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ORIGINAL PAPER

Molecular and phenotypic characterization of near isogenic lines at QTL for quantitative resistance to *Leptosphaeria maculans* in oilseed rape (*Brassica napus* L.)

R. Delourme · N. Piel · R. Horvais · N. Pouilly · C. Domin · P. Vallée · C. Falentin · M. J. Manzanares-Dauleux · M. Renard

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Abstract The most common and effective way to control phoma stem canker (blackleg) caused by *Leptosphaeria maculans* in oilseed rape (*Brassica napus*) is by breeding resistant cultivars. Specific resistance genes have been identified in *B. napus* and related species but in some *B. napus* cultivars resistance is polygenic [mediated by quantitative trait loci (QTL)], postulated to be race non-specific and durable. The genetic basis of quantitative resistance in the French winter oilseed rape 'Darmor', which was derived from 'Jet Neuf', was previously examined in two genetic backgrounds. Stable QTL involved in blackleg resistance across year and genetic backgrounds were identified. In this study, near isogenic lines (NILs) were produced in the susceptible background 'Yudal' for four of these QTL using marker-assisted selection. Various strategies were used to develop new molecular markers, which were mapped in these QTL regions. These were used to characterize the length and homozygosity of the 'Darmor-*bzh*' introgressed segment in the NILs. Individuals from each NIL were evaluated in blackleg disease field trials and assessed for their level of stem canker in comparison to the recurrent line 'Yudal'. The effect of QTL *LmA2* was clearly validated and to a lesser extent, QTL *LmA9* also showed an effect on the disease level. This work provides valuable material that can be used to study the mode of action of genetic factors involved in *L. maculans* quantitative resistance.

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R. Delourme (✉) · N. Piel · R. Horvais · N. Pouilly · C. Domin · P. Vallée · C. Falentin · M. J. Manzanares-Dauleux · M. Renard
UMR 118 Amélioration des Plantes et Biotechnologies Végétales, INRA, BP 35327, 35653 Le Rheu Cedex, France
e-mail: Regine.Delourme@rennes.inra.fr

Present Address:
N. Pouilly
Laboratoire des Interactions Plantes Micro-organismes, UMR CNRS-INRA 2594/441,
Chemin de Borde-Rouge, Auzeville,
BP 52627, 31326 Castanet Tolosan Cedex, France

Introduction

The development of near isogenic lines (NILs) for quantitative trait loci (QTL) using marker assisted selection is a reliable method for validating the additive effect of QTL, because the effect of individual or combined QTL alleles is more accurately estimated in a homogeneous genetic background. This method was successfully used to validate QTL when NILs were generated by backcrosses using either one of the QTL mapping population parents as the recurrent parent (van Berloo et al. 2001; Maeda et al. 2006; Wissuwa and Ae 2001) or new genetic backgrounds (Toojinda et al.1998; Thabuis et al. 2004; Richardson et al. 2006). With the same objective, NILs were produced from heterogeneous inbred families derived from recombinant inbred line mapping populations (Tuinstra et al. 1997; Loudet et al. 2007) or from breeding populations (Pumphrey et al. 2007). NILs are valuable material for studying QTL × environment (Steele et al. 2007) or QTL × genetic background (Lecomte et al. 2004) interactions. NILs can also be used for an improved phenotypic characterization of QTL effect (Ioannidou et al. 2003; Wissuwa and Ae 2001) or for fine mapping and identifying candidate genes underlying QTL (Quarrie et al. 2006; Wan et al. 2006; Loudet et al. 2007).

Blackleg (stem canker) of oilseed rape (*Brassica napus* L.), caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not., is a serious disease in most rapeseed producing countries (West et al. 2001; Fitt et al. 2006). In

Europe, since the mid 1980s, stem canker levels have fluctuated, possibly due to inter-year variations in climatic conditions, pathogen populations and the resistance levels of cultivated varieties. Chemical control has proven not efficient enough, so efforts aim at genetic control of the disease. In oilseed rape, several sources of resistance to blackleg are known (Delourme et al. 2006a; Rimmer 2006). Many studies on the inheritance of resistance have been carried out at both the seedling and adult plant growth stages. Two types of resistance are usually distinguished. The first type is a qualitative resistance, which is expressed in the seedling and the adult plant and is generally considered as single-gene race specific resistance. The second type is a quantitative adult-plant resistance, which is partial resistance mediated by many genes. Quantitative resistance is considered to be race non-specific and more durable than qualitative resistance and is thus of particular interest for breeding resistant varieties (Boyd 2006).

The genetic basis of quantitative resistance in the French winter oilseed rape 'Darmor', derived from 'Jet Neuf', was studied in two genetic backgrounds. In the 'Darmor-*bzh*' × 'Yudal' cross, Pilet et al. (1998) identified a total of ten resistance QTL, four of which were associated with decreased stem canker severity and decreased plant death in two seasons of field experiments. Analysis of progeny derived from a 'Darmor' × 'Samourai' cross, consisting of one double haploid (DH) population and one $F_{2:3}$ family population, identified six QTL in the DH population and four QTL in the $F_{2:3}$ families (Pilet et al. 2001). Out of 16 loci detected in all, only four QTL were common to both the 'Darmor-*bzh*' × 'Yudal' and 'Darmor' × 'Samourai' crosses.

We aimed to develop NILs for four stem canker resistance QTL in order to validate the effect of individual QTL in the susceptible background 'Yudal'. These four QTL were chosen because they were the most stably detected ones in our previous studies. Three of these QTL were stably detected in the two DH populations (on linkage groups A2, C2 and C4). The fourth QTL (on linkage group A9) was specific to the 'Darmor-*bzh*' × 'Yudal' cross but was detected throughout the two-year trial. For each QTL, we generated tall and dwarf lines since it was shown that the *Bzh* dwarf gene might have an effect on resistance to *L. maculans* (Pilet et al. 1998). In order to characterize the introgressed segment, we designed new molecular markers within the targeted QTL regions either by deriving Sequenced characterized amplified regions (SCARs); Paran and Michelmore 1993) from Random Amplified Polymorphic DNA (RAPD) or Amplified Fragment Length Polymorphism (AFLP) markers or by taking advantage of the synteny with the *Arabidopsis thaliana* (hereinafter referred to as Arabidopsis) genome (Parkin et al. 2005). The derived NILs were then evaluated for their level of stem canker resistance in the field, which allowed us to clearly validate the effect of QTL *LmA2* and to a lesser extent the effect of QTL *LmA9* in four different field sites.

