Analysis of Molecular Markers Genetically Linked to the *Leptosphaeria maculans* Avirulence Gene *AvrLm1* in Field Populations Indicates a Highly Conserved Event Leading to Virulence on *Rlm1* Genotypes

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Map-based cloning of the avirulence gene *AvrLm1* of *Leptosphaeria maculans* was initiated utilizing a genetic map of the fungus and a BAC library constructed from an *AvrLm1* isolate. Seven polymorphic DNA markers closely linked to *AvrLm1* were identified. Of these, two were shown to border the locus on its 5' end and were present, with size polymorphism, in both the virulent and the avirulent isolates. In contrast, three markers, J19-1.1, J53-1.3 (in coupling phase with avirulence), and Vir1 (in repulsion phase with avirulence), cosegregated with *AvrLm1* in 312 progeny from five in vitro crosses. J19-1.1 and J53-1.3 were never amplified in the virulent parents or progeny, whereas Vir1 was never amplified in the avirulent parents or progeny. J19-1.1 and J53-1.3 were shown to be separated by 40 kb within a 184-kb BAC contig. In addition, the 1.6-cM genetic distance between J53-1.3 and the nearest recombinant marker corresponded to a 121-kb physical distance. When analyzing a European Union-wide collection of 192 isolates, J53-1.3, J19-1.1, and Vir1 were found to be closely associated with the *AvrLm1* locus. The results of polymerase chain reaction amplification with primers for the three markers were in accordance with the interaction phenotype for 92.2% (J53-1.3), 90.6% (J19-1.1), and 88.0% (Vir1) of the isolates. In addition, genome organization of the *AvrLm1* region was highly conserved in field isolates, because 89.1% of the avirulent isolates and 79.0% of the virulent isolates showed the same association of markers as that of the parents of in vitro crosses. The large-scale analysis of field isolates with markers originating from the genetic map therefore confirms (i) the physical proximity between the markers and the target locus and (ii) that *AvrLm1* is located in (or close to) a recombination-deficient genome region. As a consequence, map-based markers pro-

vided us with high-quality markers for an overview of the occurrence of race “*AvrLm1*” at the field scale. These data were used to propose hypotheses on evolution towards virulence in field isolates.

Additional keywords: *Brassica napus*, gene-for-gene interaction, LMR1, *Phoma lingam*, resistance, repeated elements.

Since the founding work of Flor (1942, 1971), gene-for-gene interactions between plants and their pathogens, or pests, have been shown to be the rule in interactions involving bacteria, viruses, nematodes, fungi, oomycetes, and aphids (Dodds et al. 2000). Avirulence (AVR) genes specifying incompatibility in interactions where the corresponding resistance (R) gene is present were characterized at the molecular level mostly in bacterial and even viral pathogens where small genome size or nonchromosomal location facilitated direct access to incompatibility determinants (Dangl 1994; Spence 1997; Vivian and Arnold 2000). In fungi or oomycetes, reverse genetics-based strategies could only be used for a small number of species where the AVR gene expression was high enough either in vitro or during the interaction, and where the gene product was stable enough, as was the case for *Avr4* and *Avr9* in *Cladosporium fulvum* (Joosten et al. 1994; van Kan et al. 1991), *NIP1* from *Rhynchosporium secalis* (Rohe et al. 1995), or *inf1* from *Phytophthora infestans* (Kamoun et al. 1997). Following the success experienced with the cloning of AVR genes in *Magnaporthe grisea* (Orbach et al. 2000; Sweigard et al. 1995; M. H. Lebrun, *personal communication*), the construction of genetic maps and the use of map-based strategies for the cloning of AVR genes became a popular approach for genetically tractable fungal phytopathogens. In addition to the cloning of genes themselves, these approaches have the advantage of providing an insight into the genome environment of the AVR genes (Farman and Leong 1998; Orbach et al. 2000). Such an approach is currently developed for major pathogens such as *M. grisea* (Dioh et al. 2000; Farman and Leong 1998), *Mycosphaerella graminicola* (Kema et al. 2000), *Venturia inaequalis* (B. Le Cam, others? *personal communication*), *P. infestans* (van der Lee et al. 2001a), and *Leptosphaeria maculans* (Cozijnsen et al. 2000; this study). To date, only nine fungal AVR genes have been cloned and characterized at the molecu-

