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ORIGINAL PAPER

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Transfer of resistance against the beet cyst nematode from radish (*Raphanus sativus*) to rape (*Brassica napus*) by monosomic chromosome addition

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Abstract In rape (*Brassica napus*), no resistance to the beet cyst nematode (BCN) *Heterodera schachtii* is available. This study was carried out to determine the specific chromosome(s) of resistant radish (*Raphanus sativus*) carrying the gene(s) for nematode resistance as a prequisite to convert rape from a host into a trap crop for this pest. A *Raphanobrassica* progeny of 25 plants was analyzed which segregated for all nine chromosomes of the *Raphanus* genome in a genetic background of synthetic rape. The number of radish chromosomes was determined by fluorescence in situ hybridization, using the *Raphanus*-specific DNA probe pURsN; and their type was identified by chromosome-specific randomly amplified polymorphic DNA markers. Five different multiple rape–radish chromosome additions (comprising the whole set of nine radish chromosomes, a–i) were selected and crossed to rape. For each cross-progeny, the number of cysts on plant roots was counted 42 days after inoculation with a L2 larvae suspension. Simultaneously, the plants were characterized for the presence or absence of individual radish chromosomes, using sets of chromosome-specific markers. Thus, the effect of each radish chromosome on cyst number was tested. Chromosome d had a major resistance effect, whereas the presence/absence of the other radish chromosomes had nearly no influence on cyst number. Plants with added chromosome d showed a resistance level comparable with that of the radish donor parent. The analysis in the cross to rape of a plant monosomic only for chromosome d confirmed the strong effect of this chromosome on nematode resistance. A further experiment comprising seven crosses using winter rape breeding lines and monosomic addition line d as pollen parent provided the same results on a broader genetic basis. In each case, the added chromosome d in a single dosage caused nearly the full resistance of the radish donor. Resistance was independent of the glucosinolate content in the roots. The possibilities for stabilizing BCN resistance in rape and its use for other crops and nematodes are discussed.

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Introduction

The beet cyst nematode (BCN), *Heterodera schachtii* Schm. is a worldwide important pathogen in sugarbeet cropping. For its biological control, different paths of resistance breeding are followed. The transfer of resistance genes from wild beets enables direct protection against pathogen damage through the development of resistant sugarbeet cultivars (Savitsky 1975; Speckmann and de Bock 1982; Löptien 1984; Jung et al. 1986). A second strategy is the breeding of BCN-resistant cruciferous plants which are used as intercrops for trap-cropping. Such plants promote the hatching of second-stage larvae from eggs, and after penetration by the larvae into the roots, they inhibit further nematode development. Thus, growing trap crops on severely infected soils can decrease the pathogen population density. BCN resistance has been found in some varieties of oil radish (*Raphanus sativus* L. ssp. *oleiferus* DC.) and in white mustard (*Sinapis alba* L.) (Baukloh 1976; Lubberts and Toxopeus 1982). Oil radish is widely grown in Europe as a green crop in sugar beet rotations. The BCN resistance of oil radish seems to act towards an increased male/female nematode ratio (Müller 1985; Lelivelt and Hoogendoorn 1993). *R. sativus* exerts genotypic selection pressure on *H. schachtii* populations, as revealed by AFLP and RAPD markers (Kaplan et al. 1999), but no breakdown of the radish BCN resistance by virulent pathogen races has been reported.

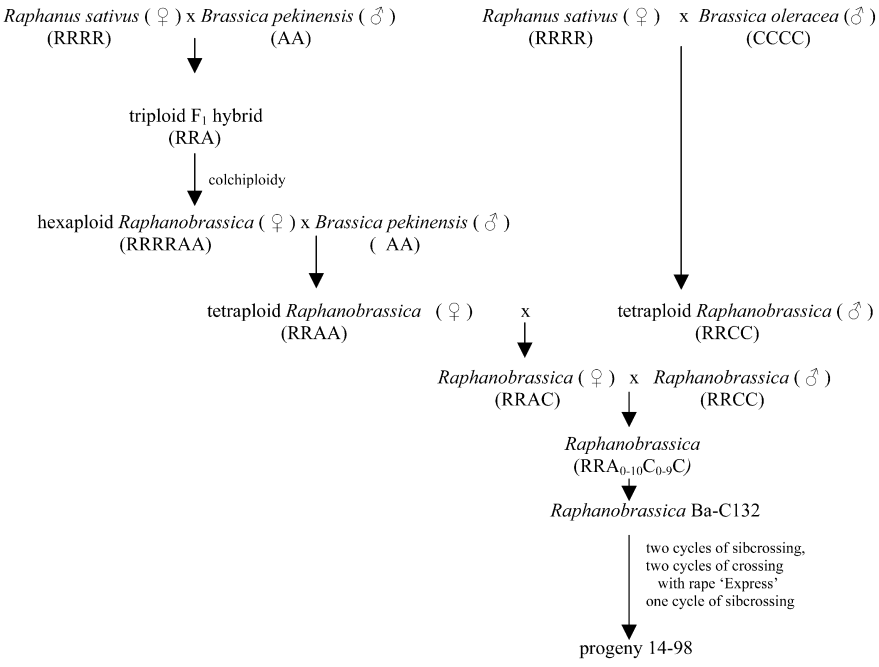

Fig. 1 Crossing scheme for the development of progeny 14-98

In the agriculturally more important oil-seed rape [*Brassica napus* L. ssp. *oleifera* (Metzg.) Sinsk.], no resistance to BCN has been found (Harrewijn 1987; Lelivelt 1995; Fatemy and Abootorabi 2002). Rape as a tolerant, good host for BCN, could not be included in rotations of dense sugarbeet cropping, because of the potential for *H. schachtii* population increase (Nielsen et al. 2003). It was proposed to develop resistant rapeseed through intergeneric hybridization, using oil radish or white mustard as donors of BCN resistance. Sexual and somatic hybrid plants between white mustard and oil-seed rape were obtained (Lelivelt et al. 1993a). Reciprocal crosses between *B. napus* and *R. sativus* were unsuccessful, as was embryo rescue (Lelivelt et al.1993b). Metz et al. (1995) showed that such hybrids could be obtained using a modified flower culture method. Radish × rapeseed hybrid plants produced by protoplast fusion were BCN-resistant (Lelivelt and Krens 1992). The resistance was also expressed in the hybrid *B. campestris* × *R. sativus*, in its F_1 and in some of the first backcross plants with *B. napus* (Dolstra 1982; Lange et al. 1989; Lelivelt 1995). Thierfelder (1994) produced resistant hexaploid radish–rape hybrids and backcross progenies with rape.

In this study, we developed additions to rape involving all radish chromosomes and characterized their response to nematode assault to determine which part of the *Raphanus* genome carries the gene(s) necessary for resistance expression in the rape genetic background, as a first step toward the stable integration of BCN resistance into rape.

