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ORIGINAL PAPER

Genetic mapping of the *Leptosphaeria maculans* avirulence gene corresponding to the *LepR1* resistance gene of *Brassica napus*

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Abstract *AvrLepR1* of the fungal pathogen *Leptosphaeria maculans* is the avirulence gene that corresponds to *Brassica LepR1*, a plant gene controlling dominant, race-specific resistance to this pathogen. An in vitro cross between the virulent *L. maculans* isolate, 87-41, and the avirulent isolate, 99-56, was performed in order to map the *AvrLepR1* gene. The disease reactions of the 94 of the resulting F_1 progenies were tested on the canola line ddm-12-6s-1, which carries *LepR1*. There were 44 avirulent progenies and 50 virulent progenies suggesting a 1:1 segregation ratio and that the avirulence of 99-56 on ddm-12-6s-1 is controlled by a single gene. Tetrad analysis also indicated a 1:1 segregation ratio. The *AvrLepR1* gene was positioned on a genetic map of *L. maculans* relative to 259 sequence-related amplified polymorphism (SRAP) markers, two cloned avirulence genes (*AvrLm1* and *AvrLm4-7*) and the mating type locus (*MAT1*). The genetic map consisted of 36 linkage groups, ranging in size from 13.1 to 163.7 cM, and spanned a total of 2,076.4 cM. The *AvrLepR1* locus was mapped to linkage group 4, in the 13.1 cM interval flanked by the SRAP markers SBG49-110 and FT161-223. The *AvrLm4-7* locus was also positioned on linkage group 4, close to but distinct from the *AvrLepR1* locus, in the 5.4 cM interval flanked by FT161-223 and P1314-300. This work will make possible the further characterization and map-based cloning of *AvrLepR1*. A combination of genetic mapping and pathogenicity tests demonstrated that *AvrLepR1* is different from each of the *L. maculans* avirulence genes that have been characterized previously.

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Introduction

Blackleg disease of canola and other *Brassica* crops is caused by the haploid ascomycete pathogen *Leptosphaeria maculans* (Sun et al. 2007; Fitt et al. 2006). The pathogen has been reported in North America (Canada, USA and Mexico), most countries in Europe, Australia, Brazil, Argentina, and a few countries in Africa (Fitt et al. 2006). Despite ongoing resistance breeding, financial losses to this disease range from 22 to 300 million dollars per year in canola-producing regions in the world (Fitt et al. 2006).

Two types of genetic resistance to *L. maculans* have been described in *Brassica* species; qualitative resistance (that is usually assayed at the seedling stage) and quantitative resistance (that is usually studied in adult-plants) (Ansan-Melayah et al. 1998; Pilet et al. 1998; Dion et al. 1995; Ferreria et al. 1995; Rimmer 2006). Qualitative or race-specific resistance follows the gene-for-gene model for plant–pathogen interactions (Flor 1971) and includes the genes *Rlm1*, *Rlm2* and *Rlm4* (Ansan-Melayah et al. 1998), *Rlm3*, *Rlm5*, *Rlm6*, and *Rlm8* (Balesdent et al. 2002), *Rlm7* and *Rlm9* (Delorume et al. 2004), *LepR1* and

LepR2 (Yu et al. 2005), and *LepR3* (Li and Cowling 2003). The genetic control of quantitative resistance is currently more poorly defined but appears to play a significant role in the protection of *Brassica* crops (Rimmer 2005).

Genetic studies in *L. maculans* have identified nine avirulence gene specificities, *AvrLm1* to *9*, corresponding to nine resistance genes of *Brassica* hosts. Most of these genes are contained within two genetic clusters, the *AvrLm1-2-6* cluster (Balesdent et al. 2002) and the *AvrLm3-4-7-9* cluster (Balesdent et al. 2005) while *AvrLm5* and *AvrLm8* are not linked to any of the other genes (Balesdent et al. 2002). Three of the avirulence genes have been cloned; *AvrLm1* (Gout et al. 2006), *AvrLm6* (Fudal et al. 2007) and the dual avirulence gene, *AvrLm4-7* (Parlange et al. 2009), that is recognized by two distinct resistance genes from *Brassica napus*, *Rlm4* and *Rlm7*. The proteins predicted from the coding sequences of all three genes are small and secreted. The products of *AvrLm6* (Fudal et al. 2007) and *AvrLm4-7* (Parlange et al. 2009) are both cysteine-rich, while the protein encoded by the *AvrLm1* gene is predicted to have a paucity of disulphide bridges (Gout et al. 2006).

