Theor Appl Genet (2005) 111: 281–290
DOI 10.1007/s00122-005-2022-8

ORIGINAL PAPER

B. Saal · D. Struss

RGA- and RAPD-derived SCAR markers for a *Brassica* B-genome introgression conferring resistance to blackleg in oilseed rape

Received: 23 December 2004 / Accepted: 30 March 2005 / Published online: 11 May 2005
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Abstract An introgression derived from the B genome of *Brassica juncea* in spring-type oilseed rape (*B. napus*) conferring recessively inherited cotyledon resistance against several pathotypes of the blackleg fungus *Leptosphaeria maculans* was mapped using PCR-based molecular markers. Resistance-associated B-genome-specific randomly amplified (RAPD) and resistance gene analog (RGA) DNA polymorphisms were converted into three sequence-specific markers (SCARs; B5-1520, C5-1000, RGALm). The flanking sequence of the RGALm locus was determined by genomic walking, leading to a 1,610-bp *Eco*RV fragment which showed extensive homology to known and putative resistance genes of a cluster on *Arabidopsis* chromosome 5. Partial sequence analysis of the genomic RAPD segment OPC-05-1700 revealed strong homology to the gibberellin 2-oxidase gene of *Arabidopsis*. The SCAR markers were analyzed in two segregating populations and were found to be linked in coupling to each other, and in repulsion to the resistance locus. In both populations, markers deviated significantly from a monogenic 3:1 segregation ratio, with plants lacking the markers being more frequent than expected. Although the mode of introgression is yet unknown, the recombinant individuals observed among susceptible progeny suggest homeology between the B-genome-specific segment and its *B. napus* counterpart. This would offer prospects for reducing the size of the introgression and further fine mapping of the resistance locus.

Communicated by C. Möllers

B. Saal · D. Struss
Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstr. 3, 06449 Gatersleben

Present address: B. Saal (✉)
Institut für Pflanzenzüchtung und Pflanzenschutz, Martin-Luther-Universität Halle-Wittenberg, Ludwig-Wucherer-Strasse 2, 06099 Halle-Saale, Germany
E-mail: saal@landw.uni-halle.de

Introduction

Oilseed rape (*Brassica napus* L.) is among the major oil-producing crops grown in the temperate climates of the Northern and Southern hemisphere. It is host to a variety of fungal pathogens, the most important of which is the teleomorph ascomycete *Leptosphaeria maculans* (anamorph *Phoma lingam*) causing blackleg disease and stem canker. At the seedling stage, gene-for-gene relationships between *L. maculans* isolates and cultivated varieties of oilseed rape have been demonstrated. Using a set of differential cultivars, investigators have identified several pairs of corresponding resistance/avirulence genes (Mengistu et al. 1991; Balesdent et al. 2001). In addition, partial adult plant resistance to most pathogenic races of *L. maculans* has been established with the introduction of the cultivar Jet Neuf (Renard and Brun 1979). Owing to growers' demand for high oil and protein quality (single- and double-low), most modern cultivars show an improved homogeneity but also an impoverishment of race-specific resistance genes (Rouxel et al. 2003) and hence a moderate level of resistance. Therefore, a search for new resistance sources is required.

B-genome species of *Brassica* exhibit a high level of resistance, and lines derived from interspecific crosses between *B. nigra* (BB) or *B. juncea* (AABB) and *B. napus* (AACC) have been selected on the basis of their high resistance to races of *L. maculans* virulent to *B. napus* at both the cotyledon and adult growth stages (Chèvre et al. 1996; Plieske et al. 1998; Dixelius 1999; Saal et al. 2004). Interestingly, resistance derived from the B genome has been reported to be inherited by a single dominant gene, but the various described lines seem to harbor independent genes, suggesting that each gene is effective on its own. Therefore, it is desirable to combine these genes (pyramidization) in order to obtain cultivars with enhanced durability of resistance. This process can be accelerated by using tightly linked molecular markers. Several markers for

resistance against blackleg at different developmental stages have already been reported from a number of B-genome resistance sources (Chévre et al. 1997; Barret et al. 1998; Plieske et al. 1998; Plieske and Struss 2001).

The striking similarity of hitherto cloned resistance genes (R genes) has enabled researchers to identify R gene-like sequences, also referred to as resistance gene analogs (RGAs) or resistance gene homologs (RGHs), which may provide a source for candidate genes in mapping experiments. RGAs have been mapped in various important crop species, including soybean (Kanazin et al. 1996; Yu et al. 1996), corn (Collins et al. 1998), potato (Leister et al. 1996) and oilseed rape (Joyeux et al. 1999; Fourmann et al. 2001).

In this paper, we report on the identification of molecular markers linked to the recessive resistance locus r_j *lm2* (Saal et al. 2004) derived from the B genome of *B. juncea* which has been introgressed into *B. napus*. This gene is highly effective against several pathogenicity groups of *L. maculans* at the cotyledon stage (Saal et al. 2004). Introgression-specific RGA and random amplified polymorphic DNA (RAPD) markers were converted into sequence-characterized amplified regions (SCARs) in order to facilitate marker-assisted backcross breeding.