Materials and methods

Plant material

Progeny 14-98 was developed in a *Raphanobrassica* interspecific hybridization program (Fig. 1), using separate donors of the *Brassica* genomes A and C (with radish for the R genome) in a way similar to that described by Clauß (1978).

Raphanobrassica RRCC

The tetraploid *Raphanobrassica* (2*n*=4*x*=36, genome constitution RRCC) was obtained by pollination of the nematode-resistant tetraploid oil radish strain 2655 (*Raphanus sativus* L. ssp. *oleiferus* DC.; 2*n*=4*x*=36, RRRR) with a pollen mixture of two tetraploid fodder kales [*B. oleracea* L. convar. *acephala* (DC.); 2*n*=4*x*=36, CCCC], strains nFMK and PC81.

Raphanobrassica RRAA

The tetraploid *Raphanobrassica* RRAA was produced with crosses of the nematode-resistant tetraploid fodder radish 101/77 (2*n*=4*x*=36, RRRR) with diploid Chinese cabbage [*B. pekinensis* (Lour.) Rupr. 'Nagaoka'; 2*n*=2*x*=20, AA]. The chromosome number of the triploid hybrid (2*n*=3*x*=28, RRA) was doubled by colchicine. The hexaploid hybrid (2*n*=6*x*=56, RRRRAA) was backcrossed with Chinese cabbage 'Nagaoka' to produce the tetraploid hybrid (2*n*=4*x*=38, RRAA).

Raphanobrassica Ba-C132

After crossing the Raphanobrassicas RRCC and RRAA, the resulting tetraploid form (2*n*=4*x*=37, RRAC) was backcrossed with *Raphanobrassica* RRCC. The *Raphanobrassica* Ba-C132 was developed from sib crosses, (RRAC × RRCC) × (RRAC × RRCC).

Progeny 14-98

The *Raphanobrassica* Ba-C132 was used in a population breeding program with several sib crosses and crosses to rape (*B. napus* L. ssp. *oleifera* Metzg., 'Express'; $2n=4x=38$, AACC). The last crossing step, which resulted in progeny 14-98, was the cross between a male sterile plant from *Raphanobrassica* [(Ba-C132 × 'Express') × 'Express'] and a male fertile plant obtained from the selfing of hybrid 'Express' × Ba-C132.

For this study, 25 yellow flowering plants were selected and crossed with the inbred line M1 of rape ('Madora') as male parent.

DNA isolation and RAPD reaction

Total genomic DNA was extracted from approximately 50 mg young leave tissue, according to Dellaporta et al. (1983) with minor modifications. DNA concentrations were estimated spectrophotometrically and adjusted to 8 ng/μl.

Arbitrary decamer primer kits A–I (Operon Technologies) were used for the amplification of DNA, based on the protocol of Williams et al. (1990) with minor modifications. Reactions (8 μl) contained 1× NH_4 reaction buffer (InVitek), 2.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM decamer primer, 0.4 units Taq DNA polymerase (InVitek) and 16 ng total plant DNA and were amplified in 96-well PCR plates in a GeneAmp PCR System 9700 (Applied Biosystems). Fragments of DNA were amplified after a 5-min denaturation step at 94 °C for 45 cycles (94 °C for 0.5 min, 35 °C for 0.5 min, 72 °C for 1 min), with the extension step of the last cycle extended to 10 min.

Fragment separation and silver-staining

Amplification products were mixed 1:1 with loading dye (98% formamide, 10 mM EDTA, 0.025% bromphenol blue, 0.025% xylencyanol) and denatured for 5 min at 95 °C. Then, 5 μl were loaded on a 4% denaturing polyacrylamide gel prepared in 1× Tris-borate EDTA. Electrophoresis was performed using a Sequi-Gen GT (38×50 cm; Bio-Rad Laboratories) running at 50 °C and 100 W for 3.5 h.

After electrophoresis, the gel on the outer glass plate was silver-stained. After fixation for 10 min in 10% acetic acid and three washes in deionized H_2O (3 min each), the gel was incubated for 30 min in staining solution (4 g silver nitrate, 3 ml 37% formaldehyde in 2 l deionized H_2O). After a short wash in deionized H_2O, the gel was incubated in developing solution (60 g sodium carbonate, 3.5 ml formaldehyde, 8 mg sodium thiosulfate in 2 l deionized H_2O). When band strength was optimal, the developing process was stopped by incubation in 10% acetic acid. After washing in deionized H_2O, the gel was air-dried. Gels were documented by a scanner. Size determination of individual bands was made by comparison with a 100-bp ladder (Invitrogen).

Amplification and labeling of pURsN

Based on the consensus sequence of pURsN described by Hirai et al. (1995), optimal primer sequences were determined for PCR amplification: pURsN FW (3′-TGGAACCACTAATCAGTGAG-5′), pURsN BW (3′-ATGGCATATGTCCGAACAGG-5′).

Amplification was performed in a 25-μl final volume of 1× NH_4 reaction buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.2 μM forward and backward primers, 1.25 units Taq polymerase and 15 ng genomic *Raphanus sativus* DNA. A GeneAmp PCR System 9700 was programmed for denaturation at 94 °C for 2 min and 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1.5 min, for denaturation, primer annealing and extension, respectively. A final elongation step at 72 °C for 5 min ended the program. pURsN was digoxigenin (DIG)-labeled by PCR using the same procedure as above, substituting 35% of the dTTP by DIG-dUTP.

Southern hybridization

Genomic plant DNA (5 μg) was digested with *Hin*dIII, separated on 0.8% agarose gel and blotted on positively charged Nylon membrane (Roche Diagnostics), as described by Sambrook et al. (1989). The filter was hybridized to 200 ng of DIG-labeled pURsN overnight at 37 °C. Hybridization, washing and detection were done according to the supplier's instructions.

Chromosome preparation and fluorescence in situ hybridization

Excised roots were pre-treated with 8-hydroxyquinoline (2 mM aqueous solution) at 24 °C for 2 h 40 min, fixed in a freshly prepared mixture of absolute ethanol/glacial acetic acid (3:1) for 24 h and stored in 70% ethanol at –20 °C. Before maceration, the root tips were washed three times in distilled water (30 min each) and then digested with an enzyme mixture of 4% cellulase 'Onozuka R-10' (Serva) and 1% pectolyase Y-23 (Seishin Pharmaceutical) in 75 mM KCl, pH 4.0, for 40 min at 37 °C. After a short rinse in distilled water, the root tips were softened in 45% acetic acid for 1–2 min and squashed. For meiotic analysis, anthers were prepared in the same way without pre-treatment and using a maceration time of 45–60 min.