Materials and methods

Production of NILs

Backcrosses to 'Yudal', the susceptible parent, were initiated from DH lines of the segregating population derived from the 'Darmor-*bzh*' × 'Yudal' cross. 'Darmor-*bzh*' is a dwarf isogenic line resulting from the introduction of the dwarf *bzh* allele in the resistant French winter cultivar 'Darmor'. 'Yudal' is a spring Korean line that is very susceptible to *L. maculans*. The DH lines were chosen to carry the targeted QTL and be as close as possible to the 'Yudal' genetic background. Plants were backcrossed until the BC2 and BC3 generations and then selfed to obtain NILs homozygous at the QTL. Plants were screened using markers located in the QTL regions and markers to control the genetic background. For foreground selection, the markers used at each targeted QTL are shown on Fig. 1. Markers were chosen according to their genetic determinism (dominant in 'Darmor-*bzh*' or codominant) and each QTL confidence interval. For this, the Pilet et al. (1998) genetic map was completed (Lombard and Delourme 2001) and a new QTL detection was carried out. The initial DH lines still contained 'Darmor-*bzh*' genetic

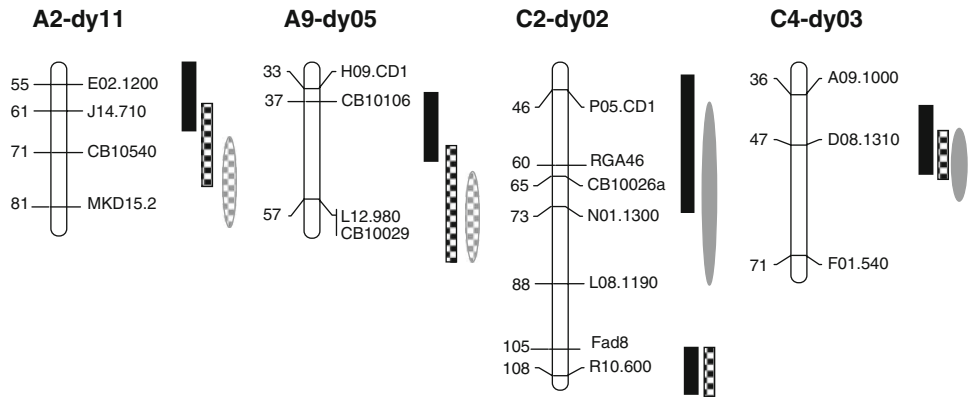

Fig. 1 Location of the markers used for NIL selection in relation to the QTL confidence intervals identified at the beginning of the study, for the four variables: disease index in 1995 (*black*) and 1996 (*gray*); plant losses in 1995 (*hatched black*) and 1996 (*hatched gray*)

information on 11–15 linkage groups (excluding the linkage group carrying the targeted QTL), which represented a minimum of 19–30% of 'Darmor-*bzh*' information based on the length of the genetic map (Table 1). Then, between 17 and 26 markers, depending on the DH lines used (Table 1), were used to control the genetic background. The markers were mainly RAPD as described by Foisset et al. (1996), Pilet et al. (1998) and Lombard and Delourme (2001) and Amplified Genetic Consensus Markers (ACGM—Brunel et al. 1999; Fourmann et al. 2002) or RGA (Resistance Gene Analogs) markers (Fourmann et al. 2001).

Dwarf and tall lines were produced for each QTL. The dwarf trait was screened using a specific PCR marker based on the sequence of the *Arabidopsis thaliana GAI* (*GIBBERELLIN-INSENSITIVE;* At1g14920) gene which corresponds to the *Bzh* gene (Renard et al. 1999). This marker was designed in collaboration with D. Brunel (INRA Versailles). (Primers: GaiUP153: ACT CGA GCA GCT TGA RRY NAT GAT G; GaiLP766: GCT TGA TTC GCN GTR AAR TG). The PCR protocol was as described in Brunel et al. (1999). Electrophoresis was performed on non-denaturing polyacrylamide gel (46 cm, 5% acrylamide-bis 29:1, 1 × TBE, 250 V for 24 h).

Development of new molecular markers at the QTL

Molecular markers were generated by various strategies. SCAR markers were derived from RAPD markers that were previously mapped in the QTL regions (Pilet et al. 1998). PCR products were cloned with the TOPO TA cloning® kit (Invitrogen, Carlsbad, USA), according to the manufacturer's instructions. Plasmid DNA extractions were carried out using the Wizard® *plus* SV minipreps DNA purification system (Promega, Madison, USA). Inserts sizes were checked using agarose electrophoresis after a 37°C, 1 h *Eco*RI restriction digest (Roche, Mannheim, Germany). Inserts were then sequenced by Cogenics Genome Express (Meylan, France).

AFLP and Sequence-Specific Amplified Polymorphism (S-SAP) markers were screened on DNA bulks built for carrying either the 'Darmor-*bzh*' or the 'Yudal' allele at the markers in each targeted QTL region. Eight bulks were then used. For AFLP markers, the restriction, ligation and preamplification reactions of the AFLP protocol were performed according to Vos et al. (1995). DNA was digested with *Eco*RI and *Mse*I restriction enzymes (Roche, Mannheim, Germany) and the corresponding adapters were ligated to the fragments. Preamplification reactions were performed with standard *EcoRI* (E + A) and *MseI* (M + G) adapter primers. Selective amplifications were performed in a 5 µL reaction mixture containing 1.5 µL diluted preamplification product, 0.06 µM IRDdye-700 or IRDye-800 labelled EcoRI primer, 0.32 µM MseI primer, 200 µM each dNTP, 2.5 mM $MgCl_2$ and 0.3 U Go Taq Flexi polymerase (Promega, Madison, USA). For S-SAP markers, selective amplifications were performed in a 5 µL reaction mixture containing 1.5 µL diluted AFLP preamplification product, 0.4 µM labelled NBS-LRR specific primer, 0.16 µM

Table 1 Characteristics of the initial double haploid lines, number of markers used to control 'Darmor-*bzh*' genetic background and results of the screen with the derived NILs and the number of retained 'Darmor-*bzh*' markers in non-targeted QTL regions (in parenthesis)

	DH				
	D11	E100	F4	C108	E63
Targeted QTL	*LmA2*	*LmA2*	*LmA9*	*LmC2*	*LmC4*
Bzh gene	Tall	Dwarf	Dwarf	Dwarf	Dwarf
# LG with remaining 'Darmor-*bzh*' segment	13	11	15	13	12
Minimum % 'Darmor-*bzh*' segments on the whole map	27	24	26	19	30
# markers to control genetic background	24	21	26	17	18
Derived NILs (# retained 'Darmor-*bzh*' markers in non-targeted regions)	NILA2.1 (1) NILA2.2 (1) NILA2.6 (1) NILA2.7 (1) NILA2.8 (1)	NILA2.3 (0) NILA2.4 (0) NILA2.5 (0) NILA2.9 (0) NILA2.10 (0) NILA2.11 (0) NILA2.12 (0) NILA2.13 (0)	NILA9.1 (0) NILA9.2 (1) NILA9.3 (1) NILA9.4 (1) NILA9.5 (1) NILA9.6 (1) NILA9.7 (1) NILA9.8 (0) NILA9.9 (1) NILA9.10 (1) NILA9.11 (0) NILA9.12 (0)	NILC2.1 (2) NILC2.2 (2) NILC2.3 (2) NILC2.4 (2) NILC2.5 (2) NILC2.6 (0) NILC2.7 (0) NILC2.8 (1) NILC2.9 (1)	NILC4.1 (1) NILC4.2 (1) NILC4.3 (1) NILC4.4 (1) NILC4.5 (1) NILC4.6 (2) NILC4.7 (2) NILC4.8 (0) NILC4.9 (0) NILC4.10 (0) NILC4.11 (0) NILC4.12 (0) NILC4.13 (1) NILC4.14 (1)

LG Linkage group

Eco + 3 primer, 200 µM each dNTP, 2.5 mM MgCl2 and 0.3 U Go Taq Flexi polymerase (Promega, Madison, USA). Three different NBS primers designed by Rocherieux (2004) were used, NBS1 (GGGGGGGTAGTGGGAAAG ACGAC), NBS2 (GGCGGTTCAGGGAAAGACATAC) and NBS1c (GTCGTCTTCCCAGCTACCCCAGTCCC).

