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ORIGINAL PAPER

Detection, introgression and localization of genes conferring specific resistance to *Leptosphaeria maculans* from *Brassica rapa* into *B. napus*

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Abstract Blackleg (stem canker) caused by the fungus *Leptosphaeria maculans* is one of the most damaging diseases of oilseed rape (*Brassica napus*). Crop relatives represent a valuable source of "new" resistance genes that could be used to diversify cultivar resistance. *B. rapa*, one of the progenitors of *B. napus*, is a potential source of new resistance genes. However, most of the accessions are heterozygous so it is impossible to directly detect the plant genes conferring specific resistance due to the complex patterns of avirulence genes in *L. maculans* isolates. We developed a strategy to simultaneously characterize and introgress resistance genes from *B. rapa*, by homologous recombination, into *B. napus*. One *B. rapa* plant resistant to one *L. maculans* isolate was used to produce *B. rapa* backcross progeny and a resynthesized *B. napus* plant from which a population of doubled haploid lines was derived after crossing with natural *B. napus.* We then used molecular analyses and resistance tests on these populations to identify and map the resistance genes and to characterize their introgression from *B. rapa* into *B. napus*. Three specific genes conferring resistance to *L. maculans* (*Rlm1*, *Rlm2* and *Rlm7*) were identified in *B. rapa*. Comparisons of genetic maps showed that two of these genes were located on the R7 linkage group, in a region homologous to the region on linkage group N7 in *B. napus*, where these genes have been reported previously. The results of our study offer new perspectives for gene introgression and cloning in *Brassicas*.

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Introduction

Stem canker (Blackleg) caused by the fungal pathogen *Leptosphaeria maculans* Desm. (Ces & de Not), affects all cultivated *Brassica* species and is responsible for severe yield losses worldwide in *Brassica napus* L. production (Fitt et al. 2006; Gugel and Petrie 1992). The most effective way to control stem canker is by genetic resistance (Rimmer and vandenBerg 1992). Two types of resistance are commonly used. On the one hand, quantitative resistance is partial, race non-specific and under polygenic control (Pilet et al. 1998) and is usually evaluated in adult plants in the field. Quantitative resistance reduces the severity of symptoms and has been suggested to be durable. On the other hand, qualitative seedling resistance is controlled by a single gene (Ansan-Melayah et al. 1998), leads to complete resistance and is race specific. Qualitative resistance may be evaluated in seedlings from the cotyledon stage to the adult stage. Several specific resistance genes (*Rlm*) have been identified in *Brassica* species (Delourme et al. 2006a). Each specific resistance gene only confers resistance against races of the fungus, which have the corresponding avirulence (*AvrLm*) gene and therefore this type of resistance gene exerts a high

selection pressure on the pathogen population. *L. maculans* populations evolve rapidly by genetic recombination during sexual reproduction, coupled with large-scale dispersal of ascospores (West et al. 2001), and adapt to major resistance genes. For example, *Rlm1*, which was used in a large proportion of French oilseed rape production areas, was overcome by pathogen populations within 3 years (Rouxel et al. 2003). More recently, in Australia, the efficacy of the "Surpass 400" specific resistance in the 'Surpass 400' cultivar was lost after only two to three growing seasons (Li et al. 2003; Sprague et al. 2006). Finally, several specific resistance genes are no longer effective when corresponding virulence genes become more common in the local pathogen population (Balesdent et al. 2005). In order to improve the durability of resistance, different specific resistance genes could be managed in time and space and used in combination with quantitative resistance and therefore, new sources of resistance are needed.

Different sources of specific resistance have already been identified. By screening a collection of oilseed rape varieties with a differential set of *L. maculans* isolates, six resistance genes were detected in *B. napus: Rlm1* was identified in 'Quinta' (Ansan-Melayah et al. 1998), *Rlm2* and *Rlm3* in 'Glacier' (Ansan-Melayah et al. 1998; Balesdent et al. 2002), *Rlm4* in 'Jet Neuf' (Balesdent et al. 2001), *Rlm7* in non-commercial lines (Balesdent et al. 2002), and *Rlm9* in 'Darmor' (Delourme et al. 2004). Five of these genes (*Rlm1*, *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9*) are on the N7 linkage group, and only *Rlm2* is on a different linkage group (Delourme et al. 2004; 2006a). Some specific resistance genes were also identified in other *Brassica* species. Cultivated *Brassica* species are organized into three homeologous genomes, named A, B and C, of which A and C are present in *B. napus*. All the resistance genes detected in *B. napus* are on the A genome, and four resistance genes were also described on the A genome in *B. rapa* (AA): *Rlm8* (Balesdent et al. 2002), *LepR1*, *LepR2* (Yu et al. 2005) and *LepR3* (Li and Cowling 2003). The majority of *Brassica* species containing the B genome also carry specific resistance to *L. maculans*. *Rlm5* and *Rlm6* were identified on the B genome of *B. juncea* (AABB) (Balesdent et al. 2002; Rimmer and vandenBerg 1992) and *Rlm10* was found in *B. nigra* (BB) (Chevre et al. 1996; Chevre, Personal Communication). To date, no specific resistance genes were detected on the C genome, in either diploid or polyploid species (Rimmer and vandenBerg 1992).