The fluorescence in situ hybridization (FISH) procedure was performed according to Schrader et al. (2000) with minor modifications. DIG-labeled pURsN was hybridized overnight at 37 °C and detected with 6 ng/µl anti-DIG fluorescein isothiocyanate (Roche Diagnostics). Chromosomes were counterstained with 1.0 ng/µl 4′,6-diamidino-2-phenylindole (Molecular Probes) for 5 min at 23 °C. Photographs were taken using a computer-assisted cooled charge-coupled device camera (Visitron Systems). Pseudo-coloration and merging of images were done with the camera's software or with Adobe Photoshop ver. 5.0.

Test for nematode resistance

One week after germination on wet paper in a Petri dish, single plantlets were planted in plastic folding boxes (5×6×8 cm) filled with soil mixed with 40% ceramic granulate. Ninety boxes were arranged in a vessel with nine rows of ten individual plants. In total, the test vessel held three rows containing 30 cross plants, together with three rows of rape and three rows of radish as controls. One week later, 2 ml larval suspension (1,000 larvae/ml) were injected into the soil. The infected plant material was cultivated in a growth chamber with a cycle of 16-h light/8-h dark and 22 °C/18 °C. The soil temperature was around 19 °C. The number of days from infection to observation was calculated using the formula of Curi and Zmoray (1966):

$$y = 465 \times \frac{1}{x-k},$$

where y is the number of days for a cycle of development, x is the mean soil temperature and k is the temperature of development termination in *H. schachtii* (8 °C). Thus, the plant reaction to nematodes was determined 42 days after infection. Loose soil was carefully separated from the roots. The roots were then washed nearly free from soil, using tap water. The cysts in water were counted and added to the number of cysts counted at the roots, using a stereo microscope.

Detection of glucosinolates

For root samples, 200 mg dry matter and for seed samples 1–10 mg (single grains) were used. Isolation of the desulfated compounds was done following the instructions of Thies (1979). The samples were heated for 3 min at 75 °C (sample concentrator). Next, 3,000 µl extracting agent (methanol/H_2O, 70/30) and 200 µl glucotropaeolin as internal standard were added and kept for 4 min at 75 °C under stirring. Then, 3,000 µl methanol/H_2O (10/90) were

added and the temperature was held for 15 min at 75 °C (sample concentrator) for extraction of glucosinolates. After 5 min centrifugation, 750 µl extract (roots) or 3,000 µl extract (seeds) were added to a Sephadex-anion exchanger (A-25, acetate form). The anion exchanger was rinsed twice with 1 ml Na-acetate buffer (pH 4.0). Then, 120 µl sulfatase and 80 µl buffer were added. The samples stayed for 16 h at 39 °C in the incubator. The glucosinolates were washed three times with 350 µl HPLC-grade H_2O from the anion exchanger and filtered with a 0.45-µm filter. HPLC analysis was carried out under the following conditions: detector Agilent DAD (series 1100), high-pressure gradient (binary) start [H_2O/CH_3CN (99/1), 17.5 min with H_2O/CH_3CN (75/25), 20 min with H_2O/CH_3CN (75/25), 25 min with H_2O/CH_3CN (99/1), 30 min with H_2O/CH_3CN (99/1)], flow 0.55 ml/min, time of analysis 30 min, column thermostat 30 °C, column ZORBAX Eclipse XDB-C18 (3.0×150 mm, 3.5 µm with low-volume column-inlet filter), wavelength of detection 229 nm.

Results

Both radish parents involved in the initial intergeneric hybridizations (Fig. 1) were known to be BCN-resistant. A preliminary study had shown segregation for nematode resistance in progeny 14-98, together with the occurrence of rape-like phenotypes. All progeny plants were male sterile. From its breeding history, progeny 14-98 could be expected to contain radish chromosomes added to a genetic background near to rape with the cytoplasm from radish.

Raphanus chromosome-specific RAPD markers

The RAPD profiles for 12 genotypes from the pedigree of progeny 14-98, representing the genome formulae RRRR, CCCC, RRCC, AA, RRAA, RRAC and AACC, were compared to search for DNA fragments usable as markers with genome R-specificity in genetic backgrounds involving the genomes A and C (Fig. 2, left panel). A total of 180 decamer primers from kits OP-A to OP-I were tested for polymorphisms discriminating genotypes with and without the genome R. Altogether, 143 primers (79.4%) produced 397 *Raphanus*-specific fragments, with a range of 1–11 fragments/primer. In Fig. 2, as an example, band OPE-14_{392} occurs in the RAPD profiles of all RRRR, RRCC, RRAA and RRAC genotypes; and it is lacking in that of genotypes without the genome R.

The *Raphanus*-specific markers were used to detect different components of genome R in progeny 14-98. A total of 25 plants, representing the morphological variation within the progeny and with different reactions to nematode infection, were analyzed. All the *Raphanus*-specific markers were found to segregate for their presence/absence in progeny 14-98 (Fig. 2, right panel). According to their distributions between the 25 plants, the markers showed nine different segregation patterns. Each marker divided unambiguously to one of the nine groups (a–i), each comprising an absolutely linked marker set, as

