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Selection of stable *Brassica napus-Brassica juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 2. A 'to and fro' strategy to localise and characterise interspecific introgressions on the *B. napus* genome

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Abstract A new strategy to localise and characterise interspecific introgressions in the genus *Brassica* is presented. It consists of the localisation of RAPD specific markers from the donor species (*B. juncea*) by RFLP on a genetic map of the recipient (*B. napus*) and on the observation of the disappearance of rapeseed markers in recombinant lines. With this method, we localised an interspecific introgression of *B. juncea*, which confers blackleg resistance at the cotyledon stage in *B. napus*, on the linkage group DY17 of the previously determined *B. napus* genetic map. The estimated size of the substituted *B. napus* fragment was 39 cM, and the resistance gene was introgressed into the rapeseed genome by homologous recombination. The significance of the different strategies used and the implication of these results in breeding programs are discussed.

Key words *Brassica napus* · *Brassica juncea* · Blackleg resistance · RFLP-RAPD markers · Microsatellite

Introduction

Blackleg, caused by *Leptosphaeria maculans* (Desm) ces. & De Not., is one of the most damaging diseases of oilseed rape (*Brassica napus* L., AA CC, 2n = 38) crops. Intraspecific oligogenic resistance at the seedling stage or polygenic resistance at the adult stage are available within oilseed rape germplasm. However, a total hypersensitive resistance can be found in *Brassica* species containing B genomes. Analysis of addition lines containing one B chromosome on an oilseed rape genetic background (Struss et al. 1991; Chèvre et al. 1996; Struss et al. 1996) indicated a mono- or oligo-genic control of this resistance.

Roy (1984) reported the creation of a *B. juncea*- *B. napus* recombinant line, Onap^{JR}, carrying *B. juncea* (JR) resistance gene(s) efficient at the adult stage. From this material, the selection of stable resistant recombinant lines and the characterisation of the resistance gene were described by Chèvre et al. (1997). For marking the introgression, specific molecular markers of *B. juncea* were obtained by the BSA (bulked segregant analysis) strategy (Michelmore et al. 1991) on backcross populations. Three RAPD markers were identified, named OPI01-*Hae*III, OPG02-800 and OPT01-800 (Chèvre et al. 1997). As the 400 RAPD primers tested identified only three markers, in spite of the high level of polymorphism expected between *B. juncea* and *B. napus*, the small size of the introgression was proposed as a hypothesis (Chèvre et al. 1997).

In the present paper, we describe the localisation and the characterisation of this introgression on the previously established (Foisset et al. 1996) oilseed rape genetic map. Two hypotheses can be proposed for the introgression of the *B. juncea* fragment into *B. napus*. The first is that the fragment was introduced into the oilseed rape genome by chromosome rearrangements, with or without replacement of a *B. napus* fragment and without homology with the target fragment. The second hypothesis is that the fragment was introduced by homologous recombination in place of a *B. napus* fragment which presents a sequence similarity to a *B. juncea* resistance gene region. To localise the introgression on the genetic map, we employed two strategies. The first, which could be efficient whatever the type of introgression, is a BSA strategy on a backcross population with one parent of the genetic map. The use of markers previously localised on the oilseed rape genetic

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map (Foisset et al. 1996) allowed us to rapidly cover the genome with a minimal number of RAPD points. The second strategy, which used the markers of the introgression, was efficient only if homology exists between the introgression and *B. napus* replaced fragment. A search for *B. juncea*-specific markers was performed on the *B. napus* genetic map. We named this first step 'to'. To confirm the location of the *B. juncea* introgression, i.e. that *B. juncea* region which substituted for a *B. napus* one, the disappearance of RAPD markers of the oilseed rape genetic map corresponding to the *B. juncea* introgression was searched for. Surrounding markers of the same linkage group were used to map the introgression. This second step was named 'fro'. The interest of BSA and of the 'to and fro' strategy in our material is discussed.

