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STS markers linked to *Phoma* resistance genes of the *Brassica* B-genome revealed sequence homology between *Brassica nigra* and *Brassica napus*

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Abstract The RFLP and AFLP techniques are laborious and expensive and therefore of limited use for marker-assisted selection, demanding a high throughput of samples in a short time. But marker-assisted selection is most useful for traits which are hard to score on single plants and influenced by environmental factors. Four RFLP and three AFLP markers have been found to be linked to genes of the B-genome of *Brassica* mediating resistance against *Phoma lingam* in oilseed rape. One RFLP and one AFLP marker were converted into three PCR-based STS markers: one of dominant, as well as one of codominant inheritance separated in a standard agarose gel and a third one of codominant inheritance to be separated in a polyacrylamide gel on an automated sequencer. As expected, the STS markers mapped at the same position as the original RFLP and AFLP markers. The STS markers are efficient in marker-assisted backcross programs of the resistant B-genome/*Brassica napus* recombinant lines with most of the tested oilseed rape varieties and breeding lines. More than 90% of the tested oilseed rape varieties and breeding lines exhibited no resistance marker alleles. The mapping results obtained with the markers, as well as comparative sequencing of the marker alleles, indicate synteny and homology between the B-genome resistance gene donors and *B. napus* in the region of the resistance genes. The location of the resistance genes in the B-genome/*B. napus* recombinant lines is most likely on the A genome. Thus the transfer of the B-genome resistance genes into *Brassica campestris* is also possible.

Keywords *Brassica napus* · *Phoma lingam* · B-genome · Resistance · Molecular markers

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Introduction

Blackleg, caused by *Phoma lingam* (Tode ex Fr.) Desm. [anamorph of *Leptosphaeria maculans* (Desm.) Ces & De Not.], is one of the most important diseases affecting oilseed rape world wide. Since resistance of cultivated oilseed rape against blackleg is polygenically controlled and only partial, the B-genome species of *Brassica*, i.e. *Brassica nigra* (BB, 2n=16), *Brassica carinata* (BBCC, 2n=34) and *Brassica juncea* (AABB, 2n=36), are used as sources of *Phoma* resistance in oilseed rape breeding. The B-genome resistance is oligogenically controlled and shows a high level of resistance (Sacristán and Gerdemann 1986; Sjödin and Glimelius 1988; Gugel et al. 1990; Chèvre et al. 1996; Struss et al. 1996). The transfer of B-genome resistance genes into oilseed rape to improve resistance or to accumulate different genes can be accelerated by using molecular markers.

Genetic marker techniques such as RFLPs and AFLPs have rapidly become indispensable tools in plant molecular-genetic analysis. They are widely used for phylogenetic studies, construction and improvement of genetic maps, mapping of important agronomic traits and particularly AFLPs for marker saturation of specific DNA regions for map-based cloning (Ballvora et al. 1995; Thomas et al. 1995; Folkertsma et al. 1996; Powell et al. 1996). Nevertheless, applications in marker-assisted breeding programs for specific genes are limited. Extensive sample preparations make both techniques time- and cost-consuming. Thus high throughput in combination with low input is impossible.

To overcome these problems, conversion of RFLPs and AFLPs into PCR-based markers is recommended (Niewöhner et al. 1995; Penner et al. 1995; Bradeen and Simon 1998; Shan et al. 1999). But often the design of PCR primers on the basis of the sequences of the RFLP probes or polymorphic AFLP fragments yields monomorphic amplification products because of predominantly homologous sequences of the different genotypes and tolerable mismatches at primer 5´-ends. Thus sequence information on regions adjacent to the marker fragment is essential.

The aim of the present study was to convert RFLP and AFLP markers linked to *Phoma* resistance genes derived from the B-genome of *Brassica* into STS markers. This was achieved by sequencing the regions adjacent to the original markers. The efficiency of the STS markers for marker-assisted breeding programs has been demonstrated.

