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Inheritance of resistance derived from the B-genome of *Brassica* against *Phoma lingam* in rapeseed and the development of molecular markers

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Abstract Genes of the B genome of *Brassica* conditioning *Phoma* resistance at the epicotyle were transferred into *Brassica napus* by interspecific hybridization. The recombinant lines expressed high resistance similar to that of the donor parents. Unlike the oligo- or polygenically inherited resistance of *B. napus* known so far, the B-genome resistance genes of the recombinant lines behaved monogenically dominant. No significant differences in the level of resistance or in the phenotype of the resistance mechanisms were observed among homozygous resistant plants when the different B-genome origins investigated, i.e. *B. nigra*, *B. juncea* and *B. carinata*, were compared. Therefore it was assumed that the resistance genes of each B-genome species and the resistance mechanisms of the species are identical. Temperature increased the expression of internal lesions caused by *Phoma lingam*. High summer temperatures in the greenhouse led to faster development of tissue damage at the epicotyle of plants, resulting in significant deviations in segregation ratios, when fixed scores were used for disease classification. Independent of origin, the three B-genome resistance genes were introgressed at the same location of the rapeseed genome. The arrangement and distances of closely linked RFLP markers on linkage-groups were similar to those of the same markers on linkage group six of the rapeseed map. It is concluded that the B-genome resistance genes were introgressed by homoeologous recombination after allosyndetical pairing of B-genome chromosomes with the A- or C-genome chromosomes.

Key words *Brassica napus* · *Phoma lingam* · *B-genome* · Resistance · Molecular marker

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Introduction

Of the phytopathogenic fungi known to affect rapeseed, *Phoma lingam* (Tode ex Fr.) Desm. [anamorph *Leptosphaeria maculans* (Desm.) Ces & De Not.] causes the highest economic losses of this crop worldwide. It was described for the first time as *Sphaeria lingam* by Tode (1791) and assigned to the genus *Phoma* by Desmaziere (1849). Müller and Tomasevic (1957) reported *P. lingam* to be the asexual form of *L. maculans*, which earlier had also been described by Desmaziere (1863). Both forms of the fungus give rise to the blackleg disease of *Brassica*. Since chemical protection is difficult and seldom economic (Ahlers 1991), genetic resistance has become an important measure of disease control. However, the genetic base of resistance in cultivated rapeseed is narrow and based primarily only on the French variety 'Jet Neuf'. This resistance is polygenically controlled (Cargeeg and Thurling 1980), non-specific, and provides only partial protection. On the other hand, several investigators have shown that the B genome of *Brassica* derived from *Brassica nigra* (BB, 2n = 16), *Brassica carinata* (BBCC, 2n = 34) and *Brassica juncea* (AABB, 2n = 36), respectively, contains a high level of resistance against *P. lingam* (Sacristán and Gerdemann 1986; Sjödin and Glimelius 1988; Gugel et al. 1990), which is oligogenically controlled and effective throughout the life of the plant (Zhu et al. 1993; Struss et al. 1996). Since B-genome *Brassica* species can be hybridized with *B. napus*, they may be applied as ready sources of *Phoma* resistance in rapeseed breeding.

In order to transfer B-genome resistance into the A- or C-genomes of rapeseed, Roy (1978, 1984) and

Sacristán and Gerdemann (1986) conducted interspecific hybridizations of the two B-genome carriers, *B. juncea* and *B. carinata*, with *Brassica napus* and *Brassica campestris*, respectively. Unfortunately, the *carinata*-resistance was lost during the backcross generations. The *juncea*-resistance remained unstable, even in F_6 and F_7, but it conditioned a high level of resistance in rapeseed with a monogenic mode of inheritance. By generating translocation lines, Chèvre et al. (1995) introduced *Phoma* resistance expressed at the cotyledon stage of *B. nigra* into *B. napus*. Delourme et al. (1995) used the stable monogenically inherited resistance of *B. juncea* to establish *Phoma* resistance in recombinant lines at the cotyledon and the adult plant stage.