Materials and methods

Plant materials

The development of the original plant material and the genetic analysis of advanced material is described in detail by Struss et al. (1991) and Saal et al. (2004), respectively. In brief, an amphitriploid interspecific hybrid (ABC) was created by crossing *B. juncea* with *B. oleracea*. After several (five to eight) backcross generations to the susceptible recurrent parent, spring-type *B. napus* cv. Andor, recombinant or addition lines, respectively, were developed. B-genome-derived seedling resistance has been identified in a segregating line, designated 937395 (Saal et al. 2004). Molecular marker investigations were carried out with the plant materials outlined in Fig. 1. A segregating line (980201), two resistant lines (980200, 980204), the three resistant B-genome species *B. nigra*, *B. juncea* and *B. carinata* and the susceptible *B. napus* cv. Andor served as initial material for the marker screening. Marker analyses for mapping were performed in 115 F_2 progeny (990149), generated by selfing a F_1 plant of a cross between the winter-type oilseed rape cv. Bristol and the homozygous resistant line 980200, as well as on 55 individuals of the segregating line 990061 derived from selfing a susceptible individual of line 980201 (Fig. 1).

Two recombinant lines have been identified as being resistant to *L. maculans* in the stem or petiole tissue (Plieske et al. 1998). Progeny of this material has also been genotyped.

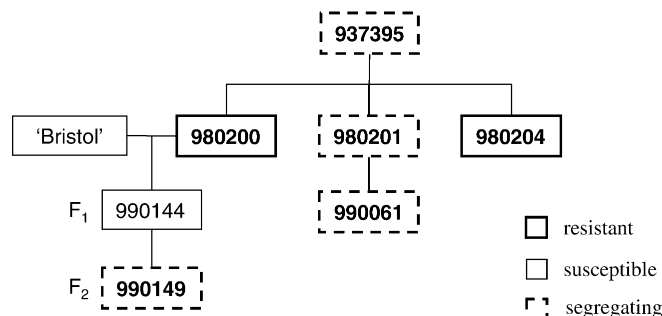


Fig. 1 Pedigree of plant materials analyzed with molecular markers. Markers were mapped to the introgression in the segregating line 990061 and F_2 population 990149

Fungal materials and resistance tests

L. maculans fungal isolates and resistance tests are described in detail by Saal et al. (2004). Visual symptom scoring followed a modified scheme of Williams and Delwiche (1979), with scores ranging from 1 (highly resistant) to 11 (highly susceptible) (Fig. 2a). Plants with a mean score above 6 were judged to be susceptible. In plants exhibiting medium lesion sizes, classification of the phenotype was corroborated by trypan blue staining of cotyledons (Fig. 2b).

DNA extraction

Genomic DNA was extracted according to the miniprep protocol of Struss et al. (1996) with modifications. Briefly, six to eight leaf discs were punched out and ground in liquid nitrogen in 2-ml reaction tubes. Following extraction in 2× CTAB lysis buffer (100 m*M* Tris-HCl pH 8.0, 20 m*M* EDTA pH 8.0, 1.4 *M* NaCl, 1% PVP, 2% CTAB), the solution was extracted with 1 vol chloroform, followed by the precipitation of DNA in the aqueous phase with 0.8 vol isopropanol. Pellets were washed in 70% ethanol, dried and resuspended in TE buffer.

Analysis of RAPDs and RGAs

RAPD experiments followed the protocol of Quiros et al. (1991) using 39 selected decamer primers—sets A, B and C from Operon Technologies (Alameda, Calif.) and UCD from the University of California, Davis—which generated RAPD amplicons that were specific for individual B-genome chromosomes according to Chèvre et al. (1996) and Struss et al. (1996).

Conserved domains of R genes of the Toll-interleukin receptor-like-nucleotide-binding site-leucine-rich repeat

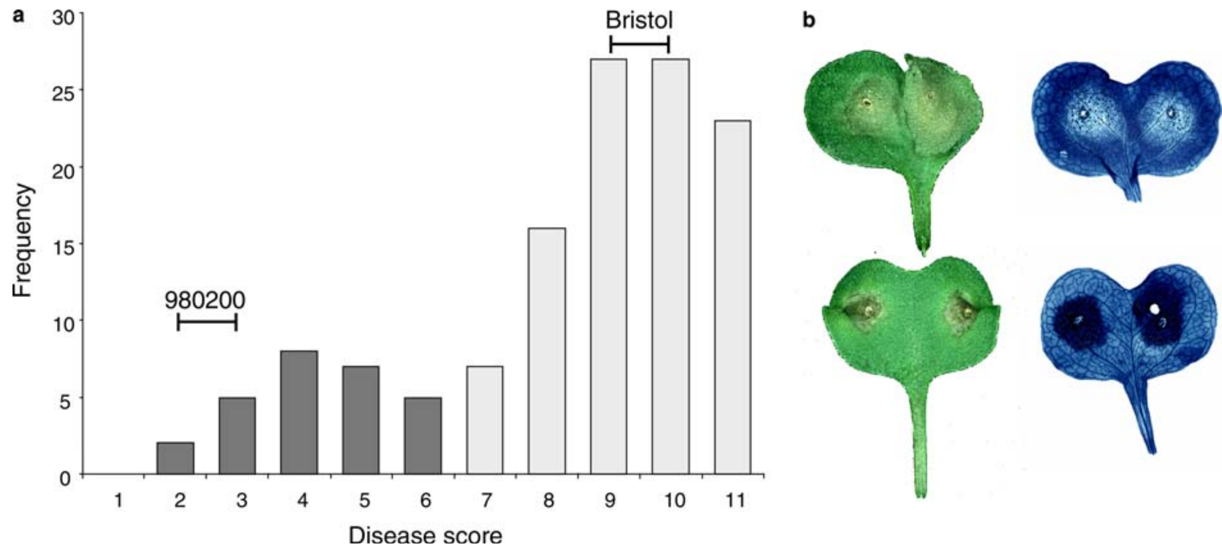


Fig. 2 a Frequency distribution of blackleg disease scores (1 = highly resistant, 11 = highly susceptible) in the segregating F_2 population 990149 (n = 127). Individuals with mean scores of 6 of lower were classified as resistant (*grey bars*); those with mean scores higher than 6 were classified as being susceptible (*light bars*). The response range is indicated for the resistant parent (980200) and the susceptible parent (cv. Bristol). **b** Cotyledon phenotype of blackleg susceptible (*top*) and resistant (*bottom*) individuals of F_2 population 990149 as