The *LepR1* gene for resistance to *L. maculans* was derived from *Brassica rapa* subsp. sylvestris and was transferred to canola using two independent crossing strategies as bridges; resynthesis of *B. napus* (Crouch 1994) and the formation of AAC allotriploids with backcrossing to *B. napus* (S. R. Rimmer, unpublished). *LepR1* controls race-specific interactions with *L. maculans* and was effective against most isolates tested, including ones collected from Canada, Australia and Europe (Yu et al. 2005). Yu et al. (2005) used a population of *B. napus* double haploid (DH) lines segregating for *LepR1* to map the gene to chromosome 2 of the *Brassica* A-genome (*B. napus* linkage group N2 corresponds to *Brassica* chromosome A2).

A rare *L. maculans* isolate, 87-41, that is virulent on *B. napus* plants carrying *LepR1* was identified by Yu et al. (2005). This made it theoretically possible to determine the genetic location in the *L. maculans* genome of *AvrLepR1*, the avirulence gene with which *LepR1* interacts. In this report, we describe the genetic mapping of *AvrLepR1* relative to marker-loci defined by sequence-related amplified polymorphism (SRAP) markers (Li and Quiros 2001) and the specificity testing of *AvrLepR1* to establish its relationship to the *AvrLm3*, *AvrLm4*, *AvrLm7* and *AvrLm9* genes which all mapped to a similar region of the *L. maculans* genome.

Materials and methods

B. napus lines and *L. maculans* isolates

The *B. napus* line ddm-12-6s-1 (S. R. Rimmer, unpublished), carries *LepR1* and is a BC_2S_2 selection from a cross between double haploid *B. napus* DH12075 (Mayerhofer et al. 2005) and *B. rapa* subsp. sylvestris (Crouch 1994), backcrossed to N–o-1, a DH line derived from 'Westar' (Sharpe et al. 1995). A distinct selection from the 'Westar' canola variety, which was described by Chen and Fernando (2006), was used as a positive control for infection by *L. maculans*. The *L. maculans* isolate, 87-41 (Yu et al. 2005), was expected to be virulent on both ddm-12-6s-1 and 'Westar', while isolates WA-74, 99-49 and 99-56 (Yu et al. 2005) were expected to be avirulent on ddm-12-6s-1 but virulent on 'Westar'. All four isolates were obtained from the collection of Dr. S. R. Rimmer. The following *B. napus* differential lines and cultivars, 22-1-1, 'Falcon', 23-1-1 and 'Darmor', which harbored *Rlm3*, *Rlm4*, *Rlm7* and *Rlm9*, respectively (Balesdent et al. 2005), were used to differentiate the corresponding *Avr* genes in the *L. maculans* parental isolates and progeny.

Inoculum preparation and virulence phenotyping

The preparation of pycnidiospores and the inoculation of *B. napus* cotyledons were performed as described previously (Ferreria et al. 1995). Each *L. maculans* isolate was tested on 12 seedlings of the ddm-12-6s-1 tester line and 12 seedlings of 'Westar' as a highly susceptible control. The disease reactions were scored 12, 14 and 21 days after inoculation and rated using the 0–9 scale described by Williams (1985).

Crossing isolates and tetrad analysis

L. maculans isolates were crossed by pairing single-pycnidiospore isolates as described by Mengistu et al. (1995) and tetrad analysis was performed following the protocol of Gall et al. (1994). Toothpicks bearing pseudothecia were stored in vials with silica gel after air-drying.

Sequence-related amplified polymorphism markers

Sequence-related amplified polymorphism (SRAP) markers were generated using the methods described by Li and Quiros (2001). Each amplification reaction used *L. maculans* template DNA, one fluorescently labeled primer and one unlabelled primer. PCR amplifications were carried out using a programmable thermal controller (BIO RAD thermal cycler gradient, BIO RAD, California, USA). Four reaction products, each using a distinct fluorescent dye, were typically pooled before size separation and detection of the fluorescently labeled DNA amplification products using an ABI 3100 Genetic Analyzer (ABI, California). The four distinguishable dyes used to label samples were FC1 (Blue), PM88 (Red), SA7 (Green) and SA12 (Yellow). The LIZ 500 (Orange) fluorescent dye was used to label

Table 1 Nucleotide sequences of primer pairs used to amplify fragments of *AvrLm1* (1/2), a region of the *L. maculans* genome 15 cM removed from *AvrLm4-7* (3/4) and SNPs primers (5/6 and 7/8) designed to preferentially amplify specific alleles at the *AvrLm4-7* locus

Primer number	Sequence (5′ to 3′)
1[a]	TCTAGCTCCCCAGCTACCAAGAAC
2[a]	GAACCACGACATCGGCTAATATTTTAG
3[b]	CTGGTAAGAACTGTACCGTAGACTC
4[b]	TAATGTTCCATCCAGGAAAACTAGA
5[c]	GGGACAAGTGCCTTGAGGATAGCT
6[c]	GCTTATTTAACAATCAAGTTGTTTACTCCTATTTT
7[d]	CATTCAGGGTCATGGTCTAAACCAGT
8[d]	TCAAGGCACTTGTCCCACAAAC