A few genes have been introduced from the B genome of *B. juncea* into *B. napus* on the DY717 linkage group (Barret et al. 1998; Chevre et al. 1997; Saal et al. 2004), but only large introgressions were obtained and no recombination was observed between the B genome introgressed fragment and its homeologous region in *B. napus*, because of the low level of homology between the genomes. Resistance genes were also introduced into *B. napus* from *B. rapa* (Crouch et al. 1994) and their introgression, via synthetic *B. napus*, was successful because *B. rapa* is a progenitor of *B. napus*. We assume that these *B. rapa* genes were introgressed by homologous recombination, but they were only mapped in *B. napus*, without information on their original position in *B. rapa* (Yu et al. 2004, 2005).

The aim of this study was to assess a method to carry out a concurrent introgression in *B. napus* and characterization of the specific resistance genes present in *B. rapa*, in spite of the heterozygosity of most *B. rapa* accessions and the complex *AvrLm* patterns of the fungus isolates. One resistant plant was selected from an old French forage *B. rapa* variety, which is a heterozygous plant population, by screening seedlings for resistance with several *L. maculans* isolates. The avirulence patterns of the isolates used were mostly representative of the most common *AvrLm* genes in the French *L. maculans* populations (Balesdent et al. 2006). Specific resistance genes carried by this plant were mapped simultaneously in *B. rapa* and in *B. napus* upon their introgression via resynthesized *B. napus*. Therefore, this is the first report of a map of the linkage group carrying resistance genes in *B. rapa*. In addition, we identified the *Rlm* genes derived from *B. rapa* and characterized their introgression in *B. napus*, using several races of *L. maculans* with known patterns of avirulence genes (*AvrLm*) and by molecular analyses.

Materials and methods

Fungal material

Ten *L. maculans* isolates were used to test the plant material for resistance genes. PHW1223 (Balesdent et al. 2005), IBCN14 (Balesdent et al. 2005), S7 (Brun et al. 2001), R2 (Somda et al. 1999), 929 (kindly provided by Dr. J.P. Tewari, Edmonton, Canada, in 1993) and R1 M1.3 (obtained at Le Rheu in 2001 from a single ascospore sampled from an infected 'SMX' plant, which harbors *Rlm1* and *Rlm6*) are natural isolates, whereas v45.57, v33.6.3, v29.3.1 and v48.2.6 are progeny isolates obtained following in vitro crosses between *L. maculans* isolates. In brief, v33.6.3 is an isolate derived from the cross 33 (Balesdent et al. 2002); v29.3.1 and 19.2.1 (Balesdent et al. 2002) were crossed to generate isolate v45.57 (Balesdent, unpublished data); and v48.2.6 was recovered from the cross 48 between 19.4.45 and v34.4.4 (Balesdent et al. 2002). The pattern of avirulence alleles (*Avrlm*), for most of the ten known *AvrLm* genes, in each isolate was previously established on an appropriate differential host set under controlled conditions (Balesdent et al. 2002; Balesdent et al. 2005; Balesdent, Personal Communication; Brun, Personal Communication). In this paper, we described isolate

avirulence patterns using the nomenclature proposed by Balesdent et al. (2005); the avirulence alleles present in the isolate are listed, only the avirulence alleles in parentheses have not been characterized.

Plant material

Seedlings of an old non homogeneous French forage *B. rapa* variety, 'Chicon', were screened with several isolates and one plant, 'C1.3', was selected for its resistance, at the cotyledon stage, to isolate S7 (Av1-5-6-7-(8)). This plant was crossed to a susceptible *B. rapa* doubled haploid line, 'Z1' (kindly provided by AAFC, Canada), to produce F1 hybrids. One F1 plant was then backcrossed to 'Z1' to produce 164 BC1 progeny, named CZB1 (Fig. 1). The same 'C1.3' plant was crossed to 'RC', a *B. oleracea* ssp. *alboglabra* doubled haploid line, and the F1 plant was colchicine-doubled to produce a resynthesized *B. napus* (RCC). The resynthesized *B. napus* was crossed to 'Darmor' to produce eight hybrids, RCCD. 'Darmor' is a winter oilseed rape variety that contains both quantitative adult-stage resistance to *L. maculans* and the specific resistance gene *Rlm9*. A population of doubled haploid lines (RCCD.DH) was produced from these hybrids using microspore cultures (Polsoni et al. 1988). More than 300 doubled haploid (DH) lines were produced either by spontaneous chromosome doubling or colchicine treatment. Thirty-one DH lines (between one to ten lines per F1 hybrid) with good seed set and showing differential reaction to isolates S7, 929 and v45.57 were used in this study (Fig. 1). In addition, 150 DH lines obtained from a *B. napus* 'Darmor-bzh' × 'Yudal' hybrid and previously described by Foisset et al. (1996) were also used in this study.