The addition of single resistance genes to a more complex partial resistance, such as that from 'Jet Neuf', or an accumulation of different resistance genes to improve the resistance level in *B. napus*, is a more difficult task than the transfer of a monogenically or oligogenically inherited resistance into susceptible plants. In such cases, selection of the desired resistant genotype by progeny analysis may be inefficient not only because of environmental effects but also because of small, if any, phenotypic differences between the various recombinant genotypes. By using molecular markers for the B-genome resistance genes, their transfer, as well as their effective addition to the resistance present in the recipient rapeseed parent, can clearly be shown.

In the present study *Phoma* resistance genes derived from all three different B genomes of *Brassica* were compared by epicotyle tests in lines of rapeseed carrying a nearly uniform genetic background. The inheritance of the genes and the phenotype of their resistance reaction was examined and molecular markers were developed for the resistance genes using RFLP and RAPD techniques.

Materials and methods

Plant materials

Phoma resistant recombinant lines had previously been developed by interspecific hybridization of the three B-genome containing *Brassica*-species with the complementary species within the triangle of U (1935) to create five different trigenomic hybrids (ABC, n = 27). These were backcrossed at least four times to *B. napus*. The recurrent parent in the backcrosses was the Canadian spring form of *B. napus* cv 'Andor' (Fig. 1). The following resistant recombinant lines (2n = 38) were used in the present investigations, selfed twice to generate segregating (F_2 and F_3) populations:

one line from AACC × BB(= *nigra*-line),
one line from CC × AABB (= *juncea*-line),
one line from AA × BBCC (= *carinata*-line).

Resistance tests

Inoculation was carried out by using a mixed-suspension of ten different single-spore isolates of *P. lingam*, collected at different regions in Germany. These isolates were kindly provided by Prof. H.-H. Hoppe, Institut für Pflanzenpathologie und Pflanzenschutz, Universität Göttingen, Germany.

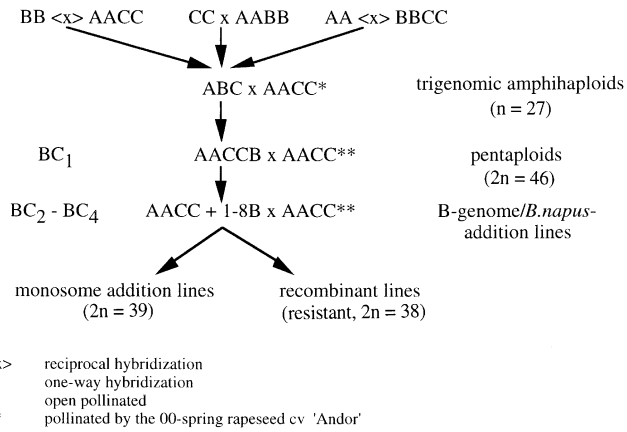

Fig. 1 Development of *Phoma*-resistant recombinant lines from interspecific hybridizations of *Brassica* species (see Struss et al. 1991)

Resistance was tested under greenhouse conditions. At the three-leaf stage, the petiole of the first true leaf was wounded by a needle and inoculated with 10 µl of a suspension of 10^7 spores/ml. For the next 3 days, the plants were placed in a growth chamber at 20°C and 100% humidity and later cultivated in a greenhouse under a 16-h light period. Lesions at the epicotyle of the plants were scored at DBCH 85, i.e. at 'first coloured seeds', 8–9 weeks after inoculation, using a modified scoring scale of Koch et al. (1989):

Scores for external necrosis:
1 – small superficial lesions around the inoculation site
2 – superficial lesions, < 25% girdling
3 – like 2, but lesions deeper
5 – < 50% girdling, stem slightly cut-in
7 – > 50% girdling, stem cut-in
8 – 100% girdling, stem strongly cut-in
9 – plants collapsed.

Scores for internal necrosis:
I – < 15% stem tissue destroyed
II – 15–30% stem tissue destroyed
III – 30–50% stem tissue destroyed
IV – 50–75% stem tissue destroyed
V – > 75% stem tissue destroyed.

For resistance tests of the parents of the recombinant lines ('Andor', *B. nigra*, *B. juncea* and *B. carinata*) and the F_1 s, only the external lesions were scored. The plants were grouped as resistant (1–5) and susceptible (7–9), respectively. For analysis of the F_3 s, the internal lesions also were scored. Plants were classified as susceptible if external necrosis scores exceeded 5 or internal necrosis scores exceeded III. Since plants may be damaged internally without showing any external symptoms (Gugel et al. 1990), internal necrosis was monitored. Analysis of F_2 genotypes was performed by testing ten F_3 plants from each F_2 plant, which was classified heterozygous resistant, if one or more F_3 plants were segregating from the rest as resistant and susceptible, respectively.