[a] Primer pair 1 and 2 amplified a 333 bp fragment in isolate 87-41 and produced no amplification product from isolate 99-56

[b] Primer pair 3 and 4 amplified a 491 bp fragment in isolate 87-41 and produced no amplification product from isolate 99-56

[c] Primer pair 5 and 6 amplified a 220 bp fragment in isolate 99-56 and produced no amplification product from isolate 87-41

[d] Primer pair 7 and 8 amplified a 291 bp fragment in isolate 87-41 and produced no amplification product from isolate 99-56

the DNA fragments of the internal size standard. All five dyes were supplied by ABI (ABI, California). Genomic DNA was extracted from parents and F_1 progeny as described by Pongam et al. (1998).

Molecular markers for the *L. maculans* mating type locus and the avirulence genes *AvrLm1* and *AvrLm4-7*

The primer pairs used to amplify the two allelic variants of the mating type locus were described by Cozijnsen and Howlett (2003). The diagnostic primer pairs 1/2 and 3/4 (Table 1) were designed from the *AvrLm1* gene sequence (EMBL database, accession number AM084345) and the BAC 28G8 sequence (EMBL database, accession number AM998637), respectively. These sequences were positioned on the same chromosome as the *AvrLm4-7* gene, ~15 cM distant from it (Parlange et al. 2009). The *AvrLm4-7* locus of *L. maculans* was amplified using the AVRint-UP/-Lo primer pair described by Parlange et al. (2009). The PCR-amplification products of *AvrLm4*-7 variants were cloned using TOPO TA cloning kits (Invitrogen, USA) and submitted for contract sequencing. The primer 5/6 amplified the 99-56 allele at nucleotide 212 of the *AvrLm4-7* gene (SNP^{212}) while the primer pair 7/8 amplified the 87-41 allele at nucleotide 241 (SNP^{241}) (Table 1).

Genetic map construction

Linkage analysis was performed using the computer program JoinMap version 3.0 (Van Ooijen and Voorrips 2001). Minimum LOD (logs of the odds ratios of linkage vs. no linkage) scores of 4.0 (maximum recombination fraction of 0.4) were used to group loci. Recombination frequencies were converted to centi-Morgan (cM) map distances using Kosambi's mapping function (Kosambi 1944).

Results

Selecting the parental isolates for mapping *AvrLepR1*

An experimental system for the genetic mapping of *AvrLepR1* required a pair of *L. maculans* isolates exhibiting contrasting abilities to infect a *B. napus* line carrying

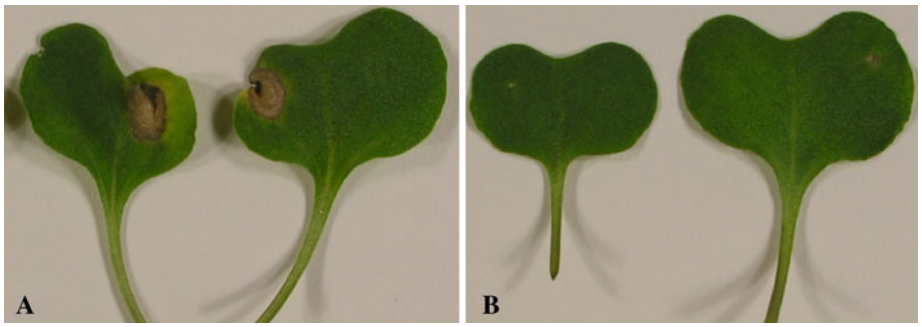

Fig. 1 The aerial portions of four seedlings of *B. napus* line ddm-12-6s-1, which carries the *LepR1* gene for resistance to *L. maculans* (the fungus that causes blackleg disease in canola). One cotyledon of each seedling has been wounded and inoculated with *L. maculans* pycnidiospores as described by Williams (1985). The two seedlings on the right were inoculated with the avirulent isolate 99-56 which carries the common, active form of the *AvrLepR1* gene and which elicited a rapid resistance reaction from line ddm-12-6s-1, corresponding to a disease rating of 1 (Williams 1985). The two seedlings on the left were inoculated with the virulent isolate 87-41 which carries the uncommon, inactive form of the avirulence gene (*avrLepR1*) and which produced a conspicuously successful infection on line ddm-12-6s-1, corresponding to a disease rating of 8 (Williams 1985)