Materials and methods

Plant materials

One resistant MXS heterozygous plant (Chèvre et al. 1997) was used to produce a MXS segregating population (Fig. 1). In the latter, one resistant plant was crossed to 'Yudal', one of the oilseed rape parents used for the construction of the genetic map (Foisset et al. 1996). The F_1 hybrid (MXY) was backcrossed to 'Yudal' to obtain the segregating population named 'MXYY' (Fig. 1). Simultaneously, MXS plants heterozygous for the resistance gene were either backcrossed four times to the 'Eurol' variety and then selfed twice, or else were selfed twice to produce nearly isogenic lines on a 'Eurol' or 'Samouraï' oilseed rape genetic background, respectively (Fig. 1). In the selfing progeny of MXS, plants homozygous and heterozygous for the resistance gene were analysed. A blackleg resistance test was performed at each generation.

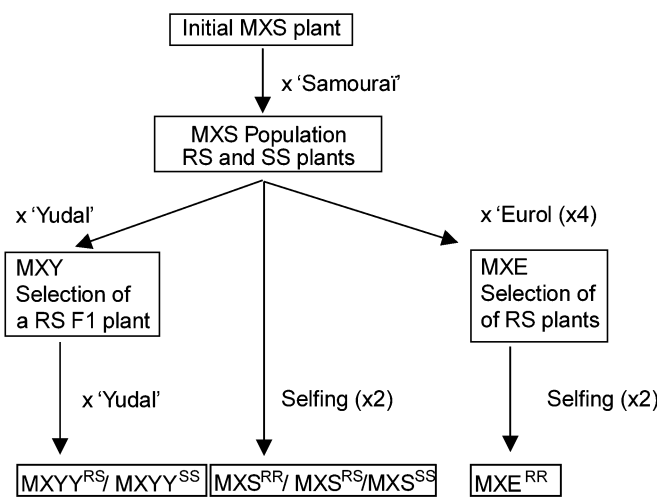

Fig. 1 Presentation of the material used for the location of the *B. juncea* blackleg resistance gene on the oilseed rape genetic map. The initial resistant recombinant line, MXS, on a winter oilseed rape genetic background, 'Samouraï' was described by Chèvre et al. (1997). The number of crosses is in *brackets*. Homozygous, heterozygous resistant and susceptible plants are identified by RR, RS and SS, respectively

The controls were for the following oilseed rape varieties : 'Darmor.*bzh*' and 'Yudal', both used for the establishment of the genetic map, together with 'Samouraï', 'Eurol' and 'Crésor', used for the initial creation of the material (Roy 1984). For *B. juncea*, one variety, 'Picra', was chosen as a reference.

Blackleg resistance tests

Inoculations were performed as described by Williams and Delwiche (1979). Seeds were sown in a greenhouse. Each half-cotyledon of 7-day-old-seedlings was wounded in the center with a needle and inoculated with a 10-µl droplet of inoculum (10^7 pycnidiospores/ml). One highly virulent isolate (#314) belonging to the A-group and obtained from an oilseed rape leaf lesion was used (Brun, comunication personal). It was representative of the fungal population pathogenicity under our field conditions. After incubation in darkness for 24 h at 20°C in a saturated humidity, seedlings were kept in a growth chamber (20°C, 16 h light/8 h night). Symptoms were scored 14 days after inoculation using a 1–9 scale according to lesion size, the occurrence of necrosis or chlorosis, and the presence of pycnidia (Williams and Delwiche 1979). Plants of classes 1–5 were considered as resistant and plants of classes 6–9 as susceptible. For the plants of this last group, a collapse of tissue with abundant pycnidial formation around the inoculation point was observed. The highest score out of the four inoculation points was considered as representative of plant behaviour.

DNA amplification

DNA was extracted from young leaves according to the method of Doyle and Doyle (1990).