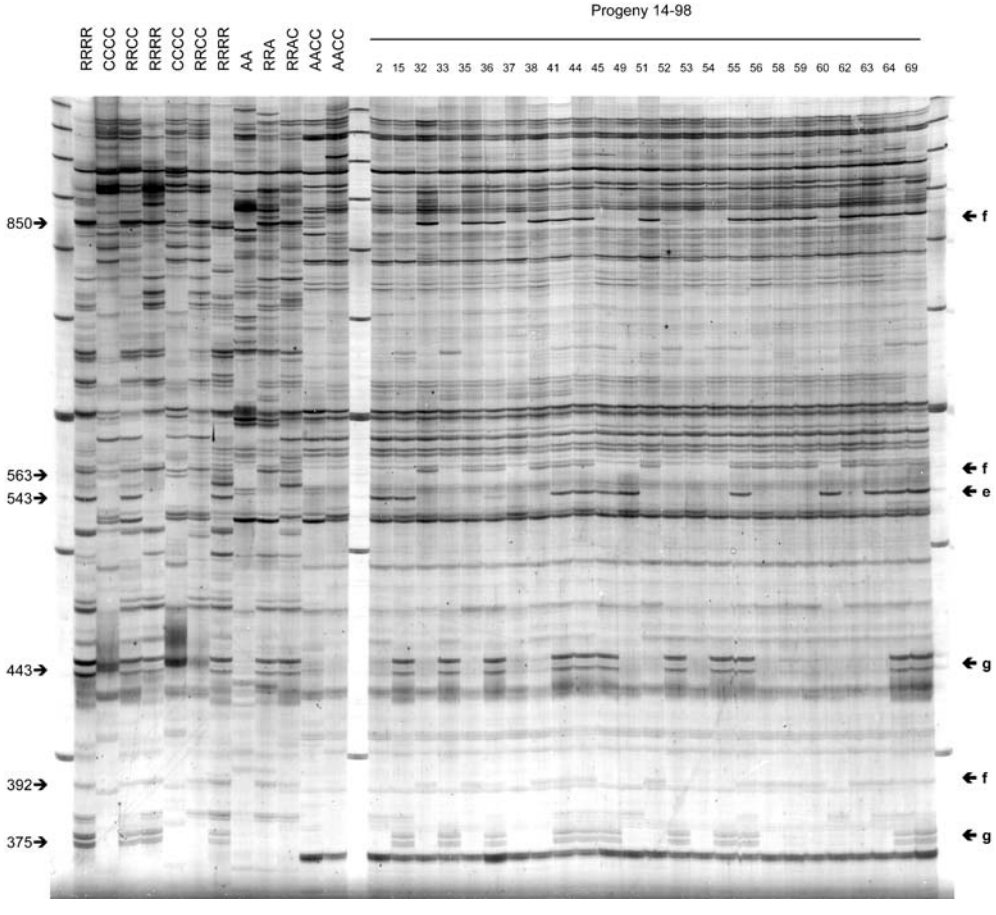

Fig. 2 A study of *Raphanus*-specific DNA fragments and markers. *Left panel* Determination of *Raphanus*-specific DNA fragments by comparison of randomly amplified polymorphic DNA (RAPD) profiles of 12 genomic types: *RRRR* (oil radish strain 2655), *CCCC* (fodder kale strain nFMK), *RRCC* (their hybrid), *RRRR* (oil radish strain 2655), *CCCC* (fodder kale strain PC 81), *RRCC* (their hybrid), *RRRR* (fodder radish 101/77), *AA* (Chinese cabbage 'Nagaoka'), *RRA* (their hybrid), *RRAC*, *AACC* (rape 'Madora') and *AACC* (rape 'Express'). *Right panel* Analysis of *Raphanus*-specific markers for 25 plants of progeny 14-98. Primer OPE-14 gives six specific fragments in three marker groups characterized by their independent plant distributions (group names *at right*, sizes *at left*): one fragment for *group e* at 543 bp, three fragments for *group f* at 392, 563 and 850 bp and two fragments for *group g* at 375 bp and 443 bp

Table 1 Distribution of the 397 *Raphanus*-specific DNA markers obtained from randomly amplified polymorphic DNA analyses with 143 different primers to nine groups (a–i), according to their single-plant segregation pattern, as observed in progeny 14-98

Marker group	Number of markers
a	51
b	51
c	53
d	55
e	53
f	42
g	33
h	34
i	25
Total	397

could be expected from segregation of the nine complete chromosomes of radish. The highest number of markers (55 markers, 13.9%) was classified to group d. The lowest number of markers (25 markers, 6.3%) was found for group i (Table 1).

The classification of the 25 plants of progeny 14-98 analyzed for the nine molecular groups is given in Table 2.

Specificity of probe pURsN for *Raphanus* chromosomes in FISH

Hirai et al. (1995) showed that pURsN hybridizes on all 18 chromosomes of *R. sativus*. If pURsN is specific for the R genome and does not hybridize on chromosomes of the A and C genomes, it would be a useful tool for the cytogenetic characterization of *Raphanobrassica* progenies. The Southern hybridization pattern of the probe was analyzed for genotypes having different genomic constitutions: AA, CC, AACC and RR (Fig. 3). The ladder-shaped profile typical for repetitive DNA appeared for the R genome, but not for the A or C genomes. Therefore, the probe was studied further by FISH.

In agreement with Hirai et al. (1995), the metaphase chromosomes of *Raphanus sativus* showed up to 18 FISH signals near the centromere (Fig. 4A). In the *Raphanobrassica* hybrid RRAA, the 18 R chromosomes showed signals, whereas the 20 chromosomes of the A genomes did not give any signal (Fig. 4B). In the *Raphanobrassica* hybrid RRCC also, not more than 18 R chromosomes hybridized with the probe, the 18 chromosomes of the C genomes remaining free of signals (Fig. 4C). Thus, the probe pURsN could be used to recognize added *Raphanus* chromosomes in progeny 14-98 on a *Brassica* background with both A and C genomes.

Comparison of molecular and cytological analyses of progeny 14-98

In each of the 25 plants of progeny 14-98, *Raphanus*-specific DNA fragments were identified belonging to groups a–i, which are defined according to their single

Table 2 Molecular and cytological identification of *Raphanus* genomic components for 25 individuals of progeny 14-98

Plant number	*Raphanus*-specific molecular marker groups										Number of *Raphanus*-specific FISH signals	Number of seeds after backcrossing	
	Number	Type										Per siliqua	Total
		a	b	c	d	e	f	g	h	i			
2	4	−	+	+	+	+	−	−	−	−	4	2.0	1,005
15	6	+	+	+	−	+	−	+	−	+	6	0.8	340
32	2	−	−	+	−	−	+	−	−	−	2	3.8	375
33	5	−	+	+	+	−	−	+	+	−	5	0.6	43
35	5	−	+	+	−	−	+	−	+	+	3	2.9	460
36	2	−	−	+	−	−	+	−	−	−	2	5.3	495
37	2	−	+	−	−	−	−	−	+	−	2	1.4	160
38	7	+	+	+	+	−	+	−	+	+	7	1.0	65
41	8	+	−	+	+	+	+	+	+	+	8	0.7	88
44	8	+	+	+	+	+	+	+	−	+	8	0.6	29
45	4	−	+	+	−	+	−	+	−	−	4	1.7	59
49	3	−	−	+	−	+	−	−	−	+	3	1.8	127
51	6	+	+	+	+	+	+	−	−	−	5	0.9	45
52	3	−	+	−	+	−	−	+	−	−	4	1.6	488
53	1	−	−	−	+	−	−	−	−	−	1	3.4	381
54	5	−	+	+	+	−	−	+	−	+	6	0.8	138
55	7	−	+	+	+	+	+	+	−	+	6	1.5	300
56	3	−	−	+	+	−	+	−	−	−	3	3.2	282
58	5	+	−	+	+	−	+	−	−	+	4	0	0
59	4	−	+	+	+	−	+	−	−	−	4	0.9	45
60	4	−	−	+	+	+	−	−	−	+	3	1.9	80
62	3	−	−	+	+	−	+	−	−	−	3	1.2	7
63	5	−	+	+	+	+	+	−	−	−	5	0.6	37
64	9	+	+	+	+	+	+	+	+	+	8	0.1	2
69	5	−	+	−	+	+	+	+	−	−	5	0.9	47

