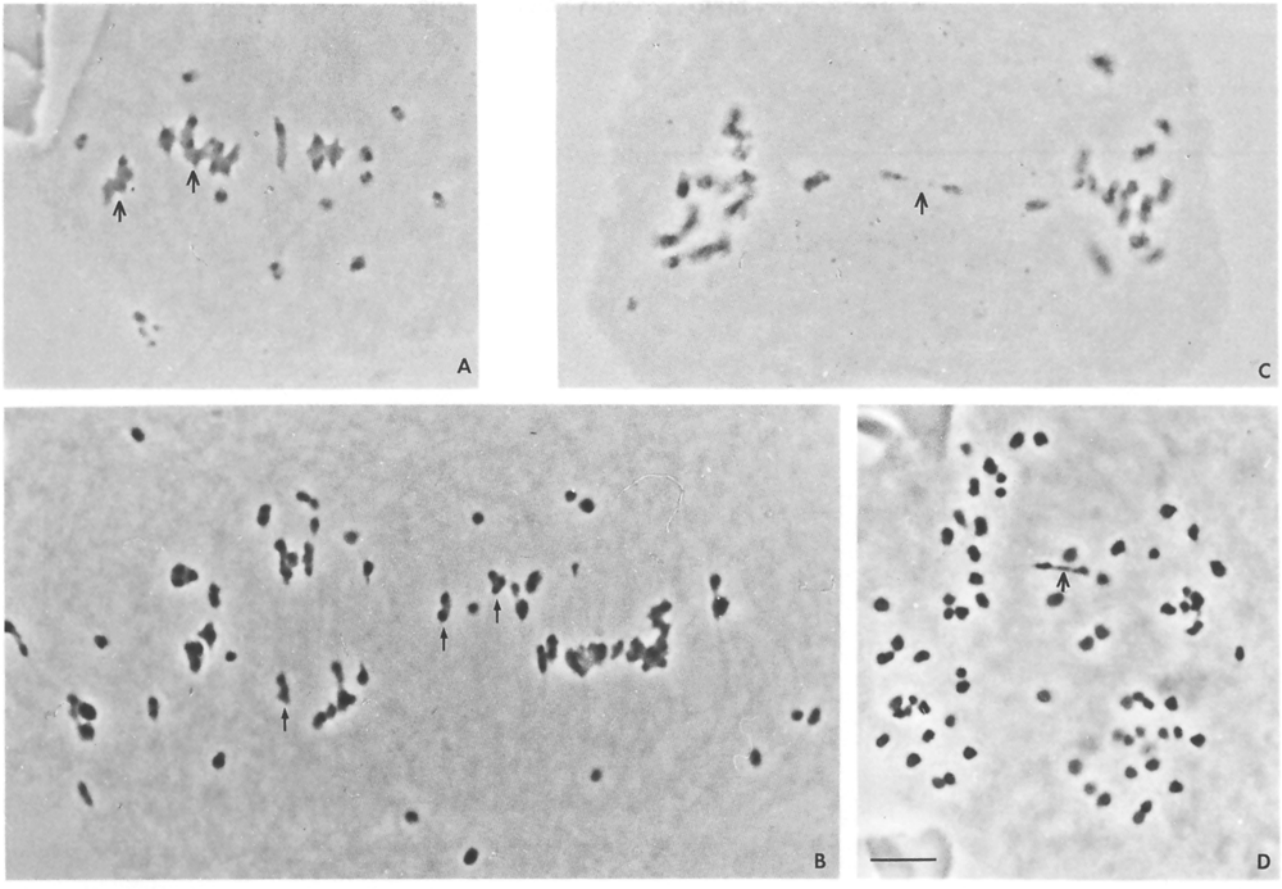

Fig. 1A–D. Meiosis in sexual and somatic hybrids of *S. alba* and *B. napus*. **A** MI in sexual hybrid showing 11 univalents + 6 bivalents + 2 quadrivalents (*arrows*). **B** MI in somatic hybrid showing prevailing bivalents (*arrows*). **C** AI in sexual hybrid showing a precociously dividing univalent (*arrow*). **D** Early AI in somatic hybrid showing a precociously dividing univalent (*arrow*). *Bar*: 5 μm