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Nucleotide sequence data have been submitted to the EMBL database as accessions numbers. AJ294758 (AD1-1.53), AJ300477 (J19-1.1), AJ300478 (Smex-2), AJ300479 (Vir1), AJ421013 (GTG5-0.4), AJ421014 (J53-1.3), and AL732383 (BACA2), AL732384 (BACA3), AL732385 (BACJ53).

lar level, and even fewer sequences have been deposited in data bases. Characterized fungal *AVR* genes encode proteins acting as elicitors or toxins, or have a still uncharacterized function, but are putatively involved in pathogenicity, as is often the case in bacteria (Laugé and de Wit 1998; Kjemtrup et al. 2000; Vivian and Arnold 2000). No functional homology between fungal *AVR* genes from one fungal species to the other has been identified so far; thus, it has become a major challenge to identify the function and structure of more *AVR* genes in more plant-fungus models, allowing a thorough overview of the signaling strategies hidden behind the generic “avirulence” term. In addition, one rarely developed prospect concerns the use of *AVR* genes, or markers linked to *AVR* genes, for a practical identification of virulent races and study of the evolution of *AVR* alleles in the field (van der Lee et al. 2001b).

L. maculans (anamorph *Phoma lingam*) is a filamentous ascomycete responsible for one of the most damaging diseases of oilseed rape (*Brassica napus*), stem canker. This pathogen name encompasses at least two distinct species (Morales et al. 1993a; Rouxel et al. 1994) from which only one, termed A/Tox⁺ species, has been shown to develop gene-for-gene relationships with *B. napus* (Ansan-Melayah et al. 1998). To date, four *AVR* genes (*AvrLm1*, *AvrLm2*, *AvrLm4*, and *alm1*) have been characterized following classical genetic studies on both the plant and the pathogen (Ansan-Melayah et al. 1995, 1998; Balesdent et al. 2001; Pongam et al. 1998). In France, the *Rlm1* resistance gene (present, for example, in cvs. Quinta, Doublol, Vivol, Capitol, and Columbus) was introduced in commercial cultivars in the early 1990s (Ansan-Melayah et al. 1997; Balesdent et al. 2001). At that time, the ratio of the corresponding avirulent races (i.e., possessing the *AvrLm1* allele) was higher than 95% in natural populations (Ansan-Melayah et al. 1997), therefore rendering the *B. napus* cvs. harboring *Rlm1* highly successful and profitable. The high frequency of the *AvrLm1* race in France fully justified the development of efforts towards developing efficient markers of the “*AvrLm1* race” to study its evolution at the field level when submitted to the presence of the corresponding *R* gene, *Rlm1*. Due to the ease with which the sexual stage of *L. maculans* can be obtained in vitro (Gall et al. 1994), a map-based cloning strategy was initiated for *AvrLm1* following in vitro pairing (Ansan-Melayah et al. 1995; Balesdent et al. 2001) (Fig. 1).

In this article, identification of markers genetically linked to *AvrLm1* was primarily used to physically define the genome region likely to encompass the gene. In a second step, the usefulness of such markers for polymerase chain reaction (PCR)-race identification at the field scale is evaluated. In a third step, evolution toward virulence at the *AvrLm1* locus is further evaluated by analyzing combinations of linked markers in virulent and avirulent natural isolates collected in different European regions. The results suggest that the gene environment corresponds to a recombination-deficient region of the fungal genome and that one main genomic event is responsible for the loss of avirulence function.

RESULTS

The *AvrLm1* linkage group.