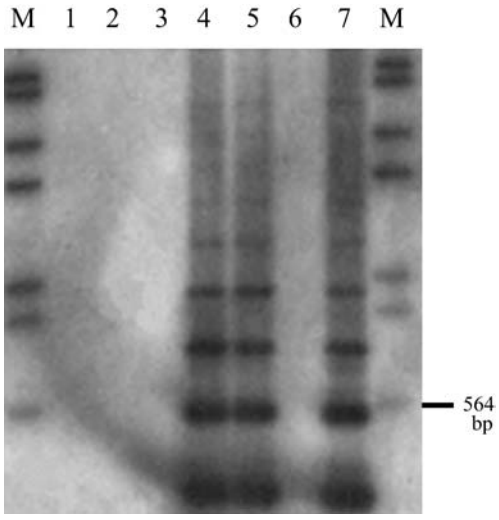

Fig. 3 Southern hybridization using probe pURsN of Hirai et al. (1995). *Lanes 1–7 Brassica pekinensis* (AA), *B. pekinensis* × *B. oleracea* (AACC), *B. oleracea* (CC), *R. sativus* var. *niger* (RR), *R. sativus* var. *sativus* (RR), *B. napus* (AACC) and *R. sativus* var. *oleiferus* (RR). Note no hybridization to A and C genomes. *M* Marker (DNA molecular weight marker III digoxigenin-labeled; Roche Diagnostics)

plant segregation (Table 2). Markers of group a, for instance, occur in these seven plants: 15, 38, 41, 44, 51, 58 and 64. Plant 64 had markers of all nine groups a–i, whereas plant 53 showed only fragments of one group, d. The number of *Raphanus*-specific marker groups in the plants of progeny 14-98 and the number of FISH signals for the cytological chromosome counts using the probe pURsN are in close agreement (Table 2). The few deviations could be due to variable signal detection of the cytological analysis (especially in plants with a high number of radish chromosomes) and/or the inability of the molecular analysis to detect the presence of two homologous chromosomes, which is probable from the preceding sib cross. The conclusion drawn from the combined molecular and cytogenetic results was that progeny 14-98 contained all nine radish chromosomes. Therefore, a few selected markers for each type of marker group could be taken to analyze efficiently the distribution and transmission frequency of radish chromosomes in further crosses with rape. All 25 plants of progeny 14-98 were crossed with rape 'Madora' as pollinator. The female fertility as seed set per siliqua was negatively correlated with the number of radish chromosomes in the female plant (Table 2). Plant 64 with nine radish chromosomes and

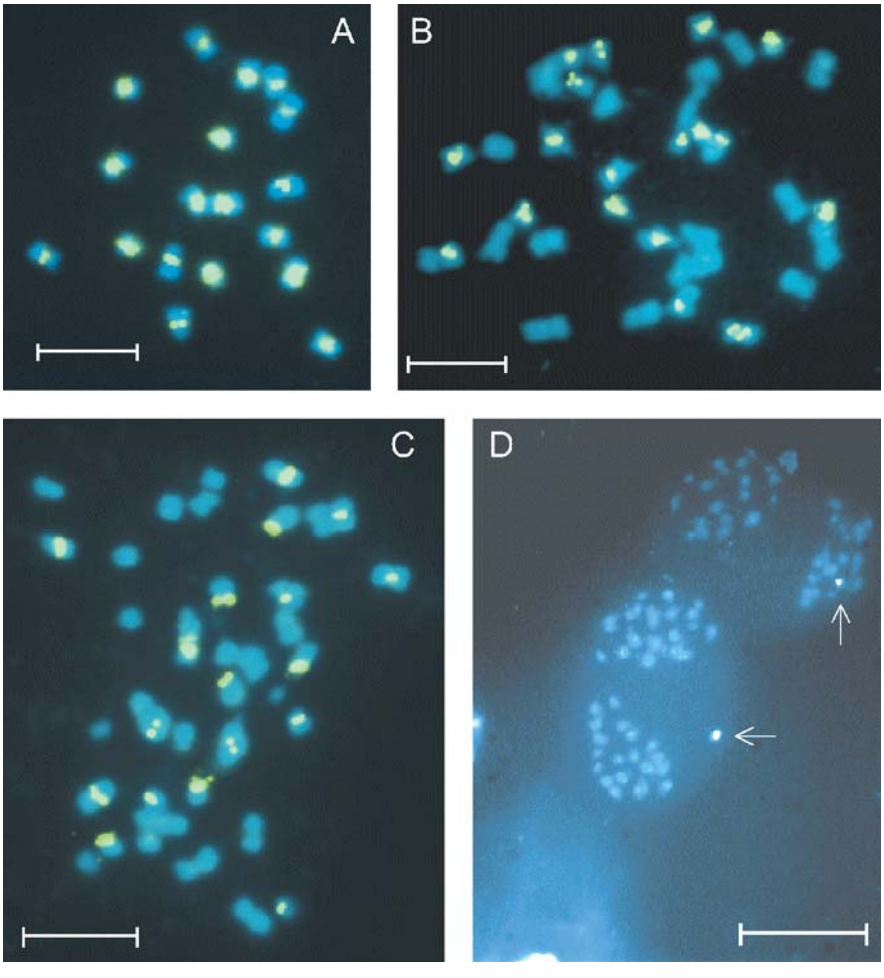

Fig. 4 Fluorescence in situ hybridization at mitotic metaphase (**A–C**) and meiotic first anaphase (**D**) chromosomes with probe pURsN (Hirai et al. 1995) for *Raphanus* chromosome identification (*yellow*/*white*). **A** *R. sativus* (RR, 2*n*=2*x*=18), **B** *Raphanobrassica* (RRAA, 2*n*=4*x*=38), **C** *Raphanobrassica* (RRCC, 2*n*=4*x*=36), **D** two PMCs of plant 53 having added monosomic radish chromosome d (*arrows*) (2*n*=4*x*=38+1). Note position of extra chromosome within and outside a daughter nucleus

Table 3 Female transmission rate of radish chromosomes in five crosses with multiple additions from progeny 14-98 and rape

Backcross of plant number	Rate of transmission for radish chromosome								
	a	b	c	d	e	f	g	h	i
15	0.96	0.39	0.64		0.56		0.16		0.36
35		0.31	0.56			0.52		0.33	0.51
45		0.39	0.14		0.43		0.18		
51	0.52	0.36	0.80	0.36	0.36	0.44			
55		0.47	0.74	0.37	0.42	0.43	0.24		0.38

plant 53 with one added chromosome had 0.1 seeds and 3.4 seeds per siliqua, respectively.