Table 2 Virulence/avirulence phenotypes of four *L. maculans* isolates (87-41, WA-74, 99-43 and 99-56) used to inoculate *B. napus* cotyledons of a susceptible control line ('Westar') and a plant line carrying *LepR1* (ddm-12-6s-1)

Isolates	Replication 1			Replication 2		
	Mean rating on Westar	Mean rating on ddm-12-6s-1	Lowest/highest rating on ddm-12-6s-1	Mean rating on Westar	Mean rating on ddm-12-6s-1	Lowest/highest rating on ddm-12-6s-1
87-41	7.5	7.5	5/9	7.6	7.6	5/9
WA-74	7.9	4.7	3/7	8.1	4.9	3/7
99-43	8.1	5.6	3/7	8.1	5.3	3/7
99-56	9	1.6	1/5	9	2.1	1/5

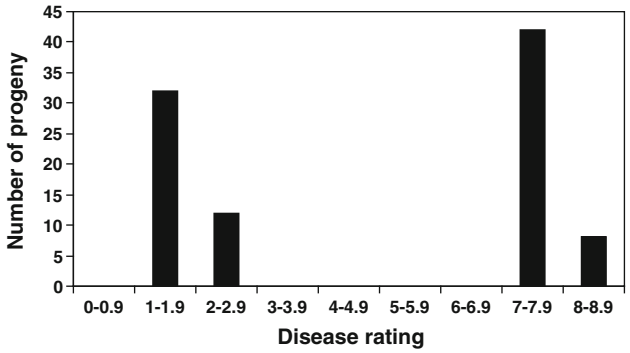

Fig. 2 The frequency distribution of disease ratings within the *L. maculans* population of 94 single-ascospore isolates derived from the 87-41 (virulent) × 99-56 (avirulent) cross. The population was segregating for virulence on *B. napus* ddm-12-6s-1, controlled by *AvrLepR1*, and the disease ratings were scored 14 days post-inoculation using the 0–9 rating scale described by Williams (1985)

LepR1. *B. napus* lines ddm-12-6s-1, carrying *LepR1*, and 'Westar' were used to test the virulence of the four *L. maculans* isolates. As expected, isolate 87-41 was virulent on both genotypes (Fig. 1; Table 2). Of the three isolates expected to be avirulent, WA-74 and 99-43 produced intermediate reactions on ddm-12-6s-1 while 99-56 was obviously avirulent (Fig. 1; Table 2). All four *L. maculans* isolates were highly virulent on the 'Westar' positive control (Table 2). Repeating the whole experiment yielded essentially identical results (Table 2). The four *L. maculans* isolates were tested with primers diagnostic for the mating type locus. Isolates 87-41, WA-74 and 99-43 yielded the amplification products expected for the *MAT1-1* mating type while 99-56 yielded those expected for the *MAT1-2* mating type.

L. maculans mating and virulence/avirulence phenotyping of the resulting recombinants

Based on the above results, 87-41 and 99-56 were crossed to produce a population segregating for *AvrLepR1*. A total of 94 single-ascospore progenies were isolated from the *L. maculans* cross and each isolate was tested for virulence on *B. napus* line ddm-12-6s-1 (that carries *LepR1*) and 'Westar' (the susceptible control). The population of *L. maculans* isolates segregated for virulence (50 isolates) and avirulence (44 isolates) and the segregation ratio approximated to the 1:1 ratio ($\chi^2 = 0.38$, $P = 0.66$) expected for genetic control of the phenotype by a single gene (Fig. 2). The 1:1 segregation ratio was supported by the analysis of complete sets of ascospores from two asci, each segregated 1:1 avirulent/virulent. All 94 progenies were virulent on 'Westar'.

Identifying SRAP markers informative for the *L. maculans* cross

SRAP primer pairs were screened using DNA of the two parental isolates (87-41 and 99-56) separately. Informative primer pairs that amplified one or more DNA fragments that were specific to one or the other of the parental isolates were selected for further use. In addition, bulked segregant analysis (BSA; Michelmore et al. 1991) employing contrasting bulked samples, one containing equal amounts of DNA from 12 avirulent isolates and the other containing equal amounts of DNA from 12 virulent isolates, was used to identify SRAP markers closely linked to *AvrLepR1*. Most primer pairs produced 30-40 amplification products with lengths ranging from 50 to 600 base pairs (bp). The average number of informative amplification products per primer pair was 1.1, with the most informative primer pair producing six products that were polymorphic between the two parents.

The segregation patterns of 283 informative SRAP polymorphisms were scored in the 94 isolates of the *L. maculans* mapping population. The informative DNA fragments were scored as either present or absent. Of the 283 loci assayed with SRAP markers, 254 loci (~90%) had balanced 1:1 segregation ratios ($P > 0.05$) while the remaining 29 loci (~10%) exhibited distorted segregation ratios ($P < 0.05$). This indicated that the 94 single-ascospore progenies represented a fairly balanced population with only an approximately twofold over representation of loci with distorted segregation ratios. Of the 29 distorted loci ($P < 0.05$), only four were highly distorted ($P < 0.01$) and of these only one had a probability <0.001.