The a.2 (*AvrLm1*) × H5 (*avrLm1*) (cross #11) (Fig. 1) *L. maculans* genetic map currently comprises 301 linked molecular markers, defining 18 major linkage groups (i.e., comprising more than 5 markers and covering more than 55 cM) (M. H. Balesdent and M. L. Kühn, unpublished data). The *AvrLm1* locus is located within a 116.8-cM linkage group composed of 14 markers, of which 7 markers are tightly clustered around the *AvrLm1* locus (Fig. 2). AD1-1.53 and J19-1.1 initially were

identified as random amplified polymorphic DNA (RAPD) fragments of 1.53 and 1.1 kb, respectively, and amplified with primers OPAD1 and OPJ19 (Table 1). GTG5-0.4 was initially a 0.45-kb polymorphic band generated by the GTG₅ intersimple sequence repeats (ISSR) primer (Table 1). Smex-2, F4R10-3.2, and Vir1 were generated by repetitive-element-based PCR (Rep-PCR) markers using primers derived from the *L. maculans*-specific repeated element LMR1 (Taylor and Borgmann

Fig. 1. Diagrammatic representation of the main in vitro crosses described in this study. Terminology for the name of isolates is described by Balesdent and associates (2001). Interaction phenotypes of the isolates are indicated as *AvrLm1*, avirulent on *Rlm1* plant genotypes; *avrLm1*, virulent on *Rlm1* genotypes; *AvrLm4*, avirulent on *Rlm4* genotypes; *avrLm4*, virulent on *Rlm4* genotypes. Isolates whose names are underlined are field isolates. Isolates within a square are Mat⁺; within a circle, Mat⁻. The dotted arrow indicates a series of crosses not detailed here.

1994; GenBank M77515) (Table 1). These primers were chosen so that they may amplify fragments between two tandemly repeated LMR1 elements (Jedryczka et al. 1999). All six markers were transformed into sequence characterized amplified regions (SCARs) following sequencing and design of more specific primers. Finally, availability of the BAC contig sequence (discussed below) enabled us to generate the marker J53-1.3. Of the seven markers, Vir1 and F4R10-3.2 were in repulsion phase with avirulence, whereas Smex-2, J19-1.1, and J53-1.3 were in coupling phase with avirulence. GTG5-0.4 and AD1-1.53 were amplified in both the virulent and the avirulent parents, and showed size polymorphism between the parents. Among the seven linked markers, five markers (Smex-2, AD1-1.53, Vir1, J19-1.1, and J53-1.3) showed no recombination events with *AvrLml* in the 98 progeny of cross #11 (Fig. 2). Four out of the five cosegregating markers, AD1-1.53, J19-1.1, Vir1, and J53-1.3, did not show any recombination event with *AvrLml* in 89 progeny of cross #23 (Fig. 2). In contrast, two recombination events were observed between Smex-2 and *AvrLml*. The marker GTG5-0.4, situated at 6.4 cM from *AvrLml* in cross #11, cosegregated with *AvrLml* in cross #23 (Fig. 2).

The order of the markers and their relative distance was further evaluated on 125 progeny from a series of backcrosses with parent isolate v23.1.3 (Fig. 1). In these crosses, J19-1.1, J53-1.3, and Vir1 still did not show any recombination event with *AvrLml*. In contrast, two recombination events were observed between *AvrLml* and GTG5-0.4 and between *AvrLml* and Smex-2 among the 26 progeny from cross #26 (corresponding to 8.4 cM; data not shown). These recombination events occurred in the same two progeny, indicating that these two markers were located on the same side of the *AvrLml* locus (Fig. 2). In addition, two recombination events were observed between AD1-1.53 and *AvrLml* in all 125 progeny (one in cross #27 and one in cross #36), indicating a genetic distance of 1.6 cM. However, in cross #27 and subsequent backcrosses, GTG5-0.4 and Smex-2 were no longer polymorphic, preventing us from locating AD1-1.53 on one side or the other of the *AvrLml* locus.

Characterization of the cosegregant markers.

All but one (AD1-1.53) of the six markers cosegregating with *AvrLml* in crosses #11 and 23 strongly hybridized to all chromosomes of the electrokaryotypes and hybridized strongly to numerous bands of restricted DNA (data not shown). Sequence analyses confirmed that they contain repeated sequences, either derived from the *L. maculans* dispersed repeat element LMR1 (Vir1, accession number AJ300479; Smex-2, accession number AJ300478), indicative of the occurrence of transposable elements (J19-1.1, accession number AJ300477; J53-1.3, accession number AJ421014), or corresponding to micro- and mini-satellites (GTG5-0.4, accession number AJ421013; AD1-1.53, accession number AJ294758). All attempts to generate single-copy sequences from markers Smex-2, Vir1, and J19-1.1 failed. Only one single-copy sequence, corresponding to part of marker AD1-1.53 (AD1F-AD1R7 fragment, Table 1), was identified (Attard et al. 2001).

