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ORIGINAL PAPER

Molecular mapping in oil radish (*Raphanus sativus* L.) and QTL analysis of resistance against beet cyst nematode (*Heterodera schachtii*)

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Abstract The beet cyst nematode (*Heterodera schachtii* Schmidt) can be controlled biologically in highly infected soils of sugar beet rotations using resistant varieties of oil radish (*Raphanus sativus* L. ssp. *oleiferus* DC.) as a green crop. Resistant plants stimulate infective juveniles to invade roots, but prevent them after their penetration to complete the life cycle. The resistance trait has been transferred successfully to susceptible rapeseed by the addition of a complete radish chromosome. The aim of the study was to construct a genetic map for radish and to develop resistance-associated markers. The map with 545 RAPD, dpRAPD, AFLP and SSR markers had a length of 1,517 cM, a mean distance of 2.8 cM and consisted of nine linkage groups having sizes between 120 and 232 cM. Chromosome-specific markers for the resistance-bearing chromosome *d* and the other eight radish chromosomes, developed previously from a series of rapeseed-radish addition lines, were enclosed as anchor markers. Each of the extra chromosomes in the addition lines could be unambiguously assigned to one of the radish linkage groups. The QTL analysis of nematode resistance was realized in the intraspecific F_2 mapping population derived from a cross between varieties 'Pegletta' (nematode resistant) x 'Siletta Nova' (susceptible). A dominant major QTL $Hs1^{Rph}$ explaining 46.4% of the phenotypic variability was detected in a proximal position of chromosome *d*. Radish chromosome-specific anchor markers with known map positions were made available for future recombination experiments to incorporate segments carrying desired genes as $Hs1^{Rph}$ from radish into rapeseed by means of chromosome addition lines.

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Introduction

The sedentary beet cyst nematode (BCN), *Heterodera schachtii* Schmidt, is a widespread and serious pest in beet cultivating areas of the world. The host range of this parasite includes arable crops belonging to plant families *Brassicaceae* and *Chenopodiaceae*. To reduce the concentration of this pathogen in highly infected soils, BCN-resistant varieties of oil radish (*Raphanus sativus* (L.) var. *oleiferus* (Reihb.) Metzg.) are used extensively as catch crop (Smith et al. 2004). It was assumed that a major dominant gene controls the resistance reaction against BCN in radish (Baukloh 1976). Resistant plants stimulate infective juveniles to invade roots, but prevent them after their penetration to complete the life cycle. BCN- resistance results from degradation of the nematode feeding sites (Wyss et al. 1984). Associated juveniles suffer from a lack of nutrients and fail to develop to the adult stage similar to the mechanism in resistant sugar beet (Holtmann et al. 2000).

Despite the increased agronomical importance, genetics of BCN-resistance in radish was not studied extensively compared to that in sugar beet or to resistance against other cyst nematodes as *Heterodera avenae* in cereals, *Heterodera glycines* in soybean and *Globodera rostochiensis* in potato (reviewed by Cook and Rivoal 1998). Molecular aspects of nematode-plant interaction related to syncytium formation are studied now intensively (Gheysen and Fenoll 2002; Lilley et al. 2005).

Breeding of oil radish varieties with BCN-resistance is a process comprising several steps of screening which requires great effort. Genetic markers linked to a resistance gene would allow to decrease the extent and to improve the quality of selection, considerably. Thus, mapping in a population segregating for BCN-resistance/susceptibility should be useful to develop markers for marker-assisted selection.

A second occasion for genetic analysis and mapping of BCN-resistance results from introgression experiments between radish and rapeseed. In the economically more important oilseed rape (*Brassica napus*) no resistance to the BCN is available (Harrewijn 1987; Fatemy and Abootorabi 2002). Rapeseed is more or less tolerant to BCN, allowing the multiplication of the pathogen and must therefore be omitted hitherto from crop rotations with sugar beet in highly infected areas. The transfer of the BCN-resistance from radish to rapeseed could convert it to a catch crop like the donor species radish and would allow the uptake of oilseed rape in crop rotation systems with sugar beet. Interspecific hybrids of rapeseed and BCN-resistant radish were produced and showed resistance expression (Lelivelt and Krens 1992; Lelivelt and Hoogendoorn 1993; Thierfelder 1994).