Genetic linkage analysis and mapping the *AvrLepR1* avirulence gene of *L. maculans*

Genetic linkage analysis was carried out on the 283 SRAP-defined marker-loci. This established 36 linkage groups each containing at least two loci and together representing 259 loci (Fig. 3) with 24 marker-loci remaining unlinked. The linkage groups spanned 2076.4 cM of *L. maculans* genome with individual linkage groups ranging from 13.1 to 164.8 cM in length (Fig. 3).

The segregation pattern of the molecular markers specific for the *L. maculans* mating type gene positioned the mating type locus (*MAT*) 15.8 cM from the top of linkage group 7 (Fig. 3). PCR amplifications with primers specific for *AvrLm1* (Table 1) yielded a 333 bp fragment using DNA from isolate 87-41 and produced no amplification product from isolate 99-56 DNA confirming the presence of *AvrLm1* in isolate 87-41 and its absence from isolate 99-56. Segregation for the *AvrLm1* marker positioned the gene 61.3 cM from the top of linkage group 13 (Fig. 3).

Segregation for virulence/avirulence on *B. napus* line ddm-12-6s-1 identified the *L. maculans* locus (*AvrLepR1*) that controls avirulence against the host's *LepR1* gene. The *L. maculans AvrLepR1* gene was located 10.1 cM from the top of linkage group 4 in the 13.1 cM interval flanked by, SBG49-110 and FT161-223 (Fig. 3). Linkage group 4 was 164.8 cM in length and carried 21 SRAP-defined loci. It also carried the 28G8-490 and P1314-300 marker-loci that are diagnostic of the chromosome carrying the *AvrLm4-7* gene. This suggested that *AvrLepR1* could be a new member of the *AvrLm3-4-7-9* genetic cluster (Balesdent et al. 2005; Van de Wouw et al. 2008) or even a new allele of the *AvrLm4-7* gene. Both the parental isolates (87-41 and 99-56) were avirulent on *B. napus* plants carrying either *Rlm4* or *Rlm7*, indicating that each isolate carried a functional *AvrLm4-7* gene and that the *AvrLm4-7* locus could not be mapped in the segregating population based on the segregation of differential disease reactions. In order to test the possibility that the (functionally indistinguishable) alleles of *AvrLm4-7* in isolates 87-41 and 99-56 could be distinguished at the level of precise nucleotide sequence, the *AvrLm4-7* locus of each parental isolate was amplified (using the AVRint-UP/-Lo primer pair: Parlange et al. 2009), cloned and sequenced with fivefold replication. A comparison of the sequences of the two parental alleles and the published sequence of *AvrLm4-7* gene (Parlange et al. 2009) revealed that the 99-56 allele carried SNP^{212} (a substitution of A for C at nucleotide 212 of the amplification product) while the 87-41 allele carried SNP^{241} (a substitution of C for G at nucleotide 241 of the amplification product). Genetic markers based on the differential PCR-amplification of these SNP's using primer pairs 5/6 and 7/8 (Table 1) allowed segregation at the *AvrLm4-7* locus to be scored in the mapping population and the *AvrLm4-7* locus to be positioned 6.8 cM below the *AvrLepR1* locus on linkage group 4 (Fig. 4) and in the 5.4 cM interval flanked by FT161-223 and P1314-300. To confirm this result, the *AvrLm4-7* gene was amplified, cloned and sequenced in six recombinant isolates carrying crossovers between the FT161-223 and P1314-300 marker-loci flanking to *AvrLm4-7*. The *AvrLm4-7* gene regions from six non-recombinant isolates, three avirulent and three virulent, were also cloned and sequenced as controls. In every case, the gene-sequence analysis of the *AvrLm4-7* alleles confirmed the earlier analysis based on SNP markers.