Marker studies

Genomic plant-DNA was extracted according to Uzunova (1994) using a modified protocol of Rogers and Bendich (1988).

RAPD-analysis was carried out according to Hu and Quiros (1991); 340 decamer primers were used from Operon Technologies,

Almeda, California (USA): A, B, C, D, Q, R, S, T, U, AG, AH, AI, AJ, AK, AL, AM, AN (groups of 20-decamer primers).

RFLP-analysis was done using *Eco*RI- and *Hin*dIII-digested genomic DNA and the protocol of Uzunova (1994). The probes were kindly provided from Dr. Milena Uzunova and Dr. Wolfgang Ecke, Institut für Pflanzenbau und Pflanzenzüchtung, Universität Göttingen, Germany. A total of 79 probes was used: 57 genomic probes from Dr. M. Uzunova (pRP), 19 genomic probes from Prof. T. Osborn (Madison, USA) and three cDNA probes from Dr. R. Töpfer (Max-Planck-Institut für Züchtungsforschung, Köln).

Statistics

For linkage analysis, data were transferred into a binary 1/0 matrix with 1 = fragment present and plant resistant, 0 = fragment absent and plant susceptible, respectively. The arrangement of the markers and resistance genes on linkage groups, as well as the calculation of their distances were analyzed using Mapmaker (Lander et al. 1987), version V 2.0 NDP for Macintosh [transformation function Kosambi (1944), maximum recombination frequency $\leq 40\%$ and minimum LOD-score $\geq 3{,}0$]. Mendelian segregation of resistance genes was proved using the chi-square goodness-of-fit test. The correlation of internal and external necrosis scores was calculated using the Spearman rank correlation for non-parametric data with StatView, version 4.0 FPU for Macintosh.

Table 1 Mean scores of resistance of each of ten plants of 'Andor', the three B-genome species, as well as F_1 plants of the three B-genome/*B. napus* recombination lines, respectively, 6 weeks after *Phoma* inoculation

'Andor'	*B. nigra*	*B. juncea*	*B. carinata*
7,8	1,0 *nigra*-line 3,1	1,2 *juncea*-line 1,3	1,0 *carinata*-line 2,8

Fig. 2 Resistance phenotype of 'Andor' **(A)** and the B-genome species *B. nigra* **(B)**, *B. juncea* **(C)** and *B. carinata* **(D)**, 6 weeks after *Phoma* inoculation

Results

In all resistance tests the high susceptibility of 'Andor' was evident in contrast to the very high resistance of the B-genome species (Table 1, see Fig. 2). About 20% of the infected 'Andor' plants were collapsed before scoring and all other plants of this cultivar showed the typical symptoms of strong blackleg disease. Lesions of the B-genome species, however, were small and superficial, and plant development was not affected by the fungus. Resistance of the recombinant lines was similar to that of the B-genome species (Table 1, see Fig. 3). F_1 plants from recombinant lines $\times$ cv 'Andor' crosses, showed high resistance to *Phoma*, indicating a dominant inheritance of the *Phoma* resistance.

Based on the results obtained from F_3 plants, the F_2 from both crosses with the *juncea*-line and the *carinata*-line segregated 1 : 2 : 1 (homozygous resistant: heterozygous resistant: homozygous susceptible; Table 2). Since the F_1 s demonstrated no segregation and exhibited high resistance, these data suggest that *Phoma* resistance of the *juncea*- and *carinata*-line is controlled by a single dominant gene.

The *nigra*-line progeny showed a significant deviation from the expected 1 : 2 : 1 ratio, due to a reduced number of homozygous resistant plants ($P < 5\%$). A possible explanation of this difference may be the fact that for technical reasons the resistance test of the recombinant lines had to be carried out under two conditions: the progenies of the *nigra*-line were tested in the summer at greenhouse temperatures mostly over 30°C during the day, whereas the *juncea*- and *carinata*-lines were tested under controlled temperature conditions between 18 and 20°C in the winter months.