For both AFLP and S-SAP markers, the amplification conditions were: 1 cycle of 3 min at 94°C, 13 cycles of 10 s at 94°C, 30 s at 65°C (reduced 0.7°C per cycle), 1 min at 72°C, followed by 25 cycles of 10 s at 94°C, 30 s at 56°C, 60 s (extended 1 s per cycle) at 72°C and a final elongation step of 5 min at 72°C. The PCR products were resolved in 41-cm gels containing 5.5% Long Ranger acrylamide gel solution (BMA, Rockland, USA), 7 M urea and 1× TBE buffer. Electrophoresis was performed in a LI-COR DNA analyzer (LI-COR, Lincoln, USA), at 2,000 V for 6 h, following the manufacturer's instructions. The AFLP and S-SAP fingerprint patterns were analyzed with AFLP-Quantar-Pro software v1.04 (Keygene, Wageningen, The Netherlands).

The AFLP and S-SAP fragments that mapped in the QTL regions were then cloned and sequenced to produce SCAR markers. The PCR products were separated by electrophoresis at 1500 V for 2.5 h on a 6% acrylamide gel buffered with 1× TBE, and revealed by silver nitrate staining as follows: the acrylamide gel was incubated for 5 min in 10% ethanol, 3 min in 1% nitric acid, and then 45 min in 7.65 mM silver nitrate. Between incubations, the gel was carefully rinsed in distilled water. Bands were detected by incubation with 0.05% formaldehyde in 0.28 M sodium carbonate and the detection reaction was stopped by adding 10% acetic acid. The gel was then rinsed for 5 min in distilled water and air-dried at room temperature. The amplified bands were excised from dried acrylamide gel with a sterile scalpel, suspended in 30 µL 5× Go Taq Flexi buffer (Promega, Madison, USA), submitted to four freezing/defrosting cycles and re-amplified by PCR using the conditions described above except for the $MgCl_2$ concentration (1.5 mM) and the PCR profile (1 cycle of 4 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C, and a final elongation step at 72°C for 5 min).

The PCR products were cloned into the pGEM T Easy vector (Promega, Madison, USA), according to the manufacturer's instructions. The AFLP or S-SAP fragment DNA inserts were checked by PCR and sequenced by Cogenics Genome Express (Meylan, France). Nucleotide sequences were aligned using the GENEDOC program v2.6.002 (http://www.psc.edu/biomed/genedoc).

Compatible primer pairs for SCARs were designed using the primer3 web interface (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi, (Rozen and Skaletsky 2000).

SCARs were amplified in a 17 µL reaction mixture containing 20 ng template DNA, 0.2 µM each primer, 200 µM each dNTP, 1.5 mM $MgCl_2$ and 0.6 U *Taq* polymerase (Promega, Madison, USA) and using the following PCR profile: 1 cycle of 4 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at annealing temperature, 30 s at 72°C, with a final elongation step of 5 min at 72°C. Amplification products were separated on 3% agarose gel buffered with 1× TBE and visualized by ultraviolet illumination after ethidium bromide staining.

Finally, primers were designed on *A. thaliana* gene sequences in regions identified as potentially colinear with the QTL regions. Primers were designed on exon sequences surrounding one or two introns to maximize the chances of successful amplification and polymorphism identification. Amplifications were performed in a 17 µL reaction mixture containing 20 ng genomic DNA, 0.3 µM primers, 200 µM each dNTP, 1.5 mM MgCl2 and 0.6 U Go Taq Flexi polymerase (Promega, Madison, USA) and using the following PCR profile: 1 cycle of 4 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at annealing temperature, 30 s at 72°C, with a final elongation step of 5 min at 72°C. Electrophoresis on agarose or acrylamide gels was performed as described above.

Genetic mapping and QTL detection

AFLP and S-SAP markers as well as SCAR markers derived either from RAPD, AFLP or S-SAP markers and the markers generated from Arabidopsis sequences were mapped on the updated 'Darmor-*bzh*' × 'Yudal' map (Delourme et al. 2006b) using MAPMAKER/EXP 3.0 (Lincoln et al. 1992). A multipoint analysis was performed to place the loci on the framework linkage map with the commands 'assign' and 'place' (LOD = 3.0). Centimorgan distances were expressed with the Kosambi function (Kosambi 1944). QTL detection was performed using Composite Interval Mapping (CIM) with QTL Cartographer software (Basten et al. 1997). Ten markers, selected by a forward-backward stepwise regression analysis, were used as cofactors in the CIM procedure, with a 10 cM window size and $P_{in/out} = 0.05$. The LOD threshold was estimated at 3.0 after 500 permutation tests for each variable. We used the linkage group nomenclature that was recently proposed as a reference by the Multinational *Brassica* Genome Project Steering Committee where the *B. napus* N1–N19 nomenclature is replaced by A1–A10 and C1–C9 designations (http://www.brassica.info/information/lg_assigments.htm). Thus the QTL were named according to their location on each linkage group i.e. *LmA9* for QTL of resistance to *L. maculans* located on linkage group A9.

Molecular characterization of the NILs

The markers used to characterize the length and homozygosity of the 'Darmor-*bzh*' introgressed segment in the

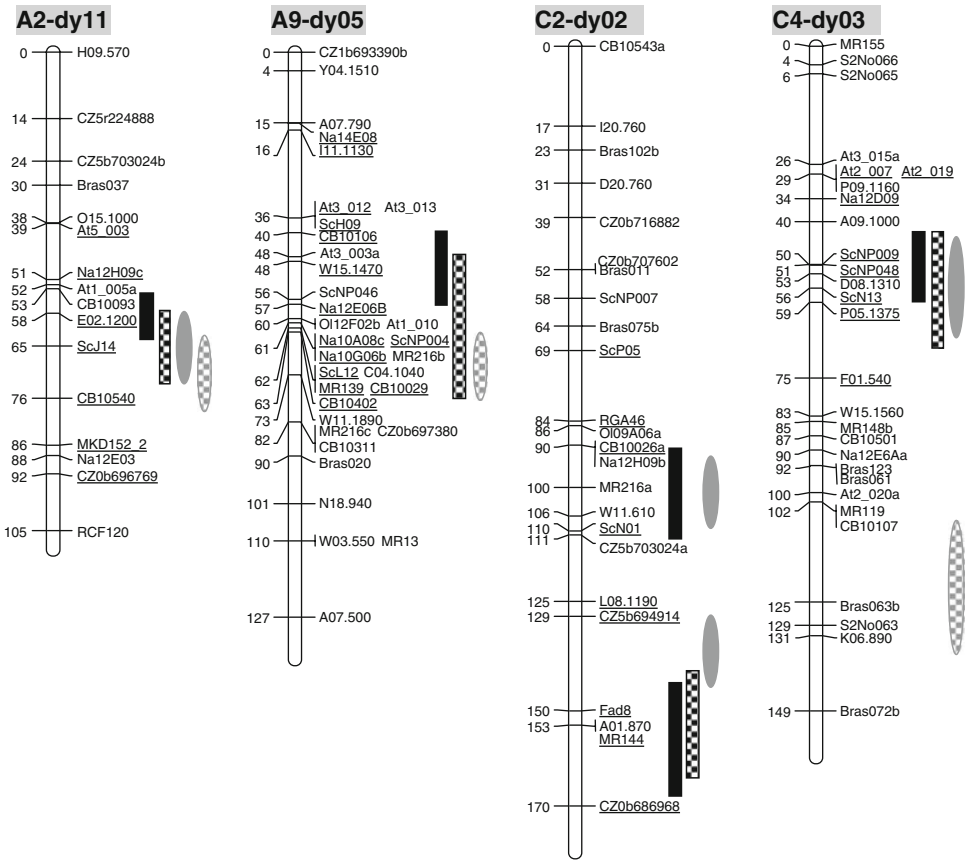

Fig. 2 New QTL positions for the four variables [disease index in 1995 (*black*) and 1996 (*gray*); plant losses in 1995 (*hatched black*) and 1996 (*hatched gray*)] obtained with the markers developed in this study. The *underlined markers* were used for the molecular characterization of the derived NILs

derived NILs are shown in Fig. 2. These were the markers developed in this study and SSR and PFM markers from Delourme et al. (2006b) that were previously mapped to the QTL regions.