AvrLepR1 has a specificity distinct from those of *AvrLm3, AvrLm4, AvrLm7 and AvrLm9*

With *AvrLepR1* possibly mapping to the *AvrLm3-4-7-9* genetic cluster, it was considered prudent to investigate whether *AvrLepR1* interacted with *Rlm3*, *Rlm4*, *Rlm7* or *Rlm9*. The interactions of each parent of the *L. maculans* mapping population (87-41 and 99-56), together with a random sample of 30 of the single-ascospore progenies, were tested with a set of six *B. napus* lines. The lines 22-1-1 (*Rlm3*), cv. 'Falcon' (*Rlm4*), 23-1-1 (*Rlm7*) and cv. 'Darmor' (*Rlm9*) were each reported to carry a single distinct gene for resistance to *L. maculans*, while ddm-12-6s-1 carried *LepR1* and cv. 'Westar' was used as the susceptible control. The results are summarized in Table 3. The two parental isolates and the 30 progenies all interacted identically with line 22-1-1, cv. 'Falcon', line 23-1-1, cv. 'Darmor' and 'Westar' (the isolates were either uniformly virulent or uniformly avirulent: Table 3). Only on ddm-12-6s-1 did the *L. maculans* isolates exhibit variation. Isolate 87-41 and 15 of the recombinants were virulent on ddm-12-6s-1, while isolate 99-56 and the remaining 15 recombinants were avirulent on this line. These results indicated that the *LepR1—AvrLepR1* gene interaction was distinct from those involving *Rlm3*, *Rlm4*, *Rlm7* or *Rlm9*.

Discussion

LepR1 is a new gene for resistance to *L. maculans* that was introduced into amphidiploid *B. napus* (canola) recently from the related diploid species, *B. rapa*, and that is harbored by the *B. napus* line ddm-12-6s-1 (Yu et al. 2005). Several gene-for-gene interactions involving specific *L. maculans* avirulence genes and the corresponding resistance genes of the *B. napus* host have already been described (Ansan-Melayah et al. 1995; Balesdent et al. 2001, 2005). The genetic mapping of *AvrLepR1* positioned