Material and methods

The plant material used for this investigation consisted of two different B-genome/*B. napus* recombinant lines carrying *Phoma* resistance genes either of *B. juncea* (juncea-line) or of *B. carinata* (carinata-line). The development of the recombinant lines and segregating populations (F_2s) was previously described (Plieske et al. 1998). Furthermore, 32 oilseed rape cultivars and breeding lines, provided by European breeders, were used in this study. In order to transfer the *Phoma* resistance genes into winter oilseed rape, cultivars were crossed with the resistant recombinant lines and F_1s were selfed to obtain segregating populations. The resistance of plants was tested and plant DNA isolated, the RFLP technique being performed according to Plieske et al. (1998).

The AFLP method was used following the protocol of KeyGene (Vos et al. 1995) with minor modifications. AFLP fragments were isolated from polyacrylamide gels by re-moisturing the gel matrix and scratching the polymorphic fragments from the glass plate. Each fragment was dissolved in sterile water, re-amplified according to the AFLP protocol with the same primer combinations (PCs), separated on a 4% NuSieve GTG (Biorad) agarose gel and extracted with the Gel Extraction Kit (Qiagen). Finally, the fragments were cloned into the pGem-T Easy vector (Promega) and sequenced using the ABI 377 sequencing system. The polymorphic RFLP probes were sequenced as well.

The regions adjacent to the polymorphic fragments were cloned and sequenced using the GenomeWalker-Universal Kit (Clontech) following the manufacturer's protocol. In detail, genomic DNA libraries were constructed by digestion of plant DNA of the susceptible oilseed rape parent and the resistant recombinant lines with different restriction enzymes leaving blunt-end restriction sites. Subsequently, adapters were ligated to both ends of the restriction fragments. PCR was carried out with adapter-specific and fragment/probe-specific primers amplifying sequences upstream of and downstream from the polymorphic fragment or probe. The amplified locus-specific fragments were isolated from agarose gels, extracted with the Gel Extraction Kit (Qiagen), cloned into the pGem-T Easy vector (Promega) and sequenced using the ABI 377 sequencing system. Sequences of the susceptible and resistant genotypes were compared using the Sequencher 3.0 program (Gene Codes Corporation). Primers were designed using Primer 0.5 (E. Lander, Cambridge, Mass., USA).

For PCR with STS markers 100–150 ng of template DNA, 250 nM of each primer, 200 µM of dNTPs, 0.8 U of *Taq* Polymerase and 1.5 mM of $MgCl_2$ were mixed. Forty five cycles of amplification (denaturing at 94°C for 1 min; annealing at 55°C, 60°C or 65°C, depending on the primer pair, for 1 min; extension at 72°C for 2 min) were followed by a final extension step at 72°C for 1 h. The amplification products were either detected on 36-cm 4.5% polyacrylamide gels (29:1 acrylamide:bisacrylamide) using the ABI 377 sequencing system or on 2% agarose gels using a Gibco BRL electrophoresis unit. The forward primer of the marker for analysis on the ABI 377 sequencer was labeled with FAM and the results were analyzed using GeneScan 3.0 and Genotyper 2.0 (Perkin Elmer). The fragments separated on agarose were stained with ethidium bromide and the results were digitalized using a black and white camera under UV-illumination.

For linkage analysis, data were transferred into a binary 1/0 matrix (1=fragment present and plant resistant, 0=fragment absent and plant susceptible). The arrangement of the markers and resistance genes within the 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), max. recombination frequency ≤40% and min. LOD score ≥3,0]. Mendelian segregation was checked using the chi-square goodness-of-fit test.

Results

The AFLP technique was used to saturate the DNA region of the *Phoma* resistance genes, which had already been mapped by four RFLP markers (Plieske et al. 1998). Two hundred and five different AFLP primer combinations (PCs) were screened in bulked segregant analyses with segregating populations of the B-genome/*B. napus* recombinant lines. Three PCs were identified in both populations showing polymorphisms between resistant and susceptible genotypes. These markers were mapped between 1 and 12.1 cM from the resistance genes using 92 F_2 plants of each of the segregating populations (Fig. 1). Comparing both lines, the arrangement of all markers within the linkage groups and the distances between them, as well as to the resistance genes, were similar.