of a highly unbalanced meiosis. Most hybrid PMCs contained predominantly bivalents and some univalents; multivalents were not observed (Fig. 1B). PMCs of $H_{som}1$ cuttings with 62–71 chromosomes contained 30–35 bivalents and up to 5 univalents. A study of 25 PMCs of $H_{som}2$-1 with 86–90 chromosomes showed 37–44 bivalents and up to 10 univalents. At AI laggards were observed in some PMCs, and an unequal chromosome distribution at the two poles was generally found. In some cells a precocious division of a univalent at AI was observed (Fig. 1D). At the sporad stage dyads, triads, tetrads and pentads were detected. Pollen fertility ranged from 0–3% in $H_{som}1$ and $H_{som}2$.

BC_1 progeny

Eight hundred backcrosses of the sexual hybrid plants with several *B. napus* accessions resulted in the formation of three BC_1 plants, one from each of $H_{sex}4$, $H_{sex}5$ and $H_{sex}6$. All three BC_1 plants were obtained without the use of embryo rescue techniques. Three hundred backcrosses by means of embryo rescue did not result in plant formation, which is possibly due to the fact that these crosses were performed in the winter. The morphology of BC_1 plants closely resembled that of the *B. napus* parent, and they were found to have 50 chromosomes. The plants appeared to produce fertile pollen, since 20–30% of the pollen stained with acetocarmine. Four hundred and seventy backcrosses of somatic hybrids $H_{som}1$ and $H_{som}2$ with *B. napus* did not result in plant formation, despite the in vitro culture of 254 siliquae of them.

Beet cyst nematode resistance

In both *S. alba* cultivars used for the hybridization experiments a low frequency of not completely BCN-resistant plants was present. Therefore, not only BCN-resistant but also BCN-susceptible hybrid plants are to be expected from sexual and somatic hybridization experiments with BCN-susceptible *B. napus* (Table 4).

Sexual hybrids $H_{sex}1$, $H_{sex}2$ and $H_{sex}4$ were found to be as BCN resistant as the *S. alba* parent. Further-

Table 4. Level of BCN resistance in cuttings from parental species *B. napus*, *S. alba* and their sexual and somatic hybrids

Genotype	Average number of cysts (range)	Significant different from[a]	
		B. napus	*S. alba*
S. alba			
cv Maxi	0.0 (0)	Yes	No
cv Emergo	0.2 (0–4)	Yes	No
B. napus			
cv Jet Neuf	57.7 (13–167)	No	Yes
cv Tantal	59.6 (35–79)	No	Yes
acc K1	42.2 (3–101)	No	Yes
cv Tower	46.9 (28–68)	No	Yes
S. alba × *B. napus*			
$H_{sex}1$	0.0 (0)	Yes	No
$H_{sex}2$	1.2 (0–11)	Yes	No
$H_{sex}3$	26.7 (4–56)	Yes	Yes
$H_{sex}4$	0.1 (0–1)	Yes	No
$H_{sex}5$	51.3 (25–88)	No	Yes
$H_{sex}6$	17.4 (5–36)	Yes	Yes
$H_{sex}4$ × *B. napus*	0.0 (0)	Yes	No
$H_{sex}5$ × *B. napus*	6.0 (6)	Yes	No
S. alba (+) *B. napus*			
$H_{som}1$	1.9 (0–7)	Yes	No
$H_{som}2$–1	0.9 (0–5)	Yes	No
$H_{som}2$–2	5.9 (0–20)	Yes	Yes
$H_{som}2$–3	2.4 (0–6)	Yes	No
$H_{som}2$–4	16.2 (1–40)	Yes	Yes
$H_{som}2$–5	2.3 (0–25)	Yes	No

[a] Average number of cysts significantly different from that of the parental *B. napus* and *S. alba* species at $P<0.05$

more, no large differences could be observed between the numbers of cysts produced on different cuttings from each BCN-resistant sexual hybrid (Table 4). One backcross plant, derived from crossing $H_{sex}4$ with *B. napus* was also found to be BCN resistant. The plant obtained from backcrossing the susceptible $H_{sex}5$ hybrid with *B. napus* was found to produce only a few cysts per plant. However, only very few cuttings were tested (Table 4). Cuttings from a backcross plant derived from crossing the susceptible $H_{sex}6$ hybrid with *B. napus* died prior to evaluation of the BCN resistance tests.