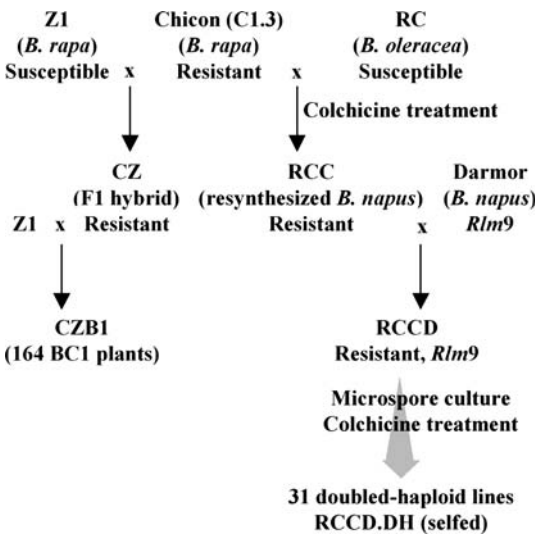

Fig. 1 Scheme of plant material production

Resistance tests at the seedling stage

A cotyledon test derived from the test of Williams and Delwiche (1979) was performed following the protocol described by Somda et al. (1999). A plastic cover was placed over the inoculated plants to create an atmosphere with 100% relative humidity (RH) and the plants were incubated in the dark at 20°C for 24 h in a growth chamber. Inoculated plants were then placed in an atmosphere with 80–90% RH (without a plastic cover) and a 16-h photoperiod at 20°C (±1°C). Symptoms on cotyledons were scored 14 and 21 days after inoculation using the 0 (no visible reaction) to 11 (total collapse of the tissue) scale. Plants were considered to be susceptible to an isolate when all replicates had a score higher than or equal to 9, at 21 days after inoculation, and resistant when all replicates had a score less than or equal to 3. When intermediate scores were obtained, we considered that the genotype had an intermediate behavior. In *B. rapa* BC progeny, each plant was inoculated with two isolates, one per cotyledon, and each cotyledon lobe was treated as a replicate, therefore, making two inoculation sites per plant and per isolate. For doubled haploid lines, each isolate was inoculated on three plants of each line and on the four cotyledon lobes of each plant. Each cotyledon lobe was treated as a replicate; therefore, 12 inoculation sites per isolate and per line were used.

Molecular analyses

Genomic DNA was extracted from the young leaves of all individual plants according to the method of Doyle and Doyle (1990). The DNA concentration was adjusted to 10, 50 and 1 ng/µl for RAPD, AFLP and SSR assays or specific markers, respectively.

Bulked segregant analysis

Sixteen plants showing resistance and 11 plants showing susceptibility to both R2 and S7 isolates were selected from the CZB1 population and their genomic DNA pooled to form the resistant (RB1) and the susceptible (SB1) bulks, respectively (Michelmore et al. 1991). These bulks were used to determine the linkage group carrying the resistance genes. As a second step, two additional bulks, RB2 and SB2, were constituted from the CZB1 population in order to cover a larger genomic region with molecular markers: the RB2 bulk was composed of the pooled genomic DNA from 12 plants showing resistance to the R2 isolate, and carrying the 'C1.3' allele for four molecular markers that were detected as linked to the resistance in the first bulked segregant analysis, or that were carried by the same linkage group as these markers; the SB2 bulk was composed of seven plants showing susceptibility to the R2 isolate and the 'Z1' allele at these four loci.

Random amplified polymorphic DNA (RAPD) markers

The RAPD primers (Operon Technologies) covering several A genome chromosomes were selected from published reference maps (Lombard and Delourme 2001). The PCRs were performed using the protocol of Foisset et al. (1996). Amplified products were separated on 1.8% agarose gels and images acquired after staining with ethidium bromide. The RAPD markers were named by combining the primer code and the size of the amplified DNA fragments (for example, W11.1690).

Simple sequence repeat (SSR) markers

The SSR primers used in this study were obtained from the UK Cropnet Database (prefixed with OI; http://www.ukcrop.net/perl/ace/search/BrassicaDB) or were developed by Celera AgGen (prefixed with CB or Bras) and are publicly available (Piquemal et al. 2005). The PCRs were performed in a total volume of 5 µl, containing M13 universal fluorescent tag to label the amplified DNA fragments. The amplified products were electrophoresed through 6.5% denaturing polyacrylamide gels and detected with an automated DNA sequencer (LI-COR Biosciences).

Amplified fragment length polymorphism (AFLP) and sequence-specific amplification polymorphism (SSAP) markers

Amplified fragment length polymorphism assays were conducted essentially as described in a previous study (Vos et al. 1995). For AFLP and SSAP, DNA was digested by *Eco*RI plus *Mse*I. After ligation of double-stranded adaptors to the ends of the restriction fragments, pre-amplification was performed with *Eco*RI and *Mse*I primer pairs with one selective nucleotide at their 3′-end. For AFLP, the selective amplification was performed with *Eco*RI and *Mse*I primer pairs with three selective nucleotides at their 3′-end. The *Eco*RI + 3 primers were labeled by IRD700 or IRD800 fluorochrome (MWG Biotech). For SSAP, the selective amplification was performed with one unlabeled *Eco*RI primer with three selective nucleotides at its 3′-end and one primer designed in the nucleotide binding site (NBS) conserved region of NBS-LRR resistance genes (Meyers et al. 1999) and labeled by IRD700 or IRD800 fluorochrome (MWG Biotech). Three different NBS primers designed by Rocherieux (2003) were used, NBS1 (GGGGGGGTAGTGGGAAAGACGAC), NBS2 (GGCG GTTCAGGGAA-AGACATAC) and NBS1c (GTCGTCTT CCCAGCTACCCCAGTCCC). For AFLP and SSAP, the amplified products were separated on 5.5% denaturing acrylamide gels and detected with the same sequencer as SSR markers. AFLP and SSAP markers were named using the international code for each primer pair and the size of the amplified fragment. AFLP markers that revealed polymorphisms between the RB2 and SB2 bulks were then checked on all the 19 individuals making up the bulks. Markers, which showed a co-segregation ratio with the resistance phenotype equal to or greater than 15 out of 19 were retained and tested on 116 additional plants from the BC1 *B. rapa* population.