(TIR-NB-LRR) class (*Rpp5*, *N*, *L6*; Hammond-Kosack and Jones 1997) were used as the source for the design of degenerate sense and antisense primers for RGA amplification. Codon preference in the *Arabidopsis* and *Brassica* genes was taken into consideration in primer design, thus keeping the degree of degeneration low. Sense and antisense primer sequences, melting temperatures and domains are shown in Table 1. A 25-µl PCR reaction contained 50 ng template DNA, 1 μM each primer, 0.2 m M each dNTP, 1× reaction buffer (Qiagen, Hilden, Germany), a final $MgCl_2$ concentration of 2.5 m M and 1 U *Taq* polymerase (Qiagen). The PCR profile consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 45 s at 94°C, 45 s at 50°C (after a 2-min ramp time), 1 min 20 s at 72°C, with a final extension step at 72°C for 7 min. Reactions were carried out in a GeneAmp PCR system 9600 or 9700 (Applied Biosystems, Darmstadt, Germany). Amplification products were separated on 1.5% standard agarose or 3% NuSieve agarose gels (Biozym Scientific, Hess. Oldendorf, Germany), respectively, in 1× TAE.

Cloning of PCR products and development of SCAR markers

B-genome-specific RAPD and RGA amplicons associated with blackleg resistance were excised from the gel, diluted in ultra-pure water and re-amplified in a PCR using the conditions described above except for the 60-

Table 1 Degenerate sense and antisense RGA primers derived from conserved motifs of the TIR and NB-ARC region of plant disease resistance genes *Rpp5*, *N*, *L6* (Hammond-Kosack and Jones 1997). The theoretical melting temperature range was used in determining appropriate annealing temperatures for PCR

Strand	Primer ID	Sequence (5 → 3′)[a]	T_m range (°C)[b]	Domain in R genes[c,d]
Sense	s1	AATTATGCTTCTTCGACGTGSTG	55.3	TIR region 4/region 5
	s2	GTGATKCCRRTTTTCTACGACGTT	52.8–57.4	TIR region 5
	s3	GGAAGTWAGRAAACAGAMSGGMGA	53.7–55.7	TIR region 5/region 6
Antisense	as1	GACARYAGAASCCAAAGGRAGAC	51.0–58.0	NB-ARC HD motif
	as2	GAAGAARCATGCWATRTCYAAAAA (as2-1)	47.7–54.4	NB-ARC motif 1
		GAARAAACATGCWATGCACTTAAA (as2-2)	50.6–53.2	
	as3	TAACCYATCGTAGCTGATTTTKAG (as3-1)	47.4–54.4	NB-ARC motif 2
		TAAYCYATCGTAACCAACTCTKAG (as3-2)	44.8–54.2	
	as4	ACATCATCAAGAAGRATAAGRACYYTC	52.5–58.0	NB-ARC Kinase 2
	as5	ATTCTGCTWCCATTWCCAAACCA	52.3	NB-ARC Kinase 3a

[a]Letters for degenerate nucleotides are according to the IUPAC nucleotide code

[b]Calculated according to the formula T_m (°C) = 81.5°C + 16.6(log$_{10}$[Na$^+$]) + 0.41(%[G + C]) – 600/L, with L = oligonucleotide length (Sambrook et al. 1989)

[c] TIR = Toll and Interleukin-1 receptor domain; regions were defined according to Hammond-Kosack and Jones (1997)

[d]NB-ARC, Nucleotide binding adaptor domain. Motif designations are as described in van der Biezen and Jones (1998)

Table 2 Primer sequences, annealing temperatures of SCAR markers B5-1520, C5-1000 and RGALm, and sizes of amplification products in the investigated plant materials

Marker	Primer sequence ($5' \rightarrow 3'$)[a]	Annealing temperature (°C)	Product size/sizes (bp) in investigated plant materials	
B5-1520	f: TGCCTTTCTCACTTCTTCTCTC r: AGCGTCTATGTCGGTCTTTCAA	53	Approx. 1,520	Introgression in 980200, 980204 980201, 990061, 990149 *Brassica nigra*, *B. juncea*, *B. carinata*
C5-1000	f: GTGGAAGAAGAGTTAGGGATAGAGC r: TTGGATAGAGAAAATGGAAGTTGTT	55	Approx. 1,000	Introgression in 980200, 980204 980201, 990061, 990149 *B. nigra*, *B. juncea*, *B. carinata*
RGALm	f: TTCGAAAACAGAGCGGTGATTT r: CCCCTTCATCGTCCCTAATAAA	52	288[b], 290	Introgression in 980200, 980204 980201, 990061, 990149
			290	*B. juncea*, *B. carinata*
			287	*B. nigra*
			288[b], 305	*B. napus* cv. Andor,
			288[b], 316	*B. napus* cvs. Bristol, Falcon, Lirabon

[a]f, Forward; r, reverse
[b]Only visible after denaturing polyacrylamide gel electrophoresis

min final extension phase in the PCR in order to create A-overhangs. Purified PCR products (QIAquick kit, Qiagen) were cloned into the pGEM TEasy vector (Promega, Mannheim, Germany) according to the manufacturer's instructions. DNA inserts were checked for the appropriate size by PCR, and positive clones were sequenced using BigDye Terminator cycle sequencing on an ABI 377 Genetic Analyzer (Applied Biosystems). Compatible primer pairs for SCARs were designed using the primer3 web interface (http://frodo.wi.mit.edu/cgi-bin/primer3; Rozen and Skaletsky 2000). SCARs were amplified in a 25-μl reaction mixture containing 50 ng template DNA, 0.25 μM of each primer, 0.1 m M each dNTP, 1.5 m M $MgCl_2$ and 1U *Taq* polymerase and subjected to the following PCR profile: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 1 min, with a final elongation step at 72°C for 7 min. Annealing temperatures for the individual SCAR primer pairs are given in Table 2. Products were separated on an agarose gel as described above or on 6% denaturing polyacrylamide gel on an ABI 377 Genetic Analyzer.