The somatic hybrids $H_{som}1$ and $H_{som}2$ had on average a high level of BCN resistance. Some hybrid cuttings were as resistant as the *S. alba* parent and the three resistant sexual hybrids (Table 4). There was no relation between the level of BCN resistance of the hybrid cuttings and the number of chromosomes in root meristem cells or PMCs.

Sexual hybrids $H_{sex}3$ and $H_{sex}6$ seemed to show an intermediate level of resistance, since the number of cysts produced on these hybrids was significantly higher than that produced on resistant *S. alba* but also significantly lower than that found on susceptible *B. napus* and $H_{sex}5$ cuttings (Table 4). The roots from cuttings of the sexual hybrids were well developed and had a more abundant growth than those of *S. alba* and *B. napus* cuttings. The root size of the somatic hybrid cuttings varied and the root system was less profound and not as well developed as those of the parental species or the sexual hybrids.

Discussion

All F_1 sexual hybrids were obtained from crosses between diploid cultivars of *S. alba* and *B. napus* and these had 31 chromosomes. Most likely these plants had the genome constitution ACS_{al}. Southern blot analysis of total plant DNA probed with the rDNA sequence confirmed the hybrid nature of the sexual hybrids.

Crosses with *B. napus* as the female parent resulted in matromorphic plants only, a result that has been reported earlier for interspecific and intergeneric hybridizations in the *Cruciferae* family (Eenink 1974; Nishi et al. 1964). Unilateral crossing barriers might have been involved; these have been described not only for intergeneric crosses in the *Cruciferae* family, but also in other plant families (Abdalla and Hermsen 1972).

At meiotic MI, hybrid PMCs showed in addition to quadrivalents the presence of trivalents and hexavalents (Table 3), which had not been observed for the *S. alba* × *B. napus* hybrids produced by Ripley and Arnison (1990). Trivalent formation, however, has been found in ACS_{ar} hybrids of *Sinapis arvensis* and *B. napus* (Mizushima 1980) and in ACD_e hybrids of *Diplotaxis erucoides* and *B. napus* (Delourme et al. 1989).

Only limited information is available on the pairing behaviour and relationship of chromosomes from the S_{al} and AC genomes. U et al. (1937) reported the exclusive occurrence of 21 univalents at MI in an amphihaploid hybrid between *S. alba* and *B. oleracea*, but these results cannot exclude allosyndetic pairing in other hybrids having the A and S_{al} genomes. The degree of autosyndesis in the S_{al} genome is unknown, while the maximal number of bivalents observed in AC hybrids (8 II:Mizushima 1980) or haploids of *B. napus* (7.73 II:Tai and Ikonen 1988) was found to be similar to the number of bivalents observed in the ACS_{al} hybrids examined in this study (Table 3). This would suggest that allosyndetic pairing between either A or C chromosomes and S_{al} chromosomes might not have occurred. However, our observation of fewer than 12 univalents and the formation of multivalents, which were not observed in AC hybrids or haploids of *B. napus* (Mizushima 1980), strongly suggests that S_{al}

chromosomes in the ACS_{al} plants were involved in allosyndetic pairing.

A high frequency, up to 25%, of dyads was found in $H_{sex}1$, $H_{sex}3$, $H_{sex}4$ and $H_{sex}5$. Presumably these 2n gametes were more vital than the n gametes, since all BC_1 plants from sexual hybrids are thought to have resulted from the fusion of an unreduced female F_1 gamete (ACS_{al}, 2n = 31) and a *B. napus* gamete (AC, n = 19), which has also been reported by Ripley and Arnison (1990).