In order to develop STS markers for the resistance genes, RFLP probes and polymorphic AFLP fragments were sequenced. On the basis of the sequence information, locus-specific PCR primer pairs were designed. However, the amplified PCR fragments were all monomorphic and the DNA sequences of the respective fragments of the resistant and susceptible genotypes were identical. Thus, the DNA regions adjacent to the original RFLP and AFLP markers were sequenced.

Using the restriction enzymes *Dra*I, *Eco*RV, *Pvu*II, *Sca*I and *Stu*I genomic DNA libraries were constructed and served as templates for PCR. Amplification products polymorphic between susceptible and resistant genotypes were isolated and sequenced for the following markers.

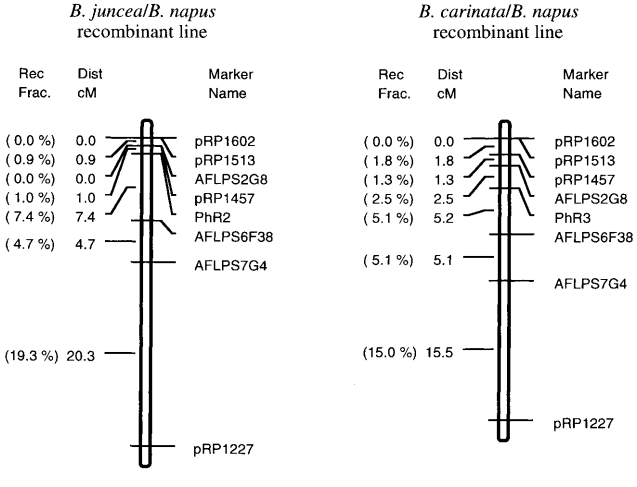


Fig. 1 Linkage of polymorphic markers and resistance genes (PhR 2+3) in F_2 plants of cross progenies of the recombinant lines. Distances between loci are in cM and recombination frequencies in %