Transmission rate of radish chromosomes

The rate of female transmission of all radish chromosomes was studied using markers of the molecular defined groups (a–i) in crosses of five selected plants from progeny 14-98 with rape 'Madora' (Table 3). The calculated transmission rates are in most cases lower than the theoretical maximum value (0.5) for univalent chromosomes. Some exceptions for chromosomes a and c, which had higher values in some crosses, can be explained through their disomic state in the mother plants 15 and 51, respectively. The occurrence of disomic additions is probable for progeny 14-98, because it originated from the cross of sister plants which could have had some chromosomes in common. The monosomic female transmission rates of most chromosomes were quite uniform with values higher than 0.3, with the exception of chromosome g.

Effects of added radish chromosomes for BCN resistance

The five crosses from multiple addition plants 15 (a-b-c-e-g-i), 35 (b-c-f-h-i), 45 (b-c-e-g), 51 (a-b-c-d-e-f) and 55 (b-c-d-e-f-g-i) with rape were used for an estimation of the main chromosomal effects for resistance after infection with second-stage larvae. The segregation for plants with and without an addition chromosome allowed 28 single comparisons, involving all nine radish chromosomes. For chromosome a, there were two comparisons; and for chromosomes b, c, d, e, f, g, h and i, there were 5, 5, 2, 4, 3, 3, 1 and 3 comparisons, respectively. Because of the imbalance within a comparison for the residual chromosomes, no unbiased effects of single chromosomes could be estimated. However, these comparisons could show which of the radish chromosomes carries the major genetic factor(s) for BCN resistance. In each comparison, the mean number of cysts was separately calculated for the cross plants with and without the added chromosome. This was also done for the corresponding rape and radish control plants to scale for the different infection levels in each experiment (Table 4). The mean cyst number for rape 'Madora' varied strongly between 106.8 and 212.6 cysts. The resistance of radish '2655' did not suppress cyst development completely, but gave means between 1.9 and 17.0 cysts. From the 28 chromosomal comparisons carried out, those for chromosome d showed the maximal difference (110.9 and 133.4 cysts). The summarized comparisons for each radish chromosome gave a high significance (P=0.0001) for chromosome d. The 89 plants without chromosome d had a mean of 146.8 cysts, which is comparable with susceptible rape (151.9), whereas the 52 plants with chromosome d had a mean of 17.5 cysts, which is comparable with resistant radish (14.0). Therefore, radish chromosome d was determined to carry the gene(s) controlling BCN resistance in oil radish.

A minor significant difference for chromosome h resulting from a single comparison was ignored.

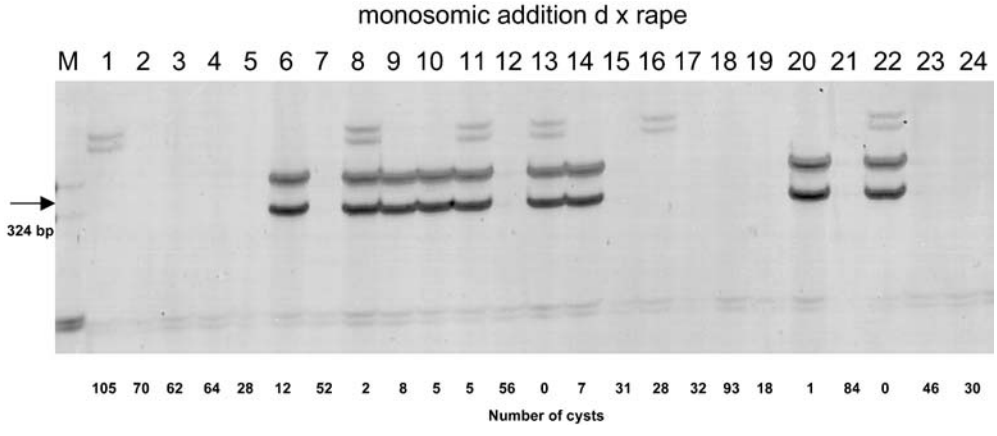

Fig. 5 Single plant cosegregation of the OPA10-324 RAPD fragment specific for radish chromosome d and the plant cyst number after infection with *Heterodera schachtii* in the cross of plant 53 (monosomic addition d) with rape 'Madora'

Table 4 Evaluation of the nine radish chromosomes for effects on nematode resistance by comparing mean cyst numbers on plants from crosses of five multiple radish–rape chromosome additions from progeny 14-98 with rape 'Madora' after classification for presence (+) and absence (–) of radish chromosomes (number of plants in parentheses). *n.s.* Not significant, * significant, *** highly significant

Back-cross of plant number	Mean cyst number																Rape 'Madora'	Radish strain 2655
	Plants with radish chromosome (a–i)																	
	a		b		c		d		e		f		g		h		i			
	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+	–	+		
15	54.0 (2)	110.7 (52)	124.6 (31)	113.2 (20)	112.5 (20)	114.7 (36)			123.6 (25)	104.2 (30)			111.8 (46)	119.2 (9)			116.3 (35)	107.3 (20)	162.4 (60)	7.1 (59)
35			170.9 (38)	98.7 (17)	131.2 (25)	163.0 (30)					124.3 (23)	167.5 (25)			167.5 (37)	109.8 (18)	153.5 (27)	143.9 (28)	212.6 (57)	9.7 (60)
45			88.9 (17)	92.1 (11)	87.4 (24)	106.8 (4)			95.3 (16)	83.3 (12)			91.1 (23)	85.8 (5)					106.8 (29)	4.3 (30)
51	119.0 (12)	44.3 (13)	70.2 (16)	97.9 (9)	94.2 (5)	76.7 (20)	120.1 (16)	9.2 (9)	113.4 (16)	21.0 (9)	95.6 (14)	60.5 (11)							119.3 (30)	1.9 (30)
55			110.8 (62)	90.6 (54)	89.4 (30)	105.9 (86)	152.6 (73)	19.2 (43)	94.2 (67)	115.4 (49)	99.5 (65)	101.8 (50)	104.7 (89)	95.9 (28)			104.4 (72)	101.1 (44)	160.0 (120)	17.0 (120)
Mean	109.7	97.4	121.1	96.7	103.6	114.1	146.8	17.5	102.8	99.7	104.6	115.6	104.8	99.7	167.5	109.8	117.4	115.5	161.3	10.8
Difference	–12.3 n.s.		–24.4 n.s.		10.5 n.s.		–129.3***		–3.1 n.s.		11.0 n.s.		–5.1 n.s.		–57.7*		–1.9 n.s.			