Phenotypic evaluation of the NILs

Plants of each NIL were evaluated in blackleg disease field trials and assessed for their level of stem canker in comparison to the recurrent susceptible line 'Yudal'. The field disease trials and scoring of blackleg severity (G2 disease index) were conducted as previously described in Pilet et al. (1998). Forty plants per plot were uprooted and crown canker was assessed on a 1–6 scale as follows: 1 = no disease, 2 = 1–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–100% of crown section cankered. An additional disease score category of 6 was used to indicate that the plant had broken at the crown from severe canker. All crown canker data were transformed to a standardized 1–9 disease severity scale using the formula: G2 index = $[(N1 \times 0) + (N2 \times 1) + (N3 \times 3) + (N4 \times 5) + (N5 \times 7) + (N6 \times 9)]/Nt$, where N1,2…6 = the number of plants with canker scores of 1, 2,….6, respectively, and Nt = the total number of plants assessed. In experiments carried out in 1995 and 1996, the proportion of plant losses was also assessed in each plot by first counting the total number of plants at the stem extension stage, and then a second time before maturity (Pilet et al. 1998).

NILs for the four QTL (QTL *LmA2*, *LmA9*, *LmC2* and *LmC4*) were evaluated at one location with three replications (INRA Le Rheu) in 2004–05. NILs for QTL *LmA2* and QTL *LmA9* were evaluated at four locations in 2005–2006 (INRA Le Rheu, CETIOM Grignon, PIONEER Génétique Blois & EURALIS Semences Blois). Eight replicates were performed at each field site to increase the test accuracy. The control for the evaluation of the NILs was the recurrent line 'Yudal'. The other controls ('Eurol', 'Falcon', 'Goeland', 'Darmor', 'Darmor-*bzh*' in 2004–2005 at Le Rheu and 'Eurol', 'Falcon', 'Goeland' in 2005–2006 at the four locations) were included as references for disease severity on winter oilseed rape. In order to make a good comparison with 'Yudal', which is a very susceptible line, the disease assessment was performed on 23rd of May in 2004–2005 and between 16th and 30th of May depending on the location in 2005–2006. For each trial, the analysis of variance (ANOVA; proc GLM of Statistical Analysis System-SAS—SAS Institute Inc., 1989) partitioned total variation into line, replicate and error effects ($P_{ij} = \mu + L_i + R_j + e_{ij}$ where P_{ij} is the G2 index of the ith line located in the jth replicate, μ the mean of all the data, L_i the line i effect, R_j the replicate j effect and e_{ij} the residual). For the 2005–2006

trials, a global analysis was also performed partitioning total variation into line, location, replicate and error effects ($P_{ijk} = \mu + L_i + T_j + R_{k/j} + e_{ijk}$ where P_{ijk} is the G2 index of the ith line located in the kth replicate of the jth location, μ the mean of all the data, L_i the line i effect, T_j the location j effect, $R_{k/j}$ the replicate k effect in the jth location and e_{ijk} the residual). Comparisons of the NIL means to the 'Yudal' mean were performed with the unilateral Dunnett test ($\alpha = 5\%$).

Results

Production of NILs

The production of NILs was initiated from five DH lines from the 'Darmor-*bzh*' × 'Yudal' population with either a tall or dwarf phenotype (Table 1). The number of plants screened at each generation for each QTL is summarized in Table 2. In the BC_1F_1, BC_2F_1 and BC_3F_1 generations, 38,

Table 2 Summary of the backcross screen used to generate the NILs, number of screened plants, number and percent of selected plants and number of dwarf plants for each generation are shown

DH	D11	E100	F4	C108	E63
Targeted QTL	*LmA2*	*LmA2*	*LmA9*	*LmC2*	*LmC4*
Bzh gene	Tall	Dwarf	Dwarf	Dwarf	Dwarf
BC1F1-QTL screening					
# plants screened	24	48	48	48	48
# plants selected	9 (37%)	13 (30%)	20 (37%)	A2 (25%)	30 (57%)
# dwarf plants	–	5	4	5	12
BC2F1-QTL screening					
# plants screened	24	63	43	64	72
# plants selected	7 (39%)	27 (36%)	13 (25%)	24 (38%)	27 (38%)
# dwarf plants	–	9	3	10	nd
Genetic background					
# plants screened	13	11	13	26	27
# plants selected	2	1	2	11	3
# dwarf plants	–	1	1	3	2
BC3F1-QTL screening					
# plants screened	24	36	60	192	60
# plants selected	9 (37%)	11 (30%)	18 (30%)	63 (33%)	31 (52%)
Genetic background					
# plants screened	8	10	16	48	28
# plants selected	3	10	6	14	11
BC2F2-High plants					
# plants screened	48		48	96	42
# plants selected/QTL[a]	4 (8%)		2 (4%)	13 (13%)	20 (47%)
# plants selected/background	4		2	3	5
BC2F2-Dwarf plants					
# plants screened		48	48	144	24
# plants selected/QTL[a]		4 (8%)	9 19%)	9 (6%)	15 (62%)
# plants selected/background		4	3	3	2
BC3F2-High plants					
# plants screened	48	48	96	144	96
# plants selected/QTL[a]	14 (29%)	4 (8%)	20 (21%)	20 (14%)	13 (13%)
# plants kept	3	2	5	4	4
BC3F2-Dwarf plants					
# plants screened		48			48
# plants selected/QTL[a]		5 (10%)			8 (16%)
# plants kept		4			3

[a] Including screening for homozygosity at the targeted QTL

36 and 35% of the plants were selected, respectively. In the BC_2F_1, BC_2F_2 and BC_3F_1 generations, markers were also used to select the genetic background and monitor the recovery of 'Yudal' genetic information along the whole genome, except for the targeted QTL region. This resulted in some NILs free from all the tested background markers although one or two markers were still present in some NILs (Table 1). These remaining markers were not located in the other non-targeted QTL regions. In the BC_2F_2 and BC_3F_2 generations, markers were used to screen plants for homozygozity at the targeted QTL regions (Table 2). These markers were either codominant markers or dominant markers from 'Yudal'. In the end, 13, 12, 9 and 14 NILs were created for QTL *LmA2, LmA9, LmC2* and *LmC4*, of which 2, 3, 1 and 4 were dwarf lines, respectively.