RAPD analyses were as described by Foisset et al. (1996). The loci are designated by an OP prefix followed by the kit letter, the primer number, and the size of the band (in base pairs).

Marker identification was performed by bulk segregant analysis from MXYY backcross progeny; an equal amount of DNA of from eight resistant (classes 1–3) plants was pooled and compared to a DNA mixture of eight susceptible (classes 6–9) plants.

The microsatellite Bn 12A was obtained using the primers and amplification conditions described by Szewc-McFadden et al. (1996). Amplified products were resolved on a 5% non-denaturing acrylamide gel run in 1 × TBE and visualised by ethidium bromide coloration.

RFLP method

Probes were produced from specific *B. juncea* RAPD markers. For the bands revealed from OPI01 and OPT01, gels were de-stained in distilled water for 1 h to eliminate TAE buffer. Amplified bands were cut out from an agarose gel with a sterile cutter. DNA was recovered by extraction with a Sephaglass band preparation (Pharmacia). We tried to clone the specific OPG02-800 band using a "SureClone" ligation kit (Pharmacia). A truncated G02 fragment, named G02t, was cloned, probably because of an over activity of the exonuclease used to remove the polyA ends in the kit.

The RFLP method was previously described by Sharpe et al. (1995).

Linkage analysis

Goodness of fit to expected Mendelian ratios for each segregating locus was tested by chi-square analysis ($\alpha = 5\%$). Linkage analysis was performed on Mapmaker/exp version 3.0b (Lincoln et al. 1992).

A minimum LOD of 3.0 and a maximum recombination frequency of 0.4 were chosen.

Results

A blackleg resistance test in the segregating population MXYY obtained from the backcross to 'Yudal' gave 125 resistant and 133 susceptible plants. These data confirmed monogenic control without segregation distortion ($\chi^2 = 1.8$). All the resistant plants analysed carried the *B. juncea* introgression-specific markers OPI01-*Hae*III and OPG02-800 (Chèvre et al. 1997).

BSA analysis

For BSA analysis, we chose RAPD markers previously mapped on the *B. napus* genetic map (Foisset et al. 1996) to test for the minimal number of RAPD points giving maximal coverage of the genome. As 'Yudal' was the recurrent parent of the backcross progeny, we only tested RAPD dominant markers present in the second parent, 'Darmor.*bzh*'. The genetic background of the initial material MXS was the accession 'Samouraï' and only bands common to 'Samouraï' and 'Darmor.*bzh*' were recovered in the segregating population. 74 'Darmor.*bzh*' markers, well spread on the genome, revealed by 61 primers, were analysed on the bulks. The markers were either absent in 'Samouraï' or present in 'Samouraï' and in both bulks. No marker was present in only one bulk.

'To' strategy

For this strategy, the hypothesis was that a *B. juncea* introgressed fragment replaced the *B. napus* homologous region by homologous recombination. Fragment OPG02-800 has been partially cloned (named G02 truncated: G02t); OPT01-800 and undigested OPI01-1300 were recovered on agarose gels to produce RFLP probes. They were tested on 'Darmor.*bzh*' and 'Yudal' restriction profiles to look for hybridization and polymorphism. T01-800 gave a complex profile and no polymorphism was detected. G02t mapped to only one locus in *B. napus*, which was polymorphic with the restriction endonuclease *Dra*I. This locus was mapped on linkage group DY17 of the genetic map. I01–1300 mapped to two loci, and one of these was also polymorphic with the same restriction enzyme and was localised 17.1 cM from the G02t-*Dra*I locus. The location of the two loci on the linkage group is presented in Fig. 2. At this stage we identified a *B. napus* genomic region homologous to the *B. juncea* region carrying the resistance gene, but we did not know if the introgression was introduced in this region.