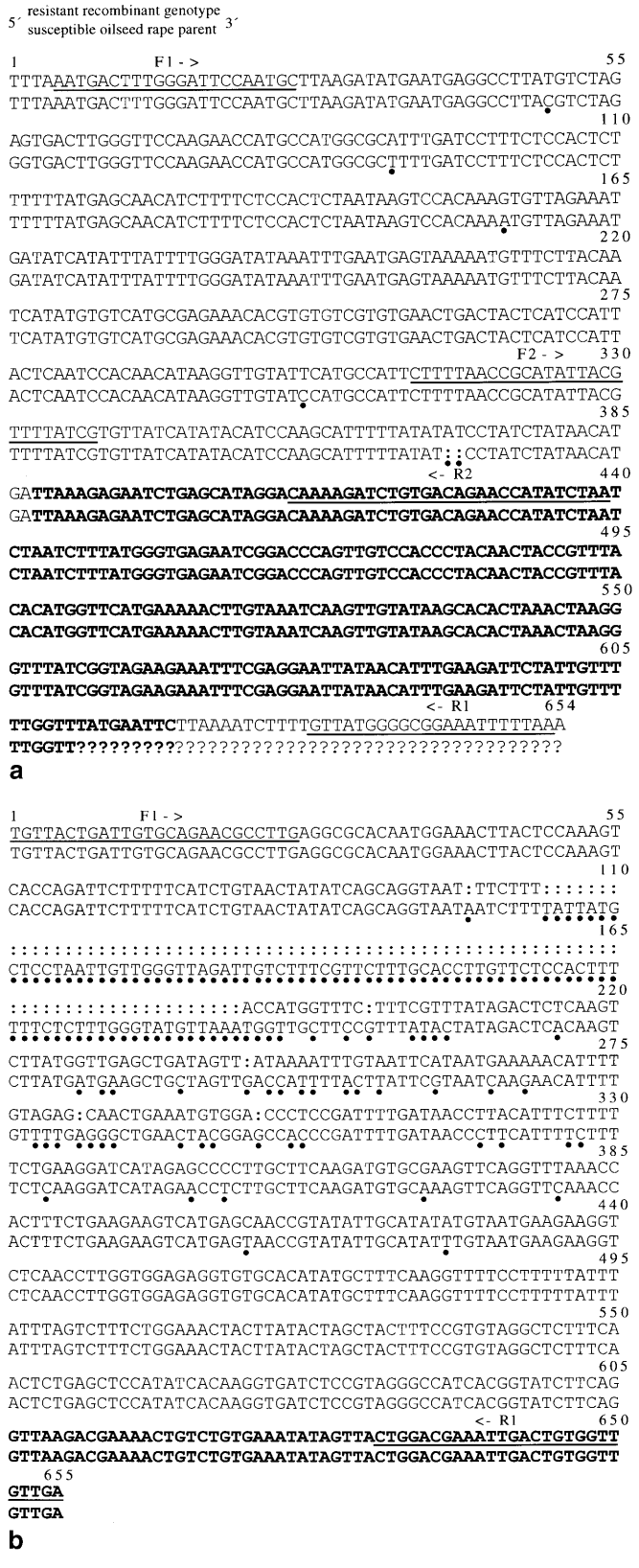


Fig. 2 Extended DNA sequence of the AFLP marker fragment S7G4 (**a**) and the RFLP probe pRP1513 (**b**). PCR primers are *underlined*, differences among the resistant recombinant line (upper sequence) and the susceptible genotype (lower sequence) are marked by *black points*, the original AFLP marker fragment and the 5´-end of the RFLP probe are marked *bold*

AFLP marker S7G4: on both sides of the fragment from the resistant recombinant genotype but only on the 3´ end from the susceptible parent,

RFLP marker pRP1513: on the 5´ end of the probe from both resistant and susceptible genotypes.

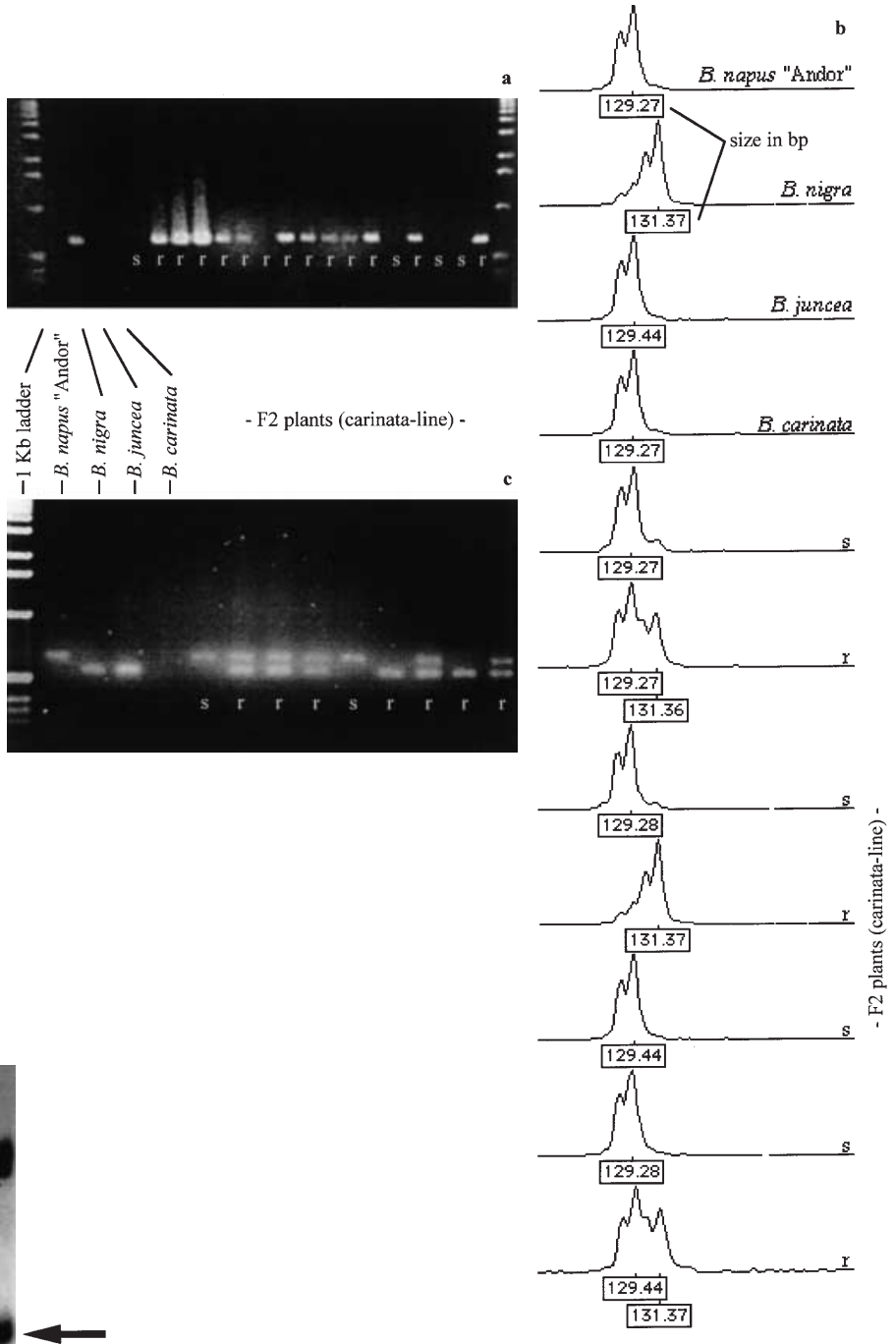
The sequences of the extended fragments are shown in Fig. 2.