Screening of the BAC library.

The AD1F-AD1R7 fragment of AD1-1.53 (Table 1) was used to probe the 3,072 BAC clones located on two high-density (HD) filters. Four clones were identified, further checked by PCR for the presence of the whole marker, and termed BACA1 (63 kb), BACA2 (62 kb), BACA3 (75 kb), and BACA4 (65 kb) (Fig. 2). The use of marker J19-1.1 to probe the HD filters gave a positive signal for 555 out of 3,072 clones (18%). Of these, only one clone, termed BACJ53 (106

kb), amplified a PCR product of the expected size using the J19F-J19R or J19F-J19R6 combinations of primers (Table 1). The clones were organized into contigs using restriction enzyme fragment profiling and hybridization (Fig. 2). Finally, complete sequencing of the three contig clones BACA2, BACA3, and BACJ53 confirmed the estimated size of the overlaps between BAC clones, and showed that the complete contig was 184,010 bp (accession numbers AL732383, AL732384, AL732385). Smex-2 could not be located in any of the five BAC clones. Vir1, which is linked to the virulent phenotype, was not identified in the contig sequence either. GTG5-0.4, AD1-1.53, J19-1.1, and J53-1.3 were located as bases 42,281 to 42,739, 57,291 to 58,824, 137,966 to 139,088, and 179,713 to 180,978, respectively (Fig. 2). The physical distance between GTG5-0.4 and AD1-1.53 was 14,559 bp, whereas it was 79,141 bp between AD1-1.53 and J19-1.1 and 40,624 bp between J19-1.1 and J53-1.3 (excluding the markers themselves). Finally, the physical distance between AD1-1.53 and J53-1.3 was 120,888 bp.

Interaction phenotypes in field isolates: frequency of *AvrLml* and *avrLml*.

The isolates from the IMAScore, CETIOM, Châlons, and Versailles collections were characterized according to their interaction phenotypes on cotyledons of Westar (susceptible control), Quinta, and Columbus (*Rlm1*). The 39 isolates of the Versailles collection were all *avrLml* isolates (i.e., virulent on Columbus and other *Rlm1*-harboring cultivars). The Châlons collection comprised eight *AvrLml* and eight *avrLml* isolates. The CETIOM collection comprised 36 *AvrLml* and 27 *avrLml* isolates. The IMAScore collection contained 48 *AvrLml* and 26 *avrLml* isolates with the following repartition: France, 7 *AvrLml* and 3 *avrLml*; Germany, 11 *AvrLml* and 9 *avrLml*; the Netherlands, 1 *AvrLml*; Portugal, 1 *avrLml*; Poland, 3 *AvrLml* and 1 *avrLml*; Sweden, 3 *AvrLml*; Switzerland, 10 *AvrLml* and 1 *avrLml*; and the United Kingdom, 13 *AvrLml* and 11 *avrLml*.

Association of the molecular markers and the interaction phenotype in field isolates.

The isolates were analyzed by PCR to evaluate the presence of the six markers Smex-2, GTG5-0.4, AD1-1.53, Vir1, J19-1.1, and J53-1.3.

Smex-2 appeared to be a rare allele, because it was present in only 11.3% of *avrLml* field isolates and 12.2% of *AvrLml* field isolates of the CETIOM and IMAScore collections (Table 2).

Six AD1-1.53 alleles were identified and corresponded to two to seven repeats of the 23-bp *MinLml* core sequence (accession number AJ294758 for the 7× repeat minisatellite sequence) (Attard et al. 2001). Due to its multi-allelic nature, no clear association with the avirulent or virulent phenotype could be deduced. However, four out of the six *MinLml* alleles (i.e., the 3×, 4×, 6×, and 7× alleles) were rare in the natural populations summing up 19.4% of the individuals. In avirulent isolates, the frequency of the 2× and 5× alleles were not significantly different ($\chi^2 = 2.57$, d. f. 1, $P = 0.109$; Fig. 3). In contrast, the 5× allele frequency was significantly higher than that of the 2× allele in virulent isolates ($\chi^2 = 20.67$, d. f., $P < 0.0001$; Fig. 3).