Specific markers

Specific molecular markers (prefixed with At) were developed from *Arabidopsis* sequences in this study, using the published alignment between N7 and chromosome 1 of *Arabidopsis* (Mayerhofer et al. 2005). Two pairs of primers, designed on At1g80400 (F: AGTCCTTGA-GGCCTT AGAGAAGA and R: CGTTTGAGGACTGTGTTGTCC) and At1g80530 (F: GACGTTCCCGCTTTACTCC and R: CTCATAGGAAAATTCCTCATATTGGT) genes, showed polymorphic loci between 'C1.3' and 'Darmor' on R7/N7. PCR was performed in a final volume of 8 µl containing 2 mM $MgCl_2$, 310 µM dNTP, 0.62 µM of each primer, 0.062 U/µl Taq polymerase (Promega), 1× Taq polymerase buffer (Promega) and 1.8 ng/µl genomic DNA. The DNA was amplified using a cycling profile of 5 min at 94°C followed by 30 cycles of 30 s at 94°C, 1 min at 55°C (for primers designed on At1g80040) or at 60°C (for primers designed on At1g80530) and 45 s at 72°C. At the end of 30 cycles, the reactions were held at 72°C for 5 min. Amplified products were separated on 2% agarose gels and images acquired after staining with ethidium bromide.

Statistical analyses and construction of genetic linkage maps

The genetic control of resistance and the segregation of molecular markers in the *B. rapa* BC progeny (CZB1) were analyzed using Chi-square tests. Segregating markers were scored for each plant and linkage analysis performed with MAPMAKER/EXP version 3.0 (Lincoln et al. 1992). Linkage groups were established with a LOD-score threshold of 4.0 and a maximum recombination frequency of 0.4. The Kosambi function (Kosambi 1944) was used to evaluate the genetic distances (in centiMorgans; cM) between linked markers.

Results

Genetic analyses of resistance in *B. rapa* progeny

As a first step in our study, a BC1 progeny was derived from 'C1.3' to determine the genetic control of resistance.

Resistance tests were performed on all 164 plants of the *B. rapa* BC1 progeny (CZB1) using the fungal isolates R2 (Av5-7-(8)-10) and S7 (Av1-5-6-7-(8)). These two isolates were chosen for their particular patterns of *AvrLm* alleles (virulent on mustard resistance genes *Rlm6* (R2) and *Rlm10* (S7)), and because they differed at the *AvrLm1* allele. Each seedling was inoculated with both isolates, one isolate on each cotyledon (Table 1). We found that 81 plants were resistant and 47 plants were susceptible to both the isolates, and 17 plants were resistant to S7 and susceptible to R2. The reaction to one of the two isolates was unclear in 19 plants. Among these, five plants were resistant and one susceptible to S7, and five plants were resistant and eight susceptible to R2. Based on the resistance segregation ratios, Chi-square test confirmed that resistance to R2 is compatible with a single dominant gene, *rR2* (86R:72S; $P = 0.26$), whereas resistance to S7 is conferred by two dominant genes (103R:48S), separated by a genetic distance of at least 21 cM ($\alpha = 0.05$). Furthermore, because no plants were both resistant to R2 and susceptible to S7, it suggested that *rR2* was closely linked or may be one of the genes governing resistance to S7 (*rS7.1*). This gene could be one of *Rlm5*, *Rlm7* or *Rlm8*, because the two fungal isolates shared *AvrLm5*, *AvrLm7*, and possibly *AvrLm8*. The second gene conferring resistance to S7, *rS7.2*, appears to also be linked to *rR2* because only 17 out of the 64 plants susceptible to R2 were also resistant to S7. If we assume that *rR2* and *rS7.1* are the same gene, the genetic distance between *rR2* and *rS7.2* is therefore estimated to be 26.6 cM, based on a sample of 64 plants.

Mapping of resistance genes in the *B. rapa* progeny

For the second step in our study, we mapped the genes conferring resistances to S7 and R2 in the *B. rapa* BC1 progeny.

First, we searched for polymorphic markers linked to the resistance genes by using 34 RAPD primers in a bulked segregant analysis. Only four markers were polymorphic in our progeny and among these, two markers, W11.1690, which was previously mapped to DY10 (corresponding to N7; Delourme et al. 2006b) in *B. napus* (Lombard and Delourme 2001), and Y13.1500, which remains unmapped in *B. napus*, were present in the resistant bulk RB1 and absent in the susceptible bulk SB1. The linkage between these markers and the resistance genes was confirmed by genotyping 135 CZB1 progeny plants.