Concerning the other RFLP and AFLP markers (see Fig. 1), either no PCR products were amplified or the DNA sequence of the amplified products of the different genotypes was identical. But, using other restriction enzymes for construction of the genomic DNA libraries and continuation of "walking", the conversion of these markers should be successful too.

As differences between the resistant genotype and the susceptible oilseed rape parent in the DNA region adjacent to the original RFLP probe, an 83-bp deletion and a number of base exchanges within an area of approximately 150 bp were identified. The rest of the region including the original RFLP probe was identical (Fig. 2b). On the 5´ side of the AFLP fragment the differences between the susceptible oilseed rape parent and the resistant genotype were represented by several point mutations and by a deletion of one AT-repeat of a short "microsatellite". On the 3´ side of the fragment there was no information about the DNA sequence of the susceptible parent because PCR products were amplified only from the resistant recombinant genotype (Fig. 2a).

PCR primer pairs were designed as indicated in Fig. 2 in order to amplify the described polymorphisms. The results are shown in Fig. 3. The polymorphism of 83 bp of the RFLP-based STS marker was amplified and discriminated the different genotypes showing codominant inheritance (Fig. 3c). The same was true for the 2-bp polymorphism of the AFLP-based STS marker (Fig. 3b). The entire extended AFLP fragment was amplified only from the resistant genotypes and showed dominant inheritance (Fig. 3a). The AFLP-based STS markers were not amplified from the resistance gene donor parents *B. juncea* and *B. carinata*, but from *B. nigra*; whereas the RFLP-based STS marker was not amplified from *B. carinata*, but from *B. nigra* and *B. juncea*. On the DNA level, *B. juncea* showed some base exchanges in comparison with the resistant genotype of the juncea-line. The RFLP-based STS marker sequence of *B. nigra* and the resistant genotypes of the juncea- and the carinata-line were identical. In contrast to the STS markers, the original RFLP marker was absent in all B-genome species (Fig. 4). But it should be noted that the tested *B. juncea* and *B. carinata* genotypes were not from the same lines as used for the initial crosses to develop the recombinant lines, since the original lines are unknown. The STS markers were tested with the same F_2 population as the original markers and they mapped to the same positions (see Fig. 1).

To investigate the efficiency of the STS markers for marker-assisted breeding, 32 oilseed rape breeding lines and varieties were assayed. Only three of them exhibited the allele linked with the *Phoma* resistance of the recom-