Table 5 Nematode resistance effect of radish chromosome d in the cross of monosomic plant 53 with rape 'Madora'

	Number of plants	Cyst number	
		Mean	Range
Controls			
Rape 'Madora' (susceptible)	60	131.5	11–365
Oil radish strain 2655 (resistant)	59	3.9	0–12
Cross plants			
Euploid (2*n*=38)	41	72.8	18–167
Monosomic addition d (2*n*=38+1)	16	4.6	0–16

Measurement of the resistance effect of chromosome d of radish

To verify the BCN resistance effect of chromosome d, which was derived above from unbalanced comparisons because of differences in number and type of residual added radish chromosomes, the effect of chromosome d was estimated in the rape cross of plant 53, monosomic only for chromosome d. A total of 57 plants were examined with the chromosome d-specific RAPD marker OPA10-324 and studied for resistance behavior after nematode infection (Fig. 5). In this progeny, 16 plants received the chromosome d, whereas it was absent in 41 plants. There was a clear single-plant cosegregation of chromosome d transmission and BCN resistance. Monosomic plants (mean of 4.6 cysts/plant) showed the resistance level of the radish control (3.9 cysts/plant; Table 5). Thus, radish chromosome d was confirmed as the conveyor of complete resistance from radish to rape.

For the evaluation of resistance expression in rape material on a broader basis containing sterile and fertile cytoplasms, the effect of chromosome d was studied in crosses between monosomic d additions as pollinators and seven breeding lines (Table 6). The rape breeding lines, V1–V5, were pollinated with pollen from plant 55/61 which had both d and f chromosomes. The five progenies were analyzed with specific markers for chromosomes d and f, respectively. In contrast to the female transmission (Table 3), chromosome f was not transferred by pollen to any of the 146 plants from crosses with V1–V5 (Table 6). Therefore, chromosome f was not considered for further analysis. The rape breeding lines V6 and V7 were crossed with pollen from plant 53/46, monosomic for radish chromosome d.

In total, the seven progenies segregated into 80 plants with and 102 plants without chromosome d (Table 6). The male transmission rate (0.44) was slightly higher than the female transmission rate (Table 3). In all progenies, the presence of chromosome d resulted in a mean cyst number per plant consistently lower than that in plants missing chromosome d. The plants without chromosome d had a similar susceptibility as the control rape 'Madora'. The plants with the radish chromosome d showed the resistance level of oil radish 2655. This implies that radish chromosome d carries the major gene(s) for the BCN resistance of radish. The gene(s) is dominant against the

Table 6 Effect of radish chromosome d on beet cyst nematode (BCN) resistance after crosses with rape breeding lines (number of plants in parentheses)

Breeding line	Pollinator[1]	Mean number of cysts			
		Radish chromosome d		Rape 'Madora'	Oil radish strain 2655
		−	+		
V1	55/61	116.9 (15)	17.0 (15)	124.9 (10)	15.4 (8)
V2	55/61	92.2 (18)	18.3 (11)	84.1 (10)	12.0 (8)
V3	55/61	66.8 (13)	16.3 (17)	62.7 (18)	17.6 (9)
V4	55/61	50.9 (13)	11.9 (18)	55.3 (13)	10.8 (5)
V5	55/61	68.8 (23)	15.0 (3)	66.6 (9)	10.9 (10)
V6	53/46	101.9 (11)	12.7 (7)	85.7 (16)	17.8 (10)
V7	53/46	110.4 (9)	7.2 (5)	91.5 (12)	20.0 (5)
Total		73.5 (102)	15.5 (80)	79.6 (88)	15.1 (55)

Table 7 Comparison of contents of total and three radish-specific glucosinolates (μmol/g dry matter) for rape plants with and without the BCN resistance transmitting radish chromosome d (number of plants in parentheses)

Plant part	Mean of backcrosses[a] with lines	Glucosinolates	Plants having radish chromosome d		Rape 'Madora'	Oil radish strain 2655
			−	+		
Roots	V1, V2	Total	10.77 (13)	10.11 (18)	8.64 (5)	27.95 (5)
		Glucoraphasatin[b]	0	0	0	18.49
		Glucoraphenin[c]	0	0	0	0.36
		Glucoerysolin[d]	0	0	0	0.32
Seeds	V1–V5, V7	Total	16.77 (57)	31.55 (57)	10.67 (19)	138.09 (19)
		Glucoraphasatin	0	0	0	1.25
		Glucoraphenin	0	0	0	92.97
		Glucoerysolin	0	0	0	34.28

[a] See Table 6 for pollinators
[b] 4-Methylthiobutenylglucosinolate
[c] 4-Methylsulphinylbutenylglucosinolate
[d] 4-Methylsulphonylbutylglucosinolate

rapeseed background, conferring the whole resistance expression with one specimen of radish chromosome d.

Glucosinolate analysis

Aliphatic glucosinolates in oil radish were suspected to have a nematocidal activity (Lazzeri et al. 1993; Sumbayak 1997). To test the hypothesis of a relationship between BCN resistance and the glucosinolates of radish, their profiles in roots (the sites for the nutrition of nematodes as they penetrate) were studied immediately after the resistance experiments. For the aim above, only the aliphatic components glucoraphasatin, glucoraphenin and glucoerysolin (produced by radish and not by rape) and the total root glucosinolates content (which differs markedly between radish and rape) were taken into further consideration. Seed glucosinolate profiles were also investigated. Glucosinolate analysis was carried out for plants with and without radish chromosome d in the rape crosses with V1 and V2 from Table 6 and have been compared with susceptible rape recipient and resistant radish donor of the chromosome d (Table 7). The cross plants with chromosome d did not show the high glucosinolate level of radish and did not differ from the levels in both BCN-susceptible plants without the additional chromosome and rape. Furthermore, the presence of radish chromosome d did not change the glucosinolate spectrum in the cross plants towards the radish-specific glucosinolates, especially glucoraphasatin, which predominated in radish roots. Thus, it was concluded that glucosinolates do not effect BCN resistance either by a high total root glucosinolate content nor by the influence of the main specific glucosinolates of radish.

The seeds of addition and non-addition plants, which were analyzed in six rape crosses (V1, V2, V3, V4, V5, V7) contained also none of the radish-specific glucosinolates (Table 7). There was a significant two-fold increase in total seed glucosinolate content caused by the presence of chromosome d, but the glucosinolate content of chromosome d additions did not reach the much higher level found in oil radish strain 2655. Thus, beside the locus for BCN resistance, a locus enhancing the total glucosinolate content in seeds and leaves (data not shown), but not that in roots, is localized on radish chromosome d.

Discussion

In spite of the common use of BCN-resistant oil radish varieties in the agricultural practice of European countries and intensive breeding activities, little knowledge is available about the genetic basis of the radish resistance to *H. schachtii*. With the aim of introducing BCN resistance into rape, we had to elucidate the number and

distribution of the loci of resistance-controlling factors in the radish donor genome. The study of alien addition lines of rape was shown to be an efficient method of identifying chromosomes carrying different genes controlling agronomic traits (Zhu et al. 1993; Chèvre et al. 1996, 1997; Snowdon et al. 2000).