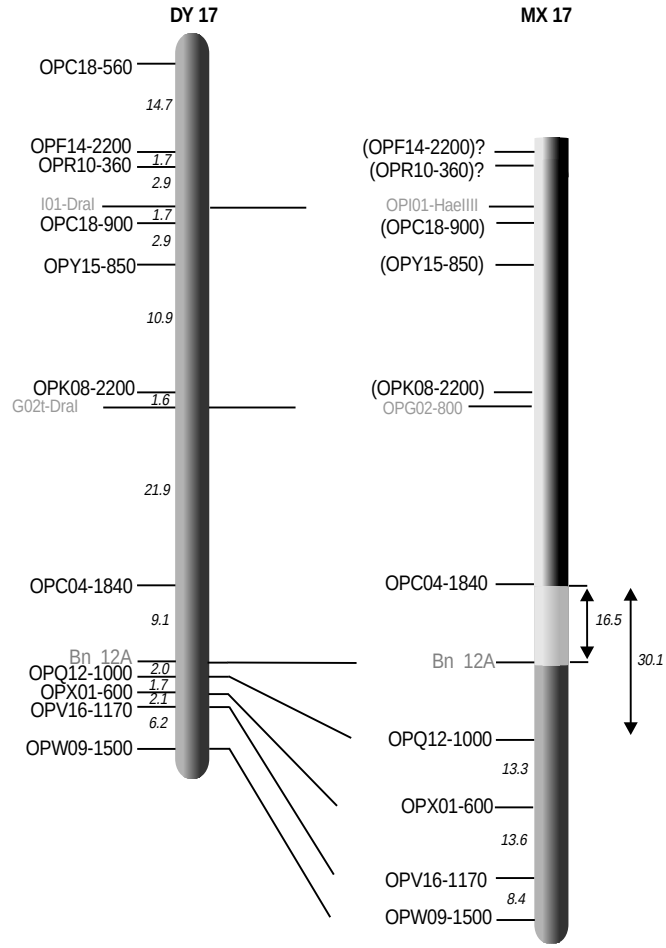

Fig. 2 Linkage groups : DY17 used as reference on which the *B. juncea* probes (I01-*Dra*I and G02t-*Dra*I) were mapped, and MX17, the corresponding group established from the resistant recombinant line

'Fro' strategy

RFLP localisation

If the introgression occurred by homologous recombination, the *B. juncea* allele would be present on plants homozygous and heterozygous for the introgression but absent in homozygous susceptible plants. Reciprocally, the corresponding *B. napus* allele must be present only in homozygous susceptible (SS) and heterozygous (RS) plants. In the second selfing progeny of MXS material (Fig. 1), homozygous (RR), heterozygous (RS) resistant, and susceptible (SS) plants were identified. The G02t probe was hybridised on RR, RS, and SS plants (Fig. 3a). We detected one locus specific for RR and RS plants, and one corresponding locus in *B. napus* specific for RS and SS plants, which was the same as the locus mapped on linkage group 17 (Fig. 3b). As the G02t RAPD fragment used as a probe was shown to be

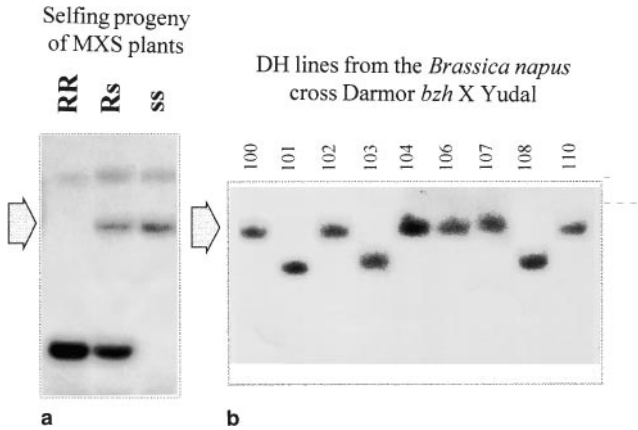

Fig. 3a,b RFLP identification of the G02t-*Dra*I locus on the homozygous (RR), heterozygous (RS) resistant and susceptible (SS) lines (**a**) and mapping of this locus on the *B. napus* segregating population used to establish the genetic map (**b**)