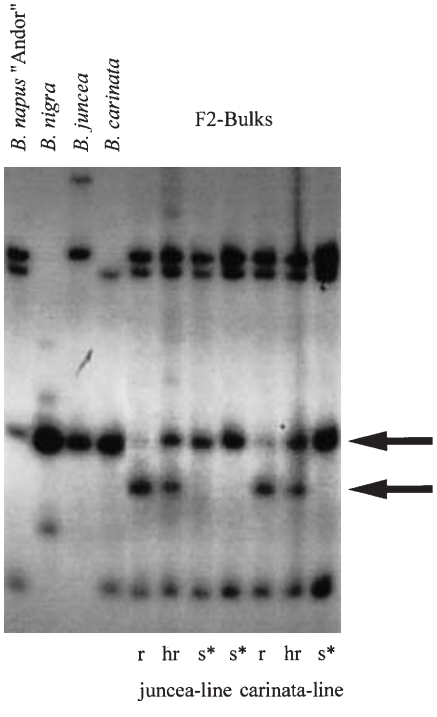


Fig. 3a–c PCR amplification products of the STS markers of the susceptible oilseed rape parent, the B-genome species and F_2 plants of the carinata-line. **a** a dominantly inherited agarose marker (AFLP-based), **b** a codominantly inherited marker, separated on an automated sequencer (AFLP-based), **c** a codominantly inherited agarose marker (RFLP-based)

Fig. 4 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 5–8* juncea-line; *lanes 9–11* carinata-line. *r*=bulk of homozygous resistant plants, *h*=bulk of heterozygous resistant plants, *s**=DNA of single susceptible plants. *Arrows* show the two polymorphic fragments

binant lines (data not shown). But it is not known whether these lines contain B-genome introgressions or not. However, two oilseed rape varieties showing the "susceptible allele" were chosen and crossed with the resistant *B. carinata*/*B. napus* recombinant line. The F_1s were selfed and the F_2s were screened with the STS markers. In these progenies the STS markers showed Mendelian segregation (Table 1) in contrast to a significant deviation from the expected segregation ratios for most of the original RFLP and AFLP markers.

Table 1 Segregation of the resistance reaction and the molecular markers in F_2 plants of the B-genome/*B. napus*-recombination lines and test-crosses between the carinata-line and winter oilseed rape varieties as well as the χ^2-values for the deviation from the expected segregation ratios of 1:2:1 and 3:1, respectively (+=P<0,1, *=P<0,05, **=P<0,01)

Loci	juncea-line	χ^2	carinata-line	χ^2	Test-crosses carinata–line	χ^2
PhR–gene	26:47:30	1.1	23:40:29	2.4	–	–
pRP1602	14:56:30	6.6*	14:57:30	6.7*	–	–
pRP1513	14:56:30	6.6*	14:57:30	6.7*	–	–
pRP1457	16:53:32	5.3+	15:54:32	6.2*	–	–
pRP1227	21:58:21	2.6	15:52:34	7.2*	–	–
AFLP S2G8	55:29	4.1*	51:31	7.2**	–	–
AFLP S6F38	52:30	5.9*	52:28	4.3*	–	–
AFLP S7G4	49:28	5.3*	52:29	5.0*	–	–
AFLP–STS ag.	55:29	4.1*	56:36	9.8**	63:21	0.0
AFLP–STS pa.	17:41:29	3.6	13:42:36	12.1**	21:44:19	0.3
RFLP–STS	13:54:29	6.8*	13:55:28	6.7*	19:42:27	1.6

Discussion

The transfer of *Phoma* resistance genes derived from the B-genome of *Brassica* into our B-genome/*B. napus* recombinant lines had probably occurred by homoeologous recombination after allosyndetical pairing of B-genome chromosomes with A- or C-genome chromosomes (Plieske et al. 1998). The question of whether the linked molecular markers are located on the rapeseed chromatin or on the B-genome introgression could not be finally answered. The absence of most of the original markers in the B-genome parents indicated a location on the rapeseed chromatin. According to our present data, all RFLP- and AFLP-based STS markers are at least present in *B. nigra*, which is the most likely ancestor of all *Phoma* resistance genes of the B-genome. Comparative sequencing of the RFLP-based STS marker fragments of *B. nigra* and of the resistant recombinant lines showed identical sequences. The entire extended sequence of the susceptible oilseed rape parent was identical to *B. nigra* as well, except for the described polymorphisms (Fig. 2b). Moreover, mapping of the four linked RFLP markers in the *B. nigra* map by the group of C. Dixelius at the University of Uppsala, Sweden, showed similar arrangements and distances of the markers within linkage group G3 (C. Dixelius, personal communication) as in all of our recombinant lines and as in the *B. napus* map of Uzunova et al. (1995) within linkage group 6. These results indicate synteny and homology between the B-genome parents and *B. napus*, at least in the region flanking the resistance genes and the linked molecular markers. This supports our assumption concerning the transfer mechanism of homoeologous recombination after allosyndetical pairing, as mentioned above.