Genomic walking

Based on the sequence of a 422-bp-sized RGA PCR product (s2/as5, Table 1), we followed the protocol of Siebert et al. (1995) and designed two primers near each end of the clone in the outward direction (genome-specific primers) in order to obtain flanking sequence information through genomic walking. Genomic libraries were generated with the restriction enzymes *Eco*RV, *Pvu*II, *Sca*I and *Ssp*I. The sequences of adapter and adapter primer were modified in order to accommodate genome-specific primers. Specific products were excised from the gel, purified, cloned and sequenced as described above.

Data analysis and database searches

Cross tabulation of marker associations versus cotyledon disease phenotype was performed using the TABLE function of the R software package (Ihaka and Gentleman 1996).

Nucleotide sequences were aligned using the BIOEDIT program v4.8.6 (T. Hall, North Carolina State University; http://www.mbio.ncsu.edu/BioEdit).

Homology searches against non-redundant nucleotide and protein databases at NCBI (http://www.ncbi.nlm.gov) were performed with the BLASTN or BLASTX (version 2.2.9) algorithm (Altschul et al. 1997), respectively, with the following parameter settings: for BLASTN, a word size of 7, gap penalties of 5 for existence and 2 for extension; for BLASTX, the BLOSUM80 substitution matrix and gap penalties of 10 for existence and 1 for extension.

Results

Identification of introgression-specific RAPDs and development of SCARs

For RAPD marker screening, the analysis was limited to a set of decamer primers which amplified B-genome-specific RAPD markers in previous studies (Chèvre et al. 1996; Struss et al. 1996). None of the reported markers could be found in resistant plants of lines 980200, 980201 and 980204 and in the B-genome species *B. nigra*, *B. juncea* and *B. carinata*. However, two B-genome-specific bands, OPB-05-2040 and OPC-05-1700, were identified from two primers, and these were also associated with the resistant phenotype in the segregating lines 980201 and 990061. These amplicons were also present in the three B-genome species. Both bands were excised from the gel, cloned, sequenced and converted into SCARs B5-1520 and C5-1000. Primer sequences and sizes of amplification products in the respective

plant materials are given in Table 2. Comparison of the two sequences spanning 650 bp from each end of the OPB-05-2040 segment among resistant recombinant lines and the B-genome species yielded more than 99% sequence identity, thus confirming B-genome-specificity. Co-segregation with their corresponding RAPD patterns and the strong association with seedling resistance in the segregating line 990061 indicated that both SCAR markers are located on the introgressed segment of the B genome that confers the resistance. Neither SCAR marker was present in the two recombinant lines developed by Plieske et al. (1998) that exhibit stem resistance, which indicates that a different B-genome segment was introgressed in their material.

Isolation and characterization of RGAs

The above panel was also screened with 12 compatible sense/antisense primer combinations derived from conserved motifs of TIR and NB-ARC domains (Table 1). Complex amplification patterns were obtained in all cases, as shown in Fig. 3. Although polymorphic, and possibly allelic, bands were observed in combinations involving primers s1 and s3, these were not associated with the resistance phenotype and therefore were not further investigated. With primer combination s2/as5, a polymorphic band of 422 bp was generated that appeared not only in resistant individuals but also in the B-genome parent *B. juncea* (Fig. 3). This band has been re-amplified, cloned and sequenced. The high similarity to the TIR and NB-ARC domains confirmed that the sequence is part of a resistance gene or RGA. Nested primers were designed so as to create a SCAR marker (RGALm; Table 2) and to amplify homologous sequences from *B. nigra* and *B. carinata*. RGALm yielded a 290-bp PCR product in the blackleg-resistant introgression lines, *B. juncea* and *B. carinata* and a 287-bp large amplicon in *B. nigra*, with sequence identities ranging between 95% and 100% (Fig. 4). As with the RAPD-derived SCAR markers, this B genome-specific segment was not observed in the stem-resistant material of Plieske et al. (1998). In addition, a PCR product of 305 bp was amplified in *B. napus* cv. Andor and the introgression lines. In *B. napus* cv. Bristol and several other winter-type cultivars (data not shown), this band was substituted by a 316-bp product (Table 2). The difference in sequence between the B-genome-specific RGA and both the 305-bp and 316-bp *B. napus*-specific RGA is accounted for by a 19-bp deletion and several nucleotide substitutions, as revealed by sequence analysis, whereas the polymorphism in the *B. napus*-specific locus is mainly due to a 12-bp insertion in cv. Bristol (Fig. 4). When products were separated by denaturing polyacrylamide gel electrophoresis, an additional band could be identified. This band, 288 bp in size, was present in the introgression lines and all of the *B. napus* cultivars tested.