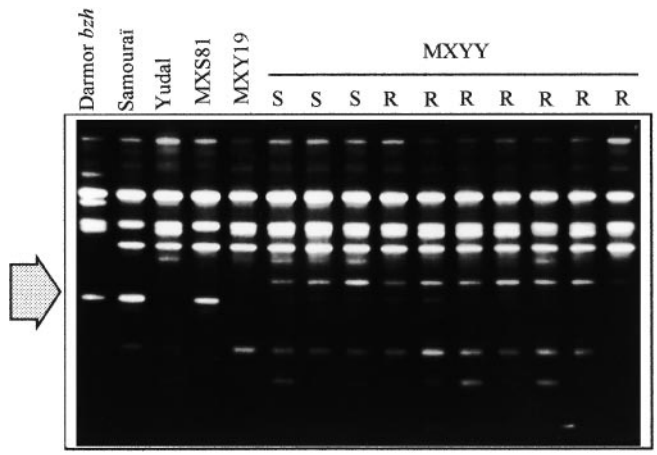

Fig. 4 Profiles obtained from OPC18 primer. The OPC18.900 marker, replaced by the introgression, was present on the oilseed rape varieties 'Darmor.*bzh*', 'Samouraï' and the heterozygous resistant mother-plant (*MXS81*) but absent on 'Yudal', the F_1 plant (*MXY19*) and the corresponding segregating population MXYY containing resistant (*R*) and susceptible (*S*) plants

B genome-specific (Chèvre et al. 1997), the G02t-*Dra*I band present in resistant plants resulted from intraspecific hybridisation, i.e. it detected a *B. juncea* locus on introgression, while the other band revealed the homologous corresponding locus of *B. napus*. This analysis was consistent with a hybridisation profile that gave a stronger (homologous) signal for the R-specific band and a faint (interspecific) signal for the S-specific band (heterologous). Hybridisation of *B. juncea* accessions would have confirmed this result, but the initial accession used for the interspecific cross was not available and the absence of intraspecific polymorphism between different *B. juncea* accessions prevented us from arriving at a conclusion (data not shown). As the *B. napus* locus has been replaced by a *B. juncea* locus, we can also conclude that the fragment has been introgressed by homologous recombination or else by total substitution of the chromosome. The latter hypothesis was excluded because of the regular meiotic behaviour of resistant plants obtained after backcrossing to oilseed rape (Chèvre et al. 1997).

RAPD and microsatellite location

To assess the location and the size of the introgression, we used RAPD markers located on linkage group 17 in the cross 'Darmor.*bzh*' × 'Yudal' (DY). First we looked for markers that gave a band in 'Darmor.*bzh*', namely OPC18-900, OPY15-850, OPK08-2200, OPC04-1840, OPQ12-1000, OPX01-600, OPV16-1170 and OPW09-1500 (Table 1). OPC18-900 (Fig. 4), OPY15-850 and OPC04-1840 bands were not present in MXYY backcross progeny. The two first markers were mapped on DY17 between G02t-*Dra*I and I01-*Dra*I, and so were replaced by the introgression in heterozygous resistant plants. The OPK08-2200 band was absent in the oilseed rape genetic backgrounds 'Yudal' and 'Samourai', and so was impossible to map. Marker OPC04-1840 was absent in the G02t-*Dra*I/I01-*Dra*I fragment. The OPC04-1840 band was present in *B. napus* var. 'Cresor', the oilseed rape variety used for the initial interspecific cross. So, the absence of this marker in the resistant plants indicated that it could have been replaced by the *B. juncea* introgression (Fig. 2). On another hand, OPQ12-1000, OPX01-600 (Fig. 5), OPV16-1170 and OPW09-1500, absent in 'Yudal', segregated in MXYY (Fig. 2). All these markers gave a normal Mendelian segregation. OPQ12-1000 was located 30.1 cM from the resistance character. As no recombination occurred between the introgressed fragment and *B. napus*, we can conclude that OPQ12-1000 was 30.1 cM from the end of the introgression. All the genetic distances were greater than those observed in the cross 'Darmor.*bzh*' × 'Yudal' (Fig. 2). This increase ranged from 35% to 682% between OPW09-1500 and OPV16-600, and between OPX01-600 and OPQ12-1000, respectively.