The location of the *Phoma* resistance genes in the B-genome/*B. napus* recombinant lines is most likely in the A genome. We analyzed re-synthesized *B. napus* as well as *B. campestris* and *B. oleracea* varieties with the RFLP-based STS marker. In none of the tested *B. oleracea* varieties were any amplification products observed, whereas both *B. napus* and *B. campestris* showed the marker alleles (data not shown). This opens up the possibility of transferring the B-genome resistance genes of our recombinant lines into *B. campestris* varieties. This is of particular interest for plant breeders because resources of *Phoma* resistance genes for *B. campestris* are rare (Rimmer and van den Berg 1992; Rimmer et al. 1995).

The *B. juncea* line tested exhibited the "resistance allele" of the RFLP-based STS marker but showed some differences on the DNA level (e.g. a 6-bp insertion). The *B. carinata* line did not exhibit this fragment. However, both lines were not the same as those used for the initial crosses to develop the recombinant lines. The recombinant lines indeed were derived from different interspecific crosses with *B. juncea* and *B. carinata*. This was already demonstrated by our RFLP analyses which clearly differentiated the recombinant lines by *B. juncea* and *B. carinata* specific markers.

The observed deviation from the expected Mendelian segregation ratios for most of the molecular markers is obviously not due to the elimination of B-genome chromatin in *B. napus*. This elimination should also be detectable also in the frequency of heterozygous genotypes, but the number of heterozygous plants was not significantly reduced. Deviations from expected segregation ratios of molecular markers are quite common (Truco and Quiros 1994; Lagercrantz and Lydiate 1995; Uzunova et al. 1995; Harushima et al. 1996). A misclassification of some of the markers can be excluded because analyses were repeated twice. The reliability of *Phoma* resistance-test results has been discussed previously (Plieske et al. 1998). Expression of the *Phoma* resistance was affected by environmental conditions. Thus, misclassification of the resistance response of some genotypes was possible. The distinction between homozygous- and heterozygous-resistant genotypes was especially difficult. Therefore, the resistance phenotype was treated as a dominantly inherited trait in linkage analysis. However, only in the genetic background of "Andor" was the number of homozygous marker genotypes carrying the allele linked to resistance reduced, whereas the segregation of the STS markers in the test-crosses between the carinata-line and winter oilseed rape varieties was not distorted. This suggests that sublethal factors caused by inbreeding depression were responsible for the reduced frequency of these homozygous marker classes in the genetic background of "Andor".

The obtained STS markers meet all breeder demands: based on PCR they can easily be adapted to automation or require only standard electrophoresis equipment. All

are useful for marker-assisted backcross programs involving the resistant B-genome/*B. napus* recombinant lines with most oilseed rape breeding lines and varieties. More than 90% of the tested breeding lines and oilseed rape varieties did not exhibit "resistance alleles" of the STS markers in our investigations. However, the distance between the AFLP-based STS markers and the resistance genes of the B-genome/*B. napus* recombinant lines is still considerable. Eight recombinant plants (susceptible plants exhibiting, or resistant plants lacking, the marker allele) were detected among 92 F_2 plants. This means that the probability of misclassification is between 5 and 10%.

The RFLP-based STS marker is more tightly linked. Two (juncea-line) and respectively four (carinata-line) recombinant plants were detected. Additionally, the marker is located on the other side of the resistance genes as is the AFLP-derived markers. This means that a recombination event between one of these two STS markers and the resistance genes can easily be detected. Since blackleg disease is not easily scorable, and is influenced by environmental factors (Badawy et al. 1992; Plieske et al. 1998), reliable resistance tests require many replications. Thus our STS markers can considerably improve the efficiency of *Phoma* resistance breeding.

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