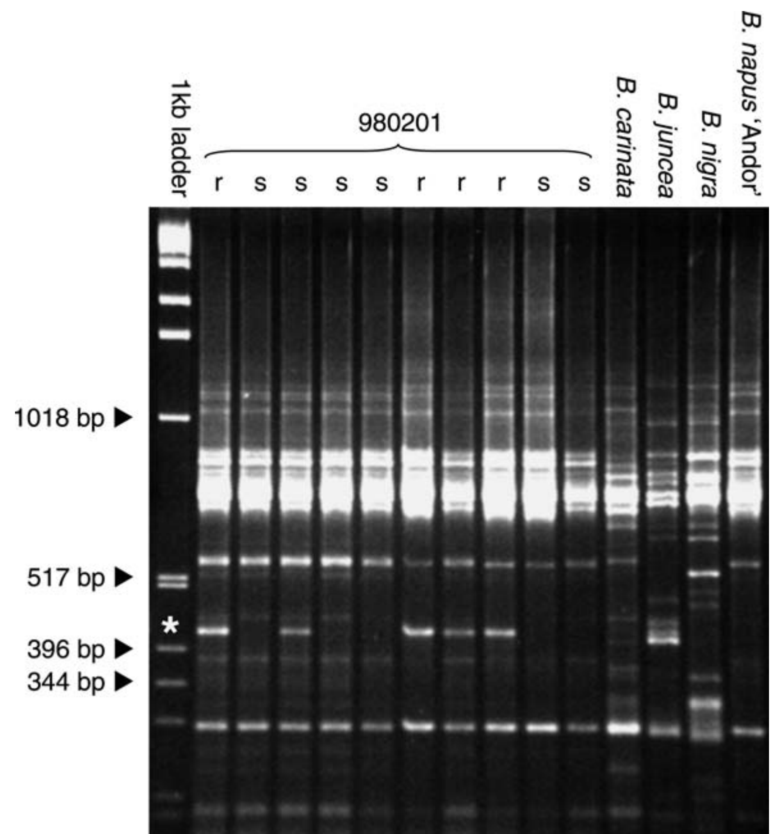


Fig. 3 Amplification pattern of RGAs generated with primer pair s2/as5. Ten individuals of the segregating line 980201 were screened. The polymorphic 422-bp band (*white asterisk*) was present in all blackleg-resistant progeny (*r*). Progeny lacking the band were susceptible (*s*). A band of similar size is present in *B. juncea*, the resistance donor

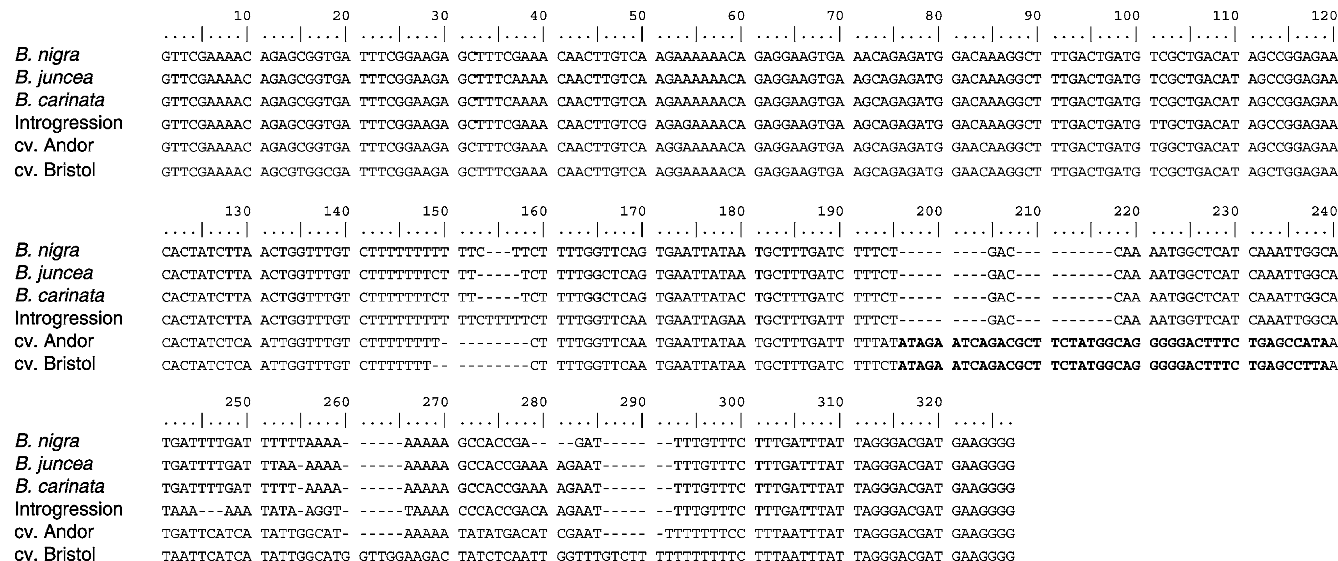


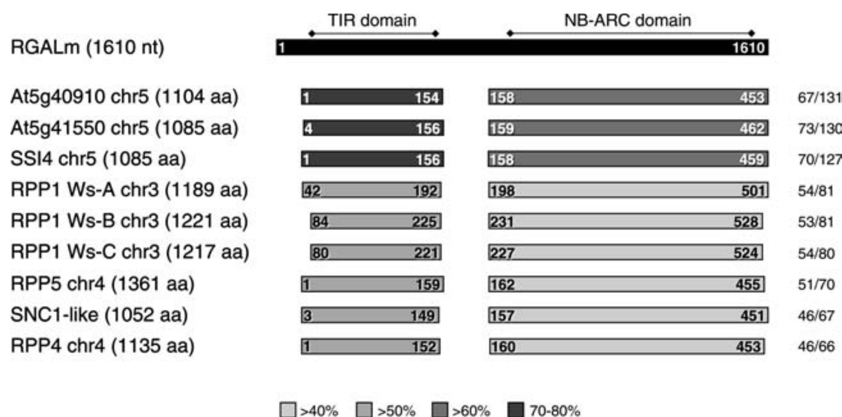
Fig. 4 Nucleotide sequence comparison of amplification products generated by the RGALm primer pair. The larger segments amplified in the *B. napus* cvs. Andor and Bristol (*grey*) are characterized by an insertion and several base substitutions (*bold*) compared to the B-genome-specific segment. The allelic sequences of cvs. Andor and Bristol differ from each other by yet other insertions and substitutions and represent a marker locus that is unlinked to the introgression site