We also analysed a Bn12A co-dominant microsatellite marker. We found an allele present in MXS homozygous and heterozygous plants for the introgression, but absent in homozygous susceptible plants (Fig. 6). This allele was not present in the *B. juncea* accession tested, and the microsatellite was not amplified in the B genome of the *B. nigra* populations tested. By contrast, this allele was recovered in the *B. napus* accession 'Cresor' (Fig. 6). We concluded that the locus was not in the introgression, but in a 'Cresor' genetic background. A common allele was identified in 'Cresor' and 'Yudal' (Table 1) and so no segregation was revealed in the MXYY backcross population. Therefore we used the initial MXS segregating population

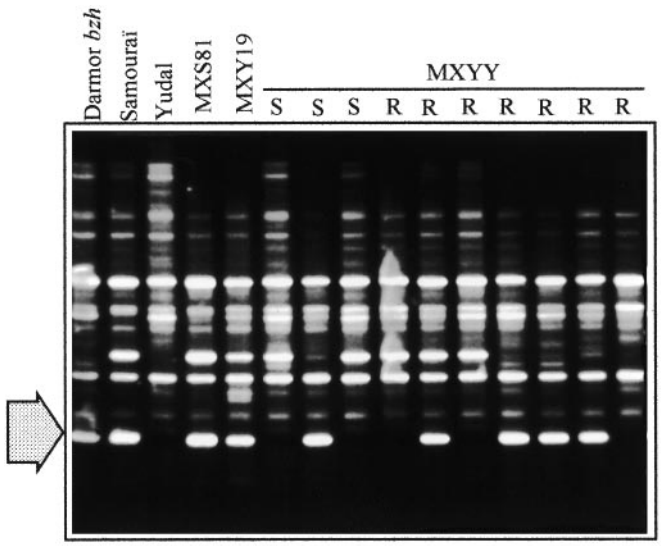

Fig. 5 Profiles obtained from the OPX01 primer. The OPX01.600 marker, placed close to the introgression, was present on the oilseed rape varieties 'Darmor.*bzh*', 'Samouraï' the heterozygous resistant mother-plant (*MXS81*) and the F_1 plant (*MXY19*) but was absent on 'Yudal'. It showed a segregation in the MXYY population containing resistant (*R*) and susceptible (*S*) plants

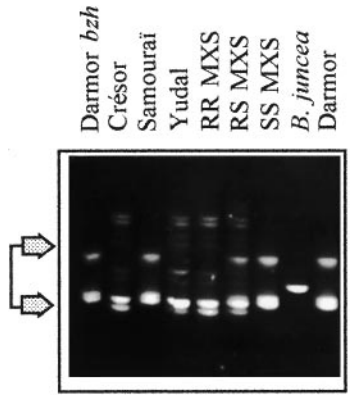

Fig. 6 Profile obtained for the microsatellite Bn12A. A specific allele was detected only on 'Cresor', 'Yudal' and MXS plants containing the resistance gene at the homozygous (*RR*) or heterozygous stage (*RS*)

(Fig. 1). Marker Bn12A was located 16.5 cM from the introgression (Fig. 2) on a residual fragment of the initial 'Cresor' genetic background in the resistant plant (Fig. 1).