Most of the field isolates displayed either the 0.42-kb allele of GTG5-0.4 present in one parental isolate of cross 11, H5, or the 0.45-kb allele present in the other cross 11 parental isolate, a.2, with very minor size variations, possibly due to the occurrence of microsatellites within the sequence. However, 10 out of 176 isolates showed an intermediate-sized GTG5-0.4 allele that could not be recognized to be the 0.45- or 0.42-kb allele,

Fig. 2. Focused view of the genetic linkage groups encompassing *AvrLm1* in crosses **A**, #11 and **B**, #23, and contig organization of **C**, the five BAC clones identified using markers linked to *AvrLm1*. **A** and **B**, Maps were constructed using multipoint linkage analysis on segregating populations of 98 progeny (cross #11) and 89 progeny (cross #23) with the Mapmaker/Exp 3.0 software. Parameters were set as follows: log of the likelihood score value = 4.0, maximum distance = 35 cM. AD1-1.53 and J19-1.1 originate from random amplified polymorphic DNA markers; GTG5-0.4 is derived from an intersimple sequence repeats marker; and Smex-2, Vir1, and F4R10-3.2 originate from repetitive-element-based polymerase chain reaction markers (Attard 2001). J53-1.3 is a marker whose primers were deduced from partial sequence data of the 5' end of clone BACJ53 (Attard 2001). Of these markers, only Vir1 and F4R10-3.2 are mapping the virulent allele. The Smex-2 relative location within linkage groups was deduced from the colocalization of the two recombinant markers Smex-2 and GTG5-0.4 in cross #26. F4R10-3.2, linked to the virulent allele in cross #11, was not analyzed further in the present study. **C**, Schematic contig organization of the five BAC clones BACA1 (63 kb), BACA2, (62 kb), BACA3 (75 kb), BACA4 (65 kb), and BACJ53 (106 kb), with location of four markers within the clones. Marker Smex-2 could not be located in any of the five BAC clones.

and were considered as missing data for the GTG5-0.4 marker. The two GTG5 alleles were evenly distributed in the collections analyzed here ($\chi^2 = 1.952$, d. f. = 1, $P = 0.162$). However, the distribution of the two alleles significantly differed from the 50:50 ratio when considering either virulent isolates (23.5:76.5 ratio, $\chi^2 = 24.366$, ddf = 1, $P < 0.0001$) or avirulent isolates (66.67:33.33 ratio, $\chi^2 = 8.999$, d. f. = 1, $P < 0.005$) (Table 2).

The three other markers, J53-1.3, J19-1.1, and Vir1, were closely associated with the *AvrLm1* gene, because their amplification (or absence of amplification) was in accordance with the interaction phenotype for 92.2, 90.6, and 88.0% of the isolates, respectively (Table 2). With the main exception of the virulent isolates of the IMASCORE collection, the ratio of association of the markers with the interaction phenotype was comparable from one collection to the other, and whatever the *AvrLm1* allele present. Mainly, J19-1.1 and J53-1.3 were amplified, and Vir1 was not amplified, in most avirulent isolates (97.8, 94.6, and 93.5%, respectively), whatever the collection they originated from (Table 2). J19-1.1 and J53-1.3 were absent but Vir1 amplified in 90.5, 94.6, and 90.5% of the virulent isolates of the CETIOM, Châlons, and Versailles collections, respectively (Table 2). In contrast, in the case of the virulent isolates of the IMASCORE collection, these three markers were associated with the *avrLm1* allele for only 65.4% (J19-1.1), 76.9% (J53-1.3), and 61.5% (Vir1) of the isolates (Table 2). Therefore, most of the discrepancies between PCR amplification of the linked markers and the interaction phenotypes were due to 11 (6 from Germany, 3 from the U.K., 1 from France, and 1 from Poland) of the 26 virulent isolates of the IMASCORE collection.

Molecular markers and interaction phenotypes in field isolates: combinations of markers.