Sequence homology to *Arabidopsis* genes

In order to further characterize the cloned and sequenced B-genome-specific, 422-bp PCR product, we obtained a flanking sequence by genomic walking, which yielded a 1,610-bp *Eco*RV fragment that stretched beyond the TIR and NB-ARC domains (Fig. 5). Homology searches using both the BLASTX and BLASTN algorithms resulted in several significant hits (*E*-values < 0.01), the most prominent being two putative resistance genes (At5g40910, At5g41550) and the *Ssi4* gene (Shirano et al. 2002) (BLASTX *E*-values $< 10^{-100}$) that reside on a R-gene cluster on *Arabidopsis* chromosome 5, called the major recognition complex MRC-J (Holub 1997). For these genes, the homologous regions overlapped with predicted or actual open reading frames coding for the TIR and NB-ARC domains and had sequence identities of greater than 70% or greater than 60%, respectively, to the *Eco*RV fragment (Fig. 5). Significant homologies were also detected to well-characterized R genes such as *Rpp1*, *Rpp4*, *Rpp5* (powdery mildew), *L6* (flax rust) and *N* (tobacco mosaic virus) (*E*-values $< 10^{-20}$).

Homology searches with BLASTN were also conducted with partial sequence information of the resistance-associated and B-genome-specific RAPD markers OPB-05-2040 and OPC-05-1700. No significant sequence homologies were found for about 1,300 nucleotides of OPB-05-2040, whereas the most significant hits for a 600-bp and a 650-bp sequence of the 5′ or 3′ end, respectively, of OPC-05-1700 were exons of the gibberellin-2-oxidase gene 3 (*E*-values: 4×10^{-56} and

Fig. 5 Results of a BLASTX homology search using the 1,610-nucleotide *Eco*RV fragment derived from RGALm as query. The reading frame translated from the query sequence that matches to the homologous regions is the same for all resistance proteins shown. The first and last amino acid positions in a homologous amino acid stretch are indicated. The *numbers* on the *right* are rounded −log *E*-values for the TIR domain (*left*) and the NB-ARC domain (*right*), respectively. The degree of amino acid sequence identity is represented by *color-gradated boxes* according to the legend at the bottom