Development of molecular markers at the QTL

SCAR markers

Nine RAPD markers located in the QTL regions were cloned and sequenced to generate SCAR markers. These were E02.1200, J14.710 (QTL *LmA2*), H09.cd1, L12.980, W15.1470 (QTL *LmA9*), P05.cd1, S18.1300, N01.1300 (QTL *LmC2*), A09.1000, D08.1310, R13.1600 and N13.1150 (QTL *LmC4*). Seven SCAR markers were obtained that were polymorphic between 'Darmor-*bzh*' and 'Yudal' (ScH09, ScJ14, ScL12, ScN01, ScN13, ScP05) or 'Darmor' × 'Samourai' (ScS18) and that mapped to the same location as the original RAPD (Table 3; Figure 2).

Eighty-six S-SAP and 30 AFLP primer combinations were screened on the 8 DNA bulks. Following this screen, 16, 16, 22 and 16 markers were polymorphic on the bulks built for QTL *LmA2*, *LmA9*, *LmC2* and *LmC4*, respectively. The polymorphic markers were mapped in the 'Darmor-*bzh*' × 'Yudal' DH population and 5 S-SAP, 10 (8 AFLP and 2 S-SAP), 14 (11 AFLP and 3 S-SAP) and 7 AFLP markers mapped in the QTL *LmA2*, *LmA9*, *LmC2* and *LmC4* confidence intervals, respectively. Twenty of these markers were cloned and sequenced, and five SCAR markers were obtained which were polymorphic between 'Darmor-*bzh*' and 'Yudal' and mapped to the original AFLP location (Table 3; Fig. 2).

Specific PCR markers derived from Arabidopsis

With progress on our *B. napus* genetic map, we were able to identify anchor points to the Arabidopsis genetic and physical maps and use the alignment between the *B. napus* and Arabidopsis maps, previously determined by Parkin et al. (2005), to identify potential Arabidopsis regions that could be syntenic to our targeted QTL regions. For each QTL, two potential syntenic Arabidopsis regions were used, within which primer pairs were designed on different genes (Table 4). For QTL

Table 3 Details of the SCAR markers designed in the study

SCARs	QTL	Original marker	Forward primer	Reverse primer	Tm (°C)	Dominance
RAPD-SCARs						
ScH09	*LmA9*	H09.cd1	AAGTTTTGGCGAATCTGTTATGCT	TGTAGCTGGGACAATGAACATTGA	50	Codom D/Y
ScL12	*LmA9*	L12.980	GGGCGGTACTAATGCAAAAGAAAA	GGGCGGTACTATATTTGAGAGAAG	60	Dom D
ScJ14	*LmA2*	J14.710	CACCCGGATGGTCGTACGAGGATA	CACCCGGATGCACTTCCTCAAGAT	60	Dom D
ScP05	*LmC2*	P05.cd1	GGTAACTACAGAAGTAGAGAT	GAGATATTGGTTGTCATATTCTT	54	CodomD/Y
ScN01	*LmC2*	N01.1300	CTCACGTTGGTTACAAGAGAGTTA	CTCACGTTGGGATACATGTTGGGT	50	Dom Y
ScS18	*LmC2*	S18.1300	CACACTCACCGCTACGAACA	TAGGCGATAAGGCTTAGAAT	60	Codom D/S
ScN13	*LmC4*	N13.A250	AGCGTCACTCAACTTACCAATCGT	AGCGTCACTCCTAGGGAGG	66	Dom D
AFLP-SCARs						
ScNP046	*LmA9*	E35M71.100	ATTCACAAGTACAACAAAGCTATGGA	TAAGGAGTTGGAAAAAGAAATAAAATG	56	Dom D
ScNP004	*LmA9*	E34M71.380	TAAGGACAAACCTGCGCTCT	GTCTAGGCATCCCTCCAGGT	56	CodomD/Y
ScNP007	*LmC2*	E36M64.300	AAATTGACTCGAGCCTGCTG	ATGCATGGCCTTTGAAGTCT	56	Codom D/Y
ScNP009	*LmC4*	E36M65.510	TCCTGTTCCGCTCTTCAACT	GAGAGAGCGATGGAGCAGTC	56	Dom D
ScNP048	*LmC4*	E46M64.100	AATTCATTTACCTCGCTTCAAA	AGACAAACTTTGGGCTTGGA	56	Dom Y

Dom dominant, *Codom* codominant, *D* 'Darmor-*bzh*', *Y* 'Yudal'

Table 4 List of the Arabidopsis genomic regions used and number of markers developed

QTL	Arabidopsis region	Number of genes used	Number of mapped loci	Number of loci within QTL regions
LmA2 & *LmC2*	At5g49A20-At5g61740	4	2	1
	At1g64280-At1g79040	8	4	1
LmA9	At3g25805-At3g58680	11	8	3
	At1g23440-At1g34120	9	4	1
LmC4	At2g25737-At2g39640	26	19	5 + 1 (*LmC2*)
	At3g51560-At3g60250	6	3	1

LmA2 and QTL *LmC2*, the same Arabidopsis regions were used since the two QTL regions are homeologous regions. Primer pairs defined on 64 genes gave rise to 40 polymorphic markers on the 'Darmor-*bzh*' × 'Yudal' DH population. From these 40 mapped markers, 13 were located within the targeted QTL regions (Fig. 2). Some of these 13 markers were based on the same Arabidopsis gene but their genetic determinism in the 'Darmor-*bzh*' × 'Yudal' DH population (dominant/codominant) or their required mode of electrophoresis (agarose/acrylamide) was different (Table 5).

Molecular characterization of the NILs

A new QTL detection was carried out using the map published by Delourme et al. (2006b) and the new markers we developed in the regions of the targeted QTL. Note that only the QTL identified on the linkage groups targeted in this study are reported in Table 6 and in Fig. 2. This analysis confirmed the position of the QTL identified by Pilet et al. (1998) and by the QTL analysis performed at the beginning of this study. In addition to Pilet et al. (1998), QTL *LmA2* was also detected for disease index in 1996. Markers which mapped to the QTL confidence intervals were then used to assess the individual NILs. Codominant markers and dominant markers which amplified a band in 'Darmor-*bzh*' were used to assess the length of the 'Darmor-*bzh*' introgressed segment in the targeted QTL region. Codominant markers as well as dominant markers which amplified a band in 'Yudal' were used to determine the homozygosity of this 'Darmor-*bzh*' segment in each line. We found that (1) different sized 'Darmor-*bzh*' introgressed segments (from 20 to 50 cM) were obtained depending on the lines; (2) the 'Darmor-*bzh*' introgressed segment covered the confidence interval of the targeted QTL except for QTL *LmC4.1* and for QTL *LmA9* for the 'plant losses' variable; (3) NILs were identified which were homozygous for the 'Darmor-*bzh*' introgressed segment (Fig. 3).