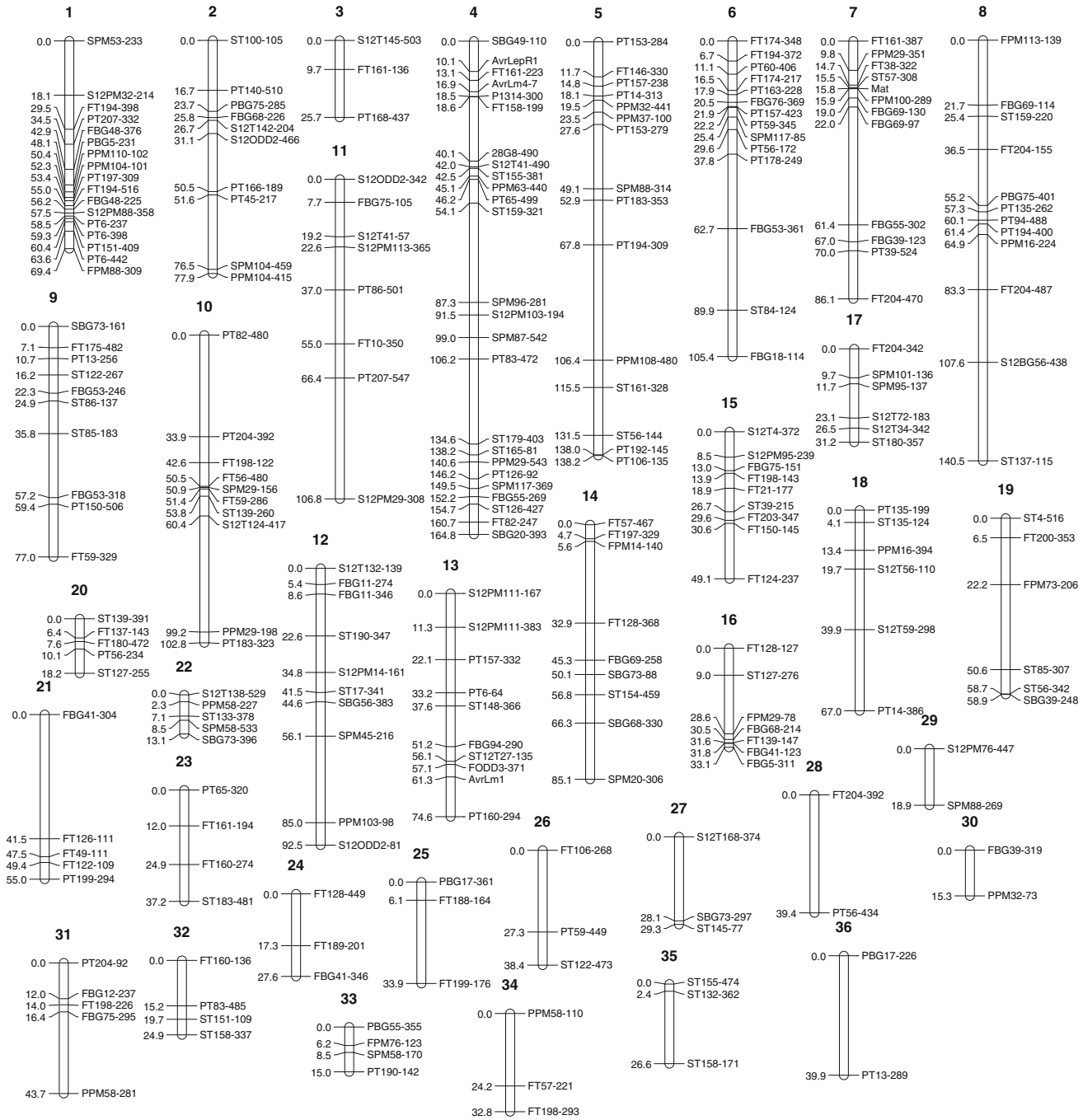

Fig. 3 Genetic map of *L. maculans* based on 264 loci associated into 36 linkage groups (LG's). The majority of loci, 259, were detected using sequence-related amplified polymorphism (SRAP) markers. The mating type locus (*MAT1*) on LG7, *AvrLm1* on LG11 and *AvrLm4-7* on LG4, were each assayed using specially designed PCR-based markers. The *AvrLepR1* locus was mapped on LG4. The locus names are shown to the right of each LG and genetic distances from the top in cM are indicated on the left of each LG

the gene on linkage group 4 close to, or part of, the *AvrLm3-4-7-9* cluster reported by Balesdent et al. (2005).

A new genetic map of *L. maculans*, based on a population of 94 single-ascospore progenies and segregating for virulence on *B. napus* line ddm-126s-1, was developed. The population was assayed with a set of informative PCR-based markers (predominantly SRAP markers) and 288 polymorphic loci were identified. Genetic linkage analysis established 36 linkage groups spanning a total of 2,076.4 cM (Fig. 3) with 24 marker-loci remaining unlinked. The relatively large number of linkage groups, 36, compared to 21 groups in the *L. maculans* genetic map

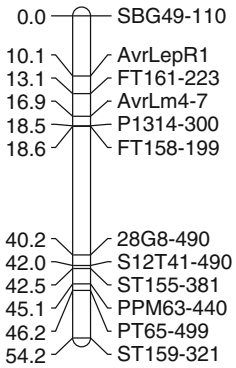

Fig. 4 A large-scale view of the top of linkage group 4 showing the relative positions of *AvrLepR1* and *AvrLm4-7*. The locus names are shown to the right of the LG and the genetic distances from the top in cM are indicated on the left of the LG

developed by Cozijnsen et al. (2000) and 15 groups (containing 5 or more markers) reported by Kuhn et al. (2006) and the high proportion of unlinked loci (24 out of 288, 8% of the loci) suggest that the marker coverage of the *L. maculans* genome was incomplete and that some chromosomes are probably represented by more than one linkage group. The *L. maculans* map described by Cozijnsen et al. (2000) was based on 159 markers mapped on 21 linkage groups with 18 loci remaining unlinked and those of Kuhn et al. (2006) were based on between 177 and 443 markers mapped on 15 major groups. Unfortunately, the use of a novel set of genetic markers makes a detailed comparison with other maps of *L. maculans* impossible.

Overall, SRAP markers provided an inexpensive system for genetic mapping in *L. maculans,* that required very little development. SRAPs are a molecular-marker system with some advantages over RAPD, RFLP, and AFLP markers (Li and Quiros 2001). They have been used for genetic mapping in plants (Li and Quiros 2001; Ferriol et al. 2003; Sun et al. 2007; Dusabenyagasani and Fernando 2008) and fungi (Dodds et al. 2004) and also in genetic diversity studies in a range of organisms (Fernando et al. 2006; Sun et al. 2006; Zhang et al. 2005). The clustering of SRAP markers (Fig. 3) was one reason for the marker coverage of the genome being incomplete despite the large number of marker-loci assayed. Marker-dense regions of the *L. maculans* genome were also reported by Kuhn et al. (2006). This marker clustering is a general feature of many PCR-based genetic markers and has been noted in genetic maps of a range of fungi, including, *Mycosphaerella graminicola* (Kema et al. 2002), *Cochliobolus sativus* (Zhong et al. 2002) and *Blumeria graminis* (Pedersen et al. 2002).

Twenty-nine or 10% of the SRAP marker-loci exhibited segregation distortion to the degree expected to occur naturally in only 5% of the markers ($\chi^2 = 16.9$, $P < 0.001$). The elevated level of segregation distortion observed in the SRAP marker-loci could have been the result of a small degree of inadvertent selection having been imposed on the population. Alternatively, it could have been caused by systematic scoring errors at a small number of loci ($\sim$14). The SRAP markers are dominant and thus prone to errors caused by failed amplification reactions. Further interrogation of the scoring and mapping data revealed that the 29 loci exhibiting segregation distortion ($P < 0.05$) were not randomly distributed but were rather clustered on 14 of the 36 linkage group and that only 2 of the distorted loci were not linked to another marker (LOD score <4.0).