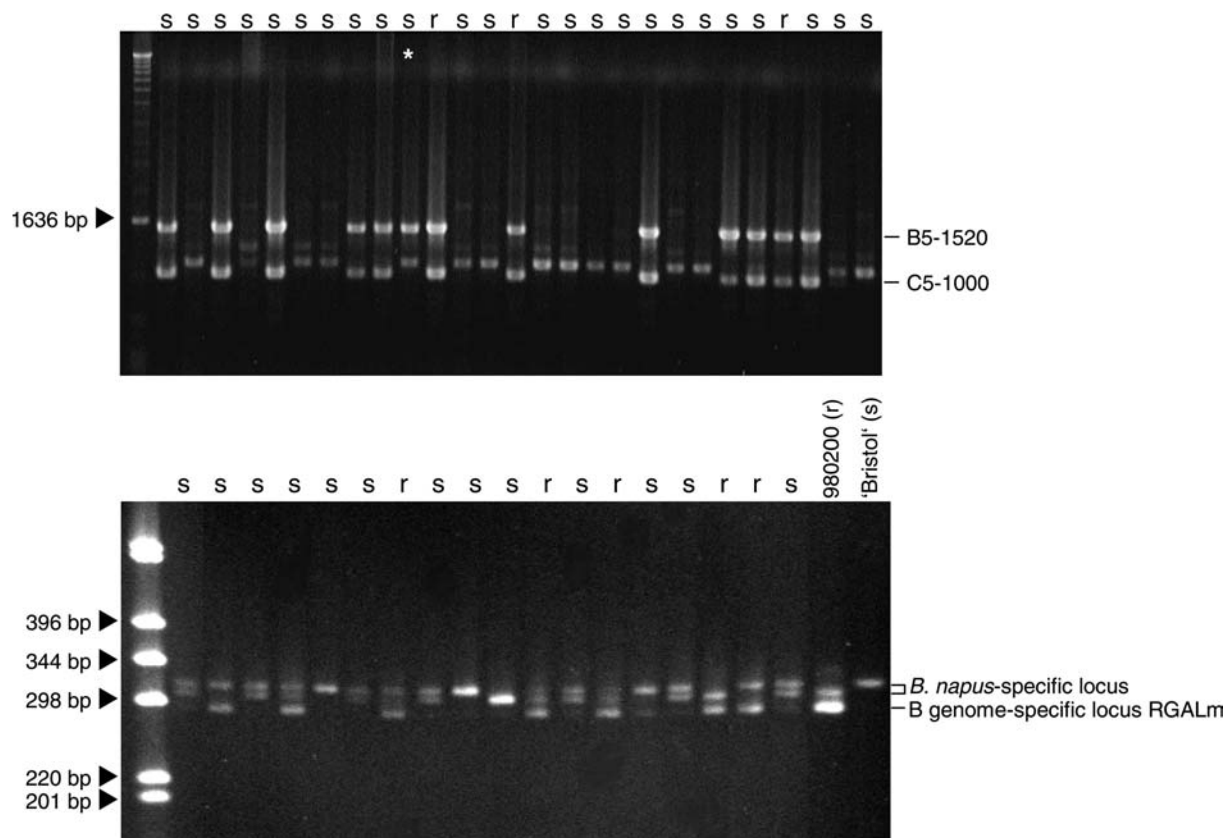


Fig. 6 Analysis of B-genome introgression-specific markers in the F_2 population 990149. *s* Susceptible, *r* resistant. *Top* RAPD-derived SCAR markers B5-1520 and C5-1000 assayed together in a 1% standard agarose gel. A recombinant individual is indicated by an *asterisk*. *Bottom* *B. napus*- and B-genome-specific amplification products generated by the RGALm primer pair, separated on a 3% NuSieve agarose gel. The patterns of the resistant (980200) and susceptible parent (cv. Bristol) are shown on the *right*

2×10^{-80}, respectively) that is located on *Arabidopsis* chromosome 2.

Analysis of SCAR markers linked to the $r_j lm2$ locus

Previous segregation analyses of cotyledon resistance (Saal et al. 2004) supported a monogenic recessive inheritance, as indicated by the histogram in Fig. 2a. The segregating line 990061, which carries the genetic background of the susceptible recurrent parent cv. Andor, and the F_2 population 990149, derived from a cross between the resistant introgression line 980200 and the *B. napus* cv. Bristol (Fig. 1), were typed with the dominant B-genome introgression-specific SCAR markers (Fig. 6). A total of 170 individuals were analyzed. Although the ratio of resistant to susceptible progeny fitted a 1:3 segregation in both populations (Saal et al. 2004), all markers of the B-genome introgression deviated from a Mendelian 3:1 ratio in so far as marker phenotypes lacking the introgressed allele were much more frequent than the expected 25% (43% in 990061, 46–56% in 990149). As expected, all resistant plants showed the

Table 3 Cross table of pairwise SCAR marker frequencies in blackleg-resistant and -susceptible progeny calculated from joined data of the segregating line 990061 and F_2 population 990149. Note that for all marker combinations, the null-allele is not observed in resistant individuals

SCAR marker[a]		Blackleg resistant[b]				Blackleg susceptible[b]			
		C5-1000		RGALm		C5-1000		RGALm	
		+	–	+	–	+	–	+	–
B5-1520	+	28	0	36	0	27	12	32	10
	–	0	0	0	0	0	57	6	52
C5-1000	+			25	0			21	1
	–			0	0			8	47

[a] +, Amplification; -, no amplification (homozygous for null-allele)
[b] Resistant, mean cotyledon disease score $\leq$ 6; susceptible, mean cotyledon disease score $>$ 6

dominant phenotype in each of the SCAR markers (Table 3, Fig. 6). The coupling phase linkage between the markers was also evident from the high frequency of null-allele associations in susceptible progeny (Table 3). The less frequent but still considerable number of susceptible progeny carrying all of the introgressed marker alleles indicated linkage in repulsion phase to the r_j *lm2* resistance locus. Due to the distorted single marker segregation ratios, standard maximum-likelihood estimates of recombination frequencies would be biased. Both gametic and zygotic selection would give rise to the data. Lorieux et al. (1995) suggested the use of the product formula (Fisher and Balmakund 1928) when the selection type is unknown. Recombination frequency estimates are 0.10 ± 0.05 for RGALm/B5-1520 and 0.05 ± 0.04 for RGALm/C5-1000 (undefined for B5-1520/C5-1000).

The 305- and 316-bp segments amplified by the RGALm primer pair in population 990149 were allelic. This codominant locus fitted a 1:2:1 ratio in 31 individuals ($P = 0.76$) and was unlinked to the B-genome introgression and the resistance locus (Fig. 6).

Discussion

The B genome of *Brassica* has been identified as a valuable source for broadening and enhancing resistance in oilseed *Brassica* crops against *L. maculans* (Roy 1978; Sacristán and Gerdemann 1986) and several researchers have succeeded in developing addition and introgression lines conferring blackleg resistance in the seedling and adult stages (Roy 1984; Zhu et al. 1993; Chèvre et al. 1996; Pang and Halloran 1996; Dixelius 1999). As in these materials resistance was inherited by one or few genes acting in a dominant manner, characterization by molecular markers was straightforward (Chèvre et al. 1997; Plieske et al. 1998; Dixelius and Wahlberg 1999). In the present investigation, near-isogenic lines showing a different response to blackleg at the seedling stage (Saal et al. 2004) were available for screening molecular markers associated with a B-genome introgression conveying resistance. As the results from previous analyses suggested recessive inheritance by a single locus, called r_j *lm2* (Saal et al. 2004), the application of PCR markers that are simple to assay, such as the SCAR markers presented in this study, could lead to the rapid introgression of this resistance into elite breeding material. B-genome chromosome-specific RAPD markers reported by Chèvre et al. (1996, 1997) and Struss et al. (1996), some of which were linked to blackleg resistance, were not detected in the screening panel of the present investigation. This result confirms the lack of reproducibility of RAPD markers often observed in plant genome analysis (Penner 1996). Conversely, RAPD markers OPB-05-2040 and OPC-05-1700, which were not detected in previous studies but showed correlation with the resistance phenotype in our material, indicated different genes involved in resistance. In the course of developing SCAR marker B5-1520, a sequence comparison to *B. nigra*, *B. carinata* and *B. juncea* provided further evidence for the B-genome origin of the introgression. Although SCAR marker C5-1000 was not sequenced, its B-genome origin is likely because the band was detected in all of the B-genome species but was absent in 12 *B. napus* cultivars (data not shown). Moreover, C5-1000 is tightly linked to B5-1520. Both markers were also absent in stem-resistant recombinant *B. napus* lines that possess B-genome introgressions derived from *B. juncea* and *B. carinata* (Plieske et al. 1998). For these lines, Plieske and Struss (2001) developed a RFLP-based sequence-tagged site (STS) marker. The B-genome-specific allele of this marker was not found in the introgression lines of our study, thereby demonstrating introgression of two different B-genome-specific segments in both materials.