Phenotypic evaluation of the NILs

A total of 48 NILs (38 tall lines, 9 dwarf lines and one line where the *Bzh* gene was segregating) were evaluated in two trials in 2004–2005 at one location. Figure 4 shows the disease level (G2 index) assessed in one trial on some of these NILs in comparison to 'Yudal' and controls. The disease level among controls was low because of the date the plants were harvested for disease assessment, which is around 3 weeks before the usual date. The comparison of the mean G2 disease index obtained for the different NILs to the 'Yudal' G2 disease index showed that the dwarf gene (*Bzh*) has a significant effect. All of the NILs carrying the *Bzh* gene (NILA2.4, NILA2.5, NILA9.5, NILA9.6, NILA9.7, NILC2.4, NILC4.7, NILC4.12, NILC4.14) or segregating for the *Bzh* gene (NILC4.6) were significantly less susceptible than 'Yudal'. No significant effect on the G2 disease index was detected for NILs carrying QTL *LmC2.1*, QTL *LmC2.2* or QTL *LmC4.2*. A significant effect was detected for QTL *LmA9* for 3 NILs (NILA9.2, NILA9.3, NILA9.11) out of 9. A significant effect was detected for QTL *LmA2* for 9 NILs (NILA2.1, NILA2.2, NILA2.3, NILA2.7, NILA2.9, NILA2.10, NILA2.11, NILA2.12, NILA2.13) out of 11.

In 2005–2006, the trial was repeated at four different field sites and included only tall NILs for QTL *LmA2* and QTL *LmA9* (Fig. 5). As in 2004–2005, the disease level of the controls was low because of the date of disease assessment. Despite different levels of disease and a significant interaction between line and location, the results of the four trials were very consistent and showed that regardless of the NIL, QTL *LmA9* does not have a significant effect on the G2 disease index. The two NILs (NILA9.2 and NILA9.3) which were significantly less susceptible than 'Yudal' in 2004–2005 at Le Rheu, were still less susceptible than 'Yudal' in 2005–2006 at this same location, but the difference was not statistically significant. Furthermore, in the other locations, these two lines were not less susceptible than 'Yudal'. A significant effect was detected for QTL *LmA2* for all the NILs at all field sites. The G2 index difference between 'Yudal' and NILs with QTL *LmA2* was estimated on average at 1.4 (1.1–2.3 depending on the location).

Discussion

In this study, we produced near isogenic lines (NILs) for four QTL involved in oilseed rape quantitative resistance to *L. maculans*, by backcross assisted breeding. In addition, new molecular markers were localised to the vicinity of the

Table 5 Details of the PCR markers designed from Arabidopsis sequences

QTL	Marker	Arabidopsis gene	Forward primer	Reverse primer	Tm (°C)	Gel	Dominance
LmA2	At5_003	At5g51120	ACTACATGGTCGTCAGATAAAGGT	CCGTCTGAACCTGGGAACTCT	53	Aga 2%	Dom D
	At1_005a	At1g73030	ACCACCATCACCAAATCCAT	CGAATGAATCCATTGTCTCTGA	53	Aga 3%	Codom D/Y
LmA9	At3_003a	At3g52300	CAGCTCCARACCAAGTTCAGC	TGTACATGTCAACAATGCCAGA	56	Acryl	Codom D/Y
	At3_012	At3g56130	TTGGGAACAGAACTTCCAGTGACG	AAGGACTTCTCCAGCTACATCTGA	53	Acryl	Codom D/Y
	At3_013	At3g56130	TTAAGCTTCTTTCAGATGACGGAG	GAGGATCTCCATAACCTACGGAGT	53	Acryl	Dom D
	At1_010	At1g31780	GCGTCGCTAAACGCTTTGTC	CATAGCCATCACTCCCATCAA	56	Acryl	Codom D/Y
LmC2	At2_009b	At2g37040	GAGAACCAAAAACGGTGTCG	TGTGTGGCTTGTTTCTTTCG	50	Acryl	Dom D
LmC4	At2_020a	At2g30490	GTCGAGAACATCAATGTCGCCG	TAGACCACAATGTTGTCTCAATCG	53	Aga 2%	Codom D/Y
	At2_018	At2g33670	ATCATCCACTTCATTCTCTTCCAG	GAAGTAGGTGATCTCAAAAGCATT	53	Acryl	Codom D/Y
	At2_019	At2g33670	CTTCAAGGTAGTCGTGGGAATAAG	AAAGGCCCATAGCTCGGGA	53	Acryl	Codom D/Y
	At2_007	At2g33670	ACGGTCCTGTTTTTCATCACC	GCCAATTCTTTTAATGCCTTTGACG	53	Aga 3%	Dom D
	At2_008	At2g36530	AACATTCAGGAGAACAAGGA	CATAGTGCTCCCAGTCATCT	48	Acryl	Dom Y
	At3_015a	At3g53480	GAAAATCAGGAGAAGAATAGACAG	TTTTACTAACTTCCATCATAATAT	51	Aga 2%	Codom D/Y

Aga agarose gel, *Acryl* acrylamide gel, *Dom* dominant, *Codom* codominant, *D* 'Darmor-*bzh*', *Y* 'Yudal'

targeted QTL and used to characterize the NILs obtained. These NILs were evaluated in field resistance trials, which clearly validated the effect of QTL *LmA2* in different field locations.

Several strategies were used to develop molecular markers in the regions of the targeted QTL. Seven and five SCAR markers were obtained from 9 RAPD and 20 AFLP markers, respectively. We then attempted to make use of the Arabidopsis—Brassica genome synteny to derive PCR-based markers since 80–90% homology is found between the exons of orthologous genes in Arabidopsis and Brassica (Schmidt 2002; Brunel et al. 1999) and extensive colinearity between Arabidopsis and Brassica genome segments was observed (Parkin et al. 2005, Schranz et al. 2006). In their comparative *A. thaliana—B. napus* genome analysis, Parkin et al. (2005) identified 21 syntenic blocks shared by the genomes of these two species that were duplicated and rearranged to cover 90% of the *B. napus* genome. To identify potential colinear Arabidopsis regions to our QTL regions, we took advantage of the partial alignment of the 'Darmor-*bzh*' × 'Yudal' genetic map with the map of Parkin et al. (2005) as well as in-house common anchor points between *B. napus* markers and the Arabidopsis genome. Using 64 Arabidopsis genes, two potential syntenic regions were chosen per QTL, which allowed 13 markers to be mapped in the targeted QTL regions. This apparent low success rate is due to the complex arrangement of the polyploid *B. napus* genome. One Arabidopsis region corresponds to many duplicated regions in each diploid *B. rapa* and *B. oleracea* species and these regions are cumulated in the *B. napus* genome (Parkin et al. 2005; Schranz et al. 2006). Moreover, even if some macrosyntenic blocks have been identified between Arabidopsis and Brassica genomes, minor deletions, insertions or translocations are relatively common at the microsyntenic level (O'Neill and Bancroft 2000; Quiros et al. 2001). These characteristics, together with the fact that we only used agarose or acrylamide electrophoresis to identify polymorphism with our markers made it difficult to successfully map markers to the targeted QTL regions. In effect, we mapped 27 additional markers in non-targeted regions, which sometimes corresponded to duplicated homeologous regions within the *B. napus* genome. For example, the region targeted for QTL *LmA2* and *LmC2*, which correspond to block E of Schranz et al. (2006) produced polymorphic markers which mapped to A7, also carrying this E segment. Similarly, one of the regions targeted for the QTL *LmC4*, which corresponds to block J of Schranz et al. (2006), gave polymorphic markers that mapped on A3 and A5, which also carry also this J segment. This illustrates the difficulties encountered for high throughput development of markers in targeted Brassica regions. The increasing availability of *Brassica* sequences in the databases especially with the International *Brassica*

Table 6 Summary of the new QTL identified on the targeted linkage groups (LG)