Second, we checked that the resistance genes are located on linkage group R7, the chromosome homologous to *B. napus* N7 (Parkin and Lydiate 1997), because this position was suggested by the linkage between resistance genes and W11.1690. The 135 CZB1 plants were therefore genotyped with CB10450 and CB10439 SSR markers that were previously mapped to N7 linkage group in *B. napus* according to Delourme et al. (2006b) and Piquemal et al. (2005). The linkage between CB10439 and *rR2*/*rS7.1* confirmed the location of the resistance genes on R7 (Fig. 2).

Third we identified additional molecular markers in the targeted genomic region, by using the second set of bulks (RB2 and SB2) built on the phenotypic resistance data and genotypic data at W11.1690, Y13.1500, CB10439 and CB10450 markers. Of the 170 AFLP and 36 SSAP primer combinations analyzed, 54 had at least one marker present

Table 1 Segregation of resistance to S7 and R2 isolates in the *B. rapa* BC progeny

	R/R[a]	R/S	R/ND	S/R	S/S	S/ND	ND/R	ND/S
CZB1-164 plants	81	17	5	0	47	1	5	8
Sample used for mapping (135 pl)	72	14	5	0	34	1	5	4

S susceptible; *R* resistant; *ND* not determined

[a] The first and second letters correspond to the phenotype obtained after inoculation by S7 and R2 isolates, respectively

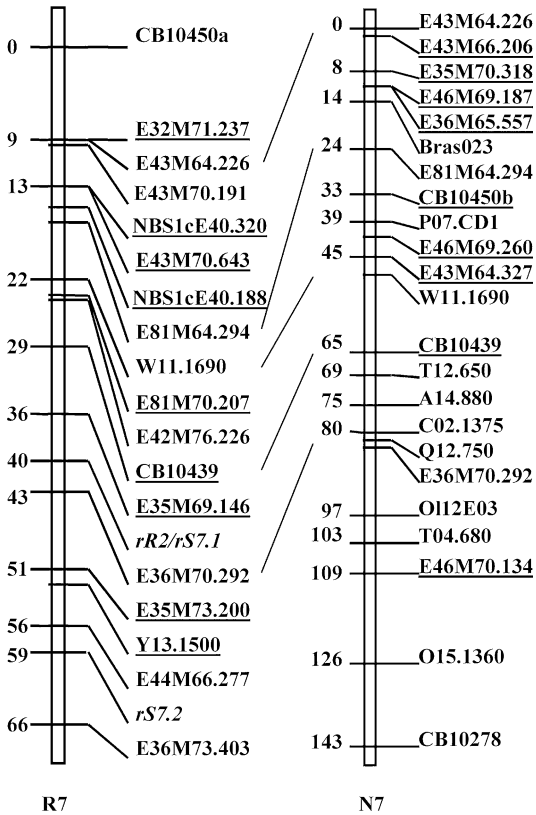

Fig. 2 Genetic map of the R7 linkage group in *B. rapa* BC progeny and its homologue N7, in the *B. napus* 'Darmor-bzh' × 'Yudal' DH population. The locus conferring resistance to the L. maculans isolate R2 was designated rR2 while rS7.1 and rS7.2 correspond to the putative position of the loci conferring resistance to the S7 isolate. The molecular markers which are *underlined* were polymorphic between 'C1.3' and 'Darmor' and were used in RCCD.DH characterization

in 'C1.3' and in the RB2 resistant bulk, and absent in 'Z1' and in the SB2 susceptible bulk. These 54 AFLP or SSAP primer combinations were then used to genotype the 19 plants making up the bulks. Thirty-five of these combinations generated markers that were linked to the resistance genes and finally, 12 markers were tested on the 135 *B. rapa* BC plants to establish the genetic map of the R7 linkage group (Fig. 2). The genes *rR2* and *rS7.1* were located at a distance of 4 and 3 cM from the markers E35M69.146 and E36M70.292, respectively. The position of *rS7.2* was estimated by only including plants susceptible to the R2 isolate in the mapping population (48 plants). The *rS7.2* gene was found to be located in a 9.9-cM interval between E44M66.277 and E36M73.403.

Identification of resistance genes in doubled haploid lines after introgression in *B. napus*

In the third step of our study, the resistant plant 'C1.3' was used to produce a resynthesized *B. napus.* This resynthesized *B. napus* was then crossed to the *B. napus* variety 'Darmor' to generate a doubled haploid segregating population, RCCD.DH (Fig. 1). As opposed to the genetically unique *B.rapa* BC1 plants, which only allowed us to test a maximum of two isolates per genotype (one per cotyledon), the use of selfed progeny of RCC.DH lines provided an opportunity to test several isolates per genotype, which was necessary to fully characterize the resistance genes carried by 'C1.3'. Therefore, selfed progeny of 31 doubled haploid lines from the RCCD.DH population was used to perform the subsequent tests.