Peterka et al. (2004) characterized multiple rape-radish chromosome additions developed from crosses between interspecific hybrids of the R genome with A and C genome, respectively. By testing the effects of radish chromosome additions in rapeseed genetic background the hypothesis for presence of a major gene for BCN-resistance was supported. From the nine chromosomes of the radish complement, one copy of chromosome *d* conferred the full resistance expression of radish to the former highly susceptible rapeseed variety 'Madora'. After backcrosses of the multiple radish chromosome additions to rapeseed, a complete series of nine individual monosomic additions of radish chromosomes (*a* to *i*) was obtained and, by subsequent selfing of monosomics, a complete set of disomic rapeseed-radish addition lines had been selected (Budahn et al. 2008). Each of the alien chromosomes was characterized by a series of chromosome-specific molecular markers. Disomic rapeseed-radish addition *d* showed high cytogenetic stability and presents a basic material for recombination experiments to introduce the BCN-resistance gene into the rapeseed genome. To recognize and manipulate radish segments carrying the BCN-resistance gene or other genes of interest, the position of chromosome-specific markers mapped together with markers and genes of unknown chromosomal location in linkage groups is of great value.

In the present study, an intraspecific radish linkage map has been constructed using RAPD and AFLP markers and anchoring the linkage groups by markers specific for the addition chromosomes. The map will be used for QTL-localization of the BCN-resistance.

Material and methods

Plant material

The *Raphanus* mapping population was developed from an intraspecific oil radish cross, with parents unrelated to the plant material of radish-rape chromosome additions. The plant A22/2 from BCN-resistant cultivar 'Pegletta' and the plant A317/1 of susceptible cultivar 'Siletta Nova' were selfed to produce the parental lines (P1, P2) and were crossed to produce the F_1 generation, respectively. One of the resulting F_1 plants showing clear resistant reaction was self-pollinated to produce the F_2 mapping population. About 245 F_2 plants were used for linkage analysis and resistance test. The parental lines and F_1 plants were also involved in the resistance test. An inbred line of winter rape variety 'Madora', chromosome receptor of rape-radish additions, was used as a susceptible control (Mousa 2004).

DNA isolation

Total genomic DNA was isolated from 50 mg of the first completely expanded leaves according to the method of Dellaporta et al. (1983). For tissue disruption mixer mill MM300 (Retsch, Haan, Germany) was used. The DNA concentration was determined spectrophotometrically and adjusted to 8 ng/µl for RAPD, dpRAPD and SRAP techniques and 125 ng/µl for AFLP analysis.

RAPD and dpRAPD analysis

Arbitrary decamer primer kits A-P (Operon Technologies, Huntsville, AL) were used for amplification of the DNA in a GeneAmp PCR System 9700 Thermocycler (Applied Biosystems, Foster City, CA) following the protocol of Williams et al. (1990). For amplification InviTaq DNA polymerase was used in combination with NH_4 reaction buffer (Invitek, Berlin, Germany). 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. Five micro liter was loaded on a 4% denaturating polyacrylamide gel prepared in 1x TBE. Electrophoresis was performed using a 38 × 50 cm Sequi-

Gen GT (Bio-Rad Laboratories, Hercules, CA) running at 50°C and 100 W for 3.5 h. After electrophoresis the gel was silver stained according to Bassam and Caetano-Anolles (1993). Air dried gels were documented by scanning. Only strong and clear bands were used for analysis. Size determination of individual bands was made by comparison to 100 bp ladder (Invitrogen, Carlsbad, CA).

For dpRAPD analysis (Budahn et al. 2008) individual primers from the Operon kits A–E and K–P were combined with the primers OPD11 and OPG19, respectively, and amplified as described for RAPD analysis. Because smaller fragments are expected for dpRAPD amplification, polyacrylamide gel electrophoresis was run for only 2 h.