GTG5-0.4 was associated to a similar extent with *AvrLm1* (71.7%), J19-1.1 (69.9%), Vir1 (70.5%), or J53-1.3 (71.7%),

therefore confirming its linkage with the whole cluster of markers, but nevertheless providing insufficient information to determine whether *AvrLm1* was located between GTG5 and J53-1.3 or outside of the whole GTG5-0.4:AD1-1.53:J19-1.1:Vir1:J53-1.3 region. The most closely associated markers were J19-1.1 and J53-1.3 because only 12 of 192 isolates (6.2%) showed recombination between these two markers (data not shown). Linkage between J53-1.3 and Vir1 was weaker with 89.6% association of the two markers, whereas Vir1 could be hypothesized to be physically closer to J19-1.1, with a 92.2% association of the two markers (data not shown).

The three markers, J19-1.1, Vir1, and J53-1.3, were associated together as in the lab crosses (encoded J19-1.1:Vir1:J53-1.3 1:0:1 or 0:1:0) for 88.0% of the isolates, whatever the collection they originated from (Table 3). For 95.3% of such isolates, the markers also were associated with the interaction phenotype (i.e., *AvrLm1*:J19-1.1:Vir1:J53-1.3, 1:1:0:1 for avirulent isolates or 0:0:1:0 for virulent isolates) (corresponding to 83.8% of the whole set of field isolates; Table 3). In the cases where markers were not associated with the interaction phenotype as they were in a2 or H5, all associations of markers were not equally represented in the field isolates. The J19-1.1:Vir1:J53-1.3 0:1:1 association of molecular markers was not observed (Table 3). Some other combinations were rare (i.e., the 0:1:0 and 0:0:0 in avirulent isolates [one isolate each], or the 1:1:1 and 1:1:0 combinations in virulent isolates [two isolates each]) (Table 3). Vir1 was present in only six avirulent isolates, all originating from the CETIOM collection, and mostly (five of six isolates) under the form of the 1:1:1 combination (Table 3). Finally, when markers were in disagreement with the interaction phenotype, the two most common combinations were 1:0:1, which was observed in seven virulent isolates (two from the CETIOM collection and five from the IMASCORE collection), and 1:0:0, which was observed in five virulent isolates (four from the IMASCORE collection and one from the Versailles collection) (Table 3).

Table 1. Primers used in this study

Name	Type	Sequence (5'–3')	Primer 5' base location ^a	References ^b
OPJ19	RAPD ^c	ggaccacct	...	4
J19F	SCAR ^d	ggaccacctcctttttaa	J19-1.1 (1)	3
J19R	SCAR	ggaccacctactaagcg	J19-1.1 (1127)	3
J19R6	SCAR	ggcatgaaggattagaggatagac	J19-1.1 (308)	3
LMR1F3	Rep-PCR ^e	aagagcacaggagagccac	LMR1 (3422)	1
LMR1F4	Rep-PCR	ggcagcttaacaagcaac	LMR1 (3747)	3
LMR1R1	Rep-PCR	atccccgcacttatagagcc	LMR1 (2409)	3
LMR1R10	Rep-PCR	cgtgtcttagtaagtcgtc	LMR1 (741)	3
vR1	SCAR	taggttatgctggagcacttg	F3R1-4 (1089)	3
OPAD1	RAPD	caaaggcgcg	...	4
AD1F	SCAR	gcggcgatctcttttctgttc	AD1-1.53 (1)	2
AD1R	SCAR	caaaggcgcgtagcggtgagatgg	AD1-1.53 (1532)	2
AD1R7	SCAR	cctatggtgtgctctagcagt	AD1-1.53 (239)	3
Ex2	Rep-PCR	agaaggagtaggagcaggag	LMR1 (4840)	3
Ex2F	SCAR	agaaggagtaggagcaggaggcg	Smex-2 (1)	3
Ex2R	SCAR	agaaagagtagggcaggag	LMR1 (1268)	3
GTG5	ISSR ^f	VHVgtgtgtgtgtgtgtg	...	3
GTG5F	SCAR	ggtggcggtgtgtgtgtgtg	GTG5-0.4 (1)	3
GTG5R	SCAR	tctgcgggggtgtgtgtgtgt	GTG5-0.4 (457)	3
J53F5	SCAR	ctactatataccagaatcct	...	3
J53R5	SCAR	attaatataggtattgtttgc	...	3

^a Numbers in parentheses indicate primer 5' base location on sequences J19-1.1 (EMBL AJ300477), LMR1 (GeneBank M77515), F3R1-4 (unpublished), AD1-1.53 (EMBL AJ294758), GTG5 (EMBL AJ421013), and Smex-2 (EMBL AJ300478). ... indicate original primers used to generate the markers

^b 1 = Jedryczka et al. 1999, 2 = Attard et al. 2001, and 3 = Attard 2001. 4 = The Qiagen Operon website. In reference 2, primers AD1F and AD1R are named AD1F1 and AD1R1, respectively.