Shoot regeneration of *S. alba* calli is difficult. It has been described by Binding et al. (1982), but without much detail on plant material and protoplast culture procedures. Primard et al. (1988) were not able to obtain plant regeneration from mesophyll protoplasts of this species. This is similar to our results, but the experiments reported here show that regeneration of hypocotyl protoplasts from *S. alba* can be achieved. Protoplast fusion of several *B. napus* cultivars with *S. alba* resulted in callus growth for all combinations, but successful plant regeneration of hybrids could be obtained only in two combinations. It is assumed that the genetic potential for protoplast division, callus proliferation and regeneration of the individual parental genotypes as well as their genetic distance will affect the production of (stable) somatic hybrid plants. This is reflected to some extend by the results from fusion experiments with *B. napus* cvs 'Barrapo', 'Tantal' and 'Jet Neuf': there was low callus growth and/or shoot regeneration in protoplast isolation experiments, from which no regenerants were obtained in the fusion experiments with two *S. alba* cultivars.

The hybrid nature of the somatic hybrids was not confirmed by Southern blot hybridization with the pea rDNA probe, which showed the *B. napus* pattern. This may be explained by the loss of some *S. alba* chromosomes in the somatic hybrid genomes. Hybridization of total DNA with cpDNA and mtDNA sequences could also not provide complete evidence for the hybrid nature, since $H_{som}1$ and $H_{som}2$ showed a *S. alba* pattern. However, morphological analyses of $H_{som}1$ and $H_{som}2$ demonstrated their hybrid nature. The somatic hybrids $H_{som}1$ and $H_{som}2$ were found to be mitotically and meiotically unstable, but in some cuttings a number of chromosomes near to the expected number of 62 was observed. The deviation from the expected number of chromosomes and the observation of univalents in hybrid PMCs at meiotic MI could be explained by the occurrence of multiple fusion events on the one hand ($H_{som}2$) and by the duplication and preferential loss of chromosomes in the hybrid calli and/or plants on the other. Chromosome instability has also been found to occur in other somatic hybrids involving *Cruciferae* species (Lelivelt and Krens 1992; Sundberg 1991). Some degree of chromosome instability might be linked to the tissue culture phase. A rather high frequency of polyploidization in hypocotyl protoplast-derived regenerants of *S. alba* was also observed in our experiments.

In the sexual hybrids $H_{sex}1$, $H_{sex}2$, and $H_{sex}4$ the level of BCN resistance was high, stable and similar to that of the resistant *S. alba* parent (Table 4). Also, a BC_1 plant obtained after backcrossing of BCN-resistant $H_{sex}4$ with *B. napus* showed a high level of BCN resistance. These results might suggest that the *S. alba* gene(s) responsible for the BCN resistance are expressed in sexual hybrids with genome constitution ACS_{al} and the backcrossing type $AACCS_{al}$.

Hybrids $H_{sex}3$ and $H_{sex}6$ were found to be BCN resistant at a level intermediate between the parental species (Table 4). It is thought that these hybrids were obtained from the fusion of a BCN-susceptible *S. alba* and *B. napus* gamete, since the number of cysts observed on BCN-susceptible *B. napus* and *S. alba* genotypes was found to also vary considerably on different root systems (Table 4).

For the cuttings from $H_{som}1$ and $H_{som}2$ the level of BCN resistance and their number of chromosomes varied, but some cuttings were found to have a level of resistance not different from that of the resistant *S. alba* parent. This was also observed in a somatic hybrid between *B. napus* and *R. sativus* (Lelivelt and Krens 1992). No correlation between BCN resistance and number of chromosomes in individual plants could be found, which is in accordance with earlier results on *B. napus* and *R. sativus* hybridization (Lelivelt and Krens 1992).

This paper reports a high level of BCN resistance in sexual as well as somatic hybrids of *S. alba* and *B. napus*. However, the somatic hybrids were mitotically unstable and sterile as opposed to the sexual hybrids. This makes the somatic hybrids obtained in this study less suitable for breeding purposes. Further backcrosses of hybrid and BC_1 plants with *B. napus* are needed to eliminate undesired *S. alba* traits and to study the possibility of introgression of BCN resistance in *B. napus* cultivars.

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