The established physical size of the *L. maculans* genome is 45 Mbp (Rouxel et al. 2011). The 36 linkage groups of the new genetic map span a total of 2,076.4 cM suggesting a physical to genetic ratio of 22 kb per cM. However, the map is incomplete so this initial figure is certainly an overestimate of the average physical size per cM for the genome. An earlier genetic investigation of *L. maculans* based on 443 loci produced a map that encompassed only 2,129 cM (Kuhn et al. 2006). Since this earlier map might be expected to represent a more complete coverage of the genome, it is possible that the meiotic divisions that produced the single-spore isolates on which the new map is based experienced an elevated recombination frequency relative to those which gave rise to the earlier population.

The current investigation located the *AvrLepR1* locus on linkage group 4 of the new genetic map of the *L. maculans* genome. With a map composed largely of SRAP markers, it was not possible to use comparative mapping to position

Table 3 Interaction phenotypes of the *L. maculans* parental isolates and of a set of 30 single-spore progenies all tested on the same panel of *B. napus* lines, each carrying a defined resistance gene

	Brassica lines and the resistance genes they are known to carry					
L. maculans isolates	Westar	22-1-1 *Rlm3*	Falcon *Rlm4*	23-1-1 *Rlm7*	Darmor *Rlm9*	ddm-126s-1 *LepR1*
99-56	V	V	A	A	V	A
87-41	V	V	A	A	V	V
Single-spore isolates[a]	0:30[b]	0:30	30:0	30:0	0:30	15:15

V virulent interaction, *A* avirulent interaction

[a] A set of 30 single-spore isolates derived from crossing *L. maculans* isolates 87-41 and 99-56

[b] The ratio of the number of avirulent isolates to the number of virulent isolate (A:V) in the 30 isolates tested

AvrLepR1 relative to other avirulence genes. A polymorphic molecular marker specific for *AvrLm1* allowed this gene to be positioned on linkage group 13, well removed from the *AvrLepR1* locus on linkage group 4. New molecular markers specific for the DNA sequence variants of the *AvrLm4-7* gene resident in the two parental isolates, 87-41 and 99-56, allowed the segregation at the *AvrLm4-7* locus to be scored in the mapping population and the positioning of the *AvrLm4-7* locus relative to the *AvrLepR1* locus (Fig. 4). The obvious recombination between *AvrLepR1* and *AvrLm4-7* demonstrated that they are distinct genes but their close linkage suggests that *AvrLepR1* is probably a new member of the *L. maculans AvrLm3-4-7-9* genetic cluster, identified previously by Balesdent et al. (2005). The reaction phenotypes of isolates 99-56 (that carries *AvrLepR1*) and 87-41 (that does not carry *AvrLepR1*) (Table 3) demonstrated that *AvrLepR1* has a distinct specificity from those of *AvrLm3*, *AvrLm4*, *AvrLm7* and *AvrLm9*. However, because both isolates were virulent on host plants carrying *Rlm3* or *Rlm9* (Table 3) it was not possible to map any of the corresponding *L. maculans* avirulence genes (*AvrLm3* or *AvrLm9*) based of segregation for the virulence/avirulence phenotypes in the new *L. maculans* mapping population. Following the finding the *AvrLm4* and *AvrLm7* are two distinct specificities of the same avirulent gene (Parlange et al. 2009), the possibility that *AvrLm3* and/or *AvrLm9* are allelic variants of *AvrLepR1* cannot be dismissed.

Nine *L. maculans* avirulence genes, each conferring specificity to one or two distinct *Brassica* resistance genes, have been analyzed genetically (Balesdent et al. 2005; Gout et al. 2006; Parlange et al. 2009). Three of these genes have been cloned; *AvrLm1* (Gout et al. 2006), *AvrLm6* (Fudal et al. 2007) and *AvrLm4*-7 (Parlange et al. 2009). We have now defined the reaction phenotype and identified the locus encoding a novel avirulence gene, *AvrLepR1*, which specifies incompatibility towards the *LepR1* resistance gene carried by *B. napus* line ddm-12-6s-1. The detailed characterization of isolate 87-41 (*avrLepR1*, *AvrLm1*) and isolate 99-56 (*AvrLepR1*, *avrLm1*) has established this pair of isolates as ideal for surveying *B. napus* germplasm for the presence of the *LepR1* and *Rlm1* resistance genes. SRAP markers tightly linked to *AvrLepR1* have been identified. These results are the first steps towards cloning *AvrLepR1*, uncovering its molecular function and defining its molecular interaction with *LepR1*.

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