Integrity of *Raphanus* genome

We obtained results about the genetic determinants of BCN resistance in the whole *Raphanus* genome by screening the effects of chromosomes added to *Brassica* genomes A and C. The high number of sexual cycles in the crossing scheme (Fig. 1), from initial wide crosses to the final step before molecular and cytological identification in progeny 14-98, raises the question about the ultimate integrity of the radish chromosomes in the analyzed chromosome addition lines. Progeny 14-98 originated from separate crosses of donors for genomes A and C, respectively, with tetraploid radish genotypes, whereas other researchers carried out sexual or somatic hybridizations directly between rape and radish to combine the three genomes at once. Because of the bivalence for the complete *Raphanus* genome in the two primary hybrids RRA and RRCC, the radish homologues paired and segregated normally. This situation held through the whole length of the hybridization program to the two rounds of crossing of *Raphanobrassica* Ba-C132 with rape (Fig. 1). There were very few chances for meiotic recombination of a radish chromosome and its homologues in genomes A and C during the hybridization program. Together with the greater distance between the R genome and the A and C genomes, respectively (Mizushima 1980), this suggested a low probability of an added chromosome to have participated in an intergenomic exchange. Therefore it could be presumed that, in progeny 14-98, the added radish chromosomes were maintained mostly unaltered in the background of the *Brassica* genomes.

Identification of *Raphanus* chromosomes

The chromosomal assignment of the BCN resistance gene(s) required both the identification of the radish chromosomes in the *Brassica* genome background and the discrimination of the nonhomologous radish chromosomes. The first part was done cytogenetically, using the radish-specific probe pURsN, and molecularly, by the identification of DNA fragments with specificity for genome R, using comparative RAPD analyses. It was more difficult to differentiate between homologous and nonhomologous radish chromosomes in the basic population of progeny 14-98. Both types had to be taken into account because of its origin from sibling and because there was no complete agreement between the cytological and molecular results in the detection of radish chromosomes. Their small size prevented unambiguous karyotypical differentiation within the chromosome set of radish. The classification of molecular markers in the basic population of progeny 14-98 into nine fragment groups of unique distribution to individual plants could also not exclude a priori the possibility of non-discrimination between nonhomologous and homologous radish chromosomes. Homologues might also have been different in their DNA sequence and in their distribution pattern to individual plants. This problem was solved by analyzing the cross of multiple addition plants 15, 35, 45, 51 and 55 with rape. Here, possible pairs of homologous radish chromosomes must have separated and distributed to different daughter plants. Therefore, the occurrence of two marker groups in the same daughter plant could be used as a criterion to show that they represented nonhomologous chromosomes. From the 28 possible pairs, 24 pairs could be tested. All of them showed common transmission, indicating nonhomology between the chromosomes a, b, c, d, e, f, g and i and nonhomology of chromosome h to b, c, f and i (data not shown). The four pairs of chromosome h with chromosomes a, d, e and g, respectively, could not been tested in this way. The nonhomology of chromosome h to the other four chromosomes was derived from a cytogenetic analysis. The karyotype of *R. sativus* contains three chromosome pairs with rDNA loci (Schrader et al. 2000). Chromosome h is the only chromosome in radish on which FISH probes of both 5S rDNA and 25S rDNA hybridize. Chromosomes a, b, e, f, g, i had no tandemly repeated sequences of ribosomal RNA-specific genes, whereas chromosomes c and d hybridized with one probe of 5S rDNA and 25S rDNA, respectively (unpublished data).

Genetics of BCN resistance

The results of this study showed clearly, despite a high level of environmental variability in the trait, that chromosome d of oil radish carries the genetic factor(s) controlling BCN resistance and all other chromosomes have no striking effect on this trait. The experiments carried out suggested that the radish resistance can be transferred completely by the addition of a single alien chromosome to rape. In a similar way, the analysis of monosomic additions of cultivated beets carrying different individual chromosomes of *Beta patellaris* revealed that gene(s) conferring full resistance to BCN were located on one chromosome, whereas the other tested chromosomes were not involved in the expression of resistance (Mesbah et al. 1997). The resistance mediated by radish chromosome d acted in a dominant manner in the genetic background of susceptible rape. The chromosomal dissolution of the experiments did not allow an estimation of gene number. From genetic analysis of intraspecific crosses between susceptible and resistant radish lines, a monogenic dominant inheritance of BCN resistance of radish was suggested (Baukloh 1976). In oil radish, susceptible varieties and those with different BCN resistance levels were bred. Therefore considerable allelic

variation at the responsible genetic locus must be taken into account. Resistance to sedentary endoparasitic cysts and root-knot nematodes in the family Heteroderidae, e.g. barley, tomato, wheat, potato and soybean, was found frequently to be controlled by single dominant or semidominant genes which could be mapped to linkage groups (for a review, see Williamson and Hussey 1996; Cook and Rivoal 1998). Plant resistance reactions to attacks of this nematode group occurred mostly with the initiation of nematode-feeding sites. The expression of many different nematode-responsive plant genes at nematode-feeding sites has been detected (for a review, see Gheysen and Fenoll 2002). Therefore, resistance in different species to the same nematode, e.g. *H. schachtii* in wild beets and oil radish, could have evolved independently under the control of diverse plant genes.

Use of the radish chromosome addition d

There are two ways from the stage of monosomic addition to genetically stabilize the resistance trait, which is needed for use in rapeseed breeding. The most convenient way is the production of disomic additions of radish chromosome d to rapeseed. Disomic additions of alien chromosomes were obtained in allotetraploid rape by Struss et al. (1991). A stable transmission should be obtained if the additional chromosome pair goes through meiosis undisturbed and in synchrony with the rape chromosomes. In rape, medium to high stability in alien chromosome transmission was observed by Chèvre et al. (1991). The second, more difficult way is the stable integration of the resistance gene by homologous recombination into one of the genomes of *Brassica napus*, as was done with the radish *Rfo* restorer gene in rapeseed (Delourme et al. 1998). The presumably monogenic dominant control of the radish BCN resistance should facilitate its stable integration. A QTL study aimed to localize BCN resistance on a radish linkage group and to develop closely linked markers for recombinant selection is underway.

H. schachtii is also a parasite of many other cruciferous plants in the cabbage group (e.g. cabbage, cauliflower, broccoli, kale, brussel sprouts) and Chinese cabbage, with great losses and no known resistance (Riggs and Schuster 1998). The radish resistance could be used for other related crops, where crosses with the radish chromosome addition line d as bridge form should be useful. *H. schachtii* has many of the same hosts as *H. cruciferae*, which may cause severe damage to cabbage and related crops. Because no resistance is available, it would be interesting to prove whether radish resistance can evade losses from this related cyst nematode. Furthermore, the methodology of radish chromosome testing could be used to search for resistance to root-knot nematodes, which were found in oil radish to be effective against *Meloidogyne hapla* and *M. incognita* (Bünte et al. 1997).

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