None of the lines tested were resistant to fungal isolates v45.57 (Av3-6-8-10), v48.2.6 (Av3-5) or IBCN14 (Av5-6-(10)) (Table 2), which indicated that the DH lines did not contain the genes *Rlm3*, *Rlm5*, *Rlm6*, *Rlm8* or *Rlm10*. By knowing that these genes were absent in the lines tested, it was possible to determine the resistance genes present in the DH line using the other isolates. The detection of lines resistant to isolates R2 (Av5-7-(8)-10), v33.6.3 (Av1-5-6-8-(10)), or PHW1223 (Av5-6-8-9-(10)) indicated that *Rlm7*, *Rlm1* and *Rlm9*, respectively, were segregating in the population. The four lines resistant to the isolate 929 (Av2-3-5-6-9-10), but susceptible to PHW1223 (Av5-6-8-9-(10)), showed that *Rlm2* was also present in the population. Finally, all the DH lines without *Rlm7* were susceptible to R1M1.3 isolate (Av4-5-7-(8)-(10)), suggesting that *Rlm4* may be absent in these lines. However, we did not have an appropriate isolate to determine whether *Rlm4* was in fact absent in the population or may have been lost due to linkage to *Rlm7*.

By combining the results of all the resistance tests (Table 2), we could deduce that *Rlm1* was present in five lines (lines 18–22) and absent in 23 lines, *Rlm2* was present in five lines (lines 20–21 and lines 28–30) and absent in 19 lines, *Rlm7* was present in 10 lines (lines 11–20) and absent in 20 lines and *Rlm9* was present in 7 lines (lines 22–28) and absent in 23 lines. Six lines carried more than one resistance gene.

According to these results, resistance to the R2 (Av5-7-(8)-10) isolate, observed in the *B. rapa* BC progeny, was conferred by *Rlm7*, which also conferred resistance to the S7 isolate (Av1-5-6-7-(8)). The *rR2* and *rS7.1* loci mapped

Table 2 Resistance phenotypes of RCCD.DH lines determined by the avirulence (*Avrlm*) patterns of *L. maculans* isolates

Isolate	Race	DH lines															
		1–10	11–15	16–17	18	19	20	21	22	23	24	25	26	27	28	29–30	31
v45.57	Av3-6-8-10 [a]	S	S	S	S	S	S	S	S	S	S	S	S	I	I	S	I
v48.2.6	Av3-5	–	–	–	–	S	S	–	–	–	–	S	–	S	S	–	S
IBCN14	Av5-6-(10)	–	–	–	–	S	S	–	S	S	S	S	S	S	I	S	S
R2	Av5-7-(8)-10	S/–	R	R	R	R	R	S	S	–	–	S	–	S	S	–	S
v33.6.3	Av1-5-6-8-(10)	S/–	S	I	R	R	R	I	R	–	–	S	–	S	S	–	S
PHW1223	Av5-6-8-9-(10)	S/–	–	–	–	S	S	S	R	I	R	R	R	R	R	S	I
929	Av2-3-5-6-9-10	S	S	S	S	I	R	R	R	R	R	R	R	R	R	R	R
R1 M1.3	Av4-5-7-(8)-(10)	S	–	–	–	–	R	S	S	S	S	S	-	S	S	S	S
S7	Av1-5-6-7-(8)	S	R	R	R	R	R	R	R	S	S	I	–	I	I	S	I
v29.3.1	Av2-7-(10)	–	–	–	–	–	R	I	I	S	–	I	–	I	R	I/–	I
Rlm pattern in DH lines			7 (4)[b]	7 (1; 4)	1; 7 (4)	1; 7 (2; 4)	1; 2; 7 (4)	1; 2	1; 9 (2)	9	9 (2)	9 (2)	9 (1; 2; 4; 7)	9 (2)	2; 9	2	(2; 9)

Each doubled haploid line was classified as resistant (*R*), susceptible (*S*), or intermediate (*I*), based on its reaction to each *L. maculans* isolate

[a] Race nomenclature according to Balesdent et al. (2005); the avirulence alleles in parentheses have not been characterized

[b] Rlm genes in parentheses have not been characterized

in the BC progeny thus corresponded to the *Rlm7* locus. The *Rlm1* gene, detected in 'C1.3', also conferred resistance to the S7 isolate, and was mapped as the *rS7.2* locus in *B. rapa* BC progeny.

Localization of specific resistance genes in doubled haploid lines after introgression into *B. napus*

In the last step of our study, we positioned resistance genes on the R7/N7 linkage group in the RCCD.DH lines, in order to assess the efficiency of resistance gene transfer from *B. rapa* to *B. napus*. The map of the N7 linkage group developed in 'Darmor-bzh' × 'Yudal' (Delourme et al. 2006b; Foisset et al. 1996; Lombard and Delourme 2001; Piquemal et al. 2005) was improved for this study by using AFLP markers (Fig. 2) and five markers common to both *B. rapa* and *B. napus* were used to align the R7 and N7 linkage groups (Fig. 2). The 31 RCCD.DH lines were genotyped by 19 molecular markers polymorphic between 'C1.3' and 'Darmor' (Fig. 3). Nine of these markers were mapped to the N7 *B. napus* linkage group (Fig. 2), among these: two were SSR markers already mapped in *B. napus* (Delourme et al. 2006b; Piquemal et al. 2005) and seven were AFLP markers added to N7 in *B. napus* in this study; eight markers were mapped on *B. rapa* R7 linkage group (Fig. 2); and two specific markers were mapped to the R7/N7 linkage group in a 'Darmor' × 'C1.3' interspecific hybrid (unpublished data). This mapping effort thus allowed us to cover, in the DH lines, a region corresponding to the *B. rapa* R7 region between E32M71.237 and Y13.1500, i.e., 64% of the R7 linkage group. The markers were ordered according to their relative position in *B. rapa* and *B. napus* and to the recombination observed in the RCCD.DH lines (Fig. 3), but genetic distances between markers were not evaluated in this population because of the limited number of lines.