LG and QTL	Trait	Position	Confidence Interval	LOD	R^2	TR^2	Additive effect
A2-DY11							
LmA2	I95	E02.1200 + 0	55–64	5.5	8.5	56	0.61
	I96	ScJ14 + 8	60–75	2.4	3.8	59	0.43
	P95	ScJ14 + 4	60–75	3.4	7.2	48	0.10
	P96	ScJ14 + 12	68–81	8.3	18.8	50	0.14
A9-DY5							
LmA9	I95	W15.1470 + 0	41–57	3.3	4.8	56	0.52
	P95	CB10402 + 4	47–77	3.5	6.4	53	0.10
	P96	CB10402 + 8	65–78	4.5	9.6	48	0.10
C2-DY2							
LmC2.1	I95	MR216a + 0	90–A20	3.1	4.9	56	−0.46
	I96	MR216a + 0	92–108	3.5	4.9	63	−0.48
LmC2.2	I95	Fad8 + 0	144–168	5.6	8.3	57	−0.59
	I96	CZ5b694914 + 10	130–144	6.7	13.3	65	−0.74
	P95	Fad8 + 0	140–160	3.0	5.4	47	−0.08
C4-DY3							
LmC4.1	I95	D08.1310 + 0	43–56	9.5	15.2	57	0.78
	I96	D08.1310 + 0	45–63	4.7	6.7	58	0.53
	P95	D08.1310 + 2	43–66	3.1	5.4	47	0.08
LmC4.2	P96	CB10107 + 22	109–135	3.4	7.7	48	0.09

R^2 is the percentage of variation explained by each QTL

TR^2 is the percentage of variation explained by each QTL and the cofactors

Additive effect is the effect of substitution of two 'Darmor-*bzh*' alleles by two 'Yudal' alleles

rapa sequencing project (http://www.brassica.info/b_rapa_sequencing_project/mbrsp.htm) will hopefully make this work easier in the future.

The production of NIL by foreground and background selection was initiated when the available markers were mainly RAPD markers. During the course of the project, SSR markers were mapped in the QTL regions, and SCARs or PCR markers were derived from Arabidopsis and finally used to characterize the length of the introgressed 'Darmor-*bzh*' segments in the 'Yudal' genome. Despite the low availability of easily workable markers, we succeeded in creating NILs for each QTL, except for QTL *LmC4.1*. Molecular characterization showed that segments covering between 20 and 50 cM were retained in the NILs and homozygosity at each introgressed QTL could be deduced from codominant markers or from markers in repulsion phase despite there being some uncertainty for a few lines. This provided material to test the effect of the introgressed 'Darmor-*bzh*' segment at each targeted QTL on the level of resistance to *L. maculans* at different field sites and years. The effect of QTL *LmA2* was clearly validated in each year and location for 9 out of 11 the NIL representatives. The additive effect of this QTL was found to be higher when comparing the NILs than that estimated in the segregating 'Darmor-*bzh*' × 'Yudal' DH population where the QTL was detected. Indeed, the introgressed QTL decreased the G2 index by up to 2.3 points while its additive effect in the DH population was estimated at 0.4 or 0.6 depending on the year. This can be explained by the change in genetic background and by the fact that estimated QTL effects in segregating populations are generally biased (Beavis 1994; Bost et al. 2001). Inversely, these hypotheses as well as possible epistatic interactions may explain the non-validation of QTL *LmC2.1*, QTL *LmC2.2* and QTL *LmC4.2*. No significant epistatic interactions were detected in the segregating 'Darmor-*bzh*' × 'Yudal' DH population but this could be due to the low power of detection of small epistatic effect in such an experimental design. For QTL *LmA9*, only some NILs (NILA9.2 and NILA9.3) were significantly less susceptible than 'Yudal' at the Le Rheu trial in 2004–2005. It decreased the G2 index of these lines by to 2 points in 2004–2005 and 0.5 points in 2005–2006 at Le Rheu, respectively, and by 0.5 points in the segregating 'Darmor-*bzh*' × 'Yudal' DH population in a one year trial. This could be due to interactions between QTL and the environment, which were found in many QTL identification experiments for many different traits including our own blackleg QTL studies (Pilet et al. 1998, 2001). The fact that some NILs appear identical to NILA9.2 or NILA9.3 (e.g. NILA9.4), from their molecular characterization, but do not show any effect on the resistance level, remains to be explained. One hypothesis is that rearrangements of the introgressed 'Darmor-*bzh*' segment have occurred but could not be detected due to the lack of a sufficient number of markers in the area.

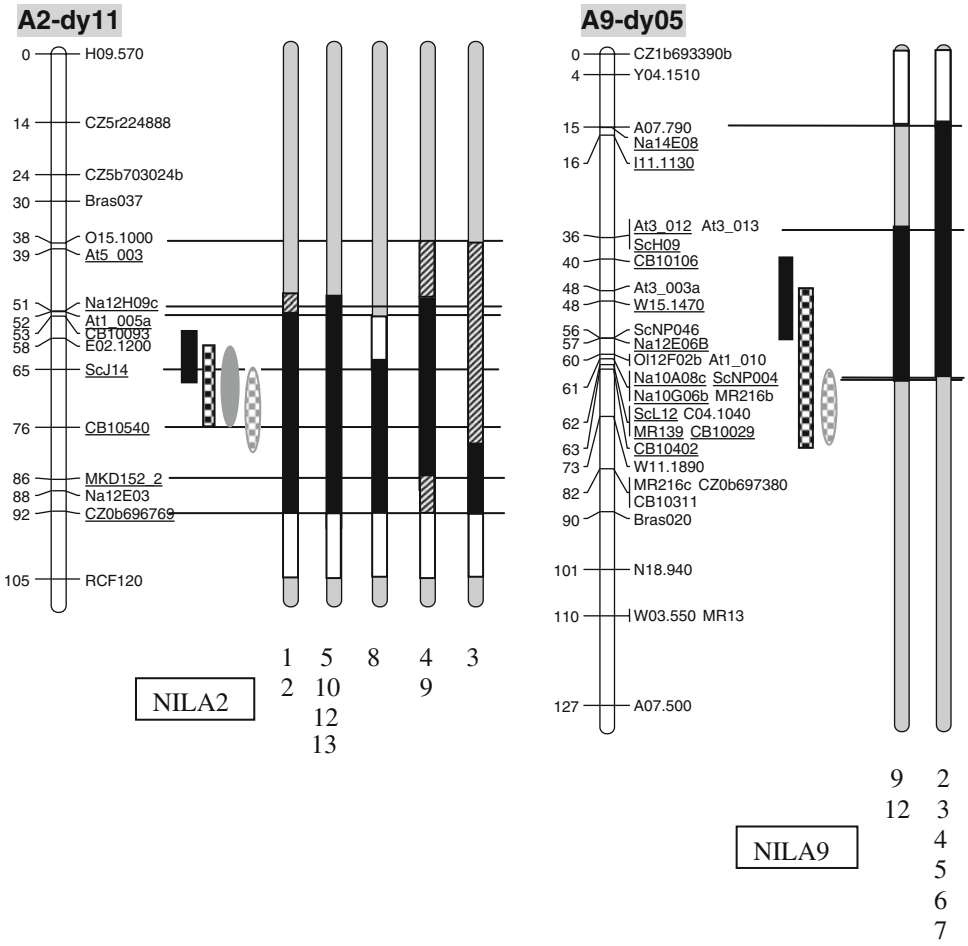

Fig. 3 Schematic representation of the NILs whose field evaluation is shown in Figs. 4 and 5. QTL positions for the four variables [disease index in 1995 (*black*) and 1996 (*gray*); plant losses in 1995 (*hatched black*) and 1996 (*hatched gray*)] are indicated. The underlined markers were used for the molecular characterization of the derived NILs. The genotype representation is the following: *black* for homozygous 'Darmor-*bzh*' segment; *hatched black* for heterozygous 'Darmor-bzh' segment; *gray* for homozygous 'Yudal' segment and *white* for missing data