Marker OPR10-360 was present in 'Yudal' and in 'Samourai' (Table 1) and so was analysed in the second selfing progeny of MXS (Fig. 1). The OPR10-360 band was present only in homozygous SS and heterozygous RS plants, and absent in 'Cresor'. Hence, OPR10-360 was either replaced by the introgression or by the 'Cresor' background. OPF14-2200 was only present in 'Yudal'. To investigate the OPF14-2200 marker, we looked for another pair of isogenic lines containing the *B. juncea* introgression, i.e. MXE^{RR} (Fig. 1). OPF14-2200 was amplified on a 'Eurol' background but was absent in MXE^{RR} plants. Like OPR10-360, OPF14-2200 was not amplified in a 'Cresor' background.

Sizing of the B. juncea introgression

RFLP, RAPD and microsatellite mapping results allowed us to conclude that the *B. juncea* introgression was located on linkage group DY17 of the *B. napus* genetic map (Foisset et al. 1996) and that an homologous recombination event occurred through at least one cross over inside of this linkage group. The size of the introgression can not be directly estimated because of the absence of recombination events between the *B. juncea* fragment and the *B. napus* background. By comparison to linkage group DY17, the minimal size of the substituted *B. napus* fragment can be estimated from the distance between I01-*Dra*I and OPC04-1840 as 39 cM.

Discussion

BSA strategy did not allow us to map the *B. juncea* interspecific introgression on the *B. napus* genetic map. Consequently, we developed a new strategy using interspecific RFLP mapping of specific *B. juncea* RAPD bands and looking for the disappearance or linkage of *B. napus* markers on the DY17 identified linkage group.

BSA strategy

No marker was found by BSA strategy on the MXYY population. One of three different hypotheses might explain this result:

(1) The constitution of the bulks was made from a segregating population obtained from a backcross to the oilseed rape parent 'Yudal'. The number of markers analysed was limited by the fact that they have to be common to 'Samourai' and 'Darmor.*bzh*'. The creation of the reciprocal backcross progeny, or of a dihaploid population, would have increased the percentage of the oilseed rape genome explored.
(2) The observation was performed only on identified markers. In fact, the disappearance of markers, due to the presence of the introgression, can be identified by BSA. This approach was supposed to compare the profiles of the two varieties used for the establishment of the genetic map to 'Samouraï' and the bulks. However, the same information can be provided by the analysis of the three oilseed rape varieties and the F_1 MXY hybrid or of isogenic lines.

(3) The BSA method can be efficient in identifying linked markers if they are close to the introgression. However, the discrepancy observed *a posteriori* between the genetic distances of the linkage group containing the interspecific introgression and the reference DY17 group indicate that no marker was found. More recombination events occurred between the oilseed rape markers in the presence of the introgression than in its absence. An increase of genetic distances at the proximity of an interspecific introgression has already been described in tomato by Alpert et al. (1995) who reported a decrease of recombination between homoeologous segments. Similarly, it has several times been described that, in the absence of the wheat *Ph1* gene mutation, recombination between genomic segments of wheat and of alien species is very rare (Jiang et al. 1994). In a like manner, we never observed crossing over between the *B. juncea* and *B. napus* fragments and recombination events seemed to be concentrated in the *B. napus* portion of the linkage group. Another possibility is that there are more recombination events for linkage group 17 in the cross MXS × 'Yudal' (MXYY) than in the cross 'Darmor.*bzh*' × 'Yudal' used for the establishment of the genetic map (Foisset et al. 1996), with no effect of the introgressed fragment but with an effect of the genetic background. Work is in progress to test DY17 markers on a 'Samourai' × 'Yudal' backcross population.

'To and fro' strategy

As RAPD markers were specific to the *B. juncea* introgression (Chèvre et al. 1997), they were used as interspecific RFLP probes on *B. napus* to rapidly localise the genomic region homologous to the introgression. RAPD markers were amplified with ten-base oligonucleotides, and a minimal change in these 20-base sequences was probably sufficient to preclude amplification. RFLP mapping is based on the hybridisation of the totality of the sequence of the RAPD band (500 base pairs for G02t and 1300 for I01). Minor changes in these sequences did not affect hybridization, although we observed a lower hybridisation signal in the *B. napus* (MXS homozygous SS in Fig. 3 a) than in the *B. juncea* introgression (MXS homozygous RR in Fig. 3 a). However, the success of this strategy depends on the number of loci revealed by the interspecific probes on the DNA of the recipient parent. Repeated sequences were unusable as observed from the OPT01-800 marker. Garcia et al. (1995) reported that all 30 RAPD bands tested revealed repeated sequences in peanut. If more than one locus is present in *B. napus* (like I01-*Dra*I), the hybridisation on RR and SS plants is necessary to detect which locus corresponds to the introgression. In our case, the interspecific hybridization of the G02t and I01 probes generated RFLP markers on *B. napus*. This strategy was the most efficient to localise the introgression.

Size of the introgression

The analysis of linkage-group-17 markers on different segregating populations allowed us to detect which markers had been replaced by *B. juncea* sequences and which remained present and linked to the introgression (Fig. 2). The minimal size of the *B. napus* substituted fragment can be estimated as 39 cM. It was impossible to determine the origin of the absence of bands OPR10-360 and OPF14-2200 which can be due either to the presence of the introgression or to the presence of the initial genetic background, i.e. 'Cresor'. RFLP mapping of these RAPD bands on isogenic lines RR, SS, on 'Cresor', and on several accessions of *B. juncea* could provide more precise information. If RFLP-specific alleles of the RR and RS lines are recovered in 'Cresor' but not in different *B. juncea* accessions, we would be able to conclude that the markers are characteristic of the 'Cresor' background. Research for new markers on the DY17 linkage group is currently in progress for the characterisation of the OPF14-2200 extremity of the recombinant consensus linkage group 17.

Homologous recombination had evidently occurred between *B. juncea* and *B. napus*. The same data were reported from interspecific introgression by Garcia et al. (1995). However, we cannot be certain that the *B. juncea* segment introgressed has the same size as the *B. napus* replaced segment even if the stability of the meiotic behavior of this material suggests a small size for the introgression. Mapping of the resistance gene within *B. juncea* will be attempted to determine whether the genetic distances are the same in *B. napus* and in *B. juncea*.

Implication in breeding programs

A number of genes other than the *Phoma* resistance gene are present in the introgressed fragment. Some of them could have dominant negative effects. Agronomic experiments are in progress to characterise recombinant lines compared to the recurrent oilseed rape variety. However, the decrease of the introgression size with marker-assisted selection in a breeding programme is difficult to determine because of the very rare recombination events between the *B. napus* and *B. juncea* segments. Several methods can be proposed: either screen large populations, or cross two recombinant lines containing overlapping introgressions and select recombination events in the backcross progeny to the recurrent oilseed rape parent, or else apply mutagenesis treatments. In all cases, a powerful PCR-based technology is necessary. For example, *B. napus* markers OPC18-900, OPY15-850, OPK08-2200 and

OPC04-1840 can be transformed into a SCAR dominant marker (Paran and Michelmore 1993) and resistant plants could be screened for the presence of these markers.

Markers identified in our work can be used to select homozygous resistant plants at an early stage in breeding programs. G02t can be a good RFLP co-dominant marker for this purpose. RAPD markers would be more useful for selection because of the ease of use of the technique and the earlier stage of application (cotyledon stage) in comparison to RFLP (three-leaf stage). For example, OPC18-900 would allow us to detect homozygous resistant plants in comparison with heterozygous/homozygous susceptible plants if the marker was present in the oilseed rape genetic background employed.

Using the synteny within the region concerned, new markers will be identified, in addition to the *B. juncea* and *B. napus* markers described, and a map-based cloning of the *B. juncea* resistance gene will be performed.

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