^c RAPD = random amplified polymorphic DNA. Operon decamer used to generate profiles.

^d SCAR = sequence characterized amplified regions; primers deduced from the marker sequences, either generated by expanding the initial nonspecific primer by a few bases or by designing new internal primers unrelated to the original ones.

^e Rep-PCR = repetitive-element-based polymerase chain reaction; primers are deduced from the LMR1 sequence (GeneBank M77515) (Taylor and Borgmann 1994).

^f ISSR = intersimple sequence repeats PCR primer; V = A, T, or C; H = A, C, or G.

Predictive value of J19-1.1, Vir1, and J53-1.3 for large-scale analysis of populations at the field scale.

Vir1, J19-1.1, and J53-1.3 were found to be high-quality PCR diagnostic tools for identification of isolates harboring *AvrLm1* among field populations. In addition, amplification with both the J19F-J19R and the LMR1F3-vR1 primer pairs generated profiles containing the polymorphic markers as well as a nonpolymorphic band that can be considered an internal control of the success of the amplification (data not shown). It was further evaluated whether these markers could allow a rapid, rough, large-scale evaluation of the race structure of the pathogen at the field scale, using large-scale DNA extraction procedures directly from infected leaf samples in the field.

DNA samples (66) extracted from leaf lesions collected in the field were analyzed for the amplification of J19-1.1, Vir1, and J53-1.3 and for identification of the AD1 allele they harbored. The association of J19-1.1 with J53-1.1 was the rule because only one out of 66 DNA samples (1.5%) showed amplification of J19-1.1 alone (Table 3). As observed in purified isolates, the association among J19-1.1, Vir1, and J53-1.3 was similar to that observed in crosses #11 and #23 in most cases, because 62 out of 66 DNA samples (93.9%) showed either the 0:1:0 or the 1:0:1 association of these markers (Table 3). The 0:1:1 combination was absent, as already was observed for pure isolates (Table 3). Finally, the 0:0:0 combination was observed for 4.5% of the DNA samples (Table 3). The most common alleles of AD1 corresponded to the 2x and 5x number of repeats of *MinLm1*. A favored association of the 2x allele (63.6% of the cases) with the J19-1.1:Vir1:J53-1.3 0:1:0 combination and of the 5x allele (70.6% of the cases) with the 1:0:1 combination was observed (Fig. 3).

On the basis of marker profiles, the *AvrLm1* allele frequency in this field could be predicted to be 16.7% (J53-1.3), 18.2% (J19-1.1), or 22.7% (Vir1). To confirm the diagnosis, 58 isolates were obtained from another set of leaf lesions collected from the same field, at the same time. Following inoculation tests, 17.2% of these isolates were shown to harbor the *AvrLm1* allele.

The molecular markers J19-1.1, Vir1, and J53-1.3 allowed an overview of the race structure at a field scale within 2 days, compared with the more than 5 weeks that is required for an inoculation test. As discussed by Balesdent and associates (2001), the interpretation of the outcome of the interaction following cotyledon-inoculation, in particular of the *AvrLm1/Rlm1* interaction, may be biased by the heterogenic nature of some *B. napus* genotypes and by the delayed expres-

sion of resistance. The reliability of the diagnosis of the avirulent race with PCR markers is therefore comparable with that of the inoculation test.

DISCUSSION

In the process toward cloning the *AvrLm1* avirulence gene of *L. maculans* using positional cloning, we observed that the target locus corresponded to a recombination-deficient area in the in vitro crosses described here. The recombination-deficient area is comprised of markers J19-1.1 and J53-1.3, along with *AvrLm1*, corresponding to a minimal physical distance of 40 kb. In addition, very few recombination events between *AvrLm1* and AD1-1.53 were observed (two out of a total of 312 in vitro progeny), suggesting that the minimal size of the recombination-deficient area may extend close to the 121 kb separating AD1-1.53 and J53-1.3.