AFLP and SSR markers

The AFLP marker analysis (Vos et al. 1995) was performed using AFLP analysis system I (Invitrogen) following suppliers instructions. For amplification steps InviTaq DNA polymerase was used. Preamplification products were diluted only 1:10 instead of 1:50. Amplification products of the selective amplification step were separated and silver stained as described for RAPD markers.

BRMS primer pairs developed for *Brassica rapa* (Suwabe et al. 2002) were amplified according to Tsuro et al. (2005) but were also separated with PAAGE and visualized by silver staining. The core sequence of BRMS 196 is $(GTG)_5$. BRMS 303 is a complex microsatellite with core sequence $(TC)_4(CTG)_5(TC)_3$.

BCN-resistance test

The nematode resistance test was performed as described by Peterka et al. (2004). One-week old seedlings of P1, P2, F_1, F_2 and rapeseed cultivar 'Madora' were inoculated with 2 ml suspension of 1,000 larvae/ml. The soil temperature was held constantly at 18°C. Cyst counting was done 47 days after inoculation.

Construction of a linkage map

The marker data were scored according to the definition of JoinMap 4 (Van Ooijen 2006) in dominant or codominant form. Using the regression mapping with Haldane's function, linkage groups were constructed with LOD threshold = 1, recombination frequency threshold = 0.4 and jump threshold = 5 with a third round of mapping.

QTL mapping

QTL analysis was done with the intervall mapping procedure of MapQTL 5 (Van Ooijen 2004) using the second round maps constructed by JoinMap 4.

Results

Marker screening

For AFLP analysis 64 EcoRI/MseI primer combinations (PCs) have been tested on parental and F_1 plants for polymorphism. About 39 PCs were selected for linkage analysis. The same has been done for the 320 decamer primers of the Operon primer sets A–P of which 35 were selected for further analysis. Additionally, 14 combinations of primer OPD11 with a second individual decamer primer and 13 combinations of OPG19 with a second primer were selected for dpRAPD marker analysis. Finally, two SSR systems from *B. rapa* were screened resulting in four SSR markers polymorphic between the parents and present in F_1.

Of special importance were markers which had been already assigned to the individual radish chromosomes in our set of interspecific rape-radish addition lines (Peterka et al. 2004). The polymorphism of the chromosome-specific markers was tested by comparisons between addition lines, the parents and F_1 of intraspecific cross used for mapping. In total, 62 markers showed polymorphism in the intraspecific mapping population and were used as anchor loci (Fig. 1).

Construction of linkage maps with chromosome-specific anchor loci

Linkage analysis has been done in the F_2 mapping population of 245 plants from cross 'Pegletta' (BCN-resistant) x 'Siletta Nova' (BCN-susceptible). Markers deviating significantly ($P < 0.005$) from expected 3:1 or 1:2:1 segregation ratio for dominant and codominant markers, respectively, were excluded from analysis. A total of 582 markers, 355 AFLP, 156 RAPD, 67 dpRAPD and four SSR markers, were used for linkage analysis. Five hundred and forty-five markers (94%) were joined into nine linkage groups (Fig. 2).

The total map length spanned 1,517 cM with a mean marker distance of 2.8 cM. The largest chromosome (*f*) had a length of 232 cM, the smallest (*g*) 120 cM (Fig. 2). There were only two gaps with over 20 cM on both ends of chromosome *b*.

As expected, the anchor markers of each specific radish chromosome were sorted into one linkage group. Because of this unambiguous assignment of linkage groups to chromosomes, the linkage groups were designated with the chromosome names (Fig. 2). By mapping of anchor markers together with the other markers on the nine linkage groups, their relative position could be determined. The number of anchor loci was between 19 for chromosome *a* and two for chromosome *h* and *i* (Fig. 2, Table 1). The

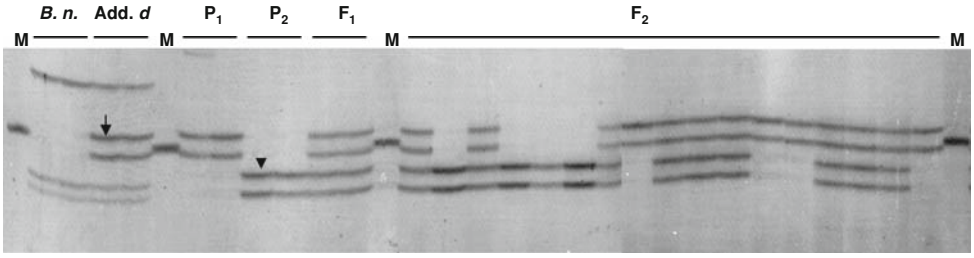

Fig. 1 Segregation of chromosome-specific marker OPC09 + OPD11-191**d* in the intrapecific radish F_2 mapping population 'Pegletta' x 'Siletta Nova'. This marker for the extra chromosome *d* in the rapeseed-radish addition line (*arrow*) is also present in the parent P1 'Pegletta' and segregates in the F_2 mapping population. The identification of the corresponding allele in P2 'Siletta Nova' (*arrowhead*) allows codominant counting of the marker. *Brassica napus* (*B. n.*), rapeseed-radish addition line *d* (Add. *d*), P1, P2 and F_1 are presented by *two lanes*. *M* 100 bp size marker

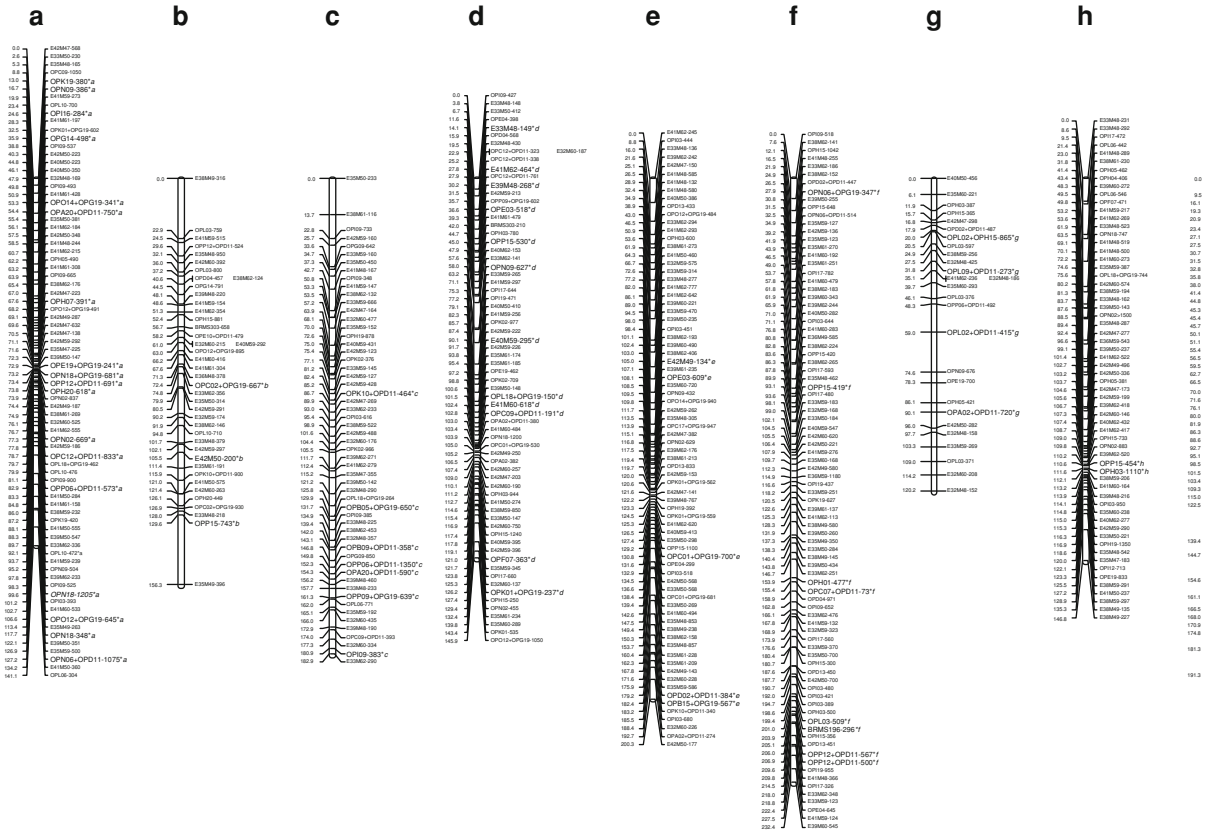

Fig. 2 Genetic map of oil seed radish (*Raphanus sativus* var. *oleiferus*) based on a F_2 population of a single plant cross between varieties 'Pegletta' and 'Siletta Nova'. The names of the linkage groups represented on the top correspond to the chromosome names assigned by the localization of chromosome-specific anchor markers (Peterka et al. 2004). Chromosome-specific anchor markers are in *bold* and designated by an *asterisk* with the chromosome letter, e. g. *a: marker for radish chromosome *a*. Distances between markers are described on the left of the linkage groups

chromosome *d* carrying BCN-resistance had 12 anchor loci distributed over the whole linkage group.

Inheritance of resistance against *Heterodera schachtii*

The mean cyst number of generations P1, P2, F_1 and F_2 was 4.1 ± 0.5, 135.5 ± 7.2, 15.7 ± 1.7 and 24.6 ± 2.0, respectively. The non-resistant parent showed a similar susceptibility as rape cultivar 'Madora' with 157.3 ± 3.2 cysts (Table 2).

Frequency distribution of cyst number in the F_2 is presented in Fig. 3. The presumed monogenic dominant inheritance of the BCN-resistance is not clearly reflected, probably due to the high non-genetic variance component giving a broad range of trait expression for the susceptible F_2 subpopulation. This phenomenon is also shown

Table 1 Mapped anchor markers for radish chromosomes *a* to *i*

Number of markers			
Chromosome	Length (cM)	Total	Anchor
a	141.0	78	19
b	156.3	41	3
c	182.9	56	7
d	145.9	69	12
e	200.3	76	5
f	232.4	86	8
g	120.2	27	4
h	146.8	62	2
i	191.3	50	2
	1,517.1	545	62

Table 2 Resistance reaction of P1, P2, F_1 and F_2 to beet cyst nematode *Heterodera schachtii*

Population	Plants	Mean cyst number	Range	Standard deviation
'Pegletta' (P1)	73	4.1	0–29	4.56
'Siletta Nova' (P2)	64	135.5	50–349	57.73
F_1	26	15.1	0–34	8.93
F_2	245	24.6	0–217	31.62
Rape 'Madora'	212	157.3	39–270	47.03

markedly in susceptible P_2 population and rape 'Madora' (Table 2). This is also indicated by an estimated heritability of $h^2 = 0.43$ (data not shown).

QTL analysis of resistance trait

For QTL analysis, the second round map (map 2) produced by JoinMap 4 for each of the nine linkage groups, *a* to *i*, was used. The highest maximum LOD value of 22.6 was calculated with the interval mapping procedure of MapQTL 5 for linkage group *d* (Table 3). This result is in agreement with the assignment of a single main genetic effect for BCN-resistance to chromosome *d* by Peterka et al. (2004) using backcross progenies of multiple rape-radish additions segregating for presence/absence or whole radish chromosomes.

The log-likelihood (LOD) plot for BCN-resistance reached the maximum score in the interval between markers E41M59-297 and OPI19-471 revealing this as the most probable location of the QTL (Fig. 3). The QTL was designated as $Hs1^{Rph}$ (quantitative trait locus for resistance against *Heterodera schachtii* from *Raphanus* genome).

The most probable location of the QTL $Hs1^{Rph}$ was at 71.0 cM. The QTL explains 46.4% of the phenotypic variance. The additive effect was −77.3 and the dominance effect −71.1, thus complete dominance of the resistant gene could be assumed. (Fig. 4)

Of the six specific markers for chromosome *d*, OPN09-627*d and OPL18 + OPG19-150*d comprise the shortest segment of 36.2 cM which contains $Hs1^{Rph}$. These markers could be used in rape-radish recombination experiments for introgression of the BCN-resistance into rapeseed by means of the intergenomic recombination between radish chromosome *d* and homoelogues in the rape genomes, A or C.

Discussion

Genetic mapping in *Raphanus*

The first aim of this study was to map the radish markers developed previously by means of rapeseed-radish chromosome additions having a chromosomal assignment, but with unknown arrangement within the corresponding linkage group. The intraspecific mapping population of 245 F_2

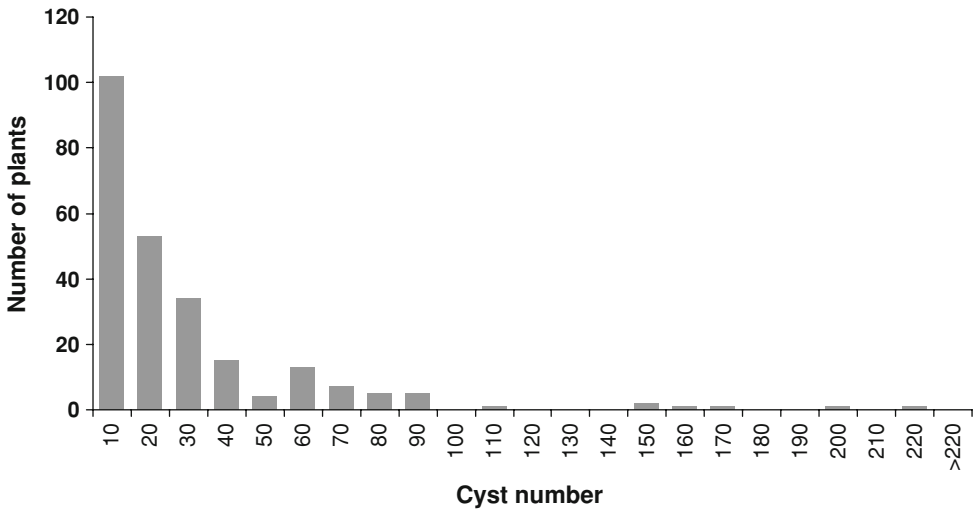

Fig. 3 Distribution of cyst number at the roots after inoculation with BCN in the F_2 mapping population of intraspecific radish cross 'Pegletta' x 'Siletta Nova'

Table 3 Maximum LOD values from interval mapping in nine radish linkage groups

Linkage group	Length of map 2 (cM)	Number of markers	Maximum LOD value
a	139.0	32	1.0
b	147.2	20	0.7
c	193.5	34	2.9
d	136.3	29	22.6
e	199.6	36	1.4
f	221.4	42	1.4
g	109.3	14	1.0
h	122.8	28	0.7
i	178.1	27	1.7

individuals was derived from a cross between two cultivated radish varieties and resulted for 545 markers in a map of 1,517 cM genetic length and nine linkage groups. Bett and Lydiate (2003) used a cross with a wild radish, *Raphanus sativus x R. raphanistrum*, to produce a map of radish in F_2 (85 individuals) and in BC_1 (54 individuals) with 236 RFLP markers of *Brassica* with a map length of 844 and 915 cM, respectively. The intraspecific radish map of Tsuro et al. (2005) having a total length of 675.8 cM was constructed with 241 markers and 94 individuals of an F_1 cross pollination population. The reason for our larger map compared to that of both groups might be a higher resolution in the present study because of higher number of individuals and markers in the mapping population. Similar distinct map length estimates from different authors were also obtained for other species as in *Brassica oleracea* with 1,606 (Cheung et al. 1997) and 893 cM (Sebastian et al. 2000), respectively.

Chromosome-specific anchor markers previously developed for the extra chromosomes in a set of rapeseed-radish chromosome additions (Peterka et al. 2004; Budahn et al. 2008) were mapped to the nine linkage groups. A physical mapping of markers on extra chromosomes could be done by means of radiation hybrids as described by Riera-Lizarazu et al. (2000) with an oat-maize addition.

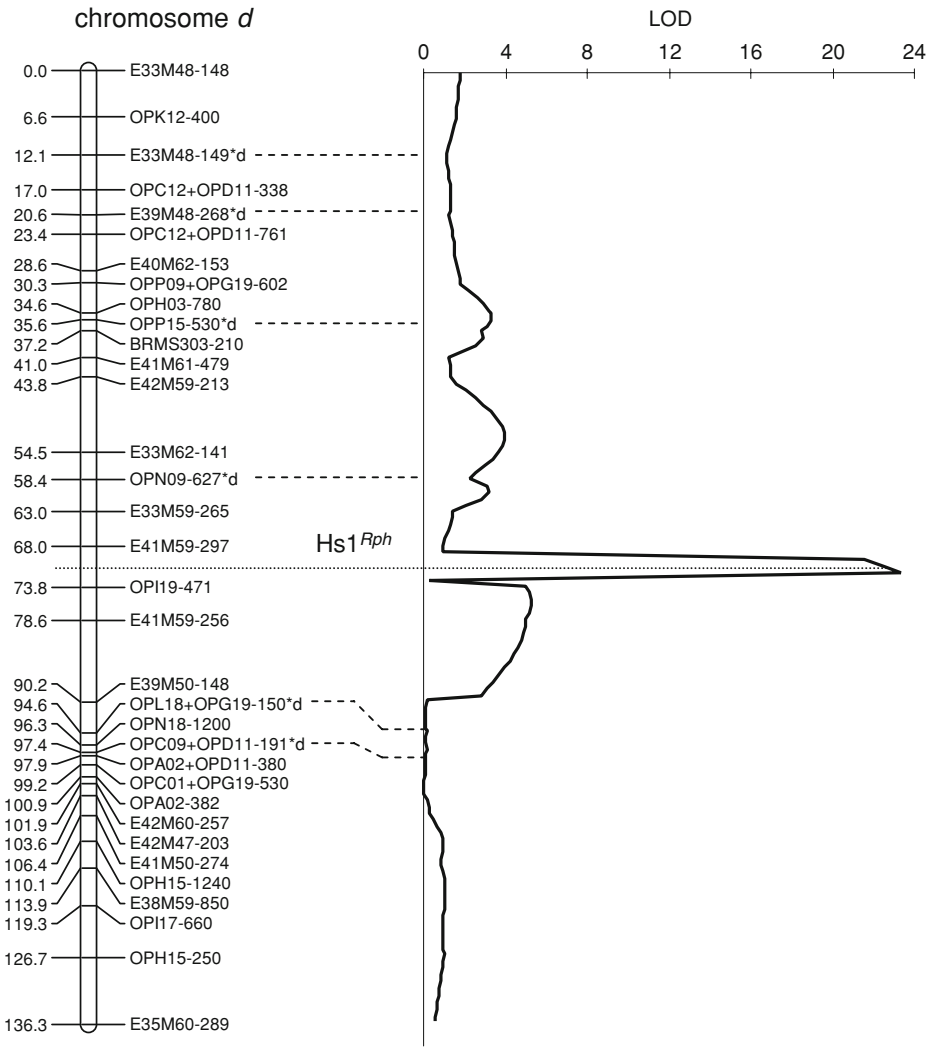

Fig. 4 LOD scan for interval mapping on radish chromosome *d* for BCN-resistance. The dotted line shows the most probable location of QTL Hs1^{Rph} at 71.0 cM on radish chromosome *d*; the dashed lines show the distance of anchor markers to the QTL for BCN-resistance

Characterization of QTL Hs1^{Rph}

The second aim was the genetic analysis and the mapping of factor(s) causing variation of BCN-resistance in oil radish. Resistant oil radish was discovered and is used already for decades. This is the first report of QTL mapping of resistance against *Heterodera schachtii* in radish.

Our study shows, that resistance to BCN is controlled by a major QTL, Hs1^{Rph}. The location of this gene on chromosome *d* revealed by the mapping analysis is in agreement with the results obtained from testing of added radish chromosomes in rapeseed (Peterka et al. 2004). Of the nine radish chromosomes, only chromosome *d* conferred the radish resistance in a rapeseed genetic background supporting the hypothesis of a single dominant gene (Baukloh 1976). Hs1^{Rph} controls the radish BCN-resistance in which the feeding site undergoes a process of degeneration which leads directly to its breakdown (Wyss et al. 1984). Similar to other nematode resistance genes, the suppression of cyst development by Hs1^{Rph} was not absolutely so that a few cysts have been developed. Resistant radish varieties show different levels of resistance expression. It is unknown if this results from allelic variation at Hs1^{Rph} or from the modifying action of minor genes. The dominant gene action of Hs1^{Rph} is in agreement with expression of resistance in the addition line *d* on susceptible rapeseed genetic background (Peterka et al. 2004). The dominance of Hs1^{Rph} could be explained through functionally relatedness to the genes which are down regulated after nematode syncytium induction (Ohl et al. 1997; Gheysen and Fenoll 2002). The gene Hs1^{Rph} probably controls a plant defense response and could not switched off by pathogen attack in contrast to the recessive allele.

Chances for integration of QTL Hs1^{Rph} into the rapeseed genome

The disomic rapeseed-radish addition line *d* with BCN-resistance (Peterka et al. 2004; Budahn et al. 2008) permits the breeding and use of rapeseed as a catch crop. Regular transmission of the extra chromosome pair on female and male side enables a consistent reproduction of the disomic state, and no essential change in the growth of the rapeseed recipient after addition of chromosome *d* had been detected. The transfer of the resistance-carrying chromosome into new open-pollinated varieties by monitoring chromosome *d*-specific markers is simple. CMS rapeseed lines possessing the *d* chromosome pair could be reproduced with a disomic maintainer and resistant monosomic hybrids were obtained by pollination of a euploid restorer line.

Effects by expression of undesired radish genes from the whole chromosome cannot be ruled out. Furthermore, creation of novel variability because of changes in gene expression after genome enhancement could appear as recently described for polyploids (Levy and Feldman 2002; Osborn et al. 2003). Therefore, the integration of a radish chromosome segment with Hs1^{Rph} into genome of rapeseed itself has to be envisaged. Intergenomic recombination between rapeseed and radish had been detected by the transfer of a fertility restorer gene (*Rf*) for cytoplasmic male sterility (Delourme et al. 1998) and recombination in rapeseed involving the radish segment was non-spontaneous induced by gamma-ray irradiation (Primard-Brisset et al. 2005). Artificial intergenomic translocations of chromosomes have been induced by chemical mutagens or radiation and played an important role in the genetics and breeding of several crop species. Successful radiation-induced translocation have been reported from wheat with different sources of resistance (Driscoll and Jensen 1963; Sears 1993; Crasta et al. 2000), from *Avena sativa* (Aung and Thomas 1976), *Beta vulgaris* (Savitsky 1975), and *Nicotiana tabacum* (Chaplin and Mann 1978). Intergenomic translocations by gamma-ray treatment of a monosomic oat-maize addition line were produced and shown to be stable (Riera-Lizarazu et al. 2000; Vales et al. 2004). The anchor marker mapped in this study allow to follow the dissection of the chromosome *d* or other radish chromosomes in such experiments and could assist in selection of introgression containing QTL Hs1^{Rph}.

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