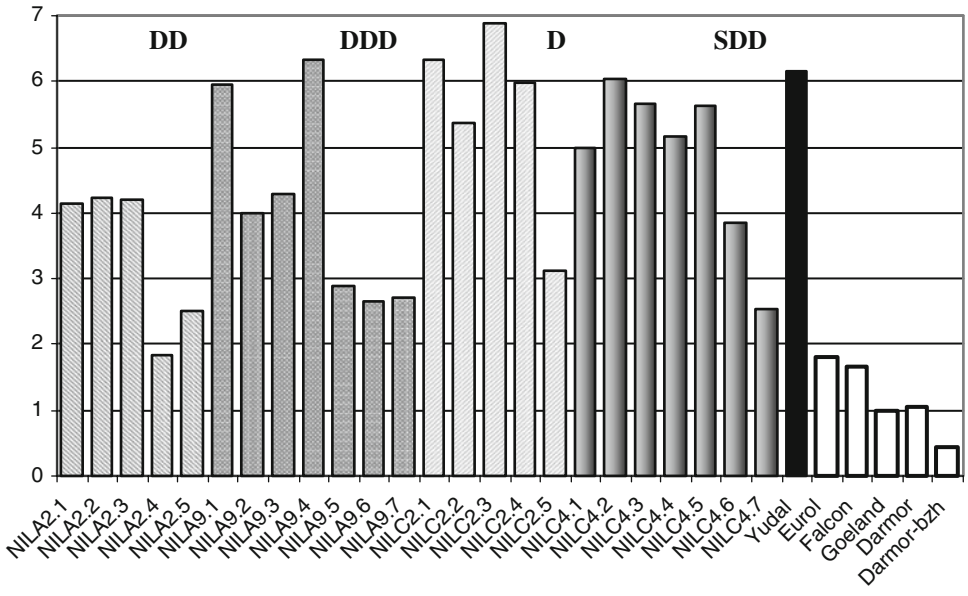

Fig. 4 The G2 disease index of NILs for QTL *LmA2*, *LmA9*, *LmC2* and *LmC4* compared to 'Yudal' and controls ('*Eurol*', '*Falcon*', '*Goeland*', '*Darmor*', '*Darmor-bzh*') in one location (INRA Le Rheu) *D* dwarf lines, *SD* semi-dwarf line

A number of studies successfully validated QTL by producing QTL-NILs after backcrosses with one of the parents of the QTL mapping population as the recurrent parent (van Berloo et al. 2001; Maeda et al. 2006; Wissuwa and Ae 2001). However, in these studies, more than 30% of the phenotypic variance was expained by the targeted QTL except in Wissuwa and Ae (2001) where the targeted QTL explained 12–27 and 10% of the phenotypic variance in the

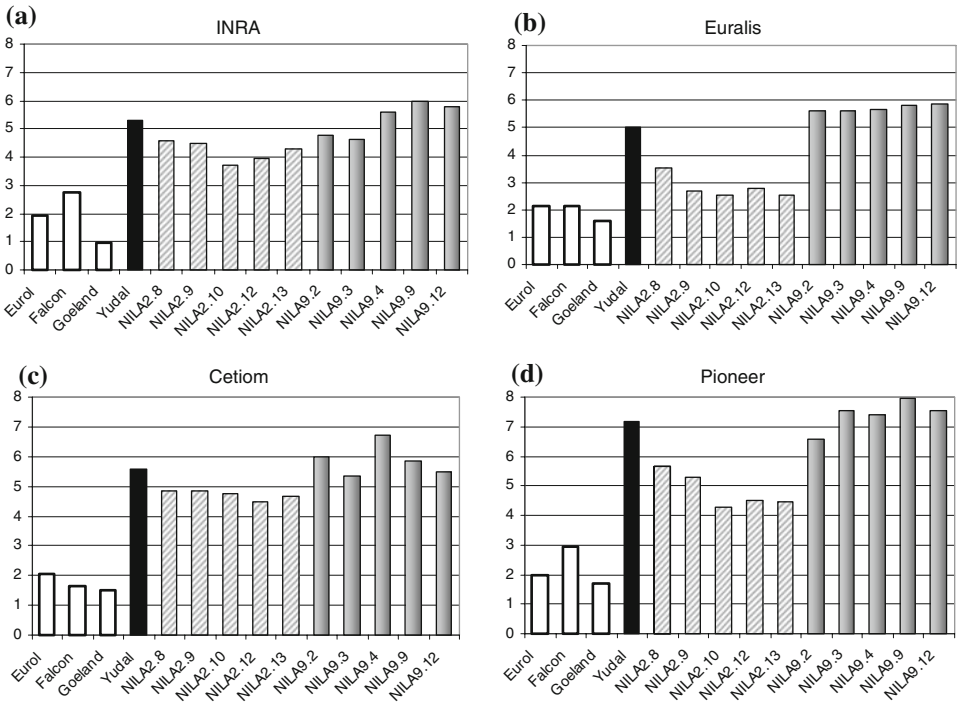

Fig. 5 The G2 disease index of NILs for QTL *LmA2* and *LmA9* compared to 'Yudal' and controls ('*Eurol*', '*Falcon*', '*Goeland*') in four locations (**a** INRA Le Rheu, **b** Euralis Blois, **c** CETIOM Grignon, **d** Pioneer Blois)

mapping population, respectively. In our study, the targeted QTL explained from 3.8 (QTL *LmA2* in 1996) to 15% (QTL *LmA9* in 1995). Thus, the validation of QTL *LmA2* and to a lesser extent QTL *LmA9* is a very positive result.

This study provides valuable material that can be used to study the mode of action of the genetic factors involved in *L. maculans* quantitative resistance, especially those underlying QTL *LmA2*. This material could also be the starting point for generating a new segregating population for finer QTL mapping as in Quarrie et al. (2006), Wan et al. (2006), Loudet et al. (2007) and Nduulu et al. (2007). The availability of a derived series of NILs within the QTL region may facilitate studies to better understand the molecular basis underlying the QTL e.g. through a candidate gene approach or by dissecting out the number of genetic factors underlying the QTL (Thompson et al. 2007). Our study also provides molecular markers that can now be used to pyramidize the QTL in order to test additive effects and epistatic interactions, and transfer the QTL to other oilseed rape varieties in order to test their effect in different genetic backgrounds. This latter point is very important since previous studies showed that the maintenance of a QTL effect could depend on the QTL and the genetic background (Hospital 2005 for review). A possible drawback for successfully transferring QTL to another background is the availability of polymorphic markers between breeding germplasms. The availability of NILs means that new molecular markers can be rapidly screened and identified at the targeted QTL without the need for mapping on the segregating population. This increases the potential for obtaining transferable markers in different germplasms.

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