Recombinant markers located on only one border of the locus were found in the contig clones; therefore, it was not possible to determine whether or not *AvrLm1* was present in the 184-kb BAC contig identified here. Therefore, one of the first steps towards positional cloning was the generation of 3' bordering markers in order to delimitate the AVR locus. For such a purpose, we chose to increase the progeny by the generation of backcrosses between sister isolates v23.1.3 and v23.1.2 (Fig. 1). This strategy was successful in generating a few recombination events between AD1-1.53 and *AvrLm1*, but remained insufficient to identify recombination events among *AvrLm1*, J19-1.1, J53-1.3, and Vir1. As an alternative, we chose to increase the number of meiotic events by analyzing the association of the available markers with *AvrLm1* in 192 European Union (EU) field isolates identified as being virulent or avirulent on *Rlm1*-harboring genotypes. Of the five markers cosegregating in lab crosses, only the three single-banding markers present (J19-1.1 and J53-1.3) or absent (Vir1) in avirulent isolates from lab crosses could be easily analyzed for their association with the interaction phenotype. Few recombination events among J19-1.1, Vir1, J53-1.3, and *AvrLm1* were observed, and it could be proposed that a quite similar distance separates *AvrLm1* from J53-1.3, on one site, and *AvrLm1* from J19-1.1, on the other site. However, the analysis still remained insufficient to effectively delimitate the genome region encompassing the gene.

The most striking feature of the present work was the high level of conservation of the genome region encompassing *AvrLm1* within EU field isolates (including leaf lesions-DNA

Table 2. Association of markers of the genetic map with the *AvrLm1* allele displayed by field isolates as a function of their collection of origin^a

Origin	Ratio of isolates (%) ^b									
	Smex-2		Vir1		J19-1.1		J53-1.3		GTG5-0.4	
	1	0	0	1	1	0	1	0	0.45	0.42
<i>AvrLm1</i>										
CETIOM (36)	16.7 (6)	83.3 (30)	83.3 (30)	16.7 (6)	97.2 (35)	2.8 (1)	94.4 (34)	5.6 (2)	66.7 (24)	33.3 (12)
IMASCORE (48)	8.7 (4)	91.3 (42)	100 (48)	0	97.9(47)	2.1 (1)	93.8 (45)	6.2 (3)	66.7 (30)	33.3 (15)
Châlons (8)	nd	nd	100 (8)	0	100 (8)	0	100 (8)	0	nd	nd
Total (92)	12.2 (10)	87.8 (72)	93.5 (86)	6.5 (6)	97.8 (90)	2.2 (2)	94.6 (87)	5.6 (5)	66.7 (54)	33.3 (27)
<i>avrLm1</i>										
Versailles (39)	nd	nd	7.7 (3)	92.3 (36)	7.7 (3)	92.3 (36)	0	100 (39)	7.9 (3)	92.1 (35)
CETIOM (27)	11.1 (3)	88.9 (24)	14.8 (4)	85.2 (23)	14.8 (4)	85.2 (23)	14.8 (4)	85.2 (23)	36.0 (9)	64.0 (16)
IMASCORE (26)	11.5 (3)	88.5 (23)	38.5 (10)	61.5 (16)	34.6 (9)	65.4 (17)	23.1 (6)	76.9 (20)	36.4 (8)	63.6 (14)
Châlons (8)	nd	nd	0	100 (8)	0	100 (8)	0	100 (8)	nd	nd
Total (100)	11.3 (6)	88.7 (47)	17.0 (17)	83.0 (83)	16.0 (16)	84.0 (84)	10.0 (10)	90.0 (90)	23.5 (20)	76.5 (65)

^a Smex-2, J19-1.1, and J53-1.3 markers are linked to the avirulent phenotype, whereas the Vir1 marker is linked to the virulent phenotype. 1 = presence of the marker, 0 = absence of the marker, 0.45 = GTG5 allele of isolate a.2, and 0.42 = GTG5 allele of isolate H5. Numbers in parentheses are the total