We followed a targeted approach by searching for RGAs as candidate genes or linked markers for blackleg seedling resistance as gene-for-gene relationships have been described in detail in the *L. maculans*-*B. napus* interaction (Rimmer and van den Berg 1992). Major classes of R genes have been identified, such as the TIR-NB-LRR, CC (coiled/coil)-NB-LRR, LRR-transmembrane (TM) and the protein kinase classes (Baker et al. 1997; Dangl and Jones 2001). As a result, researchers have focused on mapping and cloning of those RGAs mainly involving conserved NB and LRR motifs, and in several reports co-segregation between RGAs and resistance genes has been described (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Collins et al. 1998; Shen et al. 1998). In this study, we have focused on the TIR and NB domains for RGA analysis for the following reasons: (1) both domains contain well-conserved motifs; (2) this R gene class plays a role in many fungal pathogen-host interactions of *Arabidopsis*, a distant relative of *Brassica*; (3) the hypersensitive response (HR)-like response observed in the *L. maculans*-*Brassica* interaction (Saal et al. 2004); (4) the wide distribution of this class over the *Arabidopsis* genome (Meyers et al. 1999). Sequence analysis of a RGALm-derived *Eco*RV fragment revealed a resistance gene-like sequence which showed highly significant homology at the nucleotide and amino acid level to two putative R genes and the *Ssi4* gene on a resistance gene cluster on chromosome 5 of *Arabidopsis*. In *Brassica* species, RGAs have already been genetically analyzed and mapped in *B. napus*, *B. rapa* and *B. oleracea* (Joyeux et al. 1999; Sillito et al. 2000; Fourmann et al. 2001; Vicente and King 2001; Tanhuanpää 2004). However, linkage or co-segregation of RGAs to disease resistance loci has not yet been reported. Fourmann et al. (2001) found one RGA marker that maps to a region which has been identified as a quantitative trait locus in another population conferring adult resistance to blackleg.

Sequence homology searches not only identified resistance genes of the MRC-J cluster (Holub 1997) as being homeologous to the RGALm locus but also

identified the gibberellin 2-oxidase gene on *Arabidopsis* chromosome 2 as a putative homeolog of the OPC-05-1700 RAPD segment. It is speculative to argue whether both B-genome-derived sequences are orthologs of these *Arabidopsis* genes and if the introgression has a syntenic *Arabidopsis* counterpart since considerable duplication events and chromosomal rearrangements have occurred between *A. thaliana* and *B. nigra* following their divergence from a common progenitor genome (Lagercrantz 1998). Subsequent investigations will clarify if a proximal segment of *B. nigra* chromosome G8 (Lagercrantz 1998), which is composed of syntenic regions of *Arabidopsis* chromosomes 2 and 5, corresponds at least in part to the introgression.

All SCAR markers in this study were strongly associated with each other and to the $r_j lm2$ locus. Astonishingly, all markers showed an extreme distortion of the expected segregation ratio for dominant markers in the selfed progenies or F_2 populations. In general, negative selection on gametes or zygotes is held responsible for skewed segregation ratios. Since the SCAR markers in this study are dominant, it is unclear whether the frequency of homozygotes or heterozygotes were reduced. However, the higher than expected number of progeny without the introgression suggests a reduced transmission or viability of the gametes or zygotes, respectively, carrying the introgression by a yet unknown mechanism. Plieske and Struss (2001) also observed segregation distortion for restriction fragment length polymorphism (RFLP) and amplified (A)FLP markers linked to a blackleg resistance gene, with the B-genome-derived alleles being less frequent. Another—though unlikely—explanation for the marker segregation is that both progenies 990061 and 990149 were backcrosses or that inadvertent cross pollination from neighboring *B. napus* plants occurred. If either premise were valid, all plants that carried introgression marker alleles would have been heterozygous and thus would be segregating in selfed progenies. However, if this were true, markers would only be loosely associated with the resistance trait. The observed recombination between the SCAR markers could have occurred at a homeologous position of the *B. napus* genome or by the loss of introgression segments as a result of instability of a translocation. In the latter case, recombinant individuals would have arisen through breakage and fusion, which could have affected their vigor. However, we did not observe any loss in vigor.

In order to elucidate the reason for the skewed marker segregations, both the trait locus and markers will be re-analyzed in $F_{2:3}$ families. The influence of negative selection on male and/or female gametes will be studied in reciprocal backcrosses. Alternatively, large doubled haploid populations could be analyzed to see if the same direction of marker distortion would emerge. These will be main tasks in future studies as well as the identification of co-dominant markers in the region around the introgression. The question as to whether the introgression has occurred through homeologous recombination or translocation will be addressed by Southern hybridization using the SCAR markers and the *Eco*RV fragment as probes and by the identification of RFLP patterns co-segregating with the dominantly inherited PCR markers.

The fact that several recombination events were observed in all marker intervals (including the resistance locus) provides the opportunity of reducing the size of the introgression as well as the possibility of genetic and physical fine mapping.

Acknowledgements The authors greatly acknowledge financial support by the EU (FAIR-CT97-3509). We are also grateful to M. Hantschmann, B. Schmidt and B. Brückner for excellent technical assistance and to W.E. Weber for his critical reading of the manuscript.

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