Therefore, the temperature sensitivity of the resistance reaction was investigated to verify this hypothesis. About 100 F_3 plants derived from the same

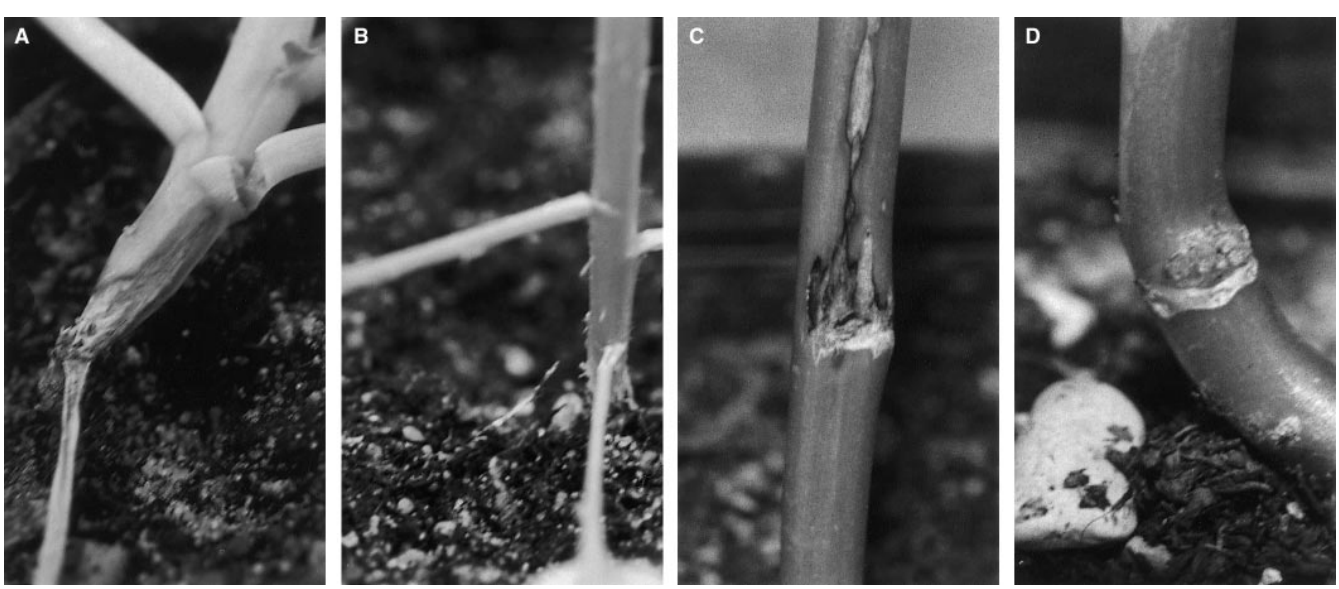

Table 2 Segregation of F_2 plants from the crosses of resistant B-genome/*B. napus* recombinant lines × 'Andor'

Genotype	Homozygous resistant	Heterozygous resistant	Homozygous susceptible	χ^2
nigra-line	11	47	31	9.3 **
juncea-line	26	47	30	1.1
carinata-line	23	40	29	2.4