The putative positions of *Rlm1*, *Rlm2*, *Rlm7* and *Rlm9* in the RCCD.DH lines were deduced from the comparison of the R7/N7 genotypes of lines with or without the *Rlm* genes (Fig. 3). Because all the RCCD.DH lines were derived from the same 'RCC' synthetic plant, produced by colchicine doubling of a single F1 hybrid ('RC' × 'C1.3'), 'RCC' was homozygous at the *Rlm1*, *Rlm2* and *Rlm7* loci. *Rlm1*, *Rlm2* and *Rlm7* originated from 'C1.3' and should be linked to 'C1.3' alleles. *Rlm9* was present in 'Darmor' and should be linked to 'Darmor' alleles.

The ten lines carrying *Rlm7* differed from the 22 lines without *Rlm7* only on the At1g80400-E35M73.200 interval, with all resistant lines containing 'C1.3' alleles on this interval and all susceptible lines containing 'Darmor' alleles. This indicated that *Rlm7* was localized in this interval (Fig. 3).

For *Rlm1*, *Rlm2* and *Rlm9*, no interval seemed to differentiate resistant from susceptible lines, in spite of regions in the linkage group common to all resistant lines either for *Rlm1*, or *Rlm9*. Indeed, for each interval included in these regions, we found some susceptible lines containing alleles from the resistant parent (Fig. 3).

Discussion

This study showed that in order to characterize three *L. maculans* specific resistance genes (*Rlm1*, *Rlm2* and

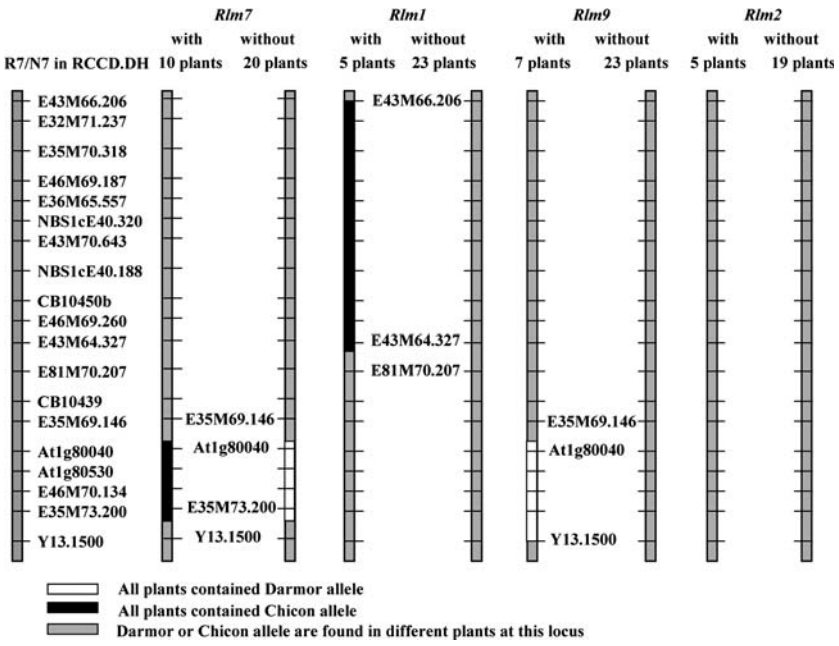

Fig. 3 Genotypes of the RCCD.DH lines *with* and *without* each *L. maculans* resistance gene (*Rlm*)

Rlm7) present in the *B. rapa* 'C1.3' plant it was necessary to analyze two segregating populations, one BC1 *B. rapa* population and a *B. napus* doubled haploid population, derived from the same *B. rapa* resistant genotype. To identify resistance genes in *B. rapa* genetic resources, which are generally heterozygous, several fungal isolates must be tested on each plant because the fungal isolates used possess complex patterns of avirulence genes. In BC1 plants, however, only two isolates can be analyzed at one time, so plant material was produced that permitted us to simultaneously identify resistance genes and assess the efficiency of their introgression in *B. napus*. Doubled haploid lines were produced from resynthesized *B. napus* using 'C1.3' as the *B. rapa* progenitor, and by crossing it to a *B. napus* cultivar which carries a resistance gene on N7. This strategy had the added benefit of determining the respective positions of both *B. rapa* and *B. napus* resistance genes. The number of lines (31) was sufficient to segregate out all the resistance genes from 'C1.3' and to obtain various sizes of introgression.

In the *B. rapa* progeny, *Rlm1* was mapped to the R7 linkage group, between the markers E44M66.277 and E36M73.403, but due to the lack of polymorphic molecular markers between 'C1.3' and 'Darmor' in this region, it was not possible to pin point *Rlm1* in RCCD.DH lines. The position of *Rlm7* was mapped in both the *B. rapa* and the RCCD.DH populations. The comparison of the two maps indicated that the *Rlm7* locus is in the At1g80040-E36M70.292 interval. Interestingly, *Rlm1* and *Rlm7* are two resistance genes naturally present in *B. napus* and have been mapped to DY10, which corresponds to the N7 linkage group (Delourme et al. 2004, 2006b), on intervals homologous to the *B. rapa* regions in this present study. Thus our results suggest that these resistance genes have the same position in *B. rapa* and *B. napus*. Either these genes are orthologous and were present on the *B. rapa* A genome prior to the formation of *B. napus*, and were present in one of the *B. rapa* plants at the origin of *B. napus*, or these genes were transferred from one species to the other through interspecific hybridization.

