INTRODUCTION OF BEET CYST NEMATODE RESISTANCE FROM *SINAPIS ALBA* L. AND *RAPHANUS SATIVUS* L. INTO *BRASSICA NAPUS* L. (OIL-SEED RAPE) THROUGH SEXUAL AND SOMATIC HYBRIDIZATION

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Proefschrift

ABSTRACT

Experiments were performed to select for beet cyst nematode (*Heterodera schachtii* Schm., abbrev. BCN) resistant genotypes of *Brassica napus* L. (oil-seed rape), and to introduce BCN-resistance from the related species *Raphanus sativus* L. (oil-radish) and *Sinapis alba* L. (white mustard) into oil-seed rape.

Sexual hybridization between *B. napus* and *R. sativus* did not result in hybrid plants, whereas from about 800 crosses between *B. napus* and the amphidiploid x*Brassicoraphanus* Sageret 284 F_1 hybrid plants were obtained. Sexual hybridization between *B. napus* and *S. alba* was only successful when diploid accessions of *S. alba* were used as the female parent. Crossability between these species was poor; only six hybrids were obtained out of approximately 10,000 crosses. The poor crossability in the intergeneric crosses was shown to be the cause of various breeding barriers. Somatic hybridization between *B. napus* and either *R. sativus* or *S. alba* resulted in a few somatic hybrid plants. Putative F_1 hybrids and somatic hybrid plants were characterized by their morphology, cytology, by DNA-analysis and by scoring resistance to BCN. Somatic hybrid plants were found to be unstable for the number of chromosomes and for BCN-resistance. Some F_1 hybrids, somatic hybrids and BC_1 plants, derived from crossing F_1 hybrids to *B. napus* as male parent had a high level of BCN-resistance, not different from that of the resistant parental genotypes. Finally, the mechanism of resistance to BCN in resistant *S. alba*, *R. sativus* and x*Brassico-raphanus* was expressed in F_1 hybrids derived from crosses between resistant genotypes of these three species and *B. napus*.

Keywords: *Brassica napus*, oil-seed rape, *Raphanus sativus*, *Sinapis alba*, x*Brassicoraphanus*, sexual hybridization, somatic hybridization, incongruity, intergeneric hybrids, fertility, hybrid characterization, cytogenetical analysis, RFLP analysis, nematode resistance, *Heterodera schachtii* Schm., beet cyst nematode, resistance mechanism, partial resistance, introgression

Stellingen behorende bij het proefschrift getiteld:

Introduction of beet cyst nematode resistance from *Sinapis alba* L. and *Raphanus sativus* L. into *Brassica napus* L. (oil-seed rape) through sexual and somatic hybridization.

Cilia L.C. Lelivelt   Wageningen, 30 maart 1993

1. Het lage niveau van bca-resistentie in genetisch uiteenlopende herkomsten van *Brassica napus* L. reduceert in hoge mate de kans op een succesvolle veredeling binnen deze soort op volledige resistentie tegen het bca. *Dit proefschrift.*

2. Er mag worden aangenomen, dat de resistenties tegen bca in *Raphanus sativus* L. en *Sinapis alba* L. op een verschillend mechanisme berusten. *Dit proefschrift.*

3. Het is urgent om op korte termijn een algemeen aanvaarde wetenschappelij-ke aanduiding in te voeren voor somatische hybriden, analoog aan de reeds aanvaarde notatie F_1 hybride voor een sexuele hybride.

4. De geplande afschaffing van de militaire dienstplicht bevordert de emancipatie van mannen.

5. De hedendaagse veredeling kan tot een optimaal resultaat komen indien de biotechnoloog "groene" en de praktische veredelaar "witte" vingers heeft.

6. Met de toename van de kwantiteit van gegevens bij de weersverwachting op de televisie is de kwaliteit wetenschappelijk gezien toegenomen, maar praktisch gezien afgenomen.

7. Om de vooruitgang in de plaatselijke infrastructuur te illustreren wordt door "De Nederlandse Spoorwegen" veelal de spreuk: "van knelpunt naar knoop-punt" gebruikt. Volgens Van Dale betekent knooppunt figuurlijk echter ook knelpunt. Dit geeft aan dat de NS in de toekomst opnieuw beperkt zal worden door de knelpunten in de infrastructuur.

8. Het uitvoeren en afronden van een promotieonderzoek is behalve een proeve van bekwaamheid tot het zelfstandig beoefenen van de wetenschap veelal ook een proeve van iemand's doorzettingsvermogen.

9. De moderne telecommunicatie-apparatuur kan de fundamentele persoonlijke vrijheid aantasten, zelfs van de meest assertieve mensen.

10. Incongruentie, een mechanisme dat een intieme partner relatie kan verstoren, kan ook optreden bij de fusie tussen instituten voor landbouwkundig onderzoek.
    Hogenboom NG (1973) Incongruity and incompatibility in intimate partner relationships. Acad. Proefschrift, LH Wageningen.

The cover shows the variation in flower appearance and colour of several BC_1 plants, which were obtained after backcrossing the F_1 (x*Brassicoraphanus* x *B. napus*) to *B. napus* as male parent (photo: TFDL-fotodienst, established at ATO-DLO)

CONTENTS

CHAPTER 1

General introduction

Introduction

The white beet cyst nematode, *Heterodera schachtii* Schmidt 1871, (abbrev. BCN) is a serious threat in beet growing areas in North-West Europe (Heijbroek *et al*. 1988) and the USA (Steele and Savitsky 1981). The host-range of this pathogen is wide and includes arable and horticultural crops belonging to several plant families, such as *Cruciferae*, e.g. *Brassica*, *Raphanus* and *Sinapis* species, *Leguminosae* and *Chenopodiaceae*, e.g. *Beta* species (Baukloh 1976, Goffart 1943, Jones 1950, Raski 1952).

Sugar beet is the second most important crop in Dutch arable farming, after potato. Damage due to infestation with beet cyst nematode in sugar beet, *Beta vulgaris* L., may be largely ascribed to narrow crop rotations. To reduce the nematode population in the soil below the economical damage threshold level, soil treatment with nematicides can be applied. However, chemical control of BCN has been found expensive and not completely effective (Griffin 1987, Steudel and Thieleman 1967). In the future it will become unacceptable for environmental reasons.

Another way to reduce the nematode population and to prevent economical damage is to grow resistant varieties. Up to date resistant commercial varieties of the cultivated sugar beet are not available (Müller 1992). For more than fifty years much time and effort have been invested in the introduction of resistance from wild beet species into sugar beet (Golden 1958, Lange *et al*. 1990, Savitsky 1960). In addition, also breeding for high levels of partial resistance in sugar beet has been attempted, but so far this has also not resulted in resistant cultivars (Curtis 1970, Heijbroek 1977, Mesken and Lekkerkerker 1988). If chemical control of BCN cannot be applied it will be necessary, therefore, to grow sugar beet in a crop rotation that does not include any other BCN-susceptible crop.

Brassica napus L. ssp. *oleifera* (Metzg.) Sinsk. (oil-seed rape), another host plant of BCN, is an important oil-producing species in North-West Europe and Canada (Downey and Röbbelen 1989, Hatje 1989). In contrast to other European countries, the increase in acreage of oil-seed rape in The Netherlands has been limited. This is mainly due to the fact that it is cultivated only in a wide crop rotation with sugar beet or in areas where sugar beet cannot be grown. Hence, breeding for BCN-resistant genotypes of *B. napus* would resolve one of the major constraints in increasing the cultivation of this crop in The Netherlands. The problem of intercropping has also become of increasing importance in countries such as France and Germany (Thierfelder *et al*. 1992).

The aim of the present study was to explore the enhancement and/or introduction of resistance to *H. schachtii* into *B. napus*. Several methods may be applied in order to obtain BCN-resistant oil-seed rape. Selection for partial resistance to BCN in *B. napus*, but also in other host plant-pathogen interactions, is suggested to be of great value when aiming at a durable type of resistance (Harrewijn 1987, Parlevliet 1976). Alternatively, the production of hybrid plants between related *Cruciferae* species with a high level of BCN-resistance and *B. napus* may ultimately result in BCN-resistant *B. napus*. A study on mechanisms of resistance in host-parasite interactions between BCN and resistant and susceptible host plants may help to assess the value of several sources of

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resistance to BCN in *Cruciferae* species.

Beet cyst nematode and sources of resistance

The beet cyst nematode was first described by Schacht (1859). *H. schachtii* is a bisexual nematode and its multiplication depends on the development of male and female nematodes (Golden 1959). Infested plants can be recognized by the occurrence on their root systems of lemon shaped adult females or of cysts, which are actually the remains of dead female bodies filled with eggs. During ripening the colour of the cysts changes from white to brown.

The life cycle of *H. schachtii* has been described in detail by Raski (1950). The embryonic development of the eggs occurs within the cysts. First stage larvae (J_1 larvae) molt and develop into J_2 larvae. The J_2 larvae complete their development before they are hatched from the cysts under the influence of plant root exudates. After penetration into and establishment within host roots, the J_2 larvae undergo a second molt. At the J_3 stage the sex differentiation of the larvae takes place. The male larvae undergo two extra molts before they reach the free living adult stage. Similarly, female larvae undergo two more molts before maturity. After fertilization of the eggs by the males, the females die, while the eggs survive within the cysts.

In the developmental cycle a low multiplication rate of nematodes can be the resultant of one or more of the following features: a decreased number of penetrating larvae, an inhibition of nematode development, a lower proportion of differentiated female nematodes relative to the number of males, a decreased number of fertilized and developed females and of eggs per adult female. The number of cysts on a root system is often used as a criterion for the level of resistance, since it is relatively easy to observe and since it is a good measure of the major part of the life cycle of BCN (Heijbroek *et al.* 1988, Lange *et al.* 1989, Müller 1992, Toxopeus and Lubberts 1979).

In recent investigations selection for resistance to BCN in *B. napus* has been applied (Bowen *et al.* 1986, Harrewijn 1987). It is not clear whether in this predominantly cross-breeding crop genetic variation for BCN-resistance occurs, as was recorded to exist in *Beta maritima* and *Beta vulgaris* (Curtis 1970, Heijbroek 1977, Mesken and Lekkerkerker 1988). The level of partial resistance in *B. napus* or in the other *Brassica* species appears to be very low (Baukloh 1976, Bowen *et al.* 1986, Harrewijn 1987, Talatschian 1974). Good resistance to *H. schachtii* has been found in related species, e.g. *Raphanus sativus* L. ssp. *oleiferus* (DC.) Metzg. (fodder radish) and *Sinapis alba* L. (white mustard) (Baukloh 1976, Lubberts and Toxopeus 1982). Cultivation of these nematode resistant green manure crops has been proven to be successful in reducing the nematode population in the soil (Steudel and Müller 1981). Little is known about the inheritance of this resistance. For the *R. sativus* resistance a dominant and monogenic inheritance is assumed (Baukloh 1976). The inheritance of resistance in *S. alba* has not been described.

Sexual and somatic hybridization in Cruciferae

Sexual hybridization is used when possible, to transfer agronomically important traits coded by the nuclear, chloroplast or mitochondrial genomes, between species belonging to different families and different genera or between species from the same genus. The success of interspecific or intergeneric sexual hybridization may be limited or hampered due to pre- and post-zygotic barriers. In recent years, however, various in vitro methods, particularly embryo rescue, have been employed successfully in interspecific and intergeneric sexual hybridization of a broad range of genotypes belonging to species of *Cruciferae* (Gundimeda *et al.* 1992, Harberd and McArthur 1980, Namai *et al.* 1980, Namai 1987, Prakash and Hinata 1980).

Somatic hybridization has been indicated to be a promising tool for the transfer of traits between sexually incongruous species in the *Cruciferae* family and other plant families (Glimelius *et al.* 1991). However, limitations to the use of this technique have also been discerned (Bauer 1990, Glimelius *et al.* 1991, Kirti *et al.* 1992). A successful somatic hybridization may be hampered by lack of a suitable protocol for protoplast culture and plant regeneration. Moreover, combining distantly related species often may result in asymmetric, genetically unstable hybrid plants (Fahleson *et al.* 1988, Hinnisdaels *et al.* 1988). Hence, the rate of success in both sexual and somatic hybridization experiments will be influenced by the genotypes used, their genetic distance and by the experimental conditions applied.

Apart from characters encoded by the organelle DNA, both somatic and sexual hybridization may aim at the introgression of desired nuclear traits into the recipient genome. Introgression of traits via normal recombination or induced recombination using sexual hybridization has been reported (Anderson 1949, Driscoll and Jensen 1963, Savitzky 1978). Indications for DNA re-arrangements in somatic hybrids have been described more recently (Mattheij *et al.* 1992, Sjödin and Glimelius 1989, Vries *et al.* 1987, Wijbrandi *et al.* 1990).

In the present study intergeneric sexual hybridization has been applied involving BCN-susceptible genotypes from the species *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk. (AACC, $2n = 38$), and BCN-resistant genotypes from *Raphanus sativus* L. ssp. *oleiferus* (DC) Metzg. (RR, $2n = 18$), *Sinapis alba* L. 2x ($S_{al}S_{al}$, $2n = 24$), *S. alba* 4x ($S_{al}S_{al}S_{al}S_{al}$, $2n = 48$) and x*Brassicoraphanus* Sageret (AARR, $2n = 38$). Interrelationship, genome constitution and number of chromosomes of the parental species and their hybrids are presented in Figure 1 (after U 1935 and Mizushima 1980). Earlier investigations on pairing of chromosomes at meiotic MI have indicated partial homology of the genomes A and C with the genome R (Dolstra 1982, McNaughton 1973, Mizushima 1980, Namai 1976, 1978). More recently, some chromosome association was also reported between the genomes A and/or C and the genome S_{al} (Chèvre *et al.* 1991, Harberd and McArthur 1980, Ripley and Arnison 1990). These data indicate that introgression of the BCN-resistance from *S. alba* and *R. sativus* into *B. napus* is feasible.

Following the International Code of Botanical Nomenclature (1983), Oost (1984) suggested that the name for hybrids between *Brassica* and *Raphanus* species should be x*Brassicoraphanus* Sageret. Toxopeus (1985) proposed
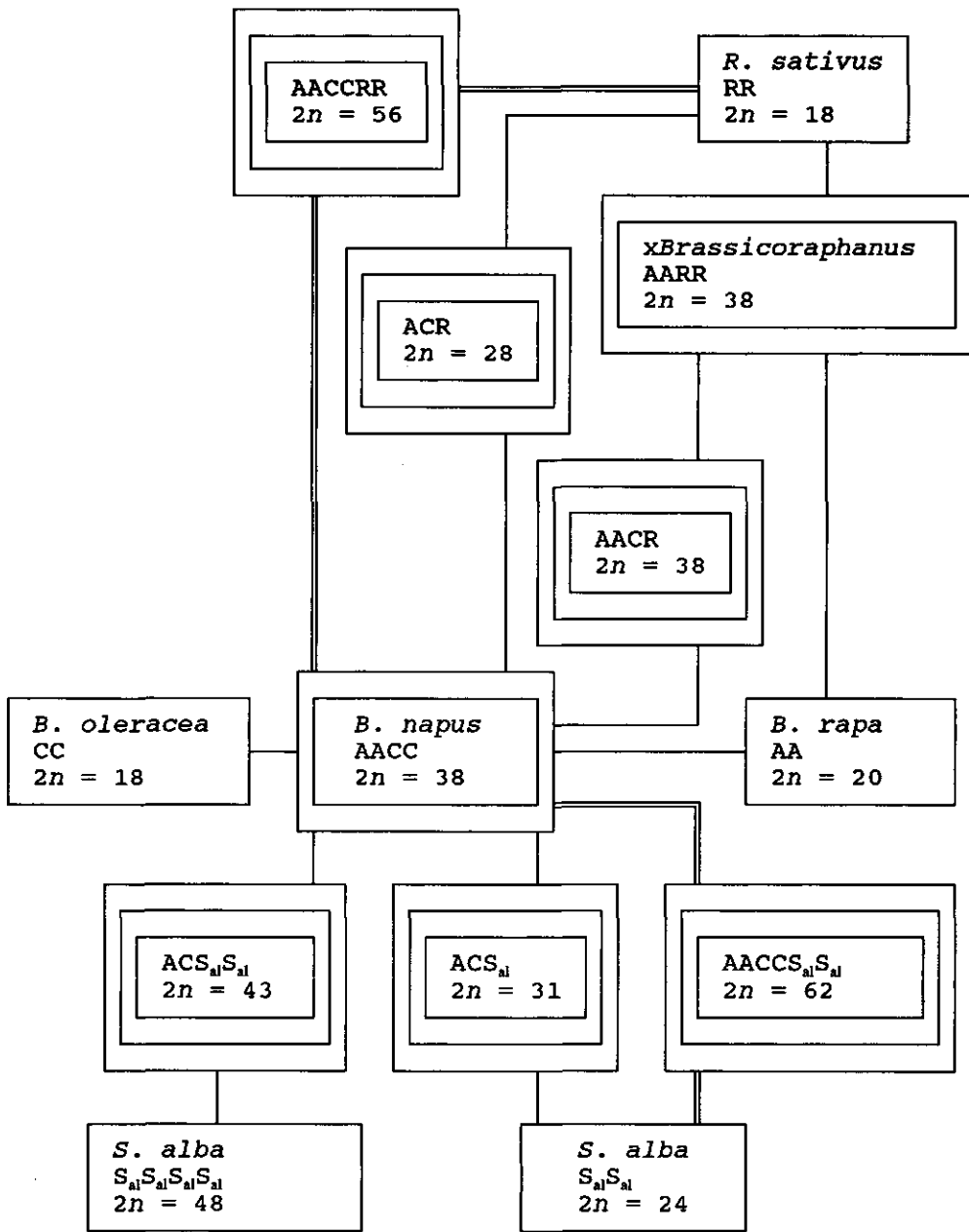
Figure 1. Interrelationship, genome designation and number of chromosomes of *Raphanus sativus*, *Sinapis alba*, *Brassica* species and their somatic and sexual hybrids. = somatic hybridization, - sexual hybridization.

Table 1. Reported studies of intergeneric sexual hybridization between *B. napus* (AACC) and diploid or tetraploid *Raphanus sativus* (RR or RRRR), between *B. napus* and x*Brassicoraphanus* (AARR), and between *B. napus* and diploid or tetraploid *Sinapis alba* ($S_{al}S_{al}$ or $S_{al}S_{al}S_{al}S_{al}$)

Cross	Number of pollinations	Number of hybrids	Reference
AACC x RR	250	0	Becker (1951)
	414	0	Nishi *et al.* (1964)
	26	0	Valdivia and Badilla (1977)
	#	>1	McNaughton and Ross (1978)
	30		Takeshita *et al.* (1980)
	6212	37	Thierfelder *et al.* (1992)
RR x AACC	263	0	Becker (1951)
	397	0	Tokumasu (1965)
	12	0	Takeshita *et al.* (1980)
	765	34	Paulmann and Röbbelen (1988)
	6155	101	Thierfelder *et al.* (1992)
AACC x RRRR	300	6	Turesson and Nordenskiöld (1943)
RRRR x AACC	#	7	Chopinet (1942) (1944)
	300	3	Turesson and Nordenskiöld (1943)
AARR x AACC	413	48*	Dolstra (1982)
AACC x AARR	349	4*	Dolstra (1982)
AACC x $S_{al}S_{al}$	148	2	Heyn (1977)
	70	36**	Valdivia and Badilla (1977)
	#	>1	Banga (1986)
	104	3	Ripley and Arnison (1990)
	12	0	Zenkteler (1990)
	586	11	Chèvre *et al.* (1991)
	#	0	Mathias (1991)
$S_{al}S_{al}$ x AACC	164	24	Ripley and Arnison (1990)
	412	9	Chèvre *et al.* (1991)
	#	4	Mathias (1991)
AACC x $S_{al}S_{al}S_{al}S_{al}$	60	0	Turesson and Nordenskiöld (1943)

* Not all seeds sown; ** Total number of seeds, hybrid seeds not mentioned; # Not mentioned

Raparadish for a cultivar group of the AARR hybrid type. McNaughton (1979) suggested the name Radicole for allotetraploids of the RRCC hybrid type, derived from reciprocal intergeneric crosses between *B. oleracea* and *R. sativus*. *B. napus* by itself is a natural amphidiploid or allotetraploid species and is assumed to be derived from hybridization between *B. rapa* (AA, $2n = 18$) and *B. oleracea* (CC, $2n = 20$) (Morinaga 1929, U 1935, Fig. 1).

Sexual hybridization between *R. sativus* and *B. napus* has often been carried out in order to transfer traits between these species, but in general with limited success (Table 1). More successful efforts have been described for intergeneric sexual hybridization between other *Brassica* species and *R. sativus* (Dolstra 1982, Mizushima 1980, Namai *et al.* 1980). Few reports describe crosses between *B. napus* or other *Brassica* species and *S. alba* (Harberd and McArthur 1980, Hinata *et al.* 1974, Mohapatra and Bajaj 1987, Nishi *et al.* 1964, U *et al.* 1937, Table 1). Reciprocal crosses between *B. napus* and *S. alba* or *R. sativus* genotypes have been performed at the diploid and tetraploid level and with a broad range of genotypes (see References in Table 1). Also, backcrosses of AACCRR hybrids, and more recently of $ACS_{al}/ ACS_{al}S_{al}$ hybrids, to *B. napus* as recurrent parent have been successful (Chèvre *et al.* 1991, Heyn 1978, Paulmann and Röbbelen 1988, Ripley and Arnison 1990). From the amphidiploid x*Brassicoraphanus*, the AARR type has been used less frequently than the CCRR type in crosses with *B. napus* (Agnihotri *et al.* 1990, Karpechenko 1937, Rousselle 1979, Table 1).

At the start of this research, only very few reports on symmetric and asymmetric somatic hybridization experiments between diploid *S. alba* or *R. sativus* and *B. napus* were known (Primard *et al.* 1988, Sakai and Imamura 1990). Currently, more information has become available on intergeneric somatic hybridizations involving *B. napus* (Table 2), or other *Brassica* species (Hagimori *et al.* 1992), with either *R. sativus* or *S. alba*. Figure 1 presents the relationship and the presumed genome constitution of symmetric somatic hybrids between diploid *S. alba* genotypes fused with *B. napus* or diploid *R. sativus* fused with *B. napus*.

Mechanisms of resistance in host plant-cyst nematode interactions

A variety of mechanisms of resistance between host species, when invaded by cyst nematodes have been described. Resistance may be brought about by lack of attraction of host plants to nematodes, by deficiencies in nutrients for the nematodes in the roots, by the lack of an effect of nematode secretions on the cells of the host plants, by browning and necrosis of host tissue at feeding sites (hypersensitive reaction) or by either induced production or constitutive presence of nematicidal/nematostatic compounds in host plant tissue(s) (Gommers 1981, Rohde 1965). Hence, the mechanism of resistance in the host-pathogen interaction may act at several stages in the life cycle of cyst nematodes. For instance, a decreased penetration of the roots by juveniles of BCN has been observed in resistant plants of *Beta* species (Johnson and Viglierchio 1969a). Also, necrosis of host plant tissue and retardation of growth of larvae has been described to occur in resistant host plants, e.g. wild *Beta*

Table 2. Reported studies of intergeneric symmetric somatic hybridization between *Brassica napus* (AACC) and diploid *Raphanus sativus* (RR), and between *B. napus* and diploid *Sinapis alba* (S$_{al}$S$_{al}$)

Type of fusion	Number of hybrids	Reference
AACC (+) RR	10	Sundberg (1991)
AACC (+) S$_{al}$S$_{al}$	14	Primard et al. (1988)

species (Heijbroek *et al.* 1988, Steele and Savitsky 1981) and *Sinapis alba* (Lubberts and Toxopeus 1982) infested with BCN, but was also found in resistant potato plants inoculated with the potato cyst nematode, *Globodera rostochiensis* Woll. (Giebel *et al.* 1971a and 1971b).

A shift in the male to female nematode sex ratio towards the development of predominantly males has been reported as a major mechanism of resistance preventing nematode multiplication. For example, it has been observed in roots of resistant plants of *R. sativus*, *S. alba* and of the wild beet species *Beta webbiana* and *B. patellaris* inoculated with BCN (Müller 1985, Talatschian 1974), in a resistant potato genotype infested with the potato cyst nematode, *Globodera rostochiensis* Pathotype 1 (Turner and Stone 1984), and it has been observed for the cereal cyst nematode, *Heterodera avenae*, parasitising resistant plants of oats and barley (Bridgeman and Kerry 1980). The cause of the shift in sex ratio and of sex determination of cyst nematodes has been subject of many studies and has not yet been clarified (Triantaphyllou 1973). It is thought that the sex determination may be controlled either environmentally (Ellenby 1954, Müller 1985, Talatschian 1974), genetically (Bridgeman and Kerry 1980, Trudgill 1967), or by both the environment and the genetic constitution of the nematode (Johnson and Viglierchio 1969b). Various environmental factors have been shown to influence the sex ratio, e.g. the nematode population density (Ellenby 1954, Johnson and Viglierchio 1969a, Janssen 1990) and the suitability and physiological state of the host plant (Betka *et al.* 1991, Kämpfe and Kerstan 1964, Müller 1985). Furthermore, the food requirements of female nematodes of BCN have been found to be much higher than those of males (Müller *et al.* 1981).

A different rate of development and/or a different survival rate of the male and female nematodes are often described as a cause of the shifted sex ratio among cyst nematodes (Bridgeman and Kerry 1980, Johnson and Viglierchio 1969a, Kerstan 1969, Sengbusch 1927, Steele and Savitsky 1981). However, these assumptions are in contrast to those of Müller (1985), who observed similar proportions of sex differentiated nematodes on both susceptible and resistant *Cruciferae* species, while the ratio of ♂ : ♀ nematodes was found to be different. An alternative explanation for the altered sex ratios might be sex reversal, which has been observed for the root knot nematodes, *Meloidogyne incognita* and *M. javanica* (Triantaphyllou 1960 and 1973). Sex reversal in cyst nematodes is unknown, although Wouts (1978) reported the existence of males

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| I Partial resistance to BCN in *B. napus* | II Somatic hybridization | Sexual hybridization |

Hybrids
AACCRR
AACCS$_{al}$S$_{al}$

Hybrids
ACR/AACCRR
ACS$_{al}$/AACCS$_{al}$S$_{al}$
AACR

Screening for BCN-resistance

Screening for BCN-resistance

Recombination and selection

Backcrossing hybrids
A to *B. napus* (*B. rapa*, *B. oleracea*) – elimination of undesired S$_{al}$/R traits
B to *S. alba/R. sativus* – enhancement of BCN-resistance

Screening for BCN-resistance

B. napus with a high level of partial resistance

B. napus and R/S$_{al}$ gene(s) with BCN-resistance

Figure 2. Schemes for enhancement (**I**) and for introgression (**II**) of resistance to *Heterodera schachtii* Schm. (abbrev. BCN) in *Brassica napus* L. (genome constitution AACC). For the introgression of resistance first a production of somatic hybrids between *B. napus* and diploid *Sinapis alba* (S$_{al}$S$_{al}$) or diploid *Raphanus sativus* (RR) is suggested, or a production of sexual hybrids between *B. napus* and diploid or tetraploid *S. alba* (S$_{al}$S$_{al}$ or S$_{al}$S$_{al}$S$_{al}$S$_{al}$), diploid or tetraploid *R. sativus* (RR or RRRR) or x*Brassicoraphanus* (AARR), both followed by elimination of R/S$_{al}$ traits by recurrent backcrosses of hybrids to *B. napus, B. oleracea* or *B. rapa* (**A**). Enhancement of the level of resistance within the somatic and sexual hybrid plants may be achieved by intercrossing or back crossing of hybrids to resistant *S. alba* or *R. sativus* (**B**).

with two testes in the parthenogenetic species *Heterodera trifolii*, the clover cyst nematode.

The above described survey illustrates that mechanisms of resistance in host-nematode interaction may vary according to the nematode and host plant species.

Outline of research

The aim of the present study was to explore the introduction of resistance to *H. schachtii* into *B. napus* L. (Fig. 2). Results of selection for partial resistance to *H. schachtii* within the species *B. napus* L. will be described in chapter 2. With the aim to transfer resistance from *S. alba* and *R. sativus*, we tried several strategies. The first approach was to use protoplast fusion between BCN-resistant *R. sativus* and *B. napus* (chapter 3) and between *S. alba* and *B. napus* (chapter 5). The second approach was sexual hybridization between *S. alba* and *B. napus* (chapter 5), between *R. sativus* and *B. napus* (chapter 4) and crossing of the bridging hybrid x*Brassicoraphanus* with *B. napus* (chapter 4). Putative hybrids were characterized by their morphology and cytology, by DNA analyses and by scoring resistance to BCN. The nature of incongruity barriers between genotypes from the four species used for sexual hybridization was studied in order to explain the obtained results in the investigated intergeneric crosses (chapter 6). In that context, pollen germination, pollen tube growth and seed development have been studied in the respective pollen-stigma combinations. Finally, the mechanism of resistance to *H. schachtii* in *S. alba* , *R. sativus* and x*Brassicoraphanus* and their intergeneric sexual hybrids with *B. napus* was investigated (chapter 7). For that purpose, the penetration and development of juveniles from *H. schachtii* was studied in susceptible and resistant genotypes from parental species and in their intergeneric hybrids.

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CHAPTER 2

Selection for partial resistance to the beet cyst nematode, *Heterodera schachtii* Schm., in *Brassica napus* L.

C.L.C. Lelivelt

Summary

Accessions of *Brassica napus* L. were examined for resistance to the beet cyst nematode by assessing the number of cysts found on the root system. The level of resistance was low. Even the most resistant accessions had on average no less than 64% of the number of cysts counted on the susceptible *B. napus* standard cultivar 'Jet Neuf'. The average number of cysts and the proportion of plants with less than 10 cysts in progenies obtained after selfing plants with less than 10 cysts was equal to that in the original accessions. The difficulties encountered in selection for partial resistance in *B. napus* are suggested to be due to the probably polygenic nature of the resistance, and high experimental variability.

Introduction

Brassica napus L. ssp. *oleifera* (Metzg.) Sinsk. (oil-seed rape) is susceptible to the beet cyst nematode, *Heterodera schachtii* Schm. (abbrev. BCN) and its cultivation may result in an increase of the population of this nematode in the soil. Because of the risk of severe damage to sugar beet due to infestation by BCN, oil-seed rape cannot be grown in narrow rotation with sugar beet. Hence, breeding for BCN-resistant oil-seed rape is of importance.

It is not clear whether useful genetic variation for BCN-resistance is available in this predominantly cross-breeding crop. Baukloh (1976) did not find any differences in susceptibility between 215 *B. napus* accessions tested, but Bowen *et al.* (1986) found differences in root growth, in hatching and in nematode multiplication on roots between 18 *B. napus* cultivars. In addition, preliminary experiments carried out by Harrewijn (1987) showed that 48 out of 196 *B. napus* accessions had an average number of cysts which was significantly lower than that observed on the susceptible standard 'Jet Neuf' and might possess partial resistance to BCN.

In this study about 100 *B. napus* accessions, including 40 of the 48 potentially resistant accessions identified by Harrewijn (1987), as well as inbred lines from partially resistant plants, were (re)tested for partial resistance to BCN. The experiments aimed at evaluating:
A) BCN-resistance of *B. napus* accessions, that had also been tested by Harrewijn (1987),
B) the level of BCN-resistance in accessions that had not been tested before,
C) the level of BCN-resistance of S_1-lines from plants with a higher level of resistance than the parental accessions,
D) the reproducibility of the resistance tests by performing replicated experiments with the same accessions.

Materials and methods

Screening for beet cyst nematode resistance

Seeds of the various *B. napus* accessions were sown in 36 ml-PVC tubes, filled with sterilized silver sand, moistened with Steiner I (Steiner 1968) nutrient solution and kept in a greenhouse at a 10 h light regime, a constant temperature of 18°C and a relative humidity of 85 to 90%. After two weeks each seedling was inoculated with 2 ml of a suspension containing approximately 300 pre-hatched J_2 larvae of *Heterodera schachtii* Schm. using a veterinary syringe. The temperature in the greenhouse was raised to 22°C during the day (Toxopeus and Lubberts 1979). At four weeks after inoculation the root systems were washed free from sand and were evaluated for the appearance of mature females, further referred to as cysts. The level of resistance of each accession was calculated either as the mean of the absolute number of cysts per root system, further referred to as 'absolute number of cysts'. In addition, the proportion of plants with less than 10 cysts was calculated as well as the mean of the absolute number of cysts relative to that found on the susceptible standard 'Jet Neuf', further referred to as 'relative number of cysts'. Differences in absolute and relative number of cysts were analysed by means of ANOVA.

Plant material

In total 100 *B. napus* accessions were evaluated for BCN-resistance, including 52 accessions which were evaluated already before by Harrewijn (1987). The material was kindly provided by the Dutch Centre of Genetic Resources (CGN/CPRO-DLO, Wageningen; 62 accessions) and by Zelder (Ottersum, The Netherlands; 38 accessions). Table 1 presents an overview of the total number of 196 accessions tested by Harrewijn (1987) and the 100 accessions tested in the experiments reported here. Harrewijn (1987) treated seeds of some accessions with fungicides and insecticides. This was not done in the present experiments.

In total four experiments were performed to screen the 100 *B. napus* accessions. The winter oil-seed rape cultivar 'Jet Neuf' was used as susceptible control. All experiments were carried out following a similar design with for each accession and for the standard cultivar 'Jet Neuf' four or five replications of 20 plants each.

Plants with less than 10 cysts on the root system were selected from all accessions with an absolute and relative number of cysts significantly lower than that on cv. 'Jet Neuf', and from one accession which was as susceptible as cv. 'Jet Neuf'. The selected plants were potted in a mixture of peat and soil. At the stage of about eight full-grown leaves the plants were vernalized at 6 to 7°C in a growth cabinet during two months, and transferred at springtime to an unheated greenhouse for flowering. Flower stalks were bagged to prevent cross-pollination. The offspring of these self-pollinated plants was tested for BCN-resistance in four replications of 20 plants each, together with the parental accessions, of which also four replications of 20 plants each were used.

Table 1. Type, origin and number of accessions of *B. napus* tested for resistance to *H. schachtii* by Harrewijn (1987) and (re)tested in the present study

Type	Collection of accessions		Number of new accessions tested	Origin
	Total tested by Harrewijn (1987)	Part retested in this study		
Fodder rape	57	15	38	N-W Europe
Swede	4	1	0	N-W Europe
Winter oil-seed rape	42	19	2	Canada, USA Europe, CIS
Spring oil-seed rape	6	3	1	Canada, Europe
Other or unspecified	87	14	7	Canada, USA Europe, CIS
Total	196	52	48	

Results

Retesting of B. napus accessions

The relative number of cysts of the 52 retested *B. napus* accessions and the level of resistance found by Harrewijn (1987) are presented in Table 2. Harrewijn (1987) had classified 12 accessions as susceptible as cv. 'Jet Neuf' and 40 accessions were considered to possess partial resistance. The absolute number of cysts on the susceptible standard cv. 'Jet Neuf' varied between experiments. This was also observed by Harrewijn (1987). Furthermore, since these 52 accessions had not been tested in a single experiment, neither by Harrewijn (1987), nor in this study, only numbers of cysts relative to those found on cv. 'Jet Neuf' are presented in Table 2, to allow comparison of the results.

The level of resistance of the 40 possibly "resistant" accessions differed greatly from that found by Harrewijn (1987), the range in relative number of cysts for the "resistant" and "susceptible" accessions being nearly equal after retesting (Table 2). Only 11 of the 40 "resistant" accessions were found to have a relative and absolute number of cysts significantly lower than that on cv. 'Jet Neuf'. Even the most resistant accession was found to have still 79% of the number of cysts observed on cv. 'Jet Neuf', as opposed to 14% found by Harrewijn (1987) (Table 2). However, the absolute number of cysts found by Harrewijn (1987) on most accessions was much lower than that observed after retesting. The absolute number of cysts observed by Harrewijn (1987) on the

Table 2. Comparison of the level of partial resistance to *H. schachtii* in *B. napus* as reported by Harrewijn (1987) and as found in the present study after retesting; cv. 'Jet Neuf' is the susceptible control

Phenotype (Harrewijn, 1987)	Number of accessions	Range of relative number of cysts[*] per root system	
		Harrewijn (1987)	Present study
BCN-susceptible	12	65-114	72-118
BCN-resistant	40	14- 68	79-122
Total	52		
B. napus 'Jet Neuf'	1	100	100

[*] (absolute number of cysts on tested accession : that on cv. 'Jet Neuf' in the relevant experiment) x 100

40 "resistant" accessions ranged from 5 to 34 cysts, whereas 22 to 78 cysts were observed in our experiments.

The retested 12 "susceptible" *B. napus* accessions were all found to be susceptible. The range in relative number of cysts was similar to that found by Harrewijn (1987) (Table 2). However, the absolute number of cysts on many accessions was higher after retesting. It ranged from 24 to 72, whereas 22 to 44 cysts were observed by Harrewijn (1987).

Evaluation of non-tested B. napus accessions

Only three out of 48 accessions, not evaluated before, were found to have an absolute and a relative number of cysts significantly lower than that found on cv. 'Jet Neuf'. These three accessions had a relative number of cysts varying from 64 to 76% of that on cv. 'Jet Neuf'.

Offspring of selected plants

In total 14 out of 100 accessions were found to have a significantly lower number of cysts than cv. 'Jet Neuf'. Plants with less than 10 cysts were selected from these 14 "resistant" accessions and from one accession being as susceptible as cv. 'Jet Neuf'. Table 3 includes these 15 accessions with their relative number of cysts and percentage of plants with less than 10 cysts. Since accessions 1 to 12 and accessions 13 to 15 were evaluated in two different experiments, results have been presented separately, together with the specific LSD values for each experiment. All material was evaluated twice (first and second test in Table 3).

Very few plants selected from these 15 accessions had no cysts or only one

Table 3. Screening for resistance to *H. schachtii* in *B. napus*. Level of resistance of 15 *B. napus* accessions evaluated in two separate tests for relative number of cysts and percentage of plants with less than 10 cysts. In the second test are also included selfed progenies (S₁) from plants with less than 10 cysts in the first test. JN = susceptible standard, cv. 'Jet Neuf'

Accession No	First test		Second test		S_1 from first test plants with < 10 cysts	
	Number of cysts relative to that on *B. napus* cv. 'Jet Neuf' (JN = 100)	Percentage of plants with <10 cysts	Number of cysts relative to that on *B. napus* cv. 'Jet Neuf'	Percentage of plants with <10 cysts	Range in number of cysts relative to that on *B. napus* cv. 'Jet Neuf'	Range in percentage of plants with <10 cysts
JN	100	3	100	0		
1	69*	8	88	4	88-91	2-5
2	65*	5	96	1	87	0
3	72*	5	58*	11	92	3
4	73*	6	87	4	65*	6
5	63*	6	93	0	99	0
6	68*	15	82	1	85	0
7	67*	16	111	0	80-109	0-6
8	51*	25	99	0	78-101	0-4
9	69*	11	76*	9	86-101	0-2
10	66*	17	73*	8	85-103	0-4
11	73*	8	109	5	89-94	1-3
12	77	9	88	3	84	0
LSD(5%)	25		21		21	
JN	100	4	100	2		
13	67*	11	119	4	93-115	4- 8
14	76*	12	100	13	78-122	1-16
15	64*	16	89	6	81	2-12
LSD(5%)	21		56		56	

*) Relative number of cysts significantly lower than the control JN = 100 (P<0.05)

cyst on their root system. "Resistant" (<10 cysts) plants from the accessions 1 to 12 were selfed and 27 S_1-lines were tested in one experiment with the corresponding parental accessions. In an other experiment, 14 S_1-lines from the accessions 13, 14 and 15 were evaluated jointly with their parental accessions. All parental material was tested twice (first and second test in Table 3). Only the parental accessions 3, 9 and 10 showed an absolute and relative number of cysts which in both tests were significantly lower than counted on cv. 'Jet Neuf'. The percentage of plants with less than 10 cysts differed greatly between experiments, and for most accessions a much lower frequency was observed in the second test (Table 3).

The progenies of the selected plants showed an equal or higher number of cysts than the parental accessions. Only the offspring of selected plants of accession 4 was found to carry on average fewer cysts than cv. 'Jet Neuf', i.e. 65%. However, the percentage of plants with less than 10 cysts in S_1-progenies from selected plants of accession 4, i.e. 6%, was equal to that observed in the original population. The selfed progeny of a plant with less than 10 cysts of the susceptible accession 13 was found to be susceptible (Table 3).

Reproducability of BCN-resistance tests

Since large differences in level of BCN-infestation were observed both in the results by Harrewijn (1987) and in those reported here, several experiments were performed to evaluate the reproducability of the BCN-resistance tests. Again, differences in absolute and relative number of cysts were found between the experiments. In some experiments the observed differences for the level of resistance between accessions was found not to be significant. In most experiments with an overall low level of infestation by BCN, more accessions with a significantly lower number of cysts than cv. 'Jet Neuf' were observed than in experiments with a generally high level of BCN-infestation.

A repeated experiment with 36 possibly "resistant" genotypes and three "susceptible" genotypes showed a significant difference from another experiment regarding absolute and relative number of cysts ($P<0.05$). The correlation coefficient (for absolute and relative number of cysts) between these two experiments was only 0.25. In addition, one experiment showed no significant difference between accessions for the absolute or relative number of cysts, whereas in the repeated experiment 11 out of 40 had a significantly lower number of cysts than cv. 'Jet Neuf'.

Furthermore, repeated testing of accessions 1 to 12, and 13 to 15 (Table 3) showed no correlation ($r=0.05$ and 0.06, respectively) between experiments for the absolute and relative number of cysts. Also, Spearman's rank correlation coefficient based on absolute and relative number of cysts in repeated tests of accessions 1 to 12 and 13 to 15 was very low (0.01-0.4).

Discussion

The level of partial resistance to BCN in the *B. napus* accessions tested in this study was found to be almost insignificant. This is in accordance with some of the earlier reports (Baukloh 1976, Bowen *et al.* 1986). The observed differences in partial resistance between several *B. napus* accessions observed by Harrewijn (1987) could not be confirmed in the experiments reported here. To a large extent this might be explained by the high experimental variability, influenced by environmental factors, the size of the experimental units, and possibly by the fungicidal treatment of the seeds used by Harrewijn (1987). Also, the overall level of infestation by BCN was found to be important: in many experiments by Harrewijn (1987) an overall lower average number of cysts was found. In our experiments, a low BCN-infestation was often associated with a larger number of accessions having a significant lower number of cysts than cv. 'Jet Neuf'. It has been shown that multiplication rates of cyst nematodes are strongly influenced by environmental factors, such as plant vigour, plant growth, plant nutrition and temperature (Curtis 1970, Johnson and Viglierchio 1969a and 1969b, Kämpfe and Kerstan 1964, Trudgill 1967), resulting in a lower number of cysts per root system under less favourable conditions for plant growth. In addition, Harrewijn (1987) evaluated per accession only one third or half of the number of plants tested in the experiments reported here.

Only few S_1 progenies from selected plants of accession 14 contained more plants with less than 10 cysts than the original population, whereas the other S_1 progenies did not. Furthermore, the average level of resistance of the S_1-progenies, with one exception, was not better than that of the parental populations. This indicates that either the selected plants within the accessions had been 'escapes' or that the level of resistance in this material is very low and under polygenic control. The observed BCN-resistance within *Brassica napus* reported here is similar to the BCN-resistance in *Beta vulgaris* (Curtis 1970, Heijbroek 1977).

Although components of resistance, such as hatching of larvae from cysts and egg-content of cysts were not evaluated with the present method, the number of cysts found on the root system is thought to be a good indicator of resistance. A study of other possible components of resistance in the present material will be difficult and is not expected to reveal significant resistance either. In general it can be stated that the *B. napus* accessions tested so far (Baukloh 1976, Bowen *et al.* 1986, Harrewijn 1987, Talatschian 1974, Table 1) included a wide range of genotypes and were all susceptible to BCN. Therefore, it seems unlikely that by accumulation of genes for resistance via crossing and selection within *B. napus* highly BCN-resistant varieties can be developed. Other types of resistance, such as the high BCN-resistance found in *Sinapis alba* (white mustard) and *Raphanus sativus* (oil-radish) must be incorporated in *B. napus* in order to obtain BCN-resistant oil-seed rape varieties that can be grown in rotation with sugar beet.

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CHAPTER 3

Transfer of resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) into the *Brassica napus* L. gene pool through intergeneric somatic hybridization with *Raphanus sativus* L.

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Summary

An intergeneric somatic hybrid was obtained through PEG-induced protoplast fusion between *Brassica napus* L. (oil-seed rape, AACC, $2n = 38$) and a beet cyst nematode resistant genotype of *Raphanus sativus* L. (fodder radish, RR, $2n = 18$). The hybrid nature of the regenerated plant was confirmed by flow cytometric analysis, RFLP-analysis and chromosome counts. Southern blot analysis of total DNA using pPhcPS1(rbc-L) as probe indicated that the somatic hybrid contains chloroplasts of *B. napus*. The mitochondrial genome of the somatic hybrid was studied more extensively using several probes and restriction enzymes. The results indicate inter- or intraspecific mitochondrial DNA recombination. Resistance to the beet cyst nematode (*Heterodera schachtii* Schm., BCN) was expressed in the hybrid at a high level.

Introduction

Brassica napus L. is an amphidiploid species. Its subspecies *oleifera* (Metzg.) Sinsk., oil-seed rape, is one of the main oil-producing crops in Western Europe and Canada. In the Netherlands, crop rotations commonly include sugar beet as a major crop. Oil-seed rape cannot be included into such a crop rotation, because it is susceptible to *Heterodera schachtii* Schm., the beet cyst nematode (abbrev. BCN). The level of resistance to BCN within *Brassica napus* L. is too low to allow selection of resistant cultivars (Harrewijn 1987). In the related species *Raphanus sativus* L. ssp. *oleiferus* (DC.) Metzg. (fodder radish), however, cultivars have been selected with a high level of resistance (Toxopeus and Lubberts 1979). The inheritance was studied by Baukloh (1976) who assumed a single dominant gene for resistance.

Studies of chromosome associations in MI in hybrids between oil-seed rape (AACC) and the bridging hybrid x*Brassicoraphanus* Sageret, which includes both AARR and CCRR genotypes, indicated partial homology between chromosomes of *Raphanus* (R) and *B. napus* (AC) (Agnihotri *et al.* 1990, Dolstra 1982, Lange *et al.* 1989). Sexual hybridization between *B. napus* and *R. sativus* has been reported, but only very few hybrid plants have been obtained due to the poor crossability between the two species (Chopinet 1944, Takeshita *et al.* 1980). Somatic cell hybridization has been found to be less impeded by pre- and postzygotic barriers, and thus this technique may result in novel nuclear hybrids and nucleus-cytoplasm combinations.

No somatic hybrids between *B. napus* and *R. sativus* have been reported as yet. However, *B. napus* has been somatically hybridized with many other species from the *Cruciferae*, and *R. sativus*, investigated less frequently, has been used as donor of cytoplasmic male sterility in asymmetric protoplast fusions with *B. napus* (Sakai and Imamura 1990).

This paper describes the production and characterization of a somatic hybrid between *B. napus* and *R. sativus*, with the view of introducing the BCN resistance of *Raphanus* into the gene pool of *B. napus*.

Materials and methods

Plant material

From *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk., cultivars 'Barrapo', 'Tantal', 'Darmor', 'Cascade', 'Jet Neuf' and accession K1 (D.J. Van der Have BV, Rilland, The Netherlands), all susceptible to *H. schachtii* Schm., and from *Raphanus sativus* L. the cv. 'Nemex', resistant to *H. schachtii* Schm., were used.

Beet cyst nematode resistance test

Seedlings and cuttings were tested for resistance to *Heterodera schachtii* Schm. according to Toxopeus and Lubberts (1979), with slight modifications. Seeds were sown in 36 ml-PVC tubes filled with silver sand, moistened with Steiner I nutrient solution (Steiner 1968) and kept in a greenhouse at a 10 h light regime, a temperature of 18°C during the day and night, and a relative humidity of 85-90%. After two weeks seedlings were inoculated with a suspension of pre-hatched J_2 larvae of *Heterodera schachtii* Schm., using a veterinary syringe to inoculate with approximately 300 larvae/plant. Subsequently, temperature in the greenhouse was raised to 22°C during the day. After two weeks 2 ml Steiner I nutrient solution was added to each plant. Four weeks after inoculation, when the female nematodes had grown into cysts and had reached their maximum size, the root system was washed free of sand and examined for the occurrence of cysts.

To test the BCN resistance of eldery plants, cuttings were made that were tested using 96 ml-PVC tubes and an inoculation density of 500 larvae/plant. Seedlings were used as control plants and were also tested using 500 larvae/plant. Experiments with cuttings and seedlings were replicated four times. In addition, the root size was also visually quantified on a scale of 1 (small) to 5 (large). Statistical analysis (t-test) was applied to the results of the BCN-resistance tests.

In vitro culture

Apical meristems, axillary buds, and ovaries were sterilized in 2% (w/v) sodium hypochlorite for 10 to 20 min, followed by washing three times with sterile tap water. Seeds were rinsed in 70% (v/v) ethanol (30 sec) and sterilized as described above. Except for the ovaries, all plant material was cultured on medium 1 (MS, Murashige and Skoog 1962; 0.8% (w/v) agar, 1% (w/v) saccharose and 1% (w/v) glucose (pH 5.8) at a 16 h light regime, 6 W/m² and 25°C. Ovaries were cultured on MS medium, 5% (w/v) saccharose, 0.8% (w/v) agar, 300 mg/l casein hydrolysate, 1 mg/l indole-3-acetic acid (IAA) and 0.5 mg/l kinetin under the same culture conditions.

Mesophyll protoplasts were isolated from in vitro grown plants. In addition, protoplasts were also isolated from plants growing on medium 1, supplemented with 1.5 mg/l norflurazon (SAN 9789) to inhibit chlorophyll formation.

Protoplasts were isolated and cultured according to Pelletier *et al.* (1983), at a density of 5×10^4 protoplasts/ml in medium Pelletier-B (2 ml per 6 ml-Petridish, Greiner, TC quality). After approximately one week, the protoplast suspension was diluted 1:1 with medium Pelletier-C and two weeks after isolation the microcallus suspension was diluted 1:1 with Pelletier-D. After three days the number of dividing cells per 1,000 cells was counted. Plating efficiency was determined three weeks after isolation by counting the number of developing microcalli per plate.

Green calli, four to five weeks old with a diameter of 0.5 to 1 mm, were transferred to medium MS 11 (MS, 1% (w/v) saccharose, 0.8% (w/v) agar, 1 mg/l 6-benzylaminopurine (BAP), and 0.1 mg/l α-naphthaleneacetic acid (NAA)) for callus growth, and transferred after one to two weeks to regeneration medium Pelletier-E (26 calli/plate). Regenerants were transferred to medium 1 and after rooting plants were transferred to the greenhouse.

Protoplast fusion

For PEG-induced protoplast fusion, SAN-treated mesophyll protoplasts were stained with fluorescein diacetate (FDA) by adding 30-40 μl FDA solution (5 mg FDA/ml in acetone) to 20 ml enzyme solution at the beginning of the protoplast isolation procedure. Isolated protoplasts of both SAN-treated and non-treated plants were resuspended each in 1 ml W5 medium (Menczel and Wolfe 1984) and after protoplast yield was determined, a total of 5×10^5 protoplasts of each parent was mixed at a 1:1 ratio. The protoplast mixture was transferred to a plastic centrifuge tube (TC quality, Greiner), washed once, pelleted at 35 x g, and resuspended in approximately 0.2 ml of W5 medium. To this protoplast suspension, 0.4 ml of a polyethylene glycol 6,000 (PEG) mixture was added in big droplets, and the tube was incubated for 10 to 25 min at 18-20°C, without shaking. Protoplasts were either incubated in a glycin/NaOH buffer (pH 10.0) containing 15% (w/v) PEG, 60 mM $CaCl_2.2H_2O$, 90 mM mannitol, 25 mM glycine and 10% (v/v) dimethylsulfoxide (DMSO) (Thomzick and Hain 1988) and subsequently rinsed thoroughly three times with washing solution (W5 medium supplemented with 50 mM morpholinoethane sulfonic acid, pH 5.5) (method A), or they were incubated in a PEG solution (45% (w/v) PEG in 12 mM $CaCl_2.2H_2O$ solution, pH 5.8) for 10 to 25 min and subsequently rinsed with a glycin/NaOH buffer (pH 10.4) containing 50 mM $CaCl_2.2H_2O$ (method B) (Uijtewaal 1987). Cells were plated at a density of 1×10^5 to 2×10^5 protoplasts/ml in medium Pelletier-B in 6-ml Petridishes (2 ml/Petridish). Fused protoplasts and calli were cultured as described above. Fusion frequency was determined as the number of fusion products per 500 cells.

Chromosome counts and pollen viability

Root tips from plants grown in the greenhouse were pretreated and fixed as described by Jochemsen and Mlyniec (1974), stained with Feulgen and examined under the light microscope. Flower buds were pretreated in 96% (v/v) ethanol:ferripropionic acid (3:1, v/v) for 3 weeks and subsequently transferred to 96% (v/v) ethanol. Anthers were either stained in Snow's solution (Snow

1963) for 6 to 8 h at 60°C or squashed directly in acetocarmine and examined under the light microscope. Pollen viability was estimated by staining freshly collected pollen in a solution containing 9% (w/v) sucrose and 0.5 mg/ml FDA. Viability frequency was determined under the UV microscope as the number of yellow/green fluorescent pollen in 200-300 pollen grains.

Southern blot analyses

Total DNA was extracted from leaves of plants grown in the greenhouse according to the method of Dellaporta et al. (1983). Total plant DNA (5-10 µg) was digested with BamHI, DraI, EcoRI, EcoRV, HindIII, SacI and XhoI according to Kreike et al. (1990). Southern blotting transfer of the DNA onto nitrocellulose (Kafatos et al. 1979) or nylon membranes (Hybond N, Amersham) and crosslin-king of the DNA was carried out according to Kreike et al. (1990).
   Several heterologous nuclear, chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA) sequences were used as probes in hybridizations. The maize cytochrome C oxidase subunit 1 (coxI), cytochrome C oxidase 2 (coxII), apocytochrome B (cob) and the alpha subunit of F1 ATPase (atpa) were provided by the University of Edinburgh, UK (Prof. Dr C. Leaver). The maize ATPase subunits 6 (atp6) and 9 (atp9) were a gift from Dr C. S. Levings III (North Carolina State University, USA) and cytochrome C oxidase 3 (coxIII) was supplied by Dr A. Bren-nicke (Institute for Gene Biological Research, Berlin, FRG). The pea ribosomal-DNA probe (rDNA, a 4.0-kb EcoRI subclone in pACycl184 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 probe DNA was labelled non-radioactively with digoxigenin-dUTP, hybridized to target DNA, and visualized by chemiluminescence according to Kreike et al. (1990).

Flow cytometry

Pieces of leaves (1 cm²) were chopped with a razor blade in 1 ml of a nuclear isolation buffer (10 mM Tris-HCl (pH 7), 10 mM spermine-tetrahydrochloride, 2.5 µg/ml 4.6-diamidino-2-phenylindole (DAPI), 10 mM NaCl, 200 mM hexyleneglycol and 0.025% Triton-X100) and filtered through a 20 µm nylon filter. After a wash with 1 ml of the same buffer, the filtrate was analyzed on a Partec PAS-II flow cytometer with a UG 5 excitation filter, TK 420, and TK 520 dichroic mirrors and a GG 435 long-pass filter. Channel analyses were performed with the standard software of the PAS-II. B. napus accession K1 was used as internal standard. For the determination of DNA content in the investigated plants, the ratio was determined of G_0 peak position(s) on the horizontal axis compared to the B. napus accession K1 G_0 peak position in each experiment. For example a peak position ratio (ppr) of 0.4 indicates that the distance of the G_0 peak of the object under study to the vertical y-axis (x=0) is 0.4 times that of the distance of the G_0 peak of B. napus accession K1 to x=0 in the same experiment.

Results

Beet cyst nematode resistance tests of parental species and sterile shoot cultures

White cysts were easy to detect on root systems of susceptible *B. napus* plants. Most seedlings of *R. sativus* cv. 'Nemex' were found to be very BCN-resistant, although a few plants were found with up to five cysts when tested with 300 larvae/seedling. Seedlings of *R. sativus* cv. 'Nemex', showing no cyst formation, were selected and used for the production of sterile shoot cultures. Shoot cultures of selected *R. sativus* cv. 'Nemex' seedlings, grown on medium 1 supplemented with SAN, were used for protoplast culture and fusion experiments. From the *B. napus* cultivars, shoot cultures, produced from seeds or, in the case of accession K1, plants, and cultured on medium 1, were used for protoplast culture and fusion experiments.

Protoplast isolation and culture

Two days after the protoplast isolation first cell divisions were visible for most of the *B. napus* cultivars. The average cell division and callus formation frequencies ranged from 8.2% and 0.09%, respectively, for *B. napus* cv. 'Darmor' to 43.3% and 1.61%, respectively, for *B. napus* cv. 'Barrapo'. Accession K1 was found to show the highest frequency of shoot regeneration (22%), whereas the other cultivars showed an average shoot regeneration frequency of less than 1 to 2%. Accession K1 was therefore chosen for fusion experiments.

The transfer of calli from medium Pelletier-D to shoot regeneration medium Pelletier-E often resulted in browning of the cells and arrest of further callus proliferation. Transfer of calli in a thin layer of liquid medium to the solidified medium MS 11 before transfer to medium Pelletier-E had a positive effect on callus growth, while shoot regeneration was also obtained.

Mesophyll protoplasts from *R. sativus* cv. 'Nemex' were viable during only one week of culture in medium B and, on average, 3.6% of the plated cells divided. Sustained cell divisions and callus formation were not observed. Addition of SAN had a negative effect on protoplast yield although cell division was not affected.

Protoplast fusion

Heterokaryons could be identified as protoplasts showing both the red autofluorescence of the *B. napus* chloroplasts as well as the yellow-green fluorescein fluorescence of *R. sativus* cytoplasm.

In order to determine favourable conditions for PEG-induced fusion, two different fusion methods were investigated. With method A a maximum heterokaryon frequency of 11% was already achieved after 15 min. of incubation. With method B the heterokaryon frequency increased with increasing PEG incubation time. Maximum heterokaryon frequencies (11%) were

obtained after 25 min. However, both methods showed a decrease in callus proliferation and regeneration after a PEG-incubation longer than 15 min.

Further fusion experiments were carried out following method A. Since the regeneration experiments had shown that *R. sativus* protoplasts did not form calli, only *B. napus* or hybrid calli and plants were expected to result from the fusion experiments. With a heterokaryon frequency of about 10% and when it is assumed that culture, proliferation, and regeneration are neither negatively nor positively affected by fusion, one out of ten plants may be expected to be a hybrid plant. In total, one plant out of 286 regenerants (Table 1) was found to be a hybrid plant, which would indicate that the regeneration capacity of the hybrid calli was negatively affected by fusion.

Table 1. Frequency of plant regeneration from protoplast derived calli of *B. napus*, *R. sativus*, and from calli obtained after PEG-induced fusion (method A)

Experiment	Fusion (%)	Number of calli		Number of hybrids
		Tested	Regenerants	
a) Regeneration				
B. napus K1	-	104	12	-
R. sativus Nemex	-	0	0	-
b) Protoplast fusion				
Expt1	8.4	2519	6	1
Expt2	8.3	2068	102	0
Expt3	10.7	1010	56	0
Expt4	9.8	2188	122	0

- = not applicable

Hybrid characterization

Morphological observations. Out of 286 regenerants, one showed hybrid characteristics such as an intermediate leaf shape and thick and wrinkled leaves. After approximately three months the putative hybrid, further referred to as SH-1, flowered. Flower buds were larger than those from the parent plants and had either the typical smooth morphology of *B. napus*, or the hairy one of *R. sativus*, or were intermediate. White *Raphanus*-like flowers appeared from buds that were morphologically nearly identical to *B. napus*. Petals of the hybrid flowers were usually longer than those of the flowers of the parental genotypes. The flowers on the hybrid varied between completely white, white with yellow

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patches, pale yellow, and even both white and yellow petals in the same flower. Purple veins, which are characteristic for *R. sativus* flowers, were not detected. Stamen development varied from normal stamens to stamens with stunted filaments and brown or rudimentary anthers. Almost all flowers possessed the full complement of six stamens and contained nectaries. Pollen production was low and less than 5% stained with FDA. The beak length of the hybrid pods was, on average, intermediate between both parental species.

Backcrosses of SH-1 hybrid cuttings with *B. napus* cv. 'Tantal' or cv. 'Jet Neuf' were not succesful. Ovary culture of 107 ovaries resulted in the formation of many aborted seeds and only one well-developed seed, which did not germinate.

The morphology of the putative hybrid indicated a highly chimeric nature. However, cuttings from this plant showed a shift towards a less chimeric phenotype, as was also indicated by flow cytometric (FCM) observations, cytological studies, and BCN tests.

Nuclear genome. Total plant DNA was isolated from two SH-1 hybrid cuttings: cutting 1 showed a highly chimeric nature (see also cytological studies) and cutting 2, made from cutting 1, displayed a less chimeric nature. Restriction patterns of total plant DNA, isolated from both cuttings, digested with *Bam*HI and *Hind*III and probed with the pea rDNA probe, showed a discrimination between the two parental species (Table 2). For *B. napus* five bands (5.2, 4.3, 2.5, 2.1 and 1.2 kb) were present in the *Bam*HI restriction pattern and for *R. sativus* four bands (4.3, 4.1, 2.5 and 1.2 kb). The somatic hybrid pattern showed the full complement of all parental bands. Digestion with *Hind*III resulted in two restriction fragments for *B. napus* (11 and 8 kb) and two for *R. sativus* (17 and 7 kb). Again, the somatic hybrid contained a summation of the parental bands. No novel bands appeared and none of the parental bands was missing. There was no difference between the restriction pattern of cutting 1 and 2.

Organelle genome. Using a chloroplast specific DNA fragment as probe (pPhcPS1(rbc-L)) and *Eco*RI or *Hind*III as restriction enzyme, it was found that the hybrid cuttings 1 and 2 contained exclusively the pattern of the *B. napus* parent (Table 2). The *Bam*HI, *Dra*I, *Eco*RV and *Xho*I digestions showed a restriction pattern that could not discriminate between the parental cpDNA (Table 2).

Hybridization experiments with mitochondrial probes indicated recombination between the parental species. Hybridization of total plant DNA from cutting 1, probed with *cox*I, *atp*a, and *atp*6 for several restriction enzymes, showed a pattern that was identical to that of the *R. sativus* parent, whereas the use of *cox*II, *cox*III, and *cob* resulted in the *B. napus* pattern. After hybridization with *atp*9, however, a novel banding pattern was observed, that was completely different from the mtDNA pattern of either parent (Fig. 1, Table 2). The occurrence of a mixture of both parental mitochondria in the hybrid is not likely, since none of the hybridization patterns showed a summation of all parental bands. Hybridization experiments of total plant DNA, isolated from hybrid cutting 2, and probed with *cox*I, *cox*III and *atp*a resulted in a restriction pattern

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that was identical to that of hybrid cutting 1.

Cytological studies and flow cytometry. The number of chromosomes of the first cuttings from the hybrid, used for backcrosses, morphological observations, and RFLP analyses, was found to show a chimeric pattern, since both cells with 54 to 56 and cells with >70 chromosomes were present in root tips. These observations were confirmed by FCM analyses of the same material. However, FCM analyses of a new series of cuttings, made from the above mentioned chimeric cuttings, displayed a less chimeric nature. Also, chromosome counts of these plants showed a shift towards a >70 or aproximately the expected number of 56 chromosomes, whereas cuttings showing tissues with both high and low numbers of chromosomes became less frequent. FCM analysis was used for detecting mixoploidy, because the number of dividing root meristem cells of some hybrid cuttings was found to be too low for the identification of chimeric tissue.

The number of chromosomes observed in 15 pollen mother cells (PMCs) varied from 58 to 62. In all cells 26 to 30 bivalents, up to two trivalents and up to six univalents were observed. Meiotic irregularities were observed. At AI unequal chromosome divisions occurred, and at MII and AII restitution nuclei were observed, resulting in more than 50% dyad formation leading to unreduced pollen.

Beet cyst nematode resistance. Cuttings of the SH-1 hybrid were tested for BCN resistance (Table 3). Much variation in level of resistance was observed between cuttings. There was no relationship, however, between number of chromosomes and level of resistance. The root size of the hybrid cuttings was usually smaller than that of cuttings of *B. napus* or *R. sativus*, but bigger than that of seedlings of *B. napus*. Significantly fewer cysts were formed on the smaller root system of *B. napus* seedlings than on the larger root system of *B. napus* cuttings ($P < 0.05$). As expected, root size of the resistant species *R. sativus* did not have a significant effect on the mean number of cysts formed ($P > 0.95$). In all experiments, the average number of cysts produced on the root systems of hybrid cuttings was lower compared to that produced on root systems of both seedlings and cuttings of *B. napus* ($P < 0.05$). Furthermore, some hybrid cuttings with well-developed roots were as resistant as the *R. sativus* parent.

Table 2. Southern blot hybridizations of total DNA of the somatic hybrid SH-1 (*B. napus* (+) *R. sativus*) digested with seven different restriction endonucleases and hybridized with several probes

Probe	type	Enzyme						
		*Bam*HI	*Dra*I	*Eco*RI	*Eco*RV	*Hind*III	*Xho*I	*Sac*I
rDNA	nucl	hybr	-	-	-	hybr	-	-
chloro	cp	*	*	BN	*	BN	*	-
*cox*I	mt	*	RS	RS	RS	RS	*	-
*cox*II	mt	*	*	*	BN	*	*	-
*cox*III	mt	*	BN	*	*	*	-	*
*atp*a	mt	RS	RS	RS	RS	RS	RS	-
*atp*6	mt	*	RS	RS	RS	RS	-	RS
*atp*9	mt	new	new	new	new	new	-	new
cob	mt	*	*	*	BN	*	-	*

rDNA = pea rDNA probe; chloro = chloroplast probe pPhcPS1(rbc-L); nucl = nuclear DNA; cp = chloroplast DNA; mt = mitochondrial DNA; BN = pattern equal to *B. napus* acc. K1; RS = pattern equal to *R. sativus* cv.'Nemex'; - no data available; * no restriction fragment length polymorphism; new = new bands; hybr = hybrid pattern
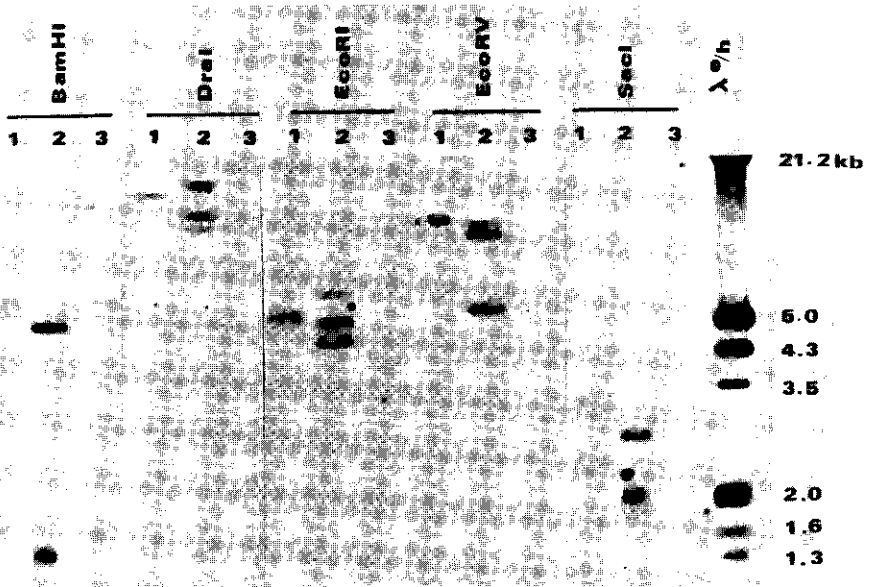

Figure 1. Characterization of mtDNA in the SH-1 hybrid (*B. napus* (+) *R. sativus*) restricted by the enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV and *Sac*I and hybridized with the heterologous *atp*9 probe. 1 = *B. napus* K1; 2 = SH-1; 3 = *R. sativus* cv. 'Nemex'. *Eco*RI/*Hind*III-digested lambda DNA was used as size marker.

Table 3. Cytological analyses and tests for resistance to the beet cyst nematode on the parental species *B. napus* and *R. sativus* and their somatic hybrid (SH-1). Data are means of four experiments. Letters indicate a significant difference at $P < 0.05$

Plant material Genotype	Propagation method	Number of plants	Number of cysts/plant	Size of roots	Number of chromosomes	Flow cytometry (ppr)
B. napus						
'Jet Neuf'	seed	55	39.3 b	2.0	38	1.0
'Jet Neuf'	cutting	59	61.7 a	4.5	38	1.0
acc. K1	cutting	44	43.9 b	4.1	38	1.0
R. sativus						
'Nemex'	seed	55	0.2 d	3.6	18	0.4
'Nemex'	cutting	53	0.1 d	4.9	18	0.4
SH-1						
mean	cutting	22	5.7 c	3.4	70[a]	1.2,2.0[a]
range			0-31	1-4	56-77	1.2-2.6

[a] number of chromosomes and ppr values which were observed most frequently in hybrid cuttings; two ppr values indicate mixoploidy

Discussion

PEG-induced protoplast fusion of *R. sativus* and *B. napus* resulted in one somatic hybrid.

Our results demonstrated that direct incubation of protoplasts in a PEG solution at high pH was preferable to using a later high pH treatment. This might be explained by factors such as the damaging effect of PEG after long incubation, the medium composition, or the positive effect of the interaction between PEG and Ca^{2+}/high pH on protoplast fusion.

It was estimated that with a heterokaryon frequency of about 10% (Table 1), and regeneration not affected by the fusion process, 10% of all regenerants should be hybrids. However, only one hybrid out of 286 regenerants was obtained. This suggests that regeneration of fusion products is reduced. In general it was found that PEG incubation had a negative effect on protoplast regeneration, since the regeneration frequency of calli from the fusion experiments was much lower than that of control *B. napus* calli (Table 1). Primard *et al.* (1988) investigated PEG somatic hybridization between *B. napus* and a non-regenerating *S. alba* accession, and found that in two out of three fusion experiments the regeneration rate was low in comparison to shoot regeneration of a mixture of untreated *B. napus* and *S. alba* calli. Another explanation of the low frequency of hybrids in our experiments might be a slower growth rate of somatic hybrid calli compared to *B. napus* calli, resulting in a greater risk to die before shoots could develop. The efficiency of protoplast fusion as a method of producing hybrids might be increased by improving protoplast regeneration efficiency or by selecting hybrids at an early stage of development by means of a micromanipulator or flow cytometric sorting.

Southern blot analysis of total DNA hybridized with a pea rDNA probe showed that the somatic hybrid SH-1 contained nuclear DNA of both parents and is apparently a true hybrid. Since mitotic chromosome numbers of about 56 (the sum of the parental chromosomes) were observed, the hybrid is thought to have resulted from a one-to-one fusion (Table 3). A possible explanation for the chimeric variation in number of chromosomes in the hybrid plant could be the occurrence of disregulated cell division and callus proliferation during in vitro culture, resulting in both a duplication of chromosomes and preferential elimination of chromosomes, including those of *R. sativus*. Similar mitotic irregularities can also have occurred at the plant level, when propagating the hybrid by means of cuttings. On the hybrid, some FDA stainable pollen was formed and meiosis studies of PMCs showed the formation of a large number of bivalents. The presence of univalents at meiosis may result from somatic chromosome elimination. Trivalent formation was observed in PMCs with more than 60 chromosomes, suggesting that either homeologous chromosome association, A and/or C with R, had occurred, since partial homology between A and R and C and R has been reported (Dolstra 1982), or that preferential association of homologous duplicated chromosomes had occurred. Marker studies and in situ hybridization studies are needed to investigate whether chromosomes from *B. napus* or *R. sativus* are duplicated or eliminated in SH-1 hybrid cuttings.

All cuttings of the hybrid tested except one showed more BCN-resistance than *B. napus*. Many cuttings were as resistant as the *R. sativus* parent. These results suggest that the expression of the resistance of the *R. sativus* in the hybrid is strong, and is not very much influenced by the presence of the *B. napus* genome. However, the resistance studies are complicated by the genetic instability of the hybrid plant, and the resulting variation in the ratio of the *B. napus* and *R. sativus* chromosomes. Chromosomal instability has often been shown to occur in somatic hybrid plants. For example in protoplast fusion products of *B. napus* (+) *E. sativa* (Fahleson *et al.* 1988) and of *S. tuberosum* (+) *S. phureja* (Puite *et al.* 1986) preferential elimination of chromosomes, resulting in asymmetric hybrids and also mixoploidy, was reported.

Analysis of cpDNA of the SH-1 cuttings suggested that the chloroplasts of the hybrid originate from *B. napus*, although definite proof of the origin of the chloroplast genome cannot be given, since only one probe was used for chloroplast analysis. However, chloroplast recombination in somatic hybrids is thought to be a rare event (Maliga *et al.* 1987).

The study of the mitochondrial genome using several probes and restriction enzyme combinations provided evidence for recombination or rearrangements in the SH-1 hybrid. Similar extensive mtDNA rearrangements, induced during tissue culture, have been reported for *Beta* (Brears *et al.* 1989) and *Brassica* (Shirzadegan *et al.* 1991). However, reports on the absence of mtDNA rearrangements in plants regenerated from protoplast-derived calli of *Brassica* are more frequent (Kemble *et al.* 1988, Morgan and Maliga 1987, Sakai and Imamura 1990). The mtDNA rearrangements reported here are more likely to have resulted from recombination between parental mtDNA in the heteroplasmic state of the hybrid cells. This is in accordance with earlier results from somatic fusion studies (Boeshore *et al.* 1983, Chetrit *et al.* 1985, Landgren and Glimelius 1990, Sakai and Imamura 1990).

Although a *Brassica-Raphanus* somatic hybrid, with a high level of BCN resistance was obtained, the hybrid genotype showed reduced fertility, and could not yet be backcrossed with *B. napus*. Therefore, further experiments are needed to optimize the production of fertile somatic hybrids between *Brassica napus* and *Raphanus sativus*, in order to be able to transfer the BCN resistance of *R. sativus* to the *B. napus* gene pool.

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CHAPTER 4

Intergeneric crosses for the transfer of resistance to the beet cyst nematode from *Raphanus sativus* to *Brassica napus*

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Summary

The possibilities to transfer important traits and in particular resistance to the beet cyst nematode (*Heterodera schachtii*, abbrev. BCN) from *Raphanus sativus* to *Brassica napus* were investigated. For these studies *B. napus*, *R. sativus*, the bridging hybrid x*Brassicoraphanus* as well as offspring from the cross x*Brassicoraphanus* x *B. napus* were used. Reciprocal crosses between *B. napus* and *R. sativus* were unsuccessful, also with the use of embryo rescue. Crosses between x*Brassicoraphanus* as female parent and *B. napus* resulted in a large number of F_1 hybrids, whereas the reciprocal cross yielded mainly matromorphic plants. BC_1, BC_2 and BC_3 plants were obtained from backcrosses with *B. napus*, which was used as the male parent. F_1 hybrids and BC plants showed a large variation for morphology and male and female fertility. Cuttings of some F_1 and BC_1 plants, obtained from crosses involving resistant plants of x*Brassicoraphanus*, were found to possess a level of resistance similar to that of the resistant parent. These results and indications for meiotic pairing between chromosomes of genome R with those of the genomes A and/or C suggest that introgression of the BCN-resistance of *Raphanus* into *B. napus* may be achieved.

Introduction

Brassica napus L. ssp. *oleifera* (Metzg.) Sinsk. (oil-seed rape and fodder rape) is a highly susceptible, but tolerant host for the beet cyst nematode, *Heterodera schachtii* Schm. (abbrev. BCN). Its cultivation results in considerable multiplication of BCN. No BCN-resistant genotypes are known for *B. napus* (Bowen *et al.* 1986, Harrewijn 1987). Sugar beet, a major crop in North-West Europe, is both susceptible and sensitive to the nematode. For this reason it is recommended not to grow oil-seed rape in a narrow rotation system with sugar beet, unless soil fumigation or nematicides are applied to control the nematode. Hence, BCN-resistant oil-seed rape would be of great value. In *Raphanus sativus* L. ssp. *oleiferus* (DC.) Metzg. (fodder radish) resistance to BCN has been found (Baukloh 1976, Toxopeus and Lubberts 1979). The inheritance of resistance in *Raphanus* is thought to be monogenic and dominant (Baukloh 1976).

Sexual hybridization of *R. sativus* and *B. napus* has been found to be difficult due to the occurrence of strong crossing barriers. In general either no or a low number of hybrids could be obtained (e.g. Chopinet 1942, Heyn 1973, McNaughton and Ross 1978, Takeshita *et al.* 1980, Turesson and Nordenskiöld 1943). However, more recently, Paulmann and Röbbelen (1988) and Thierfelder *et al.* (1991) have reported a more successful recovery of hybrid plants when embryo rescue was applied. Attempts to transfer BCN-resistance from *Raphanus sativus* to *B. napus* by means of somatic hybridization resulted in a mitotically unstable hybrid showing somaclonal variation for BCN-resistance (Lelivelt and Krens 1992).

The use of the intergeneric hybrid between *Brassica rapa* and *Raphanus sativus*, x*Brassicoraphanus* Sageret, both Raparadish (AARR, $2n = 38$) and

Radicole (RRCC, $2n = 36$), in crosses with *B. napus* has been shown to be promising for the transfer of *Raphanus* characters into *Brassica* (Agnihotri et al. 1990, Dolstra 1982). Dolstra (1982) has described successful reciprocal crosses between *B. napus* and x*Brassicoraphanus*. The BCN-resistance of the x*Brassicoraphanus* populations used for these crosses, however, has been found to be rather low (Lange et al. 1989). Recurrent mass selection for resistance within these populations of x*Brassicoraphanus* (Raparadish) has led to levels of BCN-resistance, similar to those found in fodder radish (Lange et al. 1989) and the selected populations might thus provide a better source of material for use in crosses with *B. napus*, in order to obtain BCN-resistant oil-seed rape.

The present paper describes attempts to obtain sexual hybrids between *B. napus* and *R. sativus* as well as the production and characterization of hybrids between BCN-resistant x*Brassicoraphanus* and *B. napus*. Also, the feasibility of backcrossing F_1 and BC plants with *B. napus* as recurrent parent is studied, using plants derived from F_1, BC_1 and BC_2 seeds obtained from Dolstra (1982).

Materials and methods

Testing for beet cyst nematode resistance

Seedlings or cuttings were tested for resistance to the beet cyst nematode according to the method of Toxopeus and Lubberts (1979) with slight modifications. For evaluation of F_1 and BC_1 material cuttings were used and these were propagated in vitro as described by Lelivelt and Krens (1992). In each experiment one cutting is considered a replicate. Experiments 1 to 3 were performed with F_1 hybrids, with between two to six replicates of one clone. In Experiments 4 and 5, two replicates were used for each BC_1 plant and four replicates for the F_1 hybrids. In each experiment cuttings of *B. napus* were used as susceptible control and cuttings of three populations of x*Brassicoraphanus* as resistant controls. For the susceptible and resistant controls six plants per replicate were used with for the Experiments 1 to 5, respectively, four, six, two, four and three replicates. Plants were inoculated with a suspension of pre-hatched J_2 larvae from *Heterodera schachtii.* In the Experiments 1 to 5 each plant was inoculated with approximately 500 larvae, while for the experiments with seedlings approximately 300 larvae per plant were used. At four weeks after inoculation the cysts produced on the root system of each plant were counted. A square root transformation was performed on the actual number of cysts per plant to achieve a more balanced regression analysis.

Plant material

One BCN-susceptible accession Rape Kale, and 13 susceptible cultivars of *B. napus* (oil-seed rape and fodder rape) were used. The oil-seed rape cultivars are 'Akela', 'Blako', 'Bridger', 'Cascade', 'Darmor', 'Emerald', 'Jet Neuf', 'Mansholts Hamburger', 'Petranova', 'Rapol', 'Tantal', 'Tower' and 'Zephyr'.

Seedlings, remaining free from cysts in the resistance tests were selected from the cultivars 'Nemex' and 'Pegletta' of *R. sativus* (fodder radish). In addition, seedlings with less than 6 cysts on their root system were selected from each of three populations of *xBrassicoraphanus*, described by Lange *et al.* (1989). These populations coded PH + MH, selection cycle 5(b), 5(a) and 4(a) are referred to in this paper as plants from populations A, B and C, respectively. Finally, F_1 hybrid seeds, and BC_1 and BC_2 seeds derived from backcrossing male sterile F_1 hybrids to *B. napus* cv. 'Zephyr' by insect-mediated pollination (Dolstra 1982), were sown and plants were used for further backcrosses to *B. napus*.

Sexual hybridization

Crosses. Plants were grown in pots either in a greenhouse without control of temperature and humidity during late spring and summer or in a greenhouse heated to temperatures between 20 and 22°C during early spring and autumn. Reciprocal crosses were made between *R. sativus* and *B. napus* and between the populations A, B and C of *xBrassicoraphanus* and *B. napus*. Each cross included approximately 20 plants of the cultivars and/or populations of *B. napus*, *R. sativus* or *xBrassicoraphanus*. One to two days before anthesis buds were hand-emasculated and bagged. Pollination followed two to three days later using freshly collected pollen.

F$_1$ hybrids from the crosses between *B. napus* and *xBrassicoraphanus*, as well as the F_1, BC_1 and BC_2 plants, derived from seed produced by Dolstra (1982) were backcrossed with several cultivars of *B. napus* by large-scale insect pollination. F_1 or BC plants were grown with *B. napus* plants for pollination in isolation cages in a heated greenhouse during winter or in the field during late spring and summer. Honey-bees or flies were used to pollinate the flowers without prior emasculation.

Embryo rescue. Two to 14 days after pollination developing siliquae were collected and surface-sterilized as described by Lelivelt and Krens (1992). Siliquae of reciprocal crosses between *B. napus* and either *R. sativus* or *xBrassicoraphanus* were cultured on four different media, consisting of MS minerals and vitamins (Murashige and Skoog 1962), 0.8% (w/v) agar, 5% (w/v) saccharose, 300 mg/l casein hydrolysate, set at pH 5.8; either with no hormones, or complemented with 1 mg/l indole-3-acetic acid (IAA) + 0.2 mg/l kinetin (kin), with 2 mg/l IAA + 0.5 mg/l kin or with 0.5 mg/l naphthaleneacetic acid (NAA) + 2.5 mg/l kin. The cultures were incubated at 25°C with a photoperiod of 16 h light (6 W/m^2) a day. After three to four weeks, the shrivelled ovules with small embryos, mostly at the globular stage, were excised from the siliquae and cultured on the same medium. Larger embryos, mostly at the torpedo stage, were dissected from the remains of the ovule and were propagated on Medium 1 (Lelivelt and Krens 1992). After rooting plantlets were transferred to soil and grown in a conditioned greenhouse at 18 to 22°C.

In vitro culture. Seeds were surface-sterilized, germinated and plants were propagated as described by Lelivelt and Krens (1992). Germination of seeds was carried out in the dark on a medium with MS minerals and vitamins, 0.8%

(w/v) agar, 1% (w/v) saccharose at pH 5.8. Seedlings were transplanted for propagation on Medium 1. For mass propagation, explants such as axillary buds or apical meristems of F_1 plants were sterilized and cultured on Medium 1. In addition, explants of petioles or stems were used to induce callus and shoot regeneration on Medium 1 supplemented with 1 mg/l 6-benzylaminopurine (BAP) and 0.035 mg/l gibberellic acid (GA_3). Regenerated shoots were cultured and propagated on Medium 1. Plants were potted in compost and subsequently grown in a conditioned greenhouse or F_1 and BC_1 cuttings were used to investigate BCN-resistance.

Cytological observations and pollen viability

To determine the number of chromosomes, root tips from plants grown in a greenhouse were pretreated, fixed and stained with Feulgen according to Jochemsen and Mlyniec (1974). For meiotic observations flower buds were fixed and stained in Snow's solution (Snow 1963) as described by Lelivelt and Krens (1992). Pollen viability was scored using samples of 100 to 200 pollen grains (unless otherwise mentioned). The frequency of micropollen was determined in samples of 250 to 300 pollen grains. The assessments were based on freshly collected pollen stained in either a solution containing 9% (w/v) sucrose and 0.5 mg/ml fluorescein diacetate or in an acetocarmine solution (2% (w/v) carmine in 45% (v/v) acetic acid).

Results

Sexual hybridization

Crosses. Except for the cross *R. sativus* x *B. napus*, all cross combinations yielded seeds (Table 1). Most of the seeds were large sized and plump, and were viable (average germination percentage ranged from 65 to 90%). Some of the seeds were small and shrivelled. Such seeds were sterilized and germinated in vitro, and on average 30 to 50% of these seeds resulted in the formation of plants.

Crosses between *B. napus* (♀) and *R. sativus* (♂) with or without the use of embryo rescue yielded only plants which were *B. napus*-like and presumably matromorphs. From crosses between x*Brassicoraphanus* (♀) and *B. napus* (♂) putative hybrids were obtained from both plump and small seeds. The reciprocal cross with *B. napus* as mother resulted in many *B. napus*-like plants, assumed to be matromorphs, but no hybrids, except for the crosses with x*Brassicoraphanus* population A. Again the putative hybrid plants were obtained from large and small seeds. Embryo rescue also resulted in a few putative hybrids from crosses between x*Brassicoraphanus* (♀) and *B. napus* (♂), but in general the extra input required for embryo culture did not result in improved hybrid plant production (Table 1). By far the best results were obtained when plants from x*Brassicoraphanus* population A were used as female parent in crosses with *B. napus* (Table 1).

Table 1. Seed set and number of plants and hybrids obtained without and with embryo rescue from reciprocal crosses between *B. napus* and *R. sativus* and between *B. napus* and BCN-resistant plants from the xBrassicoraphanus populations A, B and C

Cross	Without embryo rescue				With embryo rescue		
	Pollinated flowers	Seeds	Plants	Hybrids	Siliquae cultured	Plants	Hybrids
B. napus x *R. sativus*	170	41	26	0	125	12	0
R. sativus x *B. napus*	48	0	-	-	150	0	0
B. napus x x*Brassicoraphanus* A	231	138	113	18	0	-	-
B. napus x x*Brassicoraphanus* B	920	460	311	0	125	6	0
B. napus x x*Brassicoraphanus* C	629	387	242	0	50	12	0
x*Brassicoraphanus* A x *B. napus*	128	135	115	115	0	-	-
x*Brassicoraphanus* B x *B. napus*	315	175	102	101	55	4	4
x*Brassicoraphanus* C x *B. napus*	310	105	51	46	0	-	-

Hundred and fifty flowering F_1 hybrids derived from crossing resistant x*Brassicoraphanus* plants with *B. napus* yielded 447 BC_1 seeds (Table 2). Backcrosses of 54 F_1, 18 BC_1 and 16 BC_2 plants, derived from seeds obtained by Dolstra (1982), with *B. napus* as male parent resulted in 13 seeds on 3 F_1 plants, 104 seeds on 9 BC1 plants and 2277 seeds on 13 BC_2 plants (Table 2). So crossability increased with every BC generation.

Morphology. The classification of progeny from reciprocal crosses between *B. napus* and *R. sativus* or x*Brassicoraphanus* as presented in Table 1, was based on morphological observations, which clearly indicated the hybrid nature of the putative hybrid plants. Matromorphs had a morphology closely resembling the *B. napus* parent. Despite a large variation in morphology in the vegetative and generative stage, the F_1 hybrids were in many respects intermediate between the parental species, for instance in leaf morphology. Most plants grew vigorously and flowered abundantly. Flower buds of most hybrids were bigger than those of *B. napus*. Purple veins, typical for the flowers of *R. sativus* and x*Brassicoraphanus*, were not observed in flowers of F_1 hybrids. Some hybrids had a chimaeric appearance due to flowers having white petals with yellow spots or to racemes having both completely yellow and completely white flowers. Most hybrids had normally developed anthers.

BC_1 plants showed much variation in morphology, with some plants resembling very much the *B. napus* parent and others the x*Brassicoraphanus* parent. Many plants had flowers with a yellow petal colour and a petal length similar to *B. napus*. Also, plants with an aberrant morphology were observed. Most of these plants were stunted, having wrinkled leaves and flowers with rudimentary anthers. No clear morphological differences were found between the F_1 and BC_1 plants produced in this study and the respective F_1 and BC_1 plant material provided by Dolstra (1982). BC_2 plants mostly resembled the *B. napus* parent and showed a yellow petal flower colour or creamy and yellow petals. Flowers had normal anthers.

Cytological analyses and fertility

Some of the F_1 hybrids, mentioned in Table 1, and the BC_1 plants, derived from backcrossing of such F_1 hybrids with *B. napus*, were investigated cytologically (Table 3). Most F_1 hybrids were found to have a somatic number of chromosomes of 38, as expected from fusion of normal parental gametes. One plant had 57 chromosomes and the remaining hybrids were found to be hypoploid. The variation in number of chromosomes between BC_1 plants, from 36 to 70, was larger than amongst F_1 hybrids and the frequency of plants with more than 38 chromosomes was considerably higher (Table 3).

Meiosis in the F_1 hybrids was highly irregular (Table 4; Fig. 1). The degree of chromosome association in pollen mother cells (PMCs) of F_1 hybrids was found to be higher than the 10 bivalents and 18 univalents expected from pairing between the chromosomes of the genome A of *B. napus* and *B. rapa* only (Fig. 1A). In PMCs of five out of 12 F_1 hybrids on average less than 10 bivalents were observed, which, however, was associated with the occurrence of multivalents (Table 4). In 38-chromosome hybrids the average number of

Table 2. BC$_1$, BC$_2$ and BC$_3$ progenies obtained by insect-mediated backcrossing with *B. napus* as the recurrent parent. The female material consisted of F$_1$ hybrids, derived from reciprocal crosses between BCN-resistant x*Brassicoraphanus* (BCN-selected material) and *B. napus* and of F$_1$ and BC plants from Dolstra (1982)

Cross	Number of flowering maternal plants	Plants with seeds		Number of BC seeds	
		Number	Percentage	Total	Per seeded plant
BCN-selected material					
F$_1$ x *B. napus*	150	59	39.3	447	7.6
Material Dolstra (1982)					
F$_1$ x B. napus	54	3	5.6	13	4.3
BC$_1$ x B. napus	18	9	50.0	104	11.6
BC$_2$ x B. napus	16	13	81.3	2277	175.2

Table 3. Distributions for number of chromosomes in F$_1$ hybrids derived from reciprocal crosses of *B. napus* and x*Brassicoraphanus* and BC$_1$ plants derived from backcrossing F$_1$ plants to *B. napus*

Generation	Plants investigated	Number of chromosomes								
		36	37	38	39	40	42	43-45	57	65-70
F$_1$	21	3	1	16	0	0	0	0	1	0
BC$_1$	31	1	1	7	4	8	4	4	0	2

univalents was 2.1 higher than that in 36-chromosome hybrids (Table 4). The overal degrees of chromosome association in both types of hybrids was found to be similar, although the frequencies of the different types of chromosome configuration were found to vary slightly. The 36-chromosome hybrids showed on average more trivalents than the 38-chromosome hybrids. Also one PMC with a hexavalent configuration was found.

At anaphase I (AI) and metaphase II (MII) most of the 42 PMCs observed of the 36-chromosome F_1 hybrids had an 18-18, 17-19 or 16-20 distribution of chromosomes. For the 38-chromosome hybrids 182 PMCs were examined and the prevailing distributions of chromosomes in these cells were found to be 19-19 or 18-20. Laggards at AI were observed in both types of hybrid in about 12% of the PMCs studied (Fig. 1B). Mostly one or two lagging chromosomes per PMC were found. In one F_1 hybrid, 871503-7, laggards were found in as many as 20 of the 55 PMCs studied. Occasionally anaphase bridges were found. Laggards were often included in the anaphase groups, either intact or after precocious division. The second meiotic division was found to show irregularities, such as laggards and misdivisions.

A large variation between F_1 hybrids was observed with regard to frequency and types of sporad at tetrad stage. In 21 hybrids from reciprocal crosses between *B. napus* and x*Brassicoraphanus* 2652 sporads were scored. On average 78% tetrads (range 13-98%), 10% dyads (range 0-86%), 6% pentads (range 0-36%), 3% triads (range 0-9%), 2% hexads (range 0-11%), and less than 1% monads and heptads (range 0-3%) were observed. Substantial variation was found with regard to the size of the cells per sporad (Fig. 1C). Dyads mostly contained two equally sized cells and triads mostly two equally sized cells with one microgone or one large cell and two small cells. Pentads, hexads and heptads had a large variation in cell size. Dyads, triads and pentads were observed in PMCs of nearly all hybrids, but hexads and heptads in only nine out of the 21 hybrids studied. As an example hybrid 871503-7, which also has been found to show a high frequency of PMCs with laggards, showed 36% pentads, 19% hexads and 3% heptads at tetrad stage. The frequency of PMCs with microgones was relatively high and coincided at the pollen stage with micropollen formation and variation in pollen size (Table 5; Fig. 1D). Pollen production and pollen viability of the F_1 hybrids were low (Table 5).

Meiosis of BC_1 plants was studied in 43 PMCs of two genotypes with 46 chromosomes. The frequencies of univalents and bivalents were found to differ between the two genotypes, with for one genotype on average 8.88 univalents and 17.06 bivalents, whereas the other had 11.65 univalents and 15.38 bivalents. The total average of configurations in PMCs of both genotypes showed 10.56 (range 6-16) univalents, 16.05 (10-19) bivalents, 0.05 (0-1) trivalents, 0.77 (0-2) quadrivalents and 0.02 (0-1) hexavalents. Multivalents occurred in 63% of the cells and were found in cells both with low or high numbers of univalents. An increase in the number of multivalents seemed to be associated with a decrease in number of bivalents. Later stages of meiosis were not studied in detail but preliminary observations indicated the occurrence of irregularities similar to those observed in the F_1 hybrids.

A large variation for micropollen production and pollen viability was found in the BC_1 generation (Table 5). BC_1 plants with an aberrant morphology were

Table 4. Association of chromosomes in metaphase I of meiosis in pollen mother cells (PMCs) of xBrassicoraphanus, B. napus and F$_1$ hybrids from reciprocal crosses between xBrassicoraphanus and B. napus differing in number of chromosomes

Genotype	Number of PMCs analysed	Mean number (range) of configurations per cell				
		Univalents	Bivalents	Trivalents	Quadrivalents	Hexavalents
xBrassicoraphanus	20	1.80 (0-2)	18.10 (18-19)	0	0	0
B. napus	25	0	19	0	0	0
F$_1$ (2n=36)						
870047-1	10	11.30 (9-16)	8.00 (5-10)	2.50(2-3)	0.30(0-1)	0
870003-2	33	10.58 (7-15)	11.30 (6-14)	0.82(0-4)	0.06(0-1)	0.03(0-1)
870287-3	11	13.55(12-16)	7.91 (7-9)	1.36(0-2)	0.64(0-1)	0
871505-2	5	10.80 (9-12)	10.40 (9-12)	0.40(0-1)	0.80(0-1)	0
Means (Total range)	59	11.56 (7-16)	9.40 (5-14)	1.27(0-4)	0.45(0-1)	0.01(0-1)
F$_1$ (2n=38)						
870001-1	7	14.00(10-18)	10.29(7-11)	1.14(0-2)	0	0
870001-3	18	13.72(10-18)	11.28(10-13)	0.50(0-2)	0.06(0-1)	0
870022-9	12	11.42 (8-15)	12.25 (9-14)	0.25(0-2)	0.33(0-2)	0
870274-2	20	14.35(11-18)	9.95 (7-13)	1.05(0-2)	0.15(0-1)	0
871497-2	24	13.46(10-16)	11.08 (8-14)	0.54(0-2)	0.17(0-1)	0
871503-7	25	13.96(11-17)	10.84 (9-13)	0.68(0-2)	0.08(0-1)	0
871511-2	25	14.08(11-16)	9.79 (7-13)	0.29(0-2)	0.83(0-2)	0
871572-4	21	14.19(12-18)	9.29 (6-13)	0.19(0-1)	1.19(0-3)	0
Means (Total range)	152	13.65 (8-18)	10.60 (6-14)	0.58(0-2)	0.35(0-3)	0
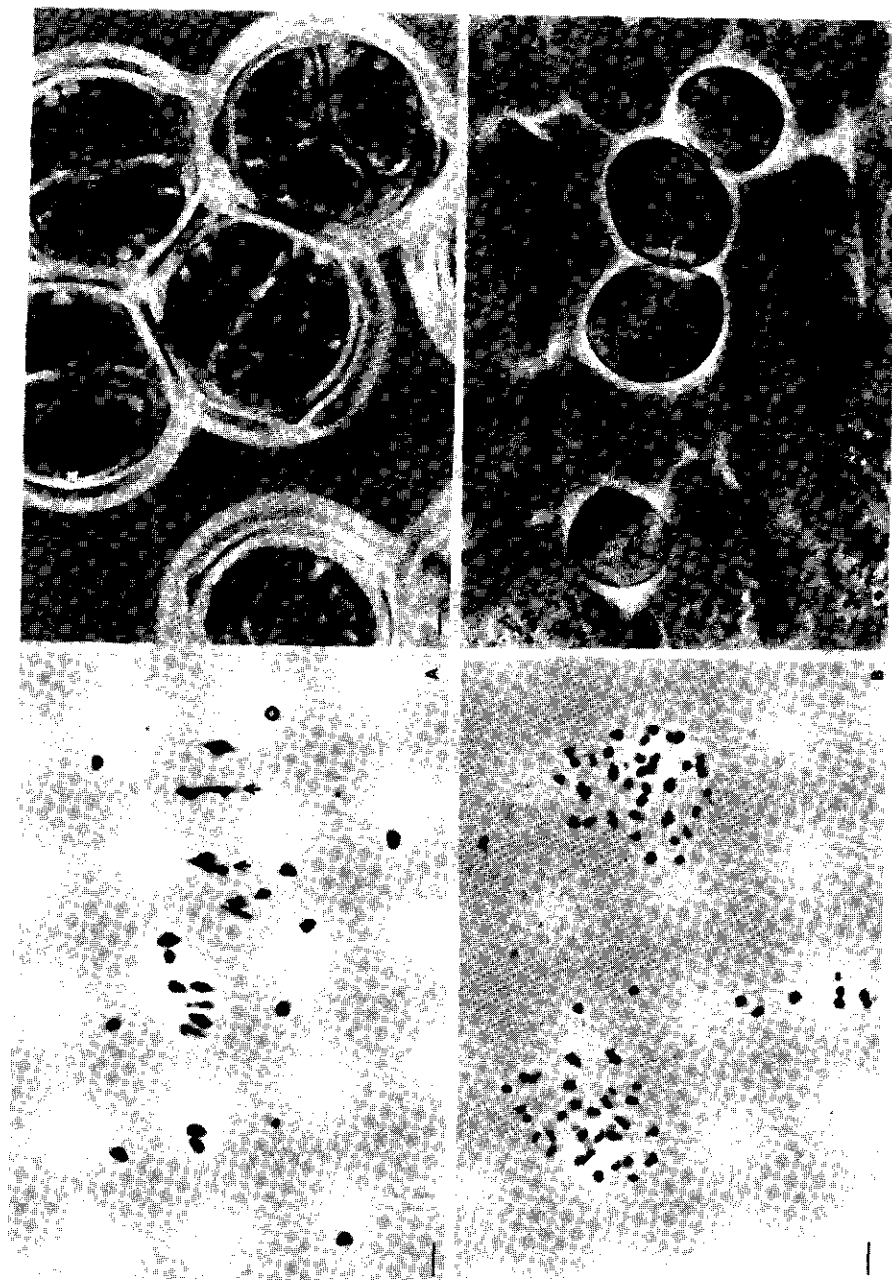
Figure 1. Meiosis in PMCs as well as pollen of F_1 hybrids of reciprocal crosses between x*Brassicoraphanus* and *B. napus* **A.** MI with 14 univalents + 8 bivalents + 2 trivalents (arrows). **B.** Lagging chromosomes at AI/MII. **C.** Tetrad stage with two dyads (DY), one tetrad (TE) and one PMC in centre with unequally sized cells. **D.** Pollen with different cell sizes, two small and two larger cells at pollen stage, probably originating from one PMC. Bar = 5 μm.

completely male sterile and were not included in Table 5. The remaining BC_1 plants produced more viable pollen as compared to the F_1 hybrids (Table 5). Flowers of BC_1 and BC_2 plants were in part female fertile, resulting in the production of BC_2 and BC_3 seeds after backcrossing with *B. napus* (Table 2).

Table 5. Frequency distribution of F_1 and BC_1 plants over pollen viability classes and micropollen classes, $F_1 =$ hybrids from reciprocal crosses between *B. napus* and x*Brassicoraphanus*; $BC_1 = F_1$ hybrids (♀) x *B. napus* (♂)

Progeny	Number of genotypes	Frequency (%)				
		Pollen viability class (%)				
		<1	1-5	6-10	11-20	>20
F_1	152[a]	63.2	19.7	11.8	4.0	1.3
BC_1	33[b]	42.4	30.3	9.1	9.1	9.1
		Micropollen class (%)				
		<1	1-5	6-10	11-20	>20
F_1	14	7.1	35.7	35.8	21.4	0
BC_1	11	9.1	81.8	9.1	0	0

[a] plants that produced very little pollen were also included;
[b] plants with aberrant morphology were excluded

BCN-resistance

The level of BCN-resistance, expressed as number of cysts per root system, was studied in 116 F_1 hybrids and is shown in Fig. 2. The susceptible control *B. napus* was found to vary considerably for the average number of cysts per plant, being 100, 34 and 37, respectively, for Experiments 1 to 3. Therefore, the results of Experiment 1 and of Experiments 2 and 3 are presented separately. The number of cysts produced on root systems of the F_1 hybrids relative to that observed on *B. napus*, however, was more consistent over experiments. The populations of x*Brassicoraphanus* also included approximately 30% plants with more than ten cysts, but the average number of cysts per plant in these three populations was low, ranging between four and ten.

A large range in average number of cysts per root system was observed in the F_1 progenies of all four crosses. The overall level of BCN-resistance of the F_1 hybrids was found to be intermediate to that of the parental species (Fig. 2).
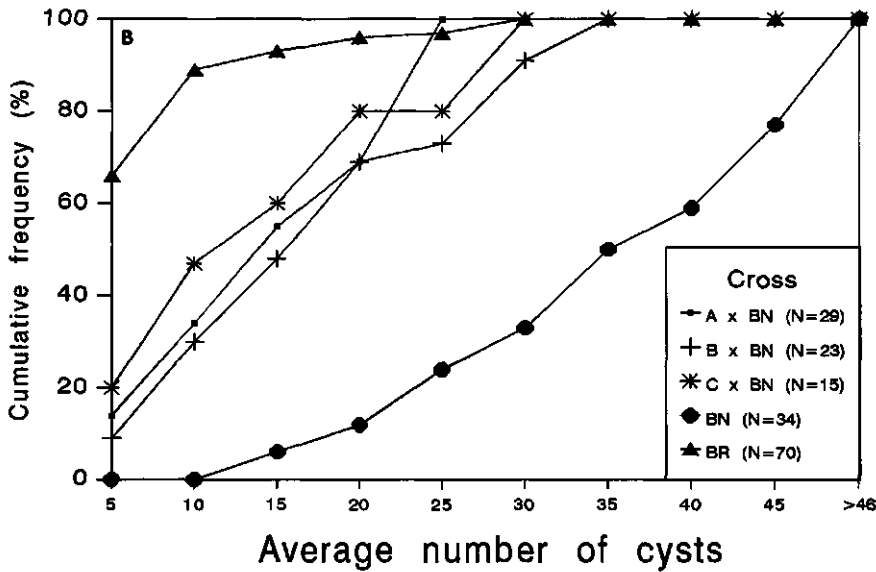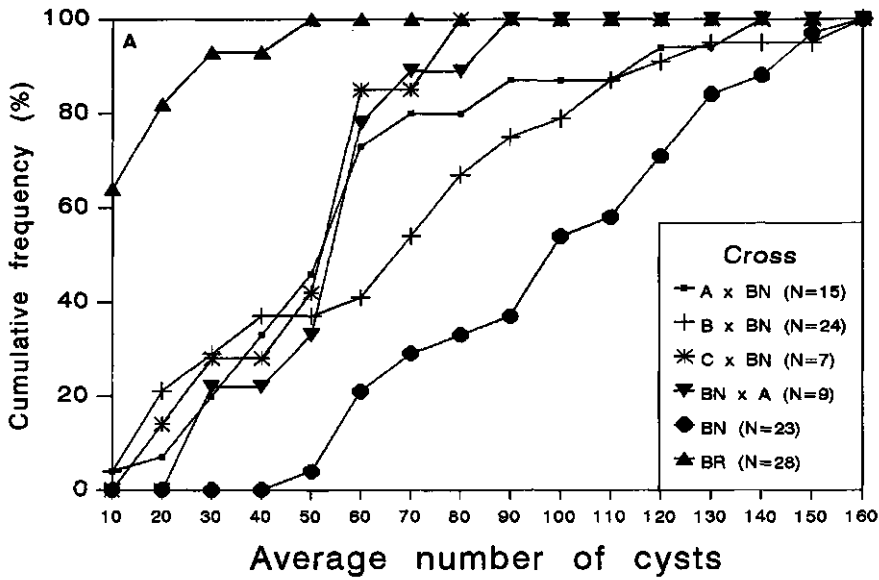
Figure 2. Cumulative frequency distributions of BCN-resistance in F_1 hybrids derived from crosses between x*Brassicoraphanus* (BR) populations A, B or C and *B. napus* (BN) (A x BN, B x BN and C x BN), and between *B. napus* and x*Brassicoraphanus* population A (BN x A). **A.** Experiment 1. **B.** Experiments 2 and 3. N = number of plants.

The proportion of highly resistant plants, i.e. plants having less than ten cysts, was not much different between the four groups of F_1 hybrids derived from crosses between the three x*Brassicoraphanus* populations and *B. napus* (Figs. 2A and 2B). In total, 27 F_1 hybrids had on average less than ten cysts on their root system. Only eight of these 27 hybrids were found to be as BCN-resistant as the x*Brassicoraphanus* parent used in crosses, i.e. having less than six cysts per root system. The number of highly resistant plants in Experiment 1 was very low for all four crosses (Fig. 2), which presumably is a result of the high level of attack by the nematode. Forty-four out of 116 hybrids (38%) showed an average number of cysts per plant being significantly lower than that observed on *B. napus* 'Tantal' or 'Jet Neuf' and did not differ in number of cysts from the resistant x*Brassicoraphanus* populations A to C. The remaining 72 F_1 hybrids were found to have an intermediate level of resistance, i.e. having significantly less cysts than the susceptible *B. napus* parent but significantly more than the x*Brassicoraphanus* populations, or had an average number of cysts not differerent from that on the susceptible *B. napus* (p > 0.95).

Thirty-two BC_1 plants, derived from three F_1 hybrids with a high level of BCN-resistance, i.e. having less than ten cysts per root system, and 18 BC_1 plants derived from susceptible F_1 hybrids were evaluated for BCN-resistance. The BC_1 progeny of susceptible F_1 plants was susceptible. Ten out of 32 BC_1 plants derived from resistant F_1 hybrids showed a high level of resistance, having two to eight cysts per root system, which is not significantly different from that observed on the x*Brassicoraphanus* populations or resistant F_1 hybrids. The remaining BC_1 plants derived from resistant F_1 hybrids were susceptible.

Discussion

The attempted direct crosses between *B. napus* and BCN-resistant *R. sativus* were unsuccessful. This was probably mainly due to incongruity barriers, as has been reported for crosses between diploid or tetraploid accessions of *R. sativus* and *B. napus* (Chopinet 1942, Fukushima 1945, Heyn 1973, Takeshita *et al.* 1980, Turesson and Nordenskiöld 1943). On the other hand, Paulmann and Röbbelen (1988) and Thierfelder *et al.* (1991) reported quite large numbers of hybrids from these intergeneric crosses but they used only a few parental genotypes. The cause of the failure in recovering hybrid plants from our experiments is unknown. It might have been due to the genotypes used in crosses or to the application of siliquae culture instead of ovule culture as was applied by Thierfelder *et al.* (1991) and Paulmann and Röbbelen (1988).

To transfer *Raphanus* characters such as BCN-resistance, many F_1 hybrids between x*Brassicoraphanus* and *B. napus* and backcross progenies of these hybrids with *B. napus* were produced. The majority of the F_1 hybrids studied had the expected number of 38 chromosomes, the sum of parental euploid gametes, which is in accordance with results of Dolstra (1982). These plants are assumed to have an AACR genome constitution. The origin of a plant with 57 chromosomes, which has also been reported by Dolstra (1982) is not clear;

it may have been derived from unreduced gametes of either *B. napus* or x*Brassicoraphanus*, resulting in an AAACCR or AAACRR genome constitution, respectively. The F_1 hybrid plants with 36 or 37 chromosomes most likely were the result of fusion of an unbalanced x*Brassicoraphanus* gamete ($n = 17$ or 18) and a normal *B. napus* gamete ($n = 19$). Dolstra (1982) showed that plants from populations of x*Brassicoraphanus* (AARR) were found to have a number of chromosomes mostly in the range of 37 to 39, and some of these plants may produce unbalanced gametes with 17 or 18 chromosomes, instead of 19.

Meiosis in F_1 plants having a similar number of chromosomes was found to be highly variable between genotypes (Table 4). The types and frequencies of chromosome associations in some 38-chromosome hybrids were similar to those in two 38-chromosome hybrids reported by Dolstra (1982). The number of univalents per cell in the 36-chromosome hybrids was on average two less than that in the 38-chromosome hybrids (Table 4). This leads to the assumption that in the 36-chromosome hybrids two chromosomes of the R genome were missing. In both types of hybrid an increase in the number of multivalents, mostly trivalents in 36-chromosome hybrids and quadrivalents in 38-chromosome hybrids, was associated with a decrease in number of bivalents. This could be explained as a result of pairing of one or two C or R chromosome(s) with a pair of A chromosomes. In AAC hybrids similar frequencies of multivalents occurred (Namai 1976, 1978), and in AAR hybrids only very few were detected (Dolstra 1982). This suggests that the extra chromosomes in multivalents were derived from pairing of chromosomes of genome A with those of genome C. However, since in AR and CR hybrids allosyndetic pairing is assumed between chromosomes of the three genomes (Dolstra 1982, McNaughton 1973, Mizushima 1980, Namai 1976), it seems likely that the decreased univalent frequency and increased multivalent formation in AACR hybrids was also caused by homoeologous pairing involving chromosomes of genome R.

The F_1 hybrids produced a high frequency of unbalanced gametes and also unreduced gametes resulting in BC_1 plants with a large variation in number of chromosomes. A few BC_1 plants had more than 60 chromosomes and were presumably derived from one or two unreduced gamete(s). Also (somatic) chromosome duplication might have occurred at a later stage of plant development. As a consequence of the variation in number of chromosomes, the types and the amount of chromosome association at meiotic MI as well as pollen viability are expected to vary between BC_1 plants. The results of studies of pollen viability and pollen production, as well as those of backcrosses with BC_1 material of Dolstra (1982), however, suggest an improvement of fertility of backcross progenies as opposed to F_1 hybrids.

Paulmann and Röbbelen (1988) and Thierfelder *et al.* (1991) also used hybrids with genome constitutions AACR and ACCR in backcrosses with *B. napus* to obtain BC progenies. The AACR and ACCR hybrids, however, were derived from crosses of AACCRR hybrids (from reciprocal crosses between *B. napus* and *R. sativus*) with *B. rapa* and *B. oleracea*, respectively, instead of using AARR or CCRR in crosses with *B. napus* (Agnihotri *et al.* 1990, Dolstra 1982, Tables 1 and 2). It remains interesting to investigate whether meiosis and the level of BCN-resistance of the material produced by Thierfelder *et al.* (1991)

and Paulmann and Röbbelen (1988) are similar to the material from this study.

The level of BCN-resistance of only a few F_1 and BC_1 plants was as high as in the x*Brassicoraphanus* plants used for crosses. It is assumed that the x*Brassicoraphanus* populations, selected for BCN-resistance, contained plants which were heterozygous for the BCN-resistance trait, and thus resulted in both susceptible and resistant F_1 hybrids when crossed with the susceptible *B. napus* parent. The fact that resistant F_1 hybrids backcrossed with susceptible *B. napus* produced a segregating BC_1 progeny, and susceptible F_1 hybrids only susceptible BC_1 progeny is evidence for a dominant behaviour of the BCN-resistance trait. From our results, however, it is not clear whether the BCN-resistance is inherited monogenically, as has been suggested by Baukloh (1976). The introduction of *Raphanus* resistance in *B. napus* might be hampered by suppression of expression of the trait by the *B. napus* genomes, or by a low degree of recombination of *Raphanus* chromosomes with those of *Brassica*. Therefore, it will be necessary to screen large numbers of backcross plants and to use a wide range of *B. napus* genotypes as the pollen parent, in order to be able to select BCN-resistant genotypes with a more balanced meiosis.

It has been shown that further backcrossing with *B. napus* results in an improved fertility of the progeny. This, and our observation that associations between chromosomes of the three genomes are likely to occur, strongly suggest that it is possible to transfer the BCN-resistance from *R. sativus* to the *B. napus* gene pool.

Acknowledgements

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CHAPTER 5

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. Also somatic hybrid plants were 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 equal to that of *B. napus* was obtained. Using cpDNA and mtDNA sequences, it was found that all sexual F_1 hybrids and somatic hybrids contain 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, 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 a potential for gene exchange.

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, is of great importance for *B. napus* in North-West Europe, since only resistant cultivars can be cultivated in a crop rotation system with sugar beet as one of the major crops. No *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 making use 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* (Mathias 1991, Ripley and Arnison 1990). 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*, presents preliminary data on the resistance of the BC_1 generation derived from the sexual hybrids, and results from studies on the morphology, cytology and from DNA analyses of the hybrids.

Materials and methods

Plant materials

Sexual hybridization. The BCN-resistant *S. alba* L. material used consisted of the diploid cultivars 'Emergo' and 'Maxi', the tetraploid cultivar 'Condor' and the tetraploid accession Oscar (Limagrain Genetics, Scheemda, The Netherlands). The accession Rape Kale and the following 13 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', 'Rapol', 'Tantal', 'Tower' and 'Zephyr'.

Somatic hybridization. For the experiments the cultivars 'Emergo' and 'Maxi' of *S. alba* L. and the cultivars '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 spring and summer, and in a heated greenhouse during 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. One to two days before anthesis, buds were emasculated by hand and bagged. Pollination was performed two to three 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* cv. 'Jet Neuf' and 'Tantal' and the four *S. alba* accessions described. Two to 14 days after pollination developing siliquae were collected,

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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 three to four 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 the 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. Ltd., Japan), while also 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 four to five weeks old, were transferred to proliferation medium MS 11 (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 30-40μl 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 showing a combination of the red autofluorescence of the mesophyll protoplasts and the yellow fluorescein fluorescence of the hypocotyl protoplasts under UV light. After two 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 for protoplasts. Putative somatic hybrids hereafter are described as $H_{som}1$, $H_{som}2$, etc.

DNA analyses

Total DNA was extracted from plants, grown in a greenhouse, according to Dellaporta *et al.* (1983). Digestion of plant DNA (5-10 μg) with the restriction

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enzymes *Hind*III, *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 hybridization experiments. The rDNA probe (a 4.0 kb *Eco*RI subclone in pACyc184 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. Dr C. Leaver). Maize ATPase subunits 6 (*atp*6) and 9 (*atp*9) 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 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 two weeks inoculated each with a suspension of approximately 500 pre-hatched J_2 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. In addition, the root size was visually quantified using a scale from 1 (small) to 5 (large). A t-test was applied for statistical analysis of the results of the BCN-resistance tests.

Results

Sexual hybridization

Out of 6717 crosses, without embryo rescue and using *B. napus* as the female parent 2010 seeds were obtained. From these seeds only *B. napus* type plants and no putative hybrids could be recovered. The reciprocal crosses with *S. alba* as the female parent, 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 (Table 1).

When embryo rescue was carried out it was found that 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 found not to be 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', $H_{sex}5$ and $H_{sex}6$ from *S. alba* cv. 'Emergo' and *B. napus* cv. 'Tantal'.

Somatic hybridization

Protoplast regeneration. Culture of mesophyll protoplasts of *S. alba* cv. 'Maxi' and 'Emergo' was unsuccessful: all cells died after two to three divisions. Division of hypocotyl protoplasts of *S. alba* cv. 'Emergo' was visible already after one day in culture. For hypocotyl protoplasts of *S. alba* cv. 'Maxi', and for mesophyll protoplasts of all *B. napus* cultivars used, cell divisions were observed only after a longer culture period. Irrespective of the two culture media used, up to 50% of the hypocotyl protoplasts of *S. alba* cv. 'Emergo' started cell division, whereas only 3 to 5% of the protoplasts of *S. alba* cv. 'Maxi' were found to divide. After four weeks the frequency of microcalli, when using 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 media according to Glimelius (1984), while *B. napus* mesophyll protoplasts performed better in media according to Pelletier *et al.* (1983). The average cell division and callus formation frequencies for *B. napus* ranged from approximately 15% and 0.2% for cv. 'Jet Neuf' to 50% and 1.5% for cv. 'Barrapo' and cv. 'Tower', respectively.

Shoot regeneration from calli of *S. alba* cv. 'Emergo' was observed on both regeneration media, with the highest frequency (5%) 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* cv. '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 one 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 chimera. 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 cv. 'Maxi', the majority of fusion experiments was

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Table 1. Seed set and plant recovery from reciprocal crosses between 14 accessions of *B. napus* and two diploid (2x = 2n = 24) or two tetraploid (4x = 2n = 48) accessions of *S. alba* (data pooled)

Cross	Without embryo rescue				With embryo rescue		
	Pollinated flowers	Seeds	Plants	Hybrids	Ovaries cultured	Plants	Hybrids
B. napus × *S. alba* 2x	6717	2010	1631	0	890	8	0
B. napus × *S. alba* 4x	386	102	87	0	388	3	0
S. alba 2x × *B. napus*	368	2	2	0	412	6	6
S. alba 4x × *B. napus*	350	0	0	0	322	0	0

Table 2. Callus formation and shoot regeneration from putative heterokaryons of *B. napus* (+) *S. alba* in media according to Glimelius (1984)

Parental genotypes			Number of hetero-karyons selected	Callus formation		Number and code of regenerating calli
B. napus	(+)	*S. alba*		Number	%	
cv. 'Jet Neuf'	(+)	cv. 'Emergo'	248	2	0.8	0
cv. 'Barrapo'	(+)	cv. 'Emergo'	143	2	1.4	0
acc. K1	(+)	cv. 'Emergo'	1448	82	5.7	2 $H_{som}1$, $H_{som}2$
cv. 'Tantal'	(+)	cv. 'Emergo'	259	57	22.0	0
cv. 'Tower'	(+)	cv. 'Emergo'	730	215	29.5	2 $H_{som}3$, $H_{som}4$

carried out with these protoplasts and mesophyll protoplasts of the five *B. napus* accessions. PEG-induced fusion yielded 8-10% heterokaryons. Cell division and callus growth of the heterokaryons was much better in media according to 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 for all combinations in callus formation, with the highest frequencies being obtained when *B. napus* cv. 'Tantal' or cv. 'Tower' were used (Table 2). Most of the putative hybrid calli produced only roots and leaf structures which lacked an apical meristem. Growth of putative hybrid calli and shoot regeneration from these calli occurred at a slower rate than that on calli of the parental species *B. napus* and *S. alba*; most shoots on the putative hybrid calli were observed after 4 to 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 to $H_{som}2$-5 were derived from fusion between *S. alba* cv. 'Emergo' and *B. napus* accession K1. $H_{som}2$-1 to $H_{som}2$-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' (Table 2).

Morphological observations and mitosis

Sexual hybrids. All putative hybrids showed intermediate morphological characteristics, e.g. beak length of the pods, but also characteristics typical for one of the parents. Petal colour, petal length and morphology of buds, for example, closely resembled the *B. napus* parent. The morphology of the first leaves looked more like the *S. alba* parent. All plants grew vigourously but sometimes they showed growth abnormalities of leaves and stems. Plants had flowers with six stamens and pollen production was, except for $H_{sex}3$, normal and similar to *B. napus* and *S. alba*. The hybrids flowered without prior vernalization, but $H_{sex}1$ and $H_{sex}2$, having the winter type *B. napus* parent, flowered approximately 1.5 months later than $H_{sex}3$ to $H_{sex}6$. The first flowers from $H_{sex}3$ showed an abberant morphology and were completely male sterile, but later flowers had a normal appearance. All plants had 31 chromosomes. Based on 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 to $H_{som}2$-5 showed irregular growth of the leaves and flowers. Leaves were dark green and in many cases wrinkled and thick. Flowers were yellow, contained six stamens and were smaller than those of the sexual hybrids. Flower petal and sepal shape, anther and stigma morphology as well as beak length varied between flowers and pods on the same hybrid plant. Bud shape and morphology also varied and resembled either *B. napus*, *S. alba* or were intermediate. Not all flowers from these plants produced pollen, and in general pollen production was low. Plants did not grow as vigourously as the sexual hybrids. The number of chromosomes in root tip cells varied between and within the plants, in total ranging from 56 to more than 90. Morphological and cytological analyses

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strongly indicated the hybrid nature of $H_{som}1$ and $H_{som}2$. $H_{som}3$ and $H_{som}4$, however, looked much like the *B. napus* parent and, based on morphology, cytological and RFLP 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 hereafter described results.

DNA analyses

Nuclear DNA. Digestion of total plant DNA with *Bam*HI and hybridization with the rDNA probe was 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 also no polymorphism was found between the two *S. alba* accessions when using this probe/enzyme combination. Hybridization of DNA from $H_{sex}1$ to $H_{sex}6$ resulted in a pattern with bands from both parental species and 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 $H_{sex}1$ to $H_{sex}6$, digested with *Hind*III 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 to $H_{som}2$-5 were found to have a cpDNA pattern equal to that of *S. alba* cv. 'Emergo'.

Table 3 presents results of the analysis of mtDNA. Combinations, that resulted in polymorphisms between the parental species, showed that the sexual hybrids $H_{sex}1$ to $H_{sex}6$ and the somatic hybrids $H_{som}1$, $H_{som}2$-1 to $H_{som}2$-5 have a mtDNA pattern equal to that of the *S. alba* parent (Table 3). No recombinant or new mtDNA restriction fragments were observed in the sexual and somatic hybrids (Table 3).

Meiosis

Sexual hybrids. The association of chromosomes at metaphase I (MI) in hybrid pollen mother cells (PMCs) showed on average 11.3 univalents (I) + 6.4 bivalents (II) + 0.3 trivalents (III) + 1.5 quadrivalents (IV) + 0.03 hexavalents (VI) (Table 4). 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 chromosome distribution. Univalents predominantly moved to the poles, however some

Table 3. Southern blot analyses of total plant DNA derived from two diploid *S. alba* cultivars, three *B. napus* accessions, the sexual hybrids $H_{sex}1$ to $H_{sex}6$ (*S. alba* x *B. napus*) and somatic hybrids $H_{som}1$ and $H_{som}2$ (*B. napus* (+) *S. alba*), digested with *Bam*HI, *Hind*III, *Eco*RI and *Eco*RV and probed with the mtDNA sequences *atpa*, *atp6*, *atp9*, *coxI* and *coxII*. 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	*atpa*	4.3	19	3.4	3.4	3.4	3.4	3.4
*Hind*III	*atpa*	11	5.2/5.3	11	18	11	18	11
*Eco*RI	*atpa*	4.9	10	6	6	6	6	6
*Eco*RV	*atpa*	12	16	16	16	16	16	16
*Hind*III	*coxI*	11	13	11	18	11	18	11
*Eco*RI	*coxI*	16	16	18	16	18	16	18
*Eco*RV	*coxI*	7	7	16	7	16	7	16
*Eco*RV	*coxII*	17/19	2.6	2.6	2.6	2.6	2.6	2.6A
*Bam*HI	*atp6*	5.3	19	5.3	5.3	5.3	5.3	5.3A
*Hind*III	*atp6*	3.4	2.3	3.4	3.4	3.4	3.4	3.4A
*Eco*RI	*atp6*	6	5.3	6	6	6	6	6A
*Eco*RI	*atp9*	2.7/2.8'	5.3	5.4	5.4	5.4	5.4	5.4A

2.7/2.8' = two bands visible at 2.7 and 2.8 kb respectively; - = not examined; A = only $H_{som}2$-1 and $H_{som}2$-5 were used

Table 4. 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		11.28	6.41	0.31	1.46	0.03

Table 5. Range in frequencies of types of sporads in sexual hybrids H_{sex}1 to H_{sex}6 and somatic hybrids H_{som}1 and H_{som}2 (pooled data)

Hybrid type	Frequencies of sporads (%)					
	Monads	Dyads	Triads	Tetrads	Pentads	Sporads with micropollen
Sexual	0-1	7-26	0-8	62-90	0-6	0-13
Somatic	0	0-26	0-12	57-93	0-6	0-6

laggards were observed. Some univalents probably divided prematurily at AI, as suggested by an increase in 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 (Table 5). 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. Study of the 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 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 to 71 chromosomes contained 30 to 35 bivalents and up to 5 univalents. Study of 25 PMCs of $H_{som}2-1$ with 86 to 90 chromosomes showed 37 to 44 bivalents and up to 10 univalents. At AI laggards were observed in some PMCs and unequal chromosome distribution at the two poles was generally found (Table 6). 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 (Table 5). Pollen fertility ranged from 0 to 3% in $H_{som}1$ and $H_{som}2$.

Table 6. Prevailing chromosome distributions and frequencies at AI of pollen mother cells (PMCs) in somatic hybrids $H_{som}1$ and $H_{som}2$

Genotype	PMCs studied	Somatic chromosomes in PMCs, range	Prevailing chromosome distribution at AI range (frequency)
H_{som} 1	13	64-71	34-36(23%), 35-36(46%)
H_{som} 2-1	30	66-9	42-44(27%), 40-46(30%)
H_{som} 2-2	18	60-70	33-33(11%), 32-24(11%)

BC1 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 with use of embryo rescue did not result in plant formation, which is possibly due to the fact that these crosses were performed in winter. BC_1 plants had a morphology closely resembling the *B. napus* parent and were found to have 50 chromosomes.
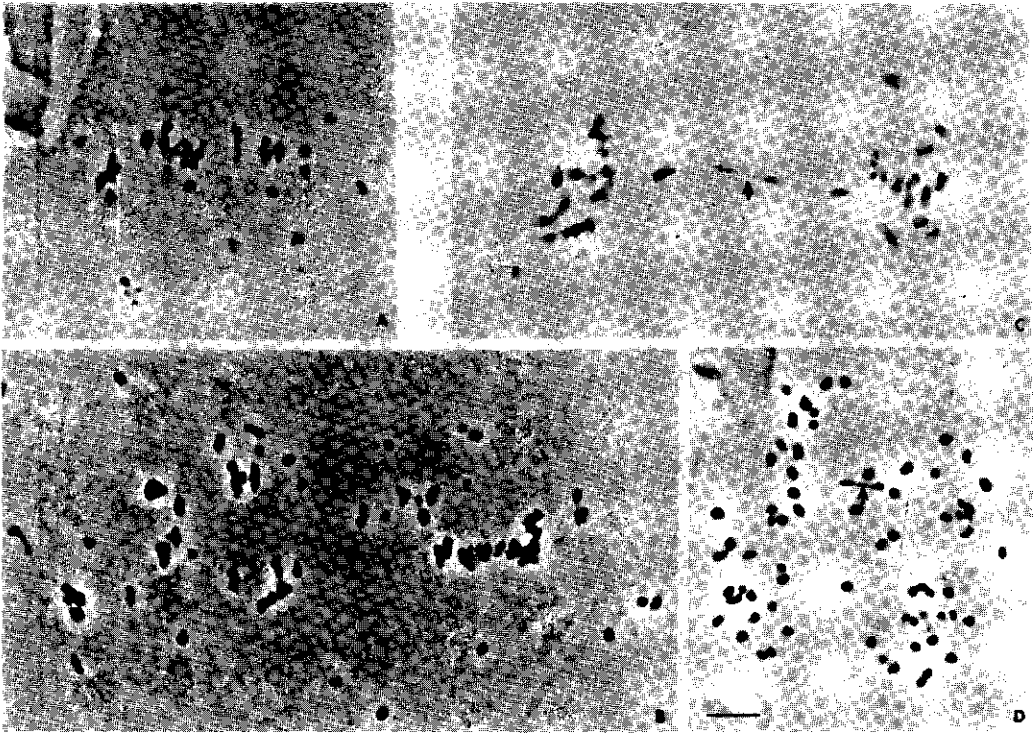
Figure 1 A-D. Meiosis in sexual and somatic hybrids of *S. alba* and *B. napus*. **A** MI in sexual hybrid showing 11 univalents + 6 bivalents + 2 quadrivalents (marked with arrows). **B** MI in somatic hybrid showing prevailing bivalents. **C** AI in sexual hybrid showing a precociously dividing univalent (arrow). **D** Early AI in somatic hybrid showing a precociously dividing univalent (arrow). Bar represents 5 μm.

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 $H_{som}2$ to *B. napus* as male parent 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 hybridization experiments a low frequency of not completely BCN-resistant plants are 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 7). Sexual hybrids $H_{sex}1$, $H_{sex}2$ and $H_{sex}4$ were found to be as BCN-resistant as the *S. alba* parent. Furthermore, no large differences could be observed between the number of cysts produced on different cuttings from each BCN-resistant sexual hybrid (Table 7). 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 give only a few cysts per plant. However, only very few cuttings were tested (Table 7). Cuttings from a backcross plant derived from crossing the susceptible $H_{sex}6$ hybrid with *B. napus* had 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 7). 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 $H_{sex}6$ seem 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 7). 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, the root system was less profound and not as well developed as those of the parental species or the sexual hybrids (Table 7).

Discussion

All F_1 sexual hybrids were obtained from crosses between diploid cultivars of *S. alba* and *B. napus* and 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, which was reported earlier for interspecific and intergeneric hybridizations in the *Cruciferae* family (Eenink 1974, Nishi *et al.* 1964). Unilateral crossing barriers might have been involved, which 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

Table 7. 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]		Average root size[b]
		B. napus	*S. alba*	
S. alba				
cv. 'Maxi'	0.0 (0)	yes	no	4.0
cv. 'Emergo'	0.2 (0- 4)	yes	no	4.6
B. napus				
cv. 'Jet Neuf'	57.7 (13-167)	no	yes	4.4
cv. 'Tantal'	59.6 (35- 79)	no	yes	4.7
acc. K1	42.2 (3-101)	no	yes	4.1
cv. 'Tower'	46.9 (28- 68)	no	yes	3.7
S. alba x *B. napus*				
$H_{sex}1$	0.0 (0)	yes	no	4.8
$H_{sex}2$	1.2 (0- 11)	yes	no	4.7
$H_{sex}3$	26.7 (4- 56)	yes	yes	4.3
$H_{sex}4$	0.1 (0- 1)	yes	no	5.0
$H_{sex}5$	51.3 (25- 88)	no	yes	5.0
$H_{sex}6$	17.4 (5- 36)	yes	yes	4.5
$H_{sex}4$ x *B. napus*	0.0 (0)	yes	no	4.8
$H_{sex}5$ x *B. napus*	6.0 (6)	yes	no	4.5
S. alba (+) *B. napus*				
$H_{som}1$	1.9 (0- 7)	yes	no	3.7
$H_{som}2$-1	0.9 (0- 5)	yes	no	3.9
$H_{som}2$-2	5.9 (0- 20)	yes	yes	3.6
$H_{som}2$-3	2.4 (0- 6)	yes	no	3.6
$H_{som}2$-4	16.2 (1- 40)	yes	yes	3.5
$H_{som}2$-5	2.3 (0- 25)	yes	no	3.5

[a] average number of cysts significant different from that on the parental *B. napus* and *S. alba* species at $P < 0.05$; [b] the root size was visually quantified using a scale from 1 (small) to 5 (large)

presence of trivalents and hexavalents (Table 4), which were not observed for the *S. alba* x *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 occurrence of exclusively 21 univalents at MI in an amphihaploid hybrid between *S. alba* and *B. oleracea*, but these results cannot exclude allosyndetic pairing in other hybrids including 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 4). This would suggest that allosyndetic pairing between either A or C chromosomes and S_{al} chromosomes might not have occurred. However, the observation of less than 12 univalents and the formation of multivalents, which were not observed in AC hybrids or haploids of *B. napus* (Mizushima 1980), strongly suggest 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 BC1 plants from the sexual hybrids are thought to have resulted from 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 details 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 for all combinations in callus growth, 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* cv. 'Barrapo', 'Tantal' and 'Jet Neuf', showing a 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 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 hand. 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. Also a rather high frequency of polyploidization in hypocotyl protoplast-derived regenerants of *S. alba* was 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 the resistant *S. alba* parent (Table 7). 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 the 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 7). It is thought that these hybrids were obtained from 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 7). 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, being in accordance with earlier results of the *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.

Acknowledgements

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CHAPTER 6

Studies of pollen germination, pollen tube growth, micropylar penetration and seed set in intraspecific and intergeneric crosses within the *Cruciferae*

C.L.C. Lelivelt

Summary

Pollen germination, pollen tube growth and micropylar penetration were investigated in intraspecific and intergeneric crosses involving *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk. (oil-seed rape or fodder rape), x*Brassicoraphanus* Sageret (Raparadish) and diploid (2x) and tetraploid (4x) accessions of *Sinapis alba* L. (white mustard). For the reciprocal intergeneric crosses between *B. napus* and x*Brassicoraphanus* no effective barriers to pollen tube growth on stigmata or in styles were observed. The resulting low frequency of hybrid plants was mainly associated with a low rate of ovules with micropylar penetration per siliqua or with embryo abortion. Hybrid plants could be obtained without use of embryo rescue. In reciprocal crosses between *B. napus* and *S. alba* 2x or 4x incongruity barriers were observed on the stigma, in the style, and in the ovary resulting in a low frequency of ovules with micropylar penetration per siliqua. Open flower pollination as compared to bud pollination generally was the more favourable procedure for pollen germination and pollen tube growth in crosses involving *S. alba*, but for micropylar penetration and seed set no differences were observed. Crosses between *S. alba* 2x (♀) and *B. napus* (♂) were found to result in a higher frequency of ovules with micropylar penetration as compared to reciprocal crosses or crosses with *S. alba* 4x. All reciprocal crosses between *B. napus* and *S. alba* 2x or 4x were unsuccessful when no embryo rescue was applied. Embryo rescue shortly after pollination, i.e. 2 to 5 days, however, resulted in hybrid seeds and plants, but only when applied to crosses between *S. alba* 2x (♀) and *B. napus* (♂). The possible effects of the genome constitution, taxonomic distance and the parthenogenetic and parthenogenesis inducing ability of the parental genotypes on the observed malfunctions at the pre- and/or post-zygotic stage of the pollen-pistil interactions are discussed.

Abbreviations: DAP - days after pollination; IAA - indole-3-acetic acid; kin - kinetin

Introduction

Many attempts to obtain intergeneric hybrids in *Cruciferae* by crossing have been unsuccessful, probably due to the existence of pre- and post-zygotic barriers (Dolstra 1982, Nishi *et al.* 1964). Recently, sexual hybrids between *Sinapis alba* L. (white mustard) and *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk. (oil-seed rape and fodder rape) could be obtained, albeit in low numbers and only when diploid (2x) accessions of *S. alba* were used as the pistillate parent. Reciprocal crosses between *S. alba* 4x and *B. napus* did not yield hybrid plants (Lelivelt *et al.* 1993). From crosses between *B. napus* (♀) and *S. alba* 2x or 4x (♂) exclusively matromorphic plants were obtained. Furthermore, crosses between x*Brassicoraphanus* Sageret (Raparadish) (♀) and *B. napus* (♂) yielded many hybrids, whereas the reciprocal cross yielded only a few hybrids and

many matromorphic plants (Dolstra 1982, chapter 4). The occurrence of matromorphic plants in interspecific and intergeneric crosses in *Cruciferae* is a common phenomenon (Nishi *et al.* 1964, Röbbelen 1966). It appears to be dependent on the genotype of both the pistillate and pollen parent and on the genetic distance between the genotypes. It may also be influenced by the temperature before, during or after meiosis and by delayed pollination (Eenink 1974a).

Hogenboom (1973) has suggested incongruity, i.e. non-functioning of the partner relationship resulting from a lack of necessary genetic infomation in one or both of the parents, as the major limiting factor in wide crosses. Incongruity is proposed to be a by-product of evolutionary divergence and is considered a mechanism entirely different from incompatibility. The specific moment, pre- or post-zygotic, at which incongruity may be expressed is thought to be based on gene(s) for gene(s) interactions of the parental species and to depend on the genotype, ploidy level and genetic distance between the parental species.

Pre-zygotic incongruity barriers may be expressed as inhibition of pollen germination on the stigma, inhibited or erratic pollen tube growth in the style, absence of micropylar penetration and of fusion of gametes. To circumvent these incongruity barriers at the pre-zygotic phase, bud pollination (Namai 1980, Sampson 1962) or polyploidisation (Akbar 1989, Nishiyama and Inomata 1966, Quazi 1988, Turesson and Nordenskiöld 1943) have been applied successfully. Embryo abortion at several stages of development may be the major cause of post-zygotic barriers. Often embryo abortion is associated with an unbalanced genome constitution in the hybrid embryo and/or endosperm. Embryo rescue, i.e. in vitro culture of ovaries, ovules or embryos may overcome these post-zygotic barriers (Harberd 1969, Zenkteler 1990).

In this study the existence of pre- and post-zygotic barriers in reciprocal crosses between *B. napus* and x*Brassicoraphanus*, between *S. alba* 2x and *B. napus* and between *S. alba* 4x and *B. napus* are investigated, with the aim to understand previously obtained positive and negative results from such wide crosses (Lelivelt *et al.* 1993, chapter 4).

Materials and methods

Plant material. Plants of the diploid cultivars 'Maxi' and 'Emergo' of *Sinapis alba* L. ($S_{al}S_{al}$, $2n = 2x = 24$), white mustard, and the autotetraploid *S. alba* ($S_{al}S_{al}S_{al}S_{al}$, $2n = 4x = 48$) cultivar 'Condor' and accession Oscar (Limagrain Genetics, Scheemda, The Netherlands) were grown in pots in a greenhouse without temperature and humidity control in spring and summer. Under similar conditions were used: the *Brassica napus* L. (AACC, $2n = 38$), oil-seed rape or fodder rape, cultivars 'Akela', 'Blako', 'Bridger', 'Cascade', 'Darmor', 'Emerald', 'Jet Neuf', 'Mansholts Hamburger', 'Petranova', 'Rapol', 'Tantal', 'Tower', 'Zephyr' and the accession Rape Kale. Furthermore, plants from a population of x*Brassicoraphanus* (AARR, $2n = 38$), Raparadish, selected for resistance to the beet cyst nematode (*Heterodera schachtii* Schm.) (Dolstra 1982, Lange *et al.* 1989) were used. Although not verified in our experiments, some

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x*Brassicoraphanus* plants may have had a number of chromosomes slightly deviating from 38, varying between 36 and 39 (Dolstra 1982).

Crosses. Reciprocal crosses were made between *B. napus* and *S. alba* 2x or 4x, and between *B. napus* and x*Brassicoraphanus*. As a control, intraspecific cross-pollinations within cv. 'Jet Neuf' and cv. 'Tantal' of *B. napus*, and within cv. 'Emergo' and cv. 'Maxi' of *S. alba* 2x were also carried out. Buds of different sizes were hand-emasculated and bagged. Usually between six and ten buds were emasculated per raceme. Pollinations were carried out between 10.00 am and 14.00 pm, 2 to 3 days after pollination (DAP) using a mixture of freshly collected pollen from several plants. Each pistil was pollinated with an abundant amount of pollen. At the time of pollination, some of the emasculated buds had reached the open flower stage; the oldest bud was marked with a label.

Preliminary experiments had shown that 1 DAP only very few pollen tubes were visible in the styles of pistils in intergeneric crosses and that no micropylar penetration of ovules had occurred, whereas 2 DAP micropylar penetration of ovules was visible. Therefore, 2, 3 and 4 DAP a maximum of four pistils per raceme, two at the "bud stage" and two at the "open flower stage", were collected for microscopic observations. The remaining pollinated pistils were used for in vitro culture or left on the plant to study seed development.

Microscopic observations on pollen tube growth and fertilization. Pollen viability was determined by staining freshly collected pollen with fluorescein diacetate (FDA) as previously described (Lelivelt and Krens 1992). Incubation with FDA indicated a viability of 75 to 95% of the pollen from *S. alba* 2x, *S. alba* 4x and *B. napus*. Only 50 to 70% of pollen from x*Brassicoraphanus* stained with FDA.

Aniline blue staining was used to study pollen-stigma interaction and pollen tube growth in the style (Dolstra 1982). Pistils were excised from pollinated buds and open flowers and after fixation and staining squashed in a drop of glycerol. For microscopic observations pistils from reciprocal crosses between *B. napus* cv. 'Tantal' and 'Jet Neuf' and either x*Brassicoraphanus* or accessions of *S. alba* 2x or 4x were used. For each cross combination and each date of harvesting at least ten pistils at the bud stage and ten at the open flower stage were examined to assess the number of germinated pollen grains on the stigma, the number of pollen tubes in the style, the site of pollen tube arrest and the occurrence of micropylar penetration of the ovules. Pollen tube arrest was described as either occurring in the style or the ovary. The buds and open flowers were randomly chosen from each raceme.

The effects of cross combination and bud or open flower pollination on the pollen-stigma interaction were analysed 2, 3 and 4 DAP. Eight cross combinations were investigated: two intraspecific crosses, *B. napus* x *B. napus* and *S. alba* 2x x *S. alba* 2x, and six intergeneric crosses, reciprocal crosses between *B. napus* and x*Brassicoraphanus*, reciprocal crosses between *B. napus* and *S. alba* 2x, and reciprocal crosses between *B. napus* and *S. alba* 4x. A regression analysis performed on the data of average number of germinated pollen grains, pollen tubes and percentage of micropylar penetration with pistils as experimental unit within cross combination, within bud pollination or open flower pollination and within DAP, resulted in large residuals and thus in an

unbalanced regression analysis. Transformation of these data only slightly improved the result, but when all values equal to 0 were omitted, a considerable decrease of the data set in the analysis occurred. Therefore, a t-test analysis was used to analyse the data from the pollen-pistil interaction.

Siliquae culture. Two to 12 DAP, developing siliquae were harvested, surface sterilized and cultured as described by Lelivelt and Krens (1992), with minor modifications. MS (Murashige and Skoog 1962) based media were supplemented either with 1 mg/l IAA and 0.2 mg/l kin, or with 2 mg/l IAA and 0.5 mg/l kin instead of 1 mg/l IAA and 0.5 mg/l kin. At the beginning of culture and just prior to excision of embryos from the siliquae, the length and width of the siliquae were measured. After 3 to 4 weeks of culture siliquae were cut open. The shrivelled ovules with small embryos were excised from the siliquae and cultured on the same medium. Larger embryos, mostly at the torpedo stage, were propagated on Medium 1 (Lelivelt and Krens 1992). Siliquae culture was applied to reciprocal crosses between *B. napus* cv. 'Tantal' and 'Jet Neuf' and the diploid and tetraploid accessions or cultivars of *S. alba*.

Seed development. To estimate the effect of post-zygotic barriers, the frequency of developing siliquae at 3 weeks after pollination, the percentage of seed set at harvest and the number of seeds per pollination was determined in reciprocal crosses between one accession and 13 cultivars of *B. napus* and either x*Brassicoraphanus* or cultivars of *S. alba* 2x. The seeds obtained were classified as small when shorter than 2 mm length, and large when longer than 2 mm. Small sized, and mainly shrivelled, seeds were rinsed, surface sterilized and germinated in vitro as described by Lelivelt and Krens (1992). Seedlings were transplanted and propagated on Medium 1. Growing plants were planted in pots with soil and transferred to a greenhouse.

Results

Pre-zygotic barriers

Pollen germination on the stigma. Since non-germinated pollen grains were washed off during the staining procedure no data on the percentage of germinated pollen grains on the stigma can be given. Also, the rate of pollen attachment was not studied but care was taken to ensure that each pistil was pollinated with an excessive amount of pollen. Pollen germination was scored as the number of pollen grains that had germinated on the stigma, with or without having penetrated the stigma papillae. The germinated pollen grains observed varied from filled pollen grains with a short pollen tube to completely empty pollen grains with a long pollen tube.

Cross-pollination within *S. alba* 2x and within *B. napus* resulted in large numbers of germinated pollen grains and numerous pollen tubes penetrating the papillae and growing into the styles. In these intraspecific crosses the majority of germinated pollen grains formed long pollen tubes. In reciprocal intergeneric

crosses between *B. napus* and *S. alba* 2x or 4x, many germinated pollen grains produced a short pollen tube, which did not enter the stigma papillae or grew only a very short distance in the papillae. Also, typical incongruity reactions, like callose deposition in the papillae and non-penetration of the papillae by pollen tubes, were observed in reciprocal crosses between *B. napus* and *S. alba* 2x or 4x, but less frequently in reciprocal crosses between *B. napus* and *xBrassicoraphanus* and they were nearly absent in intraspecific crosses.

Differences were found for pollen germination between the eight cross combinations, between 2, 3, and 4 DAP and between pollinations in the bud stage or open flower stage. The average number of germinated pollen grains observed per pistil in the six intergeneric crosses did not differ much, but was significantly lower than that in the intraspecific crosses (Table 1). For crosses *B. napus* x *S. alba* 2x, *B. napus* x *xBrassicoraphanus*, and *S. alba* 4x x *B. napus* the number of germinated pollen grains observed 3 DAP was significantly higher than 2 DAP. The slight decrease in number of germinated pollen grains observed in cross-pollination within *B. napus* and within *S. alba* 2x from 2 to 4 DAP was found not to be significant (Table 1).

In cross-pollinations within *B. napus* and within *S. alba* 2x a large number of germinated pollen grains was observed on the stigmata of all pistils. In reciprocal crosses between *B. napus* and *xBrassicoraphanus* on the majority of pistils more than 15 germinated pollen grains were observed 4 DAP. However, in reciprocal intergeneric crosses between *S. alba* 2x or 4x and *B. napus* larger differences in pollen germination between pistils were observed, with up to 28% of the pistils showing less than two germinated pollen grains 2 DAP and up to 50% of the pistils having less than 15 germinated pollen grains 4 DAP (pooled data). In crosses with *B. napus* (♀) more pistils were found to carry only a few germinated pollen grains as compared to the crosses in which *S. alba* 2x or 4x (♀) were used as the female parent. Furthermore, in the intergeneric crosses between *B. napus* and *S. alba* twice as much germinated pollen grains were observed when pollination had been carried out at the open flower stage. This was not observed for any of the other crosses.

Pollen tube growth in the style. The effects of cross combination and DAP on the number of pollen tubes in the style are presented in Table 1. For all crosses the number of pollen tubes in the style was found to be lower than the number of germinated pollen grains observed on the stigma. In all pistils of the intraspecific cross-pollinations within *S. alba* 2x and within *B. napus* the pollen tubes had reached the lower part of the ovaries already 2 DAP. Pollen tube growth was normal in all pistils and no difference between bud pollinated and open flower pollinated pistils was observed for the intraspecific crosses.

In the reciprocal crosses between *B. napus* and *xBrassicoraphanus* the number of pollen tubes in the style and ovary region 3 and 4 DAP increased to a level which was not significantly lower than that in the intraspecific situation (Table 1). As had also been observed for the intraspecific crosses, the pollen tubes in all pistils of the intergeneric crosses with *xBrassicoraphanus* had entered the ovary 4 DAP. Only very few pollen tubes had stopped their growth in the stylar tissue. The total number of pollen tubes in the open flower pollinated pistils was found to be higher than in bud pollinated pistils. In some

Table 1. Pollen germination and pollen tube growth in intraspecific crosses within *B. napus* (*B. napus* intra), within *S. alba* (2x) and in reciprocal intergeneric crosses. Data are pooled across bud pollinated and open flower pollinated pistils. Significant differences at P<0.05

Crosses	DAP	Pollen germinated		Pollen tubes in style		Percentage of pistils with pollen tubes in the ovary
		Mean number	Significantly different from *B. napus* intra at 4 DAP	Mean number	Significantly different from *B. napus* intra at 4 DAP	
B. napus x *B. napus*	2	92	no	28	no	100
	3	85	no	28	no	100
	4	74	no	27	no	100
S. alba 2x x *S. alba* 2x	2	96	no	28	no	100
	3	86	no	25	no	100
	4	80	no	26	no	100
B. napus x x*Brassicoraphanus*	2	20	yes	16	yes	100
	3	46	yes	24	no	100
	4	42	yes	19	no	100
x*Brassicoraphanus* x *B. napus*	2	39	yes	26	no	100
	3	39	yes	24	no	100
	4	43	yes	30	no	100
B. napus x *S. alba* 2x	2	27	yes	7	yes	5
	3	47	yes	12	yes	55
	4	59	yes	17	yes	38
S. alba 2x x *B. napus*	2	35	yes	11	yes	9
	3	39	yes	14	yes	33
	4	46	yes	18	yes	79
B. napus x *S. alba* 4x	2	25	yes	12	yes	10
	3	25	yes	11	yes	9
	4	23	yes	18	yes	10
S. alba 4x x *B. napus*	2	29	yes	10	yes	5
	3	42	yes	14	yes	8
	4	45	yes	12	yes	13

pistils of the reciprocal crosses between *B. napus* and x*Brassicoraphanus* an erratic, irregular pollen tube growth was observed. Most of the abnormally growing pollen tubes had thickened walls or swollen tips, which showed a strong callose deposition.

At 4 DAP, the number of pollen tubes in the reciprocal crosses between *B. napus* and *S. alba* 2x or 4x was found to be similar, but lower than that in the reciprocal crosses between *B. napus* and x*Brassicoraphanus* or in the intraspecific crosses (Table 1). In the reciprocal crosses between *B. napus* and *S. alba* 2x or 4x abnormal pollen tubes, with thickened walls or swollen tips, and abnormalities in pollen tube growth were observed frequently. The abnormalities in pollen tube growth included non-directional growth in the style, penetration of tissues outside the transmitting tissue in the middle of the style and arrest of growth in the style or near the region with the first ovules. It seems that in these crosses the pollen tubes were not attracted by the ovules, since some of the pollen tubes grew straight to the lower region of the pistil, without proceeding to the micropyle. Often an excessive, non-directional pollentube growth near the micropyle was observed.

In the reciprocal crosses between *B. napus* and *S. alba* 2x the pollen tubes grew further and in more pistils pollen tubes were found in the ovary region than in crosses involving *S. alba* 4x (Table 1). In only a few pistils pollen tubes were observed at lower parts of an ovary. On average, 15 to 35% of all pollen tubes in the four intergeneric crosses between *B. napus* and *S. alba* 2x or 4x had reached the ovaries 2 DAP, but this increased at 3 and 4 DAP to approximately 30% for crosses with *S. alba* 4x and 60% for crosses with *S. alba* 2x (data not shown). In the crosses *B. napus* x *S. alba* 4x, and *S. alba* 2x x *B. napus* more pollen tubes were found in the style when pollination was carried out at the open flower stage. For the cross *B. napus* x *S. alba* 4x a higher number of pollen tubes was also found in the ovary region of open flower pollinated siliquae. For other intergeneric crosses such a difference was not observed.

Micropylar penetration. Pollen tube penetration of the micropyle could be investigated only in those ovules of which the micropyle was visible. Therefore, the number of ovules with micropylar penetration is presented as a percentage of the total number of ovules studied in the pistils (Table 2). *B. napus* is considered to contain 42 ovules per ovary, x*Brassicoraphanus* 30 ovules, *S. alba* 2x 5 ovules and *S. alba* 4x 6 ovules (means of 10 pistils from each species). When *B. napus* or x*Brassicoraphanus* were used as pistillate parent, in intraspecific or intergeneric crosses, in general 70% of all ovules per ovary could be investigated. For *S. alba* 2x or 4x between 80 to 90% of all ovules per ovary could be evaluated for micropylar penetration. This means that some caution is required in comparing the outcome of reciprocal crosses with respect to the percentage of ovules with micropylar penetration and the percentage of pistils in which penetrated ovules were visible, presented in Table 2. Taking this deviation into account, clear differences between the crosses can still be observed.

For the intraspecific cross-pollinations within *S. alba* 2x and within *B. napus* a higher frequency of ovules with micropylar penetration was found than for all

B. napus intra '' = *B. napus* x *B. napus*; *S. alba* 4x inter = reciprocal crosses between *S. alba* 4x and *B. napus*. Data are pooled across bud pollinated and open flower pollinated pistils. Significant differences at P<0.05

Crosses	DAP	Micropylar penetration			Percentage of pistils with penetrated ovules
		Percentage of penetrated ovules	Significantly different from		
			B. napus intra 4 DAP	*S. alba* 4x inter 4 DAP	
B. napus x *B. napus*	2	49	no	yes	100
	3	71	no	yes	100
	4	80	no	yes	100
S. alba 2x x *S. alba* 2x	2	41	no	yes	85
	3	48	no	yes	91
	4	77	no	yes	100
B. napus x x*Brassicoraphanus*	2	6	yes	no	63
	3	22	yes	yes	100
	4	20	yes	yes	100
x*Brassicoraphanus* x *B. napus*	2	15	yes	yes	81
	3	22	yes	yes	72
	4	22	yes	yes	92
B. napus x *S. alba* 2x	2	0	yes	no	0
	3	1	yes	no	9
	4	2	yes	no	31
S. alba 2x x *B. napus*	2	2	yes	no	6
	3	8	yes	yes	26
	4	14	yes	yes	41
B. napus x *S. alba* 4x	2	1	yes	no	14
	3	1	yes	no	14
	4	1	yes	no	13
S. alba 4x x *B. napus*	2	1	yes	no	2
	3	0.1	yes	no	4
	4	2	yes	no	9

six intergeneric crosses. Furthermore, reciprocal crosses between *B. napus* and x*Brassicoraphanus* showed a higher percentage of penetrated ovules than reciprocal crosses between *B. napus* and *S. alba* 2x or 4x. Finally, reciprocal crosses between *S. alba* 2x and *B. napus* resulted in a higher frequency of penetrated ovules than the reciprocal cross or the reciprocal crosses between *B. napus* and *S. alba* 4x (Table 2). No difference between open flower versus bud pollinated flowers was found with regard to the frequency of micropylar penetration in the intraspecific and intergeneric crosses.

In crosses between *B. napus* and x*Brassicoraphanus* 22% of the ovules observed showed micropylar penetration at 3 and 4 DAP, which is equal to an estimated maximum of 9 ovules per ovary, when *B. napus* was used as pistillate parent, and of 7 ovules per ovary in the reciprocal cross. No reciprocal effects were observed. Micropylar penetration in reciprocal crosses between *B. napus* and *S. alba* 4x was found be very low and again no reciprocal effects were observed. In crosses between *S. alba* 2x (♀) and *B. napus* (♂) the frequency of ovules with penetrated micropyles per ovary was higher than in the reciprocal cross, and was found to be 14% at 4 DAP (Table 2). Since siliquae of *B. napus* were found to contain approximately 40 ovules, which is 8 times more than in siliquae of *S. alba* 2x, the estimated number of penetrated ovules per ovary (0.7 and 0.8) was nearly equal in the reciprocal crosses.

At 4 DAP nearly all pistils of intraspecific crosses and reciprocal crosses between *B. napus* and x*Brassicoraphanus* showed ovules with micropylar penetration. For the reciprocal intergeneric crosses between *S. alba* and *B. napus* only 9 to 41% of the pistils, in which 70 to 90% of the ovules could be observed, contained ovules with micropylar penetration 4 DAP and no reciprocal differences were found (Table 2). For all intergeneric and intraspecific crosses the percentage of pistils with pollen tubes in the ovary, presented in Table 1, showed similar trends as the percentage of pistils with penetrated ovules (Table 2).

Post-zygotic barriers

Siliquae culture and seed set. For a few reciprocal crosses between *B. napus* cv. 'Tantal' and cv. 'Jet Neuf' and either x*Brassicoraphanus* or *S. alba* 2x the effect of bud or open flower pollination on seed set was studied. Buds and flowers did not differ in seed set (data not shown).

At 3 weeks after pollination a high percentage of siliquae in reciprocal crosses between *B. napus* and x*Brassicoraphanus* or *S. alba* 2x were still green and had increased in size, although the majority of them did not contain seeds at time of harvest (Tables 3 and 4). This was also observed when siliquae culture was carried out. Many siliquae considerably increased in length and width but eventually did not yield seeds (Table 5).

When embryo rescue was not applied the mean percentage of siliquae with seeds in reciprocal crosses between *B. napus* and x*Brassicoraphanus* was much lower than the percentage of pistils showing micropylar penetration of ovules. In the cross *B. napus* x x*Brassicoraphanus*, for instance, 0 to 63% of the pollinated siliquae were found to produce seeds (Table 3), whereas the frequency of pistils with micropylar penetration in these crosses was found to

Table 3. Percentage of developing siliquae at 3 weeks after pollination and at harvest, and number of seeds per pollination in intergeneric crosses between 13 cultivars and one accession Rape Kale of *B. napus* and x*Brassicoraphanus*, from which 6 reciprocal and 8 with *B. napus* as female. Embryo rescue was not applied. Data are pooled across bud pollinated and open flower pollinated siliquae

B. napus cultivars/ accession	Percentage of developing siliquae				Number of seeds per pollination			
	At 3 weeks		At harvest		Total		Plump seeds	
	♂	♀	♂	♀	♂	♀	♂	♀
			x*Brassicoraphanus*					
cv. 'Zephyr'	69	73	43	35	0.7	0.6	0.6	0.5
cv. 'Tantal'	43	72	27	27	0.5	0.5	0.2	0.4
cv. 'Petranova'	47	60	39	37	1.6	0.7	1.5	0.6
cv. 'Tower'	90	60	53	11	1.1	0.4	1.0	0.3
cv. 'Cascade'	30	29	30	22	0.4	0.5	0.3	0.3
cv. 'Darmor'	32	-	15	20	0.3	0.3	0.1	0
cv. 'Jet Neuf'	93	-	63	-	0.9	-	0.9	-
cv. 'Rapol'	-	-	41	-	0.1	-	0.1	-
cv. 'Mansholts Hamburger'	-	-	6	-	0.3	-	0.2	-
cv. 'Akela'	-	-	0	-	0	-	0	-
cv. 'Emerald'	77	-	39	-	0.3	-	0.2	-
cv. 'Blako'	-	-	20	-	0.7	-	-	-
Rape Kale	-	-	0	-	0	-	0	-
cv. 'Bridger'	32	-	22	-	0.3	-	0.2	-

- = not determined

be 100% (Table 2). If all ovules with micropylar penetration in crosses between *B. napus* and x*Brassicoraphanus* would have developed into seeds, 9 and 7 seeds per pollination when *B. napus* was used as female and male, respectively, would have been expected. However, at harvest the number of seeds per pollination was much lower, ranging between 0 to 1.6 (Table 3). The mean number of plump seeds per pollination varied considerably between different *B. napus* accessions. A majority of seeds from crosses between *B. napus* (♀) and x*Brassicoraphanus* (♂) resulted in matromorphic plants, while the reciprocal cross yielded predominantly hybrids.

The reciprocal crosses between *B. napus* and x*Brassicoraphanus* yielded a higher percentage of siliquae with seeds as compared to crosses with *S. alba* 2x (Tables 3 and 4). The reciprocal intergeneric crosses with *S. alba* 2x resulted in a lower percentage of siliquae with seeds and in a lower number of seeds per siliqua (Table 4) than expected from the microscopic observations (Table 2). Crosses between *B. napus* (♀) and *S. alba* 2x (♂) showed a large variation in percentage of siliquae with seeds and in seed set per pollination, both being much higher as compared to the reciprocal cross. All small sized and plump seeds from crosses with *B. napus* as female parent that had germinated gave matromorphic plants. In the shrivelled seeds, which were not able to germinate, most embryos must have been aborted before the stage of maturity. Crosses with *S. alba* 2x as female parent resulted in only a few plump seeds. Plants that could be recovered from these seeds were found not to be hybrids.

Table 5 presents results of embryo rescue experiments. No effect of bud or open flower pollination on subsequent in vitro seed set in all four intergeneric crosses could be observed. Crosses between *S. alba* 2x (♀) and *B. napus* (♂) resulted in a higher frequency of siliquae with seeds than the reciprocal cross or crosses with *S. alba* 4x. Furthermore, in crosses with *S. alba* 2x (♀) the frequency of siliquae with seeds and the number of seeds per siliqua was much higher than with natural seed set when embryo rescue was performed, whereas for the reciprocal cross these were similar or lower (Table 5 vs Table 4). When embryo rescue was started 3 or 4 DAP the frequency of siliquae with seeds, i.e. between 29 and 35%, and the average number of seeds per siliqua, i.e. between 0.5 and 0.9 (Table 5) in crosses with *S. alba* 2x as female was found to be similar as expected from the microscopic observations on rate of micropylar penetration, being 26 to 41% and 0.4 to 0.7, respectively (Table 2). However, the number of mature embryos per siliqua was much lower than the total number of seeds obtained. Many embryos had been aborted at an early stage of development, but a few mature embryos could be obtained (Table 5), some of which were later on found to be hybrids. Crosses between *B. napus* (♀) and *S. alba* 2x (♂) followed by embryo rescue yielded only matromorphic plants. The reciprocal crosses between *B. napus* and *S. alba* 4x resulted in many siliquae which did not contain embryos; a few siliquae had only a few aborted embryos. The frequency of siliquae with seeds in both crosses was similar or slightly higher than expected from the results of the pistils showing ovules with micropylar penetration at 4 DAP (Table 2). Crosses performed with *S. alba* 4x did not result in hybrids.

Table 4. Percentage of developing siliquae at 3 weeks after pollination and at harvest, and number of seeds per pollination in intergeneric crosses between 12 cultivars and one accession Rape Kale of *B. napus* and the diploid cultivars 'Emergo' and 'Maxi' of *S. alba* (data pooled), of which 5 reciprocal and 8 with *B. napus* as female. Embryo rescue was not applied. Data are pooled across bud pollinated and open flower pollinated siliquae

B. napus cultivars/accession	Percentage of developing siliquae				Number of seeds per pollination			
	At 3 weeks		At harvest		Total		Plump seeds	
	♂	♀	♂	♀	♂	♀	♂	♀
				S. alba				
cv. 'Zephyr'	48	57	7	0	0.2	0	0.2	0
cv. 'Tantal'	21	96	6	0	0.2	0	0.2	0
cv. 'Petranova'	32	65	14	1	0.8	0.01	0.8	0.01
cv. 'Tower'	92	56	4	0	0.04	0	0.04	0
cv. 'Cascade'	66	34	8	0	0.3	0	0.3	0
cv. 'Jet Neuf'	91	-	30	6	1.4	0.1	1.4	0.1
cv. 'Rapol'	86	-	19	-	0.6	-	0.6	-
cv. 'Mansholts Hamburger'	36	-	15	-	1.0	-	0.9	-
cv. 'Akela'	17	-	8	-	0.2	-	0.2	-
cv. 'Emerald'	50	-	10	-	0.1	-	0.1	-
cv. 'Blako'	24	-	9	-	0.5	-	0.4	-
Rape Kale	-	-	11	-	0.2	-	0.2	-
cv. 'Bridger'	48	-	4	-	0.1	-	0.1	-

- not determined

Table 5. Percentage of siliquae with seeds at 3 weeks after pollination and number of seeds per pollination for reciprocal crosses between *B. napus* cv. 'Tantal' or cv. 'Jet Neuf' and diploid (2x) and tetraploid (4x) cultivars/accessions of *S. alba* of in vitro cultured siliquae. Initiation of culture started at different days after pollination (DAP). Data are pooled across bud pollinated and open flower pollinated siliquae

Start of in vitro culture pollination (DAP)	Percentage of siliquae with seeds 3 weeks after pollination				Number of seeds per pollination							
					Total				Mature embryos			
	S. alba 2x		*S. alba* 4x		*S. alba* 2x		*S. alba* 4x		*S. alba* 2x		*S. alba* 4x	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
2	7	19	5	3	0.2	0.4	0.1	0.1	0.1	0.03	0	0
3	7	35	0	8	0.2	0.9	0	0.2	0.04	0.02	0	0
4	4	29	15	10	0.1	0.5	0.2	0.2	0.04	0.1	0.1	0
5	8	2	22	3	0.2	0.02	0.1	0.1	0.03	0.02	0.1	0
6	6	-	-	-	0.3	-	-	-	0.1	-	-	-
7	3	9	3	4	0.1	0.2	0.1	0.1	0	0.01	0.01	0
9	11	3	4	18	0.2	0.1	0.04	0.3	0	0	0.04	0
10	-	11	0	3	0.2	0.3	0	0.1	0	0	0	0
11	-	-	-	-	0.02	-	-	-	0	-	-	-
12	-	0	-	-	-	0	-	-	-	0	-	-

- not determined

Discussion

Both pre- and post-zygotic barriers were found to be active in the intergeneric crosses between the *Cruciferae* species examined here. The incongruity barriers in reciprocal crosses between *B. napus* and the bridging hybrid x*Brassicoraphanus* were less effective than those in reciprocal crosses between *S. alba* 2x and *B. napus*. The barriers were most effective in reciprocal crosses between *B. napus* and *S. alba* 4x, from which no hybrids were obtained.

Some variation in pollen germination and pollen tube growth may have been associated with morphological characteristics of the male or female parent used in crosses and by variation between pistils of different buds or flowers. For instance, pistil length and stigma surface of buds and flowers of *B. napus* are larger than those of *S. alba*. However, since in all intergeneric crosses no reciprocal differences were observed in pollen germination and pollen tube growth in the style, the morphological differences in pistils are likely not to have had a large influence on these parameters. Also, no significant effect of length of the pistil, when using this factor as a covariable in the regression analyses, on pollen germination and pollen tube growth in all eight cross combinations could be observed (data not shown).

In some intergeneric crosses the number of germinated pollen observed was found to increase from 2 to 3 DAP, indicating that pollen grains were still able to germinate after longer periods of incubation (Table 1). It may have been likely, however, that 1 or 2 DAP similar numbers of germinated pollen grains were present on the stigma as has been found 3 or 4 DAP, but due to the inability of the pollen tubes to penetrate the papillae these pollen grains were washed off during the staining procedure. The latter presumption might also explain why at 1 DAP only very few germinated pollen grains were visible on the stigmata in the intergeneric crosses. It has been described that *Brassica* pollen is able to germinate within a few hours of incubation on the stigma (Kroh 1964).

No clear effect on pollen germination, pollen tube growth, micropylar penetration or seed set of bud pollination as compared to open flower pollinaton was observed. Higher levels of crossability with pollinations in the bud stage have been reported (Oelke 1957, Sampson 1962), although no effect or a negative effect of using young buds has also been reported (Dolstra 1982, Hinata *et al.* 1974, Namai 1980). Brown *et al.* (1991) described a positive effect of pollination of older buds or open flowers on ovule development in interspecific crosses between *B. rapa* and *B. oleracea*, and assumed that both emasculation (wounding) and immaturity of the gynoecium in buds had a negative effect on crossability. A lack of pollen tube functioning, observed in the reciprocal crosses between *B. napus* and x*Brassicoraphanus* or *S. alba* in this study appears to be largely due to incongruity barriers rather than to immaturity of the gynoecium, since both bud pollinated and open flower pollinated pistils showed a low frequency of micropylar penetration of ovules (Table 2). The contradictory results regarding the optimal developmental stage of flowers for congruity may be ascribed to effects of genotype, both at the species and cultivar level, and to environmental influences.

In the reciprocal crosses between *B. napus* and x*Brassicoraphanus* a barrier at the stigma or in the stylar tissue was apparently not effective since the observed number of pollen tubes in the style was similar to that found in pistils of the intraspecific crosses (Table 1). Furthermore, 4 DAP all pistils in these intergeneric and intraspecific crosses showed ovules with micropylar penetration (Table 2). The expression of pre-zygotic barriers in reciprocal crosses between *B. napus* and x*Brassicoraphanus* was similar and could not provide evidence for the observed reciprocal differences in hybrid seed set. The greater success in hybrid seed set in crosses with x*Brassicoraphanus* (♀) might, therefore, be explained by a higher percentage of fusion of gametes, by lower post-zygotic barriers or by an absence of development of matromorphs. Since the occurrence of illegitimate self-pollination or cross-pollination in crosses with *B. napus* (♀) was prevented by means of emasculation and bagging it is assumed that *B. napus*-like plants were matromorphs. It has been described by Noguchi (1928) that micropylar penetration, deposit of the male gamete in the embryo sac, followed by a parthenogenetic outgrowth of the unreduced egg cell without fertilization and subsequent elimination of the male gamete may lead to matromorph development. The frequency of fusion of gametes as well as the effect of development of matromorphs on hybrid seed development in crosses with *B. napus* as female parent remain to be investigated. A negative effect of the development of matromorphs on hybrid seed set could partly be explained by the fact that unreduced gametes may be involved, which in hybrids result in an unbalanced number of chromosomes in the embryo and endosperm as opposed to a situation with reduced gametes. Alternatively, a faster development of matromorphic seeds may have prevented the outgrowth of hybrid seeds (Eenink 1975).

The predominant formation of matromorphs in crosses with *B. napus* as female parent may indicate that the parthenogenetic ability in a broad sense, as defined by Eenink (1974b), is high for *B. napus*, although the parthenogenesis inducing ability of the pollen parents, x*Brassicoraphanus* and *S. alba* 2x or 4x, may have been important too. It is interesting to note that in crosses between a few *B. napus* genotypes and x*Brassicoraphanus* plants, only hybrids and no matromorphs were obtained. Also, the observation of successful crosses between *B. napus* (♀) and *S. alba* 2x (♂) (Chèvre *et al.* 1991, Heyn 1977) is in conflict with our findings. An explanation could be that the parental genotypes used in these studies had a low parthenogenetic (inducing) ability resulting in the predominant development of hybrids. It would be interesting to study whether selection of *B. napus* genotypes with a low parthenogenetic ability and *S. alba* or x*Brassicoraphanus* with a low parthenogenesis inducing ability would increase the hybrid progeny in crosses between *B. napus* and x*Brassicoraphanus* or *S. alba*.

The genetic distance between the *S. alba* genotypes and *B. napus* genotypes used in our study is rather large (Vaughan and Denford 1968) and it may therefore be difficult to obtain hybrids. Hinata *et al.* (1974) also described poor crossability for *S. alba* as male or female parent in crosses with diploid *Brassica* species. Tables 4 and 5 indicate that the hybrid embryos from intergeneric crosses with *S. alba* 2x or 4x were aborted at a very early stage of development. Only embryo rescue shortly after pollination, 2 to 5 days, resulted

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in mature seed development and in successful plant formation in crosses between *S. alba* 2x and *B. napus*, which were found to show the highest percentage of ovules with micropylar penetration. Optimization of embryo rescue techniques by culturing excised ovules instead of the siliquae, may enhance hybrid seed set in these crosses (Zenkteler 1990).

The failure of hybrid seed set in crosses between *B. napus* (♀) and *S. alba* 2x (♂) and in reciprocal crosses between *B. napus* and *S. alba* 4x as opposed to crosses between *S. alba* 2x and *B. napus* was associated with both strong pre- and post-zygotic barriers. In crosses with *S. alba* 4x aneuploid gametes, produced by tetraploid accessions of *S. alba*, may have resulted in an unbalanced number of chromosomes after gametal fusion in the zygote and the endosperm, leading to early embryo abortion. Also, when tetraploid instead of diploid genotypes are used, differences in quantitative and qualitative genome compositions in the hybrid and/or endosperm are to be expected. Crosses between diploid and autotetraploid species are reported to be less compatible than crosses at the diploid level (Dolstra 1982, Håkansson 1956, von Wangenheim 1955). However, an increase in crossability, in one or both cross directions, by using autotetraploid accessions in intergeneric crosses in *Cruciferae* has also been reported (Karpechenko 1937, Quazi 1988, Turesson and Nordenskiöld 1943).

Quantitative and qualitative differences in genomic constitution in embryo, endosperm and maternal tissue also may help to explain the observed differences in hybrid seed set in the reciprocal crosses between *B. napus* and *S. alba* 2x. Watkins (1932) suggested that interspecific crosses usually were more successful when the species with the highest number of chromosomes was used as female parent, leading to a more balanced genomic ratio of 2 : 3 of the hybrid embryo : endosperm. The results presented in this study, however, show that this is not generally valid. More recently, Johnston *et al.* (1980) proposed an EBN (Endosperm Balance Number) theory, where abnormal endosperm development may occur when the ratio of maternal : paternal chromosomes in the endosperm, irrespective of their ploidy number, deviates from 2 : 1. If it is assumed that the EBN values for *S. alba* and *B. napus* are different, the observed reciprocal difference may then be explained by their EBN ratios. Alternatively, it has also been suggested that the unilateral incongruity barriers observed in intergeneric or interspecific crosses might be linked to the use of the C genome species, i.e. *B. napus* or *B. oleracea* as the pistillate parent (Chiang *et al.* 1977, Dolstra 1982). The experiments reported here and by others (Heyn 1977, U *et al.* 1937) indicate that for *S. alba* similarly based unilateral cross incongruities can be found, since crosses with *B. napus* or *B. oleracea* as mother were less successful in contrast to the reciprocal cross. However, Turesson and Nordenskiöld (1943) found no differences between reciprocal crosses between *B. napus* and *R. sativus*, and more recently Chèvre *et al.* (1991) obtained equal numbers of hybrids between reciprocal crosses of *B. napus* and *S. alba*.

The work reported here has shown that reciprocal differences in hybrid seed set in intergeneric crosses in *Cruciferae* appear to be partly associated with differences in pollen germination and pollen tube growth. Post-zygotic barriers, involving an unbalanced genome constitution in hybrid embryo and/or

endosperm are assumed to be a major cause of failure of hybrid seed development. The use of genotypes with low parthenogenetic or low parthenogenesis inducing ability might enhance the yield of hybrid seeds due to the absence of matromorphic seeds, but this remains to be investigated. Furthermore, a more detailed study of the effects of other genotypes and environments on pollen-pistil interaction in these intergeneric crosses are needed.

Acknowledgments

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CHAPTER 7

The development of juveniles of *Heterodera schachtii* in roots of resistant and susceptible genotypes of *Sinapis alba*, *Brassica napus*, *Raphanus sativus* and hybrids

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Summary

The development of *Heterodera schachtii* Schm. (beet cyst nematode, BCN) juveniles in roots of resistant and susceptible genotypes belonging to cruciferous crop species and hybrids was studied from 4 to 28 days after inoculation. No difference in root penetration by larvae was observed between resistant and susceptible plants. The development of nematodes in roots from resistant plants of *Raphanus sativus* L., resistant x*Brassicoraphanus* Sageret and a resistant hybrid x*Brassicoraphanus* x *Brassica napus* L. was similar. BCN-resistance in these three sources of plant material appeared to be related to an increased male:female nematode ratio as compared to the ratio found in susceptible *R. sativus* plants. Also in resistant plants of *Sinapis alba* L. and a resistant intergeneric hybrid *S. alba* x *B. napus* the increase in male:female nematode ratio, as compared to the ratio found for susceptible *S. alba* cultivars and a susceptible intergeneric hybrid *S. alba* x *B. napus*, seemed to be related with the observed resistance. In roots of the resistant *S. alba* and of a resistant hybrid *S. alba* x *B. napus*, however, BCN-resistance might also be due to a slower development of larvae and increased necrosis of root cells at the site of larval penetration.

Introduction

The white beet cyst nematode, *Heterodera schachtii* Schmidt (abbrev. BCN) is an important pathogen of sugar beet (*Beta vulgaris* L.). It can cause severe crop losses in the sugar beet growing areas of North-West Europe (Heijbroek *et al.* 1988) and the USA (Steele and Savitsky 1981). At present, no resistant sugar beet varieties are available. Oil-seed rape, *Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk., is a tolerant, but good host for the multiplication of this nematode. For this reason, it is advisable only to include resistant oil-seed rape in a narrow rotation scheme with sugar beet as a main crop. High levels of BCN-resistance have been observed, but only in related species such as *Raphanus sativus* L. ssp. *oleiferus* (DC.) Metzg. (fodder radish) and *Sinapis alba* L. (white mustard) (Baukloh 1976, Lubberts and Toxopeus 1982) and not in the species *B. napus* L. itself (Harrewijn 1987).

With the aim to transfer BCN-resistance to *B. napus*, sexual hybridization between *B. napus* and resistant *S. alba*, and between *B. napus* and the resistant hybrid x*Brassicoraphanus* Sageret (Raparadish, AARR, $2n = 38$), derived from crosses between *Brassica rapa* (AA, $2n = 20$) and *R. sativus* (RR, $2n = 18$), has been carried out successfully (Lelivelt *et al.* 1993, chapter 4). F_1 hybrids and first backcross plants, with *B. napus* as the male recurrent parent, have been obtained. Some of these hybrids have demonstrated a high level of BCN-resistance, similar to the resistant *S. alba* or x*Brassicoraphanus* parent (Dolstra 1982, Lange *et al.* 1989, Lelivelt *et al.* 1993, chapter 4).

H. schachtii is a bisexual nematode and its multiplication is dependent on the development of male and female nematodes. The life cycle has been

described by Raski (1950). From the eggs, J_2 larvae hatch, penetrate root systems and establish a feeding site. After molting of J_2 larvae differentiation to male and female nematodes occurs. The J_3 male and female larvae undergo two more molts before they reach the adult stage. After fertilization of the eggs within the adult female nematodes by the adult free living male nematodes, the females die and the eggs survive within the remains of the female bodies, which are then called cysts.

The mechanism of resistance to BCN in *S. alba* and *R. sativus* is thought to be related to an increase of the male:female nematode ratio and not to an inability of larvae to penetrate the roots or to an inhibition of growth and development of male and female nematodes within the roots (Müller 1985). However, indications for the death of female larvae of *H. schachtii* in resistant *S. alba* plants as a consequence of necrosis of root tissue have also been reported (Lubberts and Toxopeus 1982). Inhibition of growth and differential death-rate of larvae of the two sexes, resulting in unbalanced sex ratios, has been described for many host-pathogen interactions with *Heterodera* species (Bridgeman and Kerry 1980, Johnson and Viglierchio 1969, Kerstan 1969, Sengbusch 1927, Steele and Savitsky 1981). However, there is much diversity of opinion as to explain the cause of the shifted sex ratios in cyst nematodes, with as a basic question if sex determination is genetically ruled (Bridgeman and Kerry 1980, Koliopanos and Triantaphyllou 1972), environmentally controlled (Ellenby 1954, Grundler *et al*. 1991, Müller 1985, Trudgill 1967) or if sex determination might be under both environmental and genetical control (Johnson and Viglierchio 1969).

In this paper studies are described on the development of juveniles of *H. schachtii* in roots of resistant and susceptible intergeneric sexual hybrids with genomes from *B. napus* and *S. alba* or *B. napus* and *R. sativus*, in comparison to the development of juveniles observed in the resistant and susceptible parental species with the aim to gain more knowledge about the cause of the low multiplication rate of BCN observed in resistant species and hybrids.

Materials and methods

Plant material. Seedlings and cuttings, propagated in- vitro through axillary bud or meristem culture were used as material to study host-pathogen interactions (see also Lelivelt and Krens 1992). Resistant plants of x*Brassicoraphanus*, plants of susceptible and resistant cultivars of *Sinapis alba* L. (white mustard), *Raphanus sativus* L. (fodder radish), *Brassica napus* L. (oil-seed rape) and their intergeneric hybrids that were used in experiments are listed in Table 1.

Beet cyst nematode resistance tests. Seeds were sown and cuttings were individually transplanted into 36 ml-PVC tubes, filled with sterilized silver sand, moistened with Steiner I (Steiner 1968) nutrient solution and kept in a greenhouse at a 10 h light regime, a constant temperature of 18°C and a relative humidity of 85 to 90%. Twenty-one rows of eight PVC tubes with seedlings or cuttings from each genotype were randomly placed within trays in

the greenhouse. After 2 weeks each seedling or cutting was inoculated with 2 ml of a suspension containing approximately 300 pre-hatched J_2 larvae of *Heterodera schachtii* Schm. using a veterinary syringe.

Table 1. Materials used in the experiments

Species	Genotype	
	Resistant	Susceptible
Raphanus sativus	'Nemex'	'Siletina'
Sinapis alba	'Maxi'	'Gisilba'
Brassica napus	-	'Jet Neuf'
x*Brassicoraphanus*[1]	pop 4(b)	-
S. alba x *B. napus*[2]		
'Maxi ' x 'Tantal'	-	$H_{sex}3$
'Emergo' x 'Jet Neuf'	$H_{sex}2$	-
x*Brassicoraphanus* x *B. napus*[3]	hybrid	-

[1] Lange *et al.* 1989; [2] Lelivelt *et al.*, 1993; [3] chapter 4

As inoculum the population that has been the standard for the last 15 years at our institute was used, a mixture from collections carried out in 1974, 1975 and 1976 at various locations in The Netherlands, and subsequently multiplied on various susceptible plant species in the greenhouse in Wageningen. Larvae were reared on susceptible *Beta vulgaris*, *Brassica napus* or *Sinapis alba* plants. After inoculation the temperature in the greenhouse was raised to 22°C during the day (Toxopeus and Lubberts 1979).

Several preliminary tests were performed to establish the optimal sampling frequency for collecting roots with larvae of different stages and to determine the optimal conditions for staining of root tissue, which has led to the following protocol: at 4, 7, 11, 14, and 18 days after inoculation (DAI) 10 to 15 seedlings and 10 cuttings from each of the parental species, and between 5 and 10 cuttings from the three intergeneric hybrids were collected. The root systems were washed free from sand, stained for 1 to 1.5 min. in an acid fuchsin-lactophenol solution at 80°C in a water bath, and subsequently rinsed thoroughly with distilled water. To achieve an optimal contrast of stained larvae within colourless root tissue, redundant pigment in the root tissue was extracted by the incubation of roots in glycerol: water (50:50, v/v) during 2 to 3 days at room temperature followed by storage in a fresh glycerol:water solution at 6-8°C until analysis (Goodey 1937). For observation each root system was gently squashed between two microscopic slides and the total number of nematodes at different developmental stages, as defined by Raski (1950), was

scored using a light microscope. Acid fuchsin-lactophenol solutions, water and glycerol solutions were also checked for the presence of nematodes, since larvae might be lost during the staining procedure (Müller 1985). At 4 DAI the number of larvae that had penetrated the root system of each seedling or cutting was scored, and at 7 to 14 DAI also the number of larvae at different developmental stages and the proportion of sex differentiated larvae were determined. At 28 days after inoculation the root systems of 10 cuttings and 10 seedlings of resistant and susceptible parental genotypes and of cuttings of intergeneric hybrids were evaluated for the appearance of mature females.

Results

Penetration and establishment of second stage larvae. Table 2 presents the number of second stage larvae (J_2 larvae) that had penetrated the roots of parental species and hybrids at 4 DAI. The observed average numbers of J_2 larvae per root system were similar for most of the susceptible and most of the resistant host plants. For all genotypes under study, a large variation between plants from the same genotype for the number of larvae penetrated into the root system was observed. Between 20 to 36 percent of the approximately 300 nematodes used to inoculate a plant had penetrated the roots of seedlings or cuttings at 4 DAI. Root systems of cuttings were usually much larger than roots of seedlings and were found to contain on average more J_2 larvae (Table 2).

The J_2 larvae either had migrated into the cortex and were oriented parallel to the root axis, or they were in perpendicular positions with or without their tails outside the cortex. Sometimes multiple invasions of larvae at one feeding site were observed in the root system. Also, larval penetration of the hypocotyl was generally visible in seedlings from all species. These larvae were often in a curled position. Most of the larvae had established their feeding site in the smaller lateral roots, while the remaining larvae had penetrated the main root or the hypocotyl. No differences between resistant and susceptible plant material for site of larval penetration could be observed.

Rate of larval development. At later observation dates the total number of larvae visible within the roots was found to have declined slightly, being 10 to 15% lower than the number of larvae observed in the roots at 4 DAI. To some extent this was found to be due to losses of larvae during the staining procedure. The proportion of larvae in solutions at 4 and 7 DAI was very low. At these observation dates the larvae were at the J_2 and J_3 stage, harbouring within the roots and not on the outside, and presumably did not come off easily by the staining procedure. At 11 DAI mainly J_4, and at 14 and 18 DAI also adult male and female nematodes, that had been washed off the roots, were present in the solutions and represented approximately 5 to 10% of the total number of larvae observed. A decrease in total number of larvae per root system, observed for all genotypes, might also be explained by migration of nematodes out of the roots or to death of larvae, which consequently could not be stained anymore. Therefore, relative numbers of larvae at different developmental stages as a

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Table 2. The number of J_2 larvae of *Heterodera schachtii* Schm. found in root systems of seedlings or cuttings of resistant and susceptible cultivars of *S. alba*, *R. sativus*, *B. napus*, of x*Brassicoraphanus* and of intergeneric hybrids. Data are means of 5 to 10 cuttings or seedlings at 4 days after inoculation with 300 larvae per plant. - = Not determined; () = 95%-confidence interval of the mean

Genotype	Mean of total number of J_2 larvae within roots	
	Cuttings	Seedlings
BCN-susceptible		
B. napus 'Jet Neuf'	81 ($\pm$38)	67 ($\pm$35)
S. alba 'Gisilba'	85 ($\pm$49)	64 ($\pm$26)
R. sativus 'Siletina'	109 ($\pm$35)	62 ($\pm$29)
S. alba x *B. napus* $H_{sex}3$	69 ($\pm$37)	-
BCN-resistant		
S. alba 'Maxi'	90 ($\pm$62)	63 ($\pm$22)
R. sativus 'Nemex'	108 ($\pm$29)	51 ($\pm$17)
x*Brassicoraphanus*	92 ($\pm$32)	59 ($\pm$18)
S. alba x *B. napus* $H_{sex}2$	80 ($\pm$59)	-
x*Brassicoraphanus* x *B. napus*	70 ($\pm$30)	-

percentage of the total number of larvae observed on each sampling date are presented in Table 3. When the data are expressed as relative frequencies, no differences between seedlings and cuttings could be observed. Therefore, since for the hybrid genotypes only cuttings were tested, only the results of cuttings are shown in Table 3.

At 4 DAI, swollen J_2 larvae, which had started their second molt, were found in root systems of all genotypes under study. In most susceptible genotypes this frequency was found to be higher than in resistant plants of species or hybrids. The frequencies of J_2 larvae presented in Table 3 also include the J_2 larvae that had started to molt. To calculate the frequency of J_3 and J_4/adult stage larvae also J_3 molting and J_4 molting larvae, respectively, have been included in the score. At 7 DAI root systems of plants from all species studied contained J_3 larvae and occasionally also J_4 male larvae. The frequency of molting J_2 larvae, as a proportion of the total number of J_2 larvae inside roots, was found to be 10% for the resistant *S. alba* cv. 'Maxi' and about 33% for the other resistant and susceptible parental species and resistant hybrid $H_{sex}2$ at 7 DAI. For the susceptible hybrid $H_{sex}3$ and the hybrid x*Brassicoraphanus* x *B. napus* about half of the J_2 larvae inside roots had

Table 3. Frequencies of various developmental stages of *Heterodera schachtii* Schm. larvae in root systems of resistant and susceptible cultivars of *S. alba*, *R. sativus*, *B. napus*, of x*Brassicoraphanus* and of intergeneric hybrids. Data are means of 5 to 10 cuttings at 4 to 14 days after inoculation (DAI) with 300 larvae per plant. () = 95%-confidence interval of the mean

Genotype	Develop-mental stage of larvae	Proportion of various stages of larvae as a % of the total number of larvae observed at different DAI			
		4	7	11	14
BCN-susceptible					
B. napus 'Jet Neuf'	J_2	100	64	16	4 (±4)
	J_3	0	35	9	3 (±3)
	J_4/adult	0	<1	75	93 (±4)
S. alba 'Gisilba'	J_2	100	66	10	3 (±9)
	J_3	0	33	14	6 (±5)
	J_4/adult	0	<1	76	91 (±10)
R. sativus 'Siletina'	J_2	100	68	14	4 (±6)
	J_3	0	32	22	8 (±8)
	J_4/adult	0	0	64	88 (±14)
S. alba x *B. napus* $H_{sex}3$	J_2	100	70	26	3 (±6)
	J_3	0	30	20	3 (±6)
	J_4/adult	0	0	54	94 (±7)
BCN-resistant					
S. alba 'Maxi'	J_2	100	94	55	44 (±39)
	J_3	0	6	35	11 (±12)
	J_4/adult	0	0	10	45 (±37)
R. sativus 'Nemex'	J_2	100	68	11	3 (±3)
	J_3	0	32	48	9 (±10)
	J_4/adult	0	0	41	88 (±10)
x*Brassicoraphanus*	J_2	100	71	11	6 (±12)
	J_3	0	29	36	7 (±8)
	J_4/adult	0	<1	53	87 (±10)
S. alba x *B. napus* $H_{sex}2$	J_2	100	75	59	32 (±22)
	J_3	0	25	29	8 (±12)
	J_4/adult	0	0	12	60 (±21)
x*Brassicoraphanus* x *B. napus*	J_2	100	77	14	7 (±9)
	J_3	0	23	52	6 (±6)
	J_4/adult	0	0	34	87 (±11)

started their second molt at 7 DAI.

At 11 DAI, the majority of larvae in root systems of susceptible genotypes and of BCN-resistant *xBrassicoraphanus*, *R. sativus* and *xBrassicoraphanus* x *B. napus* were at the J_3 molting or the J_4 stage, whereas in roots of resistant plants of *S. alba* cv. 'Maxi' and the resistant $H_{sex}2$ more than approximately 50% of the larvae were still at the J_2 stage. At 14 DAI approximately 90% of the larvae present in roots of susceptible and resistant plants of species and hybrids were at the J_4/adult stage, except for resistant *S. alba* cv. 'Maxi' and hybrid $H_{sex}2$, where 50% of the larvae had reached these developmental stages. Most adult male nematodes had left the root systems, since mainly empty skins of adult male larvae were observed in the solutions or attached to the root systems. The adult male score, therefore, includes the number of empty skins found in solutions or present in root systems. In roots of resistant plants of *S. alba* cv. 'Maxi' and $H_{sex}2$ many J_2 larvae and also J_2 molting larvae were still found at 14 DAI. The development of larvae, reflected by the frequency of J_3 and J_4/adult staged larvae at different DAI, in roots of resistant plants of *S. alba* cv. 'Maxi' seemed to be slower than in roots of susceptible plants of *S. alba* cv. 'Gisilba'. In the resistant hybrid $H_{sex}2$ this slower development was also observed, but was less conspicuous (Table 3). Also, already at 7 DAI, necrosis of root cells near the head of larvae at the site of penetration could be observed in resistant plants of *S. alba* cv. 'Maxi'. Early necrosis was less frequently seen in roots of the hybrid $H_{sex}2$ and in roots of the other resistant and susceptible genotypes. The developmental rate of larvae in roots of resistant and susceptible plants of *R. sativus* did not differ much, as could also be observed by comparison of the intergeneric hybrids *xBrassicoraphanus* and *xBrassicoraphanus* x *B. napus* (Table 3).

At 18 DAI it was difficult to determine the total number of larvae per root system, since many early staged larvae could not be recognized anymore. Also necrosis of root tissue (syncytia) around the adult male and female nematodes was observed.

Sex differentiation. Table 4 shows the proportion of sex differentiated larvae, i.e. the sum of sex differentiated J_3 to adult larvae relative to the total number of larvae observed in root systems. The root systems of susceptible plants of *B. napus* cv. 'Jet Neuf', *S. alba* cv. 'Gisilba' and *R. sativus* cv. 'Siletina', and of the susceptible hybrid $H_{sex}3$ were found to contain less male than female larvae, resulting in a ♂:♀ sex ratio at 11 DAI ranging from 0.5 to 0.8. In root systems of resistant plants of *R. sativus* cv. 'Nemex', *xBrassicoraphanus* and the hybrid *xBrassicoraphanus* x *B. napus*, the proportion of sex differentiated nematodes at 11 DAI was similar to that observed in the susceptible genotypes, but since more male than female nematodes were present in root systems, the ♂:♀ ratio varied between approximately 9 to 13. For resistant plants of *S. alba* cv. 'Maxi' and hybrid $H_{sex}2$ the frequencies of male and female nematodes at 11 DAI were much lower than those found in the other resistant and susceptible genotypes. In addition, there was an excess of male nematodes, similarly as was observed for resistant *R. sativus* and hybrids (Table 4).

The sex ratios in susceptible genotypes at 14 DAI were not much different from those found at 11 DAI. The proportion of male and female nematodes in

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Table 4. Sex differentiation of larvae from *Heterodera schachtii* Schm., in root systems of resistant and susceptible cultivars of *S. alba, R. sativus, B. napus,* of x*Brassicoraphanus* and of intergeneric hybrids. Data are means of 5 to 10 cuttings at 11, 14 and 28 days after inoculation (DAI) with 300 larvae per plant. () = 95%-confidence interval of the mean

Genotype	Sex differentiated nematodes as a percentage of the total number of larvae observed						Average number of ♀ nematodes	
	11 DAI			14 DAI			14 DAI	28 DAI
	♂	♀	ratio	♂	♀	ratio	$J_3 + J_4 +$ adult	adult
BCN-susceptible								
B. napus 'Jet Neuf'	30.5	49.4	0.62	36.8	57.4	0.64	49.5 (±28.6)	39.9 (±17.1)
S. alba 'Gisilba'	29.5	51.9	0.57	35.2	55.2	0.64	30.6 (±13.1)	23.6 (±11.3)
R. sativus 'Siletina'	34.6	42.0	0.82	44.3	46.4	0.95	27.5 (±12.0)	16.8 (±13.7)
S. alba x *B. napus* H_{sex}3	18.9	40.8	0.46	36.7	57.8	0.63	43.3 (±18.0)	37.4 (±24.0)
BCN-resistant								
S. alba 'Maxi'	10.0	0.6	16.7	39.5	5.9	6.7	3.7 (±7.5)	0.1 (±0.6)
R. sativus 'Nemex'	66.4	5.3	12.5	82.6	8.0	10.3	5.1 (±3.9)	0.2 (±1.0)
x*Brassicoraphanus*	68.3	7.7	8.9	82.9	7.1	11.7	3.7 (±4.0)	3.4 (±6.8)
S. alba x *B. napus* H_{sex}2	13.2	2.7	4.9	49.3	12.9	3.8	6.9 (±6.6)	0.1 (±0.6)
x*Brassicoraphanus* x *B. napus*	63.2	5.7	11.1	76.6	13.2	5.8	7.0 (±4.8)	2.6 (±6.4)

susceptible plants of *B. napus*, *S. alba* and $H_{sex}3$ were equal, while for *R.sativus* cv. 'Siletina' a slightly higher frequency of male nematodes and a lower frequency of females was observed. This might be due to the fact that within the cross-pollinating *R. sativus* cv. 'Siletina' some partial resistance is thought to be present (Toxopeus, personal communication). This is also indicated by the lower multiplication rate of BCN observed at 28 DAI as opposed to the other susceptible genotypes (Table 4).

The ♂:♀ ratios at 14 DAI in resistant plants of *S. alba* cv. 'Maxi' and in the hybrid x*Brassicoraphanus* x *B. napus* were found to be lower than those calculated at 11 DAI, whereas for the other resistant genotypes the ratios of ♂:♀ were similar for both dates (Table 4). The observed difference in sex ratio at 11 and 14 DAI are likely to be due to large differences in the total proportion of sex differentiated larvae at both observation dates, i.e. 10.6% at 11 DAI and 45.4% at 14 DAI for *S. alba* cv. 'Maxi', and in the large difference between the proportion of female nematodes and male nematodes in the resistant species and hybrids.

The increase in the percentage of sex differentiated nematodes from 11 to 14 DAI in resistant genotypes was mainly due to an increase in the proportion of male nematodes. The largest increase in the proportion of males was observed for the resistant *S. alba* cv. 'Maxi' and $H_{sex}2$. For the susceptible genotypes in general an increase of both male and females was observed (Table 4). The ratio of ♂:♀ nematodes was not influenced by whether seedlings or cuttings from the parental species were used for the experiments (data not shown).

At 28 days after inoculation a clear difference in the average numbers of females on root systems of resistant and susceptible species was found (Table 4). Only mature females were counted, whereas J_3 or early J_4 stage females were not included. Therefore, the average number of mature females observed at 28 DAI is lower than the total number of females observed at 14 DAI. On root systems of resistant plants occasionally a few females were visible, and in general clustered on the same lateral root.

Discussion

In the experiment described above it was investigated whether the resistance to BCN in *R. sativus* and *S. alba* can be explained by a decreased root penetration by the nematodes, an impaired larval development or a changed sex ratio.

No difference between resistant and susceptible genotypes was observed for the number of J_2 larvae of *H. schachtii* that had penetrated the root system, which is in accordance with results by Müller (1985). The differences in root size of seedlings and cuttings may explain the difference in frequency of root penetration (Table 2), but was not found to have an effect on the rate of larval development or on the ultimate ratio of male to female nematodes. This indicates that, for the method applied in this study, both seedlings and cuttings are suitable to assess the level of BCN-resistance.

The development of juveniles of *H. schachtii* in genotypes of *R. sativus* and

the intergeneric hybrids x*Brassicoraphanus* and x*Brassicoraphanus* x *B. napus* indicates that the mechanism of resistance to BCN in *R. sativus* is related to a shift towards a higher $\male:\female$ nematode ratio, but not by a decrease of the total proportion of sex differentiated nematodes, which again is in agreement with results by Müller (1985). For *S. alba* also more males than females were observed in resistant genotypes, but the frequency of sex differentiated larvae at 14 DAI in roots of resistant plants of cv. 'Maxi' and of *S. alba* x *B. napus* was lower than in the other resistant and susceptible genotypes (Tables 3 and 4). Similar results on frequency and rate of larval development have also been found in other experiments, not reported here, with resistant plants of *S. alba* cv. 'Emergo' and susceptible plants of *S. alba* cv. 'Hohenheimer'. These results are in contrast to those by Müller (1985), who indicated that the mechanism of resistance to BCN in *S. alba* and *R. sativus* was similar, and only related to a shift in the sex ratio.

The observed lower frequencies of sex differentiated nematodes in resistant *S. alba* genotypes and $H_{sex}2$, as compared to the situation in susceptible *S. alba* genotypes, might be explained by a delayed penetration by larvae or by a stronger migration of J_2 larvae in search for a suitable feeding site, which ultimately results in a lower developmental rate. An indication for these assumptions is that in resistant plants of *S. alba* or $H_{sex}2$ many larvae were still at the J_2 stage at 14 DAI.

For resistant *S. alba* plants, also necrosis of root cells at the site of penetration and subsequent deterioration of nematodes was observed. A necrotic reaction had not been found in the in-vitro experiments by Müller (1985, personal communication), but has been observed in in-vivo experiments with a similar design (Lubberts and Toxopeus 1982). The necrosis of root tissue at the site of larval penetration may be ascribed to a hypersensitive reaction of root cells during or after penetration by larvae, similarly to that described for BCN invasion in monosomic additions of *Beta vulgaris* x *B. procumbens* (Heijbroek *et al.* 1988) and for other host-parasite interactions involving *Heterodera* and *Globodera* species (Gommers 1981). From the presented results it remains unclear if predominantly males or females in resistant *S. alba* had died as a consequence of necrosis, since it is difficult to assess the sex of larvae at an early J_3 stage. The results of the proportion of male nematodes in resistant *S. alba*, being similar to that found in susceptible *S. alba* (Table 4), and the indication that female nematodes require approximately 40 times more food than males (Müller *et al.* 1981) supports the assumption of death of predominantly female nematodes as a consequence of necrosis (Lubberts and Toxopeus 1982). However, since the speed of development in resistant *S. alba* was slower than that in susceptible plants, higher frequencies of males might develop after 14 DAI. Further detailed studies on the determination of the sex of stagnated J_3 larvae will be necessary to support these assumptions.

The difference in developmental rate of larvae and the absence of necrosis of root cells in the experiments by Müller (1985, personal communication), as opposed to the experiments reported here, might be explained by the difference in the methods used to grow the inoculated plant material. This may result in a different host plant metabolism which is suggested to have a major impact on the development and the sex determination of *H. schachtii* (Betka *et al.* 1991,

Grundler *et al.* 1991). If it is assumed that the sex determination of J_2 larvae of *H. schachtii* takes place within 2 to 3 DAI and that sex determination is influenced by environmental conditions, i.e. favourable conditions resulting in relatively more female determined larvae and unfavourable conditions in more males (Betka *et al.* 1991, Grundler *et al.* 1991, Müller 1985), the present divergent results for resistant *S. alba* and *R. sativus* may be explained by a different speed of penetration and by a divergent host plant metabolism. The intermediate respons for rate of larval development and necrosis in the intergeneric hybrid between a resistant plant of *S. alba* and a susceptible plant of *B. napus* would indicate the involvement of resistance genes with additive effects.

For introgression of BCN-resistance in *B. napus* and subsequent elimination of undesired *S. alba* and *R. sativus* traits further backcrossing of hybrids with *B. napus* as the recurrent parent is required. Furthermore, it will be necessary to evaluate if backross genotypes can be selected carrying very little of the *S. alba* or *R. sativus* genome, except for those parts of the genome carrying the gene(s) for BCN resistance present in the *S. alba* or *R. sativus* parents. The results of the *S. alba* x *B. napus* hybrid and the x*Brassicoraphanus* x *B. napus* hybrid evaluated in this study indicate nevertheless that the two different mechanisms of resistance present in *S. alba* and *R. sativus* can be transferred to hybrid and possibly to backcross progeny, strongly suggesting that it may be possible to obtain BCN-resistant oil-seed rape cultivars from these intergeneric hybrids.

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CHAPTER 8

General discussion

Discussion

The main objective of research presented in this thesis was to explore the introduction of resistance to *Heterodera schachtii* Schm., the beet cyst nematode (abbrev. BCN), into *Brassica napus* L., oil-seed rape. *Brassica napus* is thought to be very susceptible to BCN, but not to suffer any yield losses due to the attack of the nematode. Breeding BCN-resistant oil-seed rape cultivars, however, would enable farmers to extend the cultivation of this crop in rotation with one of the major arable crops in North-West Europe, the sugar beet, which is highly susceptible and intolerant to BCN.

Partial resistance within B. napus

The prospects of breeding BCN-resistant oil-seed rape cultivars by hybridizati- on and selection of resistant genotypes within the species *B. napus* were found to be poor (chapter 2). To date, a wide range of genotypes of *B. napus* have been tested for BCN-resistance, but turned out to be susceptible (Baukloh 1976, Bowen *et al.* 1986, Harrewijn 1987, Talatschian 1974, chapter 2). Although a small genotypic effect on the multiplication rate of the nematodes was observed in some accessions (Bowen *et al.* 1986, Harrewijn 1987, chapter 2), it seems unlikely that from this material highly BCN-resistant *B. napus* can be developed.

Appreciable levels of BCN-resistance in the two parental species of *B. napus* (AACC, $2n = 38$), *Brassica rapa* (AA, $2n = 20$) and *B. oleracea* (CC, $2n = 18$) have not been observed (Baukloh 1976, Harrewijn unpublished data, Lubberts and Toxopeus 1982, Talatschian 1974). Therefore, resynthesis of *B. napus* is not promising as an alternative method for the introduction of this trait into oil- seed rape.

Introduction into B. napus of BCN-resistance from related species

Some BCN-resistant genotypes have been found in the two related species *Raphanus sativus* (oil-radish, RR, $2n = 18$) and *Sinapis alba* (white mustard, $S_{al}S_{al}$, $2n = 24$). The mechanisms and the mode of inheritance of BCN-resistance found in *S. alba* and *R. sativus* were not clear at the start of this research. A difference in mechanism of resistance between these two *Cruciferae* species was indicated by Lubberts and Toxopeus (1982), but contested by Müller (1985). Anyhow it was decided that hybrids of both species with *B. napus* should be produced, and the nature of resistance be studied in more detail. Whereas *R. sativus* has often been hybridized with *Brassica* species, hybridizations between *Brassica* species and *S. alba* have been carried out less frequently and predominantly in Europe and Canada (chapter 1). This is probably due to the fact that *S. alba* as a forage crop is mainly cultivated in these areas.
    For introgressing *Raphanus* genes into *B. napus*, hybridization with x*Brassicoraphanus* Sageret (an amphidiploid from *B. rapa* x *R. sativus*), and repeated backcrossing to *B. napus* was found to be more promising than direct

sexual hybridization with *R. sativus* (chapter 4). This corroborates results obtained by Dolstra (1982). Furthermore, although Paulmann and Röbbelen (1988) and Thierfelder *et al.* (1992) were quite successful in obtaining F_1 hybrids from direct crosses between *B. napus* and *R. sativus*, they also indicated that gene exchange between *Raphanus* and *Brassica* chromosomes was likely to be higher when using AACR or ACCR type hybrids in backcrosses with *B. napus* instead of ACR or AACCRR type hybrids.

However, the results presented in this thesis showed that introgression of BCN-resistance via sexual hybridization with x*Brassicoraphanus* might be hampered by several obstacles. Firstly, the BCN-resistance tests of the AACR hybrids indicated dominance of BCN-resistance, but did not provide conclusive evidence for simple monogenic inheritance, as recorded by Baukloh (1976) (chapter 4). In effect, the lowered expression of BCN-resistance in the F_1 hybrids and BC_1 plants would more likely support the model of polygenic inheritance and/or additivity of resistance genes. The inheritance of the *Raphanus* resistance has to be studied in more detail, as well as the question whether the expression of the gene(s) for resistance from the genome R can be affected, and in this particular case suppressed, by gene(s) from the *B. napus* genomes A and C.

Secondly, the rate of success for transfer of BCN-resistance from *Raphanus* to *B. napus* might also be limited by incongruity barriers and lack of meiotic pairing in the hybrids AACR and the backcross progeny (chapters 4 and 6). For instance, the recovery of offspring from backcrossing F_1 hybrids with *B. napus* or either of its parental species as male recurrent parent has been found to be more difficult than the production of the hybrids itself. This was shown for several intergeneric crosses in *Cruciferae*, such as *B. napus* x *R. sativus* and reciprocal cross (Thierfelder *et al.* 1992), *Diplotaxis erucoides* x *B. napus* (Delourme *et al.* 1989), *S. alba* x *B. napus* and reciprocal cross (Chèvre *et al.* 1991).

Nevertheless, the prospects of obtaining fertile backcross progeny from x*Brassicoraphanus* x *B. napus* appear to be good and indications of homoeologous pairing between A-, C-, and R- chromosomes have been obtained (chapter 4). In addition, x*Brassicoraphanus* x *B. napus* hybrids and BC_1 plants with a high level of BCN-resistance have been selected. This indicates that introgression of resistance genes via sexual hybridization with x*Brassicoraphanus* is feasible. This is, furthermore, corroborated by the results of the study on mechanisms of resistance to BCN, which showed that the development of juveniles within the resistant *R. sativus* parent and resistant intergeneric F_1 hybrids was similar (chapter 7).

Transfer of genes from *S. alba* was found to be much more difficult than from x*Brassicoraphanus*. This was mainly due to strong incongruity barriers (chapters 5 and 6). Although introgression of resistance into *B. napus* via *S. alba* x *B. napus* hybrids requires long-term breeding activities, the first results as described in this thesis are promising. Half of the few F_1 hybrids obtained and also one BC_1 plant out of three were highly BCN-resistant. However, the mechanism of resistance in *S. alba* was in the resistant F_1 hybrids expressed in an intermediate way only. Therefore, further studies are needed to find out if

the genes for resistance will be expressed sufficiently and similarly in a *B. napus* background.

Further backcrossing of the *S. alba* x *B. napus* hybrids to *B. napus* as recurrent male parent might result in rapid loss of *S. alba* chromosomes, owing to preferential pairing of the homologous chromosomes of *B. napus* in the AACC(S$_{al}$) hybrids (Chèvre *et al.* 1991, Ripley and Arnison 1990, Ripley *et al.* 1992). Therefore, it will be advisable to also use *B. oleracea* or *B. rapa* as male recurrent parent in backcrosses with the F$_1$ and BC$_1$ plants, resulting in ACCS$_{al}$ and AACS$_{al}$ offspring, respectively, in order to improve the potential gene exchange between A-, C- and S$_{al}$- chromosomes. Backcrosses of F$_1$ hybrids from *B. napus* x *R. sativus* and reciprocal crosses with either *B. rapa* and *B. oleracea*, aiming at improved homeoelogous gene exchange, have also been applied by Paulmann and Röbbelen (1988) and Thierfelder *et al.* (1992). However, the disadvantage of using the material from these backcrosses (ACCS$_{al}$ and AACS$_{al}$) is its larger genetic distance from *B. napus* than that of the AACCS$_{al}$ plants resulting from backcrosses with *B. napus* itself.

Somatic hybridization as a method to transfer BCN-resistance does not look as promising as sexual hybridization between the three *Cruciferae* species: the obtained somatic hybrid plants were highly sterile and unstable both for chromosome number and BCN-resistance. Genetic instability, such as polyploidy and aneuploidy is rather common in wide somatic hybrids in *Cruciferae* (Glimelius *et al.* 1991), and may be caused by either chromosome elimination, multiple fusion events or endoreduplications. On the other hand, protoplast culture has been shown to induce exchange between alien chromosomes at a rather high frequency (Larkin *et al.* 1990). Furthermore, in several asymmetric somatic hybrids indications for translocations have been found (Famelaer *et al.* 1988, Hinnisdaels *et al.* 1990). Chèvre *et al.* (1991) pointed out that the prospects for introgression of desirable traits were higher for *B. napus* (+) *S. alba* somatic hybrids than for sexual hybrids obtained, a conclusion based on pairing configurations of chromosomes, the fertility of hybrid plants and the observation of 38-chromosome plants with the desired trait, *Alternaria* resistance, after several generations of backcrossing and selfing. These results are in contrast to those presented in this thesis but stress the importance of the use of a broad range of genotypes, and also of the best method (somatic or sexual hybridization) to obtain hybrids.

In my work a reliable comparison between sexual and somatic hybridization cannot be made, since the number of sexual and somatic hybrids obtained is low. As opposed to the sexual hybrids, the use of the somatic hybrids *S. alba* (+) *B. napus* and *R. sativus* (+) *B. napus* in further resistance breeding programmes probably will be limited, although there are good indications for the expression of BCN-resistance in the somatic hybrids (chapters 3 and 5). It must be remembered, however, that it will only be possible to use these somatic hybrids when plants can be selected with a more stable genome constitution and a better female or male fertility. In order to provide more information on these intergeneric hybridizations, further analysis and additional experiments are needed.

Prospects for S. alba and R. sativus resistance

To date little attention has been given to the genetic variation within the *H. schachtii* population used for the detection of resistance in *Cruciferae* species. The previous selection efforts in *Beta* or *Cruciferae* species have nearly all been made with populations of *H. schachtii*, considered to be homogeneous (Harrewijn 1987, Heijbroek *et al.* 1988, Lange *et al.* 1989, Lubberts and Toxopeus 1982). In this thesis also mixed *H. schachtii* populations, reared on either susceptible *B. napus*, *B. vulgaris* or *S. alba*, were used to evaluate the level of resistance. Although the development of resistance breaking pathotypes of *H. schachtii* cannot be ruled out, the present investigations have shown that the *S. alba* and *R. sativus* resistance to BCN most likely is durable.

Only very recently detection of several virulent pathotypes of BCN has been described (Müller 1992). These virulent pathotypes were capable of breaking resistance in a monosomic addition line of sugar beet carrying one chromosome of *B. procumbens* (Müller 1992), but did not affect the BCN-resistance in *S. alba* and *R. sativus* (Müller, personal communication). These virulent populations, however, have been selected under laboratory conditions, which were very different from conditions in the field. They have a relatively low working spectrum. Furthermore, the study of resistance mechanisms has shown that both the *S. alba* and *R. sativus* resistance is durable, since it causes the preferential development of female nematodes. A fast multiplication of virulent pathotypes of BCN is not likely to occur, because 1) females of parasitic nematodes have a relatively low rate of multiplication, 2) virulent females most likely are fertilized by avirulent male nematodes only, 3) virulence in cyst nematodes is mainly inherited recessively, and 4) BCN are sedentary nematodes. If needed BCN can be controlled effectively by nematicidal treatment of the soil. Nevertheless, a proper use of both types of resistance to BCN in *B. napus* is advocated.

An increased knowledge on pathotypes of BCN and on resistance gene(s) in the *Cruciferae* species is necessary to estimate the value of the BCN-resistance in the intergeneric hybrids from crosses between *B. napus* and either *S. alba* or *R. sativus*.

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Summary

Brassica napus L. (oil-seed rape) is a small crop in The Netherlands. One of the important reasons for the limited interest in cultivating *B. napus* is the high multiplication rate of the beet cyst nematode, *Heterodera schachtii* Schm. (abbrev. BCN) on this crop. As a consequence, the cultivation of oil-seed rape in narrow rotation with sugar beet (*Beta vulgaris* L.), an important arable crop in The Netherlands, is not possible without soil fumigation, which is detrimental to the environment. Breeding for BCN-resistant oil-seed rape would make an increase in acreage in The Netherlands possible and is the ultimate objective of the research presented in this thesis. The aim of the present study was to explore the enhancement and the introduction of resistance to *H. schachtii* in *B. napus*. Experiments were performed aimed at selecting for partial resistance within the species *B. napus* L., and at transferring BCN-resistance gene(s) from the related cruciferous species *Sinapis alba* L. (white mustard) and *Raphanus sativus* L. (fodder radish) to *B. napus*.

Attempts to detect sources of complete or of a high level of partial resistance to BCN in the species *B. napus* were unsuccessful (chapter 2). The detection of partial resistance was found to be difficult due to high experimental variability. The rate of transmission of BCN-resistance from selected resistant plants to their progeny was very low.

Crosses between *B. napus* (AACC, $2n = 38$) and *R. sativus* (RR, $2n = 18$) failed in both directions. Sexual hybridization of *B. napus* with xBrassicoraphanus Sageret (AARR, $2n = 38$, amphidiploid from *B. rapa* (AA, $2n = 20$) x *R. sativus*) was found to be promising for the transfer of BCN-resistance from *Raphanus* to *B. napus* (chapter 4). When crossing xBrassicoraphanus as female with *B. napus* about 800 pollinations resulted into 284 F_1 hybrids. The reciprocal cross yielded mainly matromorphic plants. The intergeneric hybrids varied in their morphology at the vegetative and generative stages and in male and female fertility. Most F_1 hybrids had the expected number of 38 chromosomes. Meiosis was studied in 36- and 38-chromosome hybrids and showed much variation in pairing behaviour between hybrids. Some indications were found for pairing between chromosomes of the three genomes A, C and R. F_1 hybrids from reciprocal crosses between xBrassicoraphanus and *B. napus*, though highly sterile, could be used with some success as female in backcrosses to *B. napus*. The BC_1 plants studied had a more variable number of chromosomes than the F_1 hybrids. Several backcrosses of the F_1 hybrids to *B. napus* resulted in an increase in male and female fertility. On average, the F_1 hybrids had an intermediate level of BCN-resistance, but some plants had a level of resistance not different from that of the xBrassicoraphanus plants used for crosses. The BCN-resistance tests indicated a dominant inheritance of the BCN-resistance trait. The decreased expression of BCN-resistance in the F_1 hybrids and BC_1 plants indicates a polygenic nature of resistance and/or additivity of genes.

Intergeneric crosses between *S. alba* and *B. napus* were performed reciprocally, with both diploid (2x) and tetraploid (4x) accessions of *S. alba*. F_1 hybrid plants were recovered only from crosses with *S. alba* 2x as female

parent, with application of embryo culture shortly after pollination. Only six hybrids were obtained from approximately 10,000 crosses (chapter 5). The F_1 hybrids had the expected number of 31 chromosomes and morphological characteristics from both parents. The cytological studies of chromosome association at meiotic metaphase I of these hybrids suggested partial homology between chromosomes of the AC and S_{al} genomes and thus a potential for gene exchange. F_1 hybrid plants were highly sterile; only three BC_1 plants were obtained after backcrossing to *B. napus*. Three F_1 hybrids and one BC1 plant were highly BCN-resistant and had a level of resistance equal to that of the *S. alba* parent.

The nature of pre- and post-zygotic incongruity barriers was studied in reciprocal intergeneric crosses between *B. napus* and either x*Brassicoraphanus*, *S. alba* 2x or *S. alba* 4x (chapter 6). The low seed set in reciprocal crosses between *B. napus* and the bridging hybrid x*Brassicoraphanus* was mainly associated with a low frequency of micropylar penetration and with embryo abortion. In reciprocal crosses between *S. alba* and *B. napus* pollen germination and pollen tube growth were highly disturbed. This resulted in a low percentage of fertilized ovules. In addition embryo abortion occurred. The reciprocal differences in hybrid seed set between these intergeneric crosses appeared to be partly associated with differences in pollen germination and pollen tube growth, although post-zygotic barriers were assumed to be the main cause of the generally poor results from these intergeneric crosses. A strong induction and development of matromorphic seeds in crosses with *B. napus* as female parent was observed. This suggests a high parthenogenetic ability of the *B. napus* genotypes used and a high parthenogenesis inducing ability of the pollen parents.

To circumvent pre- and post-zygotic incongruity barriers, somatic hybridization has been applied to combine *B. napus* with *R. sativus* (chapter 3) and *B. napus* with *S. alba* (chapter 5). No regeneration of protoplasts from *R. sativus* was achieved, which allowed simple hybrid plant selection based on the regeneration capacity of only one parent (*B. napus*) in the fusion product. However, it was found that the overall regeneration capacity of cells after fusion was low, and only one somatic hybrid was obtained. Cuttings of this somatic hybrid were unstable for the number of chromosomes and BCN-resistance. Some cuttings were highly BCN-resistant. Backcrossing this hybrid to *B. napus* as male parent was not successful, probably due to the meiotic and mitotic instability of this hybrid. RFLP analysis of the nuclear genome showed a hybrid pattern and no new bands were found. RFLP analysis of the chloroplast DNA of the *B. napus* (+) *R. sativus* hybrid showed a banding pattern equal to that of the *B. napus* parent, pointing to a loss of *Raphanus* chloroplasts. RFLP analysis of mitochondrial DNA showed bands identical to those of either *B. napus* or *R. sativus* but a novel restriction pattern suggesting recombination and/or rearrangements after protoplast fusion, also occurred.

Protoplast fusion of *B. napus* with *S. alba* resulted in more somatic hybrids than fusion of *B. napus* and *R. sativus*, but also these hybrids were found to be chimeras for number of chromosomes and BCN-resistance. The somatic hybrids showed morphological characteristics of both parents. Most hybrids had a higher number of chromosomes than expected to result from fusion of one *B.*

napus protoplast and one *S. alba* protoplast. RFLP analysis of the nuclear DNA showed a pattern equal to that of the *B. napus* parent. The organelle DNA pattern of the somatic hybrids *B. napus* (+) *S. alba* was identical to that of the *S. alba* parent for both the chloroplast and mitochondrial DNA. The BCN-resistance of the somatic hybrids varied between cuttings of the same hybrid, but some were as resistant as the *S. alba* parent and the sexual hybrids from *S. alba* x *B. napus*. Backcrosses of the somatic hybrids to *B. napus* as recurrent male parent did not result in BC_1 plants, which is thought to be mainly due to the chromosomal instability of the somatic hybrids.

The mechanism of resistance to BCN in the resistant *S. alba* and *R. sativus* accessions was studied by examining the development of juveniles of *H. schachtii* in roots of resistant and susceptible intergeneric sexual hybrids, comparable with the development of juveniles observed in the resistant and in the susceptible parental species (chapter 7). Different mechanisms of resistance appeared to be present in *S. alba* and *R. sativus*. For resistant *R. sativus* a low multiplication rate of the nematode was associated mainly with a higher male:female nematode ratio than found in susceptible *R. sativus*. For *S. alba* BCN-resistance is thought also to be due to a slower developmental rate of larvae, and an increased necrosis of root cells at the site of larval penetration. The mechanism of resistance of both resistant species was expressed at an intermediate level in intergeneric sexual hybrids *S. alba* x *B. napus*, x*Brassicoraphanus* and x*Brassicoraphanus* x *B. napus*.

Although it is not easy to obtain sexual or somatic hybrids between *B. napus* and related species with resistance to the beet cyst nematode, the results of the work presented in this thesis indicate that resistance is expressed at a high level in those few hybrid plants obtained. This supports the view that BCN-resistant oil-seed rape cultivars can be obtained from these intergeneric hybrids, although much work remains to introduce the resistance into a *B. napus* background by means of backcrossing, selection and meiotic studies.

Samenvatting

Introductie van bietecystenaaltjes-resistentie uit *Sinapis alba* L. and *Raphanus sativus* L. in *Brassica napus* L. (koolzaad) via sexuele en somatische hybridisatie

Brassica napus L. (koolzaad) is een klein gewas in Nederland. Een van de belangrijkste verklaringen voor de geringe belangstelling om koolzaad te telen is de hoge vermeerderingsgraad van het bietecystenaaltje, *Heterodera schachtii* Schm. (afk. BCA) op dit gewas. Als gevolg hiervan is het telen van koolzaad in nauwe rotatie met suikerbiet (*Beta vulgaris* L.), een belangrijk landbouwgewas in Nederland, niet mogelijk zonder de toepassing van grondontsmetting, die schadelijk is voor het milieu. Het kweken van BCA-resistent koolzaad zal een verruiming van de teelt in Nederland mogelijk maken, en dit is het uiteindelijke doel van het onderzoek, dat in dit proefschrift is behandeld. Het doel van deze studie was onderzoek doen naar de mogelijkheden voor het verhogen en de introductie van resistentie tegen *H. schachtii* in *B. napus*. Er werden experimenten uitgevoerd betreffende de selectie op partiële resistentie binnen de soort *B. napus* en de overdracht van BCA-resistentie-gen(en) vanuit de verwante kruisbloemige soorten *Sinapis alba* L. (gele mosterd) en *Raphanus sativus* L. (bladrammenas) naar *B. napus*.

   Pogingen tot het vinden van bronnen met volledige of met een hoog niveau van partiële resistentie tegen BCA binnen de soort *B. napus* waren niet succesvol (hoofdstuk 2). De detectie van partiële resistentie werd bemoeilijkt door een hoge mate van variabiliteit in de proeven. De mate van expressie van BCA-resistentie van geselecteerde planten naar hun nakomelingschap was erg laag.

   Kruisingen tussen *B. napus* (AACC, $2n = 38$) en *R. sativus* (RR, $2n = 18$) mislukten in beide richtingen. Sexuele hybridisatie tussen *B. napus* en x*Brassicoraphanus* Sageret (AARR, $2n = 38$), een amphidiploid van *B. rapa* (AA, $2n = 20$) x *R. sativus*, bleek veelbelovend voor de overdracht van BCA-resistentie vanuit *Raphanus* naar *B. napus* (hoofdstuk 4). Bij het gebruik van x*Brassicoraphanus* als moeder in kruisingen met *B. napus* werden 284 F_1-hybriden verkregen uit 800 kruisingen. De reciproke kruising resulteerde overwegend in matromorfe planten. De geslachtshybriden varieerden in morfologie in het vegetatieve en generatieve stadium en in mannelijke en vrouwelijke fertiliteit. De meeste F_1-hybriden hadden het verwachte aantal van 38 chromosomen. Meiose werd bestudeerd in 36- en 38-chromosomige hybriden en gaf een grote mate van variatie in paringsgedrag te zien tussen de hybriden. Er waren aanwijzingen voor paring tussen chromosomen van de drie genomen A,C en R. F_1-hybriden, verkregen uit reciproke kruisingen tussen x*Brassicoraphanus* en *B. napus*, waren in hoge mate steriel, maar konden toch met enig succes als moeder worden gebruikt in terugkruisingen met *B. napus*. De bestudeerde T_1 (terugkruisings)-planten vertoonden meer variatie in aantal chromosomen dan de F_1-hybriden. Meerdere terugkruisingen van de F_1-hybriden met *B. napus* resulteerden in een verbetering van de mannelijke en vrouwelijke fertiliteit. De F_1-hybriden hadden gemiddeld een intermediair niveau van BCA-resistentie, maar een aantal planten had een niveau van resistentie, dat niet verschilde van dat van de

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x*Brassicoraphanus*-planten, die waren gebruikt voor de kruisingen. De resultaten van BCA-resistentietoetsen gaven aanwijzingen voor een dominante vererving van de BCA-resistentie. De verlaagde expressie van de BCA-resistentie in de F_1-hybriden en T_1-planten is een indicatie voor een polygene overerving van resistentie en/of van additiviteit van genen.

Geslachtskruisingen tussen *S. alba* en *B. napus* werden reciprook uitgevoerd, met zowel diploïde (2x) als tetraploïde (4x) herkomsten van *S. alba*. Hybride F_1-planten werden alleen verkregen uit kruisingen met *S. alba* 2x als moeder en met gebruikmaking van embryocultuur kort na bestuiving. In totaal werden slechts 6 hybriden verkregen uit ongeveer 10.000 kruisingen (hoofdstuk 5). De F_1-hybriden hadden het verwachte aantal chromosomen ($2n = 31$) en vertoonden morfologische eigenschappen van beide ouders. Cytologisch onderzoek van de chromosoomassociaties in de meiotische metafase I van deze hybriden duidde op een gedeeltelijke homologie tussen chromosomen van de genomen AC en S_a, en dus op een mogelijkheid tot uitwisseling van genen. F_1-planten waren in hoge mate steriel. Na terugkruising met *B. napus* werden slechts drie T_1- planten verkregen. Drie F_1-hybriden en één T_1-plant waren in hoge mate BCA-resistent en hadden een niveau van resistentie, dat gelijk was aan dat van de *S. alba* ouder.

De oorzaak van pre- en post-zygotische kruisingsbarrières werd bestudeerd in reciproke geslachtskruisingen tussen *B. napus* enerzijds en x*Brassicoraphanus*, *S. alba* 2x of *S. alba* 4x anderzijds (hoofdstuk 6). De geringe zaadzetting in reciproke kruisingen tussen *B. napus* en de brugsoort x*Brassicoraphanus* ging voornamelijk gepaard met een lage frequentie in micropylaire penetratie en met embryo-abortie. In reciproke kruisingen tussen *S. alba* en *B. napus* werden pollenkieming en pollenbuisgroei sterk geremd, hetgeen resulteerde in een laag percentage bevruchte zaadknoppen; ook vond embryo-abortie plaats. De reciproke verschillen in hybride-zaadzetting tussen deze geslachtskruisingen bleken gedeeltelijk geassocieërd met verschillen in pollenkieming en pollenbuisgroei, alhoewel er werd aangenomen dat post-zygotische barrières de belangrijkste oorzaak waren voor de overwegend slechte resultaten van deze geslachtskruisingen. Er werd een sterke inductie en ontwikkeling van matromorfe zaden in kruisingen met *B. napus* als moeder geconstateerd. Dit wijst op een hoog parthenogenetisch vermogen van de *B. napus*-genotypen, alsmede een hoog parthe-nogenese-inducerend vermogen van de pollenouders.

Voor het omzeilen van pre- en post-zygotische incongruentie werd somatische hybridizatie toegepast tussen *B. napus* en *R. sativus* (hoofdstuk 3) en tussen *B. napus* en *S. alba* (hoofdstuk 5). Er werd geen regeneratie verkregen vanuit protoplasten van *R. sativus*, wat een eenvoudig selectiesysteem voor hybride planten mogelijk maakte, gebaseerd op de regeneratiecapaciteit van slechts één ouder (*B. napus*) in het fusieprodukt. Echter, de regeneratiecapaciteit van cellen na fusie was laag, en slechts één somatische hybride is verkregen. Stekken van deze somatische hybride waren onstabiel in het aantal chromosomen en in BCA-resistentie. Sommige stekken waren zeer BCA-resistent. Het terugkruisen van deze hybride met *B. napus* als vader had geen succes, waarschijnlijk door meiotische en mitotische instabiliteit van deze hybride. RFLP-analyse van het kerngenoom liet een hybride patroon

zien en er werden geen nieuwe banden gevonden. RFLP-analyse van het chloroplast-DNA van de *B. napus* (+) *R. sativus* hybride liet een bandenpatroon zien, dat gelijk was aan dat van de *B. napus* ouder, hetgeen wijst op het verlies van *Raphanus*-chloroplasten. RFLP-analyse van mitochondrieel DNA liet banden zien die identiek waren hetzij met die van *B. napus*, of met die van *R. sativus*, maar ook kwam een nieuw restrictiepatroon voor, dat recombinatie en/of herrangschikking na protoplastenfusie doet vermoeden.

Protoplastenfusie van *B. napus* met *S. alba* resulteerde in meer somatische hybriden dan fusie van *B. napus* met *R. sativus*, maar ook deze hybriden bleken chimeren te zijn. De meeste hybriden hadden een hoger aantal chromosomen dan verwacht na fusie tussen één *B. napus* protoplast en één *S. alba* protoplast. RFLP-analyse van het kerngenoom liet een patroon zien dat identiek was aan dat van de *B. napus* ouder. Het organel-DNA patroon van de somatische hybriden *B. napus* (+) *S. alba* was identiek aan dat van de *S. alba* ouder voor zowel het chloroplast- als het mitochondrieel-DNA. BCA-resistentie in de somatische hybriden varieerde tussen stekken van dezelfde hybride, maar sommige waren even resistent als de *S. alba* ouder en als de sexuele hybriden *S. alba* x *B. napus*. Terugkruisingen van de somatische hybriden met *B. napus* als vader resulteerden niet in T_1-planten, hetgeen vermoedelijk te wijten is aan de chromosomale instabiliteit van de somatische hybriden.

Het mechanisme van resistentie tegen BCA in de resistente *S. alba* en *R. sativus* herkomsten werd bestudeerd door de ontwikkeling van larven van *H. schachtii* in wortels van resistente en vatbare sexuele geslachtshybriden met *B. napus*, te vergelijken met de ontwikkeling van larven in de resistente en vatbare ouders (hoofdstuk 7). Er bleken verschillende mechanismen van resistentie aanwezig te zijn in *S. alba* en *R. sativus*. Voor de resistente *R. sativus* was een lage vermeerderingsgraad van het aaltje hoofdzakelijk geassocieerd met een hogere mannetjes:wijfjes-larvenverhouding dan voorkomt in vatbare *R. sativus*. Voor *S. alba* wordt de BCA-resistentie mogelijk ook veroorzaakt door een langzamere ontwikkeling van de larven en necrose van wortelcellen op de plaats van penetratie door larven. Het resistentiemechanisme van beide resistente soorten komt tot uiting op een intermediair niveau in de sexuele geslachtshybriden *S. alba* x *B. napus*, x*Brassicoraphanus* en x*Brassicoraphanus* x *B. napus*.

Ofschoon het niet gemakkelijk is om sexuele en somatische hybriden te verkrijgen tussen *B. napus* en verwante soorten met resistentie tegen het bietecystenaaltje, geven de resultaten van het werk, vermeld in dit proefschrift, aan, dat resistentie tot expressie komt in de hybriden, die zijn verkregen. Dit ondersteunt de visie, dat BCA-resistente koolzaadrassen uit deze geslachtshybriden kunnen worden verkregen, echter met dien verstande, dat nog veel werk m.b.t. terugkruising en stabilisatie van de resistentie in de *B. napus* achtergrond nodig zal zijn.

NAWOORD

Curriculum vitae

Cilia Lelivelt werd op 12 april 1964 geboren te Barendrecht (Z-H). Zij behaalde het Gymnasium-ß diploma in 1982 aan het Ichthus College te Drachten. In 1987 werd het Ingenieursdiploma behaald in de studierichting Plantenveredeling aan de Landbouwuniversiteit te Wageningen, met als specialisaties Plantenziektekunde en Tuinbouwplantenteelt. Van maart 1987 tot juli 1991 heeft zij onderzoek verricht naar de veredeling van koolzaad op resistentie tegen het bietecystenaaltje, waarvan de resultaten zijn weergegeven in dit proefschrift. Het onderzoek is uitgevoerd op de Stichting voor Plantenveredeling SVP te Wageningen, die in 1991 is opgegaan in het Centrum voor Plantenveredelings- en Reproductieonderzoek (CPRO-DLO). Sinds september 1991 is de auteur werkzaam bij Royal Sluis te Enkhuizen.