** Significant at $P = 1\%$

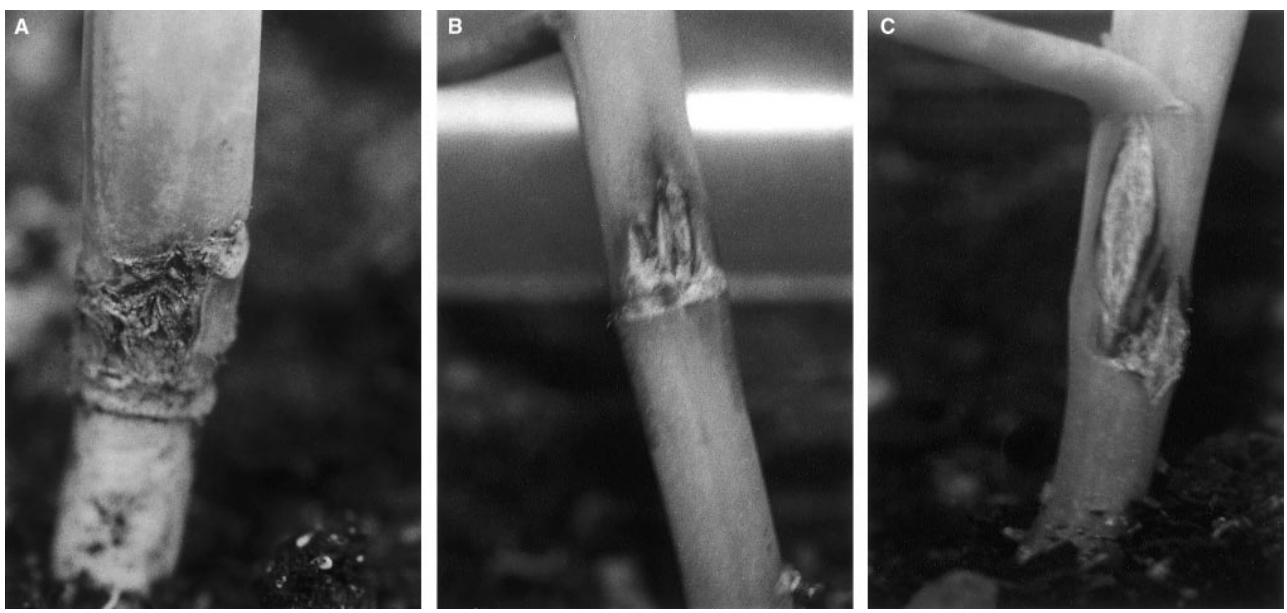

Fig. 3A–C Resistance phenotype of the B-genome/*B. napus* recombinant lines 6 weeks after *Phoma* inoculation. **A** *B. nigra*/*B. napus*-, **B** *B. juncea*/*B. napus*-, and **C** *B. carinata*/*B. napus*-recombinant lines

heterozygous F_2 plant of the *B. carinata*/*B. napus* recombinant line were grown under normal greenhouse conditions of 18–20°C until scoring, and another 100 F_3 plants from the same F_2 individual were incubated in a growth chamber at simulated greenhouse summer conditions of 30°C day, 25°C night.

All plants kept at 30°C on the average showed one score higher for external necrosis than plants grown at 18–20°C. But the ratio of 'resistant' (scoring notes 1–5) to 'susceptible' (scoring notes 7–9) was not significantly affected by the different temperatures (Table 3). The effect of temperature was more evident for the development of the internal lesions. Even plants with only small external lesions (scores 2, 3 and 5) revealed strong damage at their internal tissue at 30°C but not at 18–20°C (Fig. 4). Obviously the higher temperature affected the internal symptoms more than the external ones. This response to temperature of the *Phoma* phenotypes resulted in a significantly disturbed (i.e. 1 : 1, resistant : susceptible) segregation at 30°C. On the other hand, the infected plants grown at 18–20°C did express the expected segregation of 3 : 1 for both, the external and the internal scores (Table 3).

In order to map the resistance genes by molecular analysis a bulked segregant analysis (Michelmore et al. 1991) was carried out. Seventy four out of the 79 RFLP probes (94%), and 259 out of the 340 RAPD primers (76%) exhibited informative band patterns. More than 90% of these probes or primers showed at least one fragment polymorphic between *B. napus* and the three *Brassica*-species containing the B genome. These results are indicative of a large genetic distance between the B-genome and the A- and C-genomes of *Brassica*. Thirty eight percent of the RAPD primers and 67% of the RFLP probes also detected polymorphisms between the three different recombinant lines, a result most likely due to the different genome donors used for the interspecific hybridizations. Four RFLP probes were selected showing co-dominantly inherited polymorphisms among the DNA-pools of the three recombinant lines (Fig. 5). Finally, one RAPD primer revealed a dominantly inherited polymorphic fragment of 800-bp length existing among the DNA-pools of the *carinata*-line.

These above markers were tested with the total of 90 F_2 plants of the segregating population and analyzed for linkage with the resistance genes (Fig. 6). The arrangement and distances between the RFLP markers are similar within all linkage groups. The *Phoma* resistance genes of the *juncea*- and the *carinata*-lines are tightly linked with markers pRP1457.H, pRP1513.E

Fig. 4 Resistance phenotype of F_3 plants derived from a heterozygous resistant F_2 plant of the cross resistant *B. carinata*/*B. napus* recombinant line × 'Andor' after growth at 18–20°C (**A** and **D**) and 30°C (**B** and **C**), respectively. Plants were classified as resistant by external scores (**C** and **D**), but ranged between I **(A)** and V **(B)** by internal scores

Table 3 Segregation of the resistance phenotype reaction of F_3 plants derived from a heterozygous resistant F_2 plant of the cross resistant *B. carinata*/*B. napus* recombinant line × 'Andor' after growth at 18–20°C and 30°C, respectively

Temp. (°C)	External score								
	1	2	3	5	7	8	9	$r:s$	χ^2
18–20	25	12	26	18	18	3	2	81:23	0.5
30	0	14	54	10	4	8	14	78:26	0
	Internal score							Combined external and internal scores	
	I	II	III	IV	V	$r:s$	χ^2	$r:s$	χ^2
18–20	67	11	5	14	7	83:21	1.3	79:25	0.1
30	3	37	9	27	28	49:55	43***	49:55	43***

*** Significant at $P = 0.1\%$

and pRP1602.H. The distances and position of the *nigra*-resistance gene are different in comparison with the resistance genes in the *juncea*- and *carinata*-lines, most likely due to a deviation of segregation in the *nigra*-line resistance reaction. The RAPD-marker is located 28.5 cM from the resistance gene of the *carinata*-line.

All four RFLP-markers were mapped to the same linkage group (six) of the rapeseed linkage map of Uzunova et al. (1995). A comparison of the patterns showed that Uzunova et al. had mapped the same fragments which co-segregated with the susceptibility allele in the recombinant lines and in 'Andor' (see Fig. 5, fragment no. 2 marked by the arrow). Thus the same marker loci were mapped and the resistance genes of the B genome were all introgressed into the same linkage group of rapeseed.

Discussion

Different *Brassica* species display different levels of resistance against *P. lingam* (for a review see Rimmer and van den Berg 1992). Several major oilseed rape varieties currently exhibit polygenically controlled resistance (Dion et al. 1995; Ferreira et al. 1995) derived from the cv 'Jet Neuf'. The B genome of *Brassica* contained in *B. nigra* (BB, 2n = 16), *B. carinata* (BBCC, 2n = 34) and *B. juncea* (AABB, 2n = 36), however, controls the highest level of resistance, based on the hypersensitive reaction of the cotyledons after infection (Roy 1978; Sacristán and Gerdemann 1986; Sjödin and Glimelius 1988). This resistance has been introduced successfully into *B. napus* by interspecific hybridization (Roy 1984; Sjödin 1989; Struss et al. 1991; Zhu et al. 1993; Chèvre et al. 1995; Delourme et al. 1995). Struss et al. (1996) reported oligogenic inheritance of the B-genome resistance, a characteristic which increases the value of this source for breeding purposes. In the present study, all three potential B-genome *Phoma* resistance genes derived from *B. nigra*, *B. juncea* and *B. carinata* were compared for the first time by epicotyle tests in lines of rapeseed with a nearly uniform genetic background.

Because of the monogenically dominant inheritance of the *Phoma* resistance, only one resistance gene or

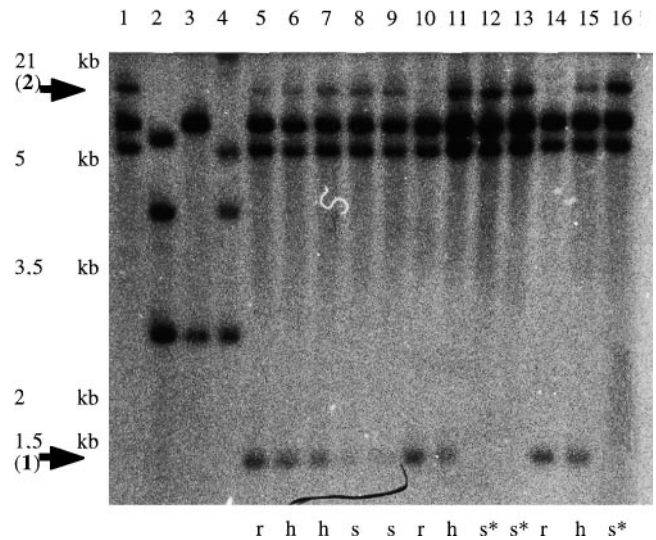

Fig 5 Bulked segregant analysis with DNA-bulks of F_2 plants, digested with *Eco*RI and hybridized with pRP1513. *Lane 1* 'Andor'; *lanes 2–4 B. nigra*, *B. juncea*, *B. carinata*; *lanes 5–9 nigra*-line; *lanes 10–13 juncea*-line; *lanes 14–16 carinata*-line. *r* = bulk with homozygous resistant plants, *h* = bulk with heterozygous resistant plants, *s* = bulk or single plant DNA (*) with susceptible plants. *Arrows* show the two polymorphic fragments

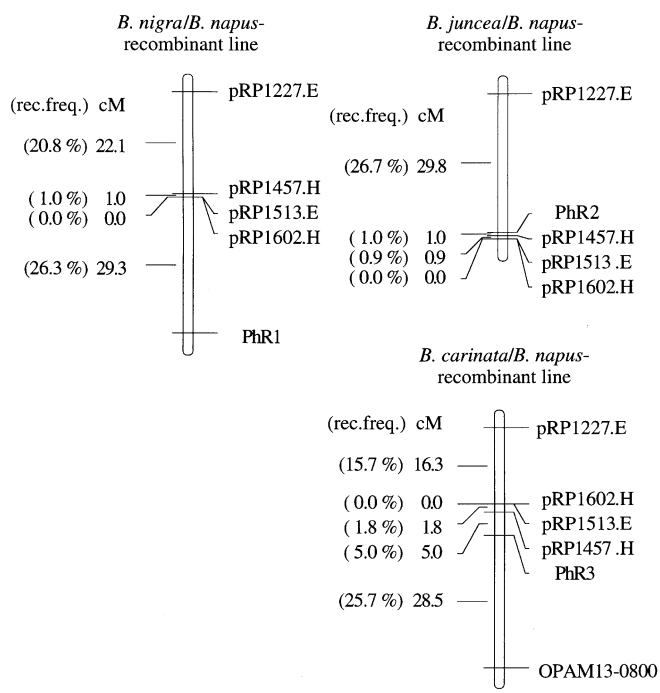

Fig. 6 Linkage of polymorphic markers and resistance genes (*PhR 1–3*) in F_2 plants of cross progenies of the three recombinant lines. Distances between loci are in cM and recombination frequencies in %

gene complex of the B genome was introduced into each of the recombinant lines. Struss et al. (1996) identified three chromosomes carrying *Phoma* resistance genes in *B. nigra* and *B. carinata* and two chromosomes in *B. juncea*. The third gene locus in *B. juncea* was not apparent, but it may be that it is located on the eighth *juncea* chromosome, absent in the investigation. In summary, each of the genes in the recombinant lines investigated here, contributed the same level of resistance as did the B-genome donor parents as such. Therefore, the data indicate that the high level of B-genome resistance is already accomplished by one single resistance gene of the possible three of each B-genome species. This supports the finding of Lagercrantz and Lydiate (1996) of a series of triplicated regions in different *Brassica* genomes, and led us to suspect that the three resistance genes of each B-genome species are identical. The present three recombinant lines also showed a high homology in the resistance mechanism against *P. lingam*. No significant differences in phenotype or level of resistance were observed among the three lines. Considering the gene for gene hypothesis, a virulent interaction between certain *Phoma* races and resistance genes is possible. Kuswinanti et al. (1995) observed first indications of this phenomenon in cotyledons of *Brassica*. But until now there has been no a report on breakdown of the resistance of *B. napus* or B-genome sources against *P. lingam* by new aggressive races of the fungus. Nevertheless, the extensive cultivation of resistant *B. napus* cultivars may easily lead to an increase in virulent races of *P. lingam*. With such a possibility in view, new sources of resistance to blackleg are essential to provide effective control of this disease in the future. The monogenically dominant inheritance and the high level of resistance confers on our present material a high value for the breeding of resistant *B. napus* varieties, especially for hybrid breeding. This resistance was successfully transmitted (data not shown), after hybridization of the recombinant lines with susceptible winter forms of different *B. napus* cultivars.

Expression of *Phoma* resistance in the investigated B-genome recombinant lines was affected by environmental conditions and decreased, in particular, by high temperatures. At 30°C the infected plants exhibited faster development of lesions and higher susceptibility to *Phoma* in comparison with the response of plants of the same line at 18–20°C. Moreover, the internal necrosis in the infected stem was more severe at 30°C even when external lesions were relatively small. Similar phenomena have been observed for rust resistance in the *Gramineae* (Browder 1980; Dyk and Johnson 1983; Gerechter-Amitai et al. 1984). Badawy et al. (1992) also reported temperature sensitivity for cotyledon resistance in *B. napus*. While cv 'Quinta' showed a high sensitivity to *P. lingam* at 18°C, it was highly susceptible at 27°C. The authors observed promotion of infection symptoms, even on the stem, by high temperature. The discrepancy between the results of the *nigra*-recombinant line and the *juncea*- and *carinata*-recombinant lines in the present study were obviously also related to the prevailing temperature conditions: the

recombinant lines derived from *B. juncea* and *B. carinata* were tested in the winter, while the resistance test with the *nigra*-recombinant line was performed during summer at temperatures over 30°C, where the resistant lines attained thinner stems and the plants were weaker in general. The skewed segregation of the F_2 population of the *B. nigra* recombinant line from the expected 1:2:1 might be related to the negative influence of high temperature on resistance expression. For reliable identification of the *Phoma* reaction in the greenhouse, the resistance tests should be performed under controlled conditions. Although the correlation between external and internal scores was high ($r = 0{,}842$, $P < 0{,}01\%$), a scoring system based on the assessment of both external and internal symptoms is preferable, since the present results confirmed those of Gugel et al. (1990) that plants may be damaged internally without showing any external symptoms.

Molecular markers for *Phoma* resistance can facilitate the utilization of these genes for resistance improvement in *B. napus*. Because the markers in the *juncea*- and *carinata*-recombinant lines are closely linked, they are useful for marker-assisted breeding programs. Whether the larger distance of markers to the *nigra*-resistance gene is due to a deviation of segregation in the resistance reaction, or is an indication of a different introgression site, is under investigation at present. Notwithstanding, the studies have shown that the arrangement and distances between the RFLP markers are similar within all linkage groups of the recombinant lines and linkage group "six" of the rapeseed linkage map of Uzunova et al. (1995). Thus these data suggest that the three B-genome resistance genes were introgressed at the same location of *B. napus* genome, indicating a transfer by homoeologous recombination after allosyndetical pairing of B-genome chromosomes with A- or C-genome chromosomes. Such allosyndetical pairing is rare (Olsson 1960; Busso 1985; Busso et al. 1987), because of structural differences (Röbbelen 1960; Armstrong and Keller 1981) and large genetic distances (Song et al. 1988; Thormann et al. 1994), as also shown by our investigations. Similar phenomena were observed for the transfer of bacterial resistance into cultivated rice from a wild form (Amante-Bordeos et al. 1992), in which allosyndetical pairing was only partial. Also for *Phoma* resistance, recombination was assumed as the mechanism for gene transfer from the B genome of *B. juncea* into *B. napus* (Roy 1978, 1984; Sacristán and Gerdemann 1986; Delourme et al. 1995).

The absence of DNA-markers in the B-genome parents suggests that all of the employed RFLP markers and the RAPD marker are most likely located on the rapeseed chromatin and not on the introgressed B-genome chromatin (see Fig. 5). If recombination frequency is depressed between rapeseed and B-genome chromatin, the physical distances between the markers and the genes are probably much larger than the distances based on recombination frequencies. Specific B-genome markers for the *Phoma* resistance genes should be more tightly linked, which would be essential for a map-based cloning approach to these genes. The development of markers based on the RFLP probes used for the construction of the *B. nigra* linkage map of Truco and Quiros (1994) is in progress.

The distances of 1.0 and 5.0 cM between RFLP markers and the *Phoma* resistance genes of *juncea*- and *carinata*-recombinant lines, respectively, make the markers suitable for marker-assisted breeding programs. But the RFLP technique is time consuming and expensive. Therefore, the development of specific PCR markers based on closely linked AFLP markers is in progress (data not shown). Also, investigations are on the way to prove the efficiency of these markers for the transfer and accumulation of B-genome *Phoma* resistance genes into susceptible or partially resistant rapeseed varieties for the breeding of stable blackleg resistance.

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