In *B. napus*, other *L. maculans* specific resistance genes have been suggested to be located on the N7 linkage group, in different cultivars (Delourme et al. 2006a, 2004; Dion et al. 1995; Ferreira et al. 1995; Mayerhofer et al. 2005; Rimmer 2006). Hence, there appears to be a cluster of resistance genes on N7 in *B. napus*. If these *B. napus* genes have orthologs in *B. rapa*, as it seems to be the case for *Rlm1* and *Rlm7*, then a cluster of resistance genes may also be present in *B. rapa*, and other resistance genes may be found on R7 among *B. rapa* species. Another hypothesis is that some resistance genes appeared in *B. napus* after its formation. Indeed, clusters of resistance genes are known to evolve rapidly through unequal crossing over, gene conversion or recombination, which may generate new alleles or new resistance genes (Noel et al. 1999).

In this study, the *Rlm*9 gene could not be mapped, however in a previous study (Delourme et al. 2004) it was positioned in *B. napus* on the N7 linkage group between the markers A14.880 and C02.1375. Two of our findings appear to confirm the position of *Rlm*9 on N7: first, *Rlm7* and *Rlm9* genes were never found simultaneously in the same plant, and second, all RCCD.DH plants carrying *Rlm9* possessed 'Darmor' alleles on N7 between the CB10439 and E46M70.134 markers. However, since plants were found that lacked *Rlm9* although they had the 'Darmor' alleles in this interval, *Rlm9* may be a major resistance gene whose expression is regulated by other genetic factors.

In our study, the *Rlm* genes detected in *B. rapa* were introgressed into *B. napus* by homologous recombination via the creation of a synthetic *B. napus*. Homologous recombination occurred at a high frequency and along the entire linkage group, because our molecular analysis of the RCCD.DH lines showed large variations in the sizes of introgressed fragments. This observation agrees with the results reported by Parkin and Lydiate (1997), which showed that R7 and N7 linkage groups are essentially the same. This may explain why some resistance genes introduced from *B. rapa* could be used successfully in *B. napus* commercial lines, such as the 'Surpass 400' resistance gene (Li and Cowling 2003), whereas resistance genes found on the Brassica B genome showed a lack of recombination when they were introgressed into *B. napus* (Barret et al. 1998; Chevre et al. 1997).

Three specific resistance genes, *Rlm1*, *Rlm2* and *Rlm7*, were detected in a single *B. rapa* plant. Further screening of the same or of other *B. rapa* cultivars with *L. maculans* isolates may detect other resistance genes and perhaps even resistance genes that do not exist naturally in *B. napus*. Resistance genes have been detected in *B. rapa*: among 555 accessions, 4.5% contained *Rlm1*, *Rlm2* or *Rlm4* (Delourme et al. 2006a). Both *Rlm7* and *Rlm8* were detected in at least one *B. rapa* accession (Balesdent et al. 2002; Delourme et al. 2006a). The *Rlm8* gene was recently described and has not yet been observed in *B. napus*. Three resistance genes, *LepR1*, *LepR2* and *LepR3*, were introduced from *B. rapa* into *B. napus* (Crouch et al. 1994). The *LepR1* gene was mapped to N2, and *LepR2* and *LepR3* to N10 (Yu et al. 2004; Yu et al. 2005), clearly indicating that they are different from *Rlm1* and *Rlm7* that are present in 'C1.3'. In our study, we did not map the *Rlm2* gene, but if its position is conserved between *B. rapa* and *B. napus*, it is expected to map to R10 which corresponds to *B. napus* LG16-N10 (Delourme et al. 2004, 2006b), so in a similar position to *LepR2* or *LepR3*. This suggests either that *Rlm*2 corresponds to *LepR2* or to *LepR3*, or that another cluster of *L. maculans* resistance genes exists on N10/R10 in *B. rapa*.

We have produced plant material that may be used to clone different *Rlm* genes identified in this study. In spite of the presence of different resistance genes on the same linkage group (N7) and the complex pattern of *AvrLm* genes in the *L. maculans* isolates, the resistant doubled haploid lines with very small introgression should allow the identification of molecular markers closely linked to each *Rlm* gene. Subsequent fine mapping would then be possible by screening the 300 DH lines produced with molecular markers and an appropriate isolate of *L. maculans*. For example, isolate R2 could be used for *Rlm7*, because *Rlm5*, *Rlm8* and *Rlm10* were not present in this material.

Last, the material characterized was produced in a 'Darmor' genetic background, which carries polygenic resistance conferred by various quantitative trait loci (Pilet et al. 1998). It has been suggested that the combination of quantitative and qualitative resistance may improve the durability of resistance (Brun et al. 2000). In the future, therefore, it should be possible to choose DH lines carrying different combinations of quantitative trait loci and *Rlm* genes effective against the local pathogen population, and to assess the genetic structure that provides the most effective and durable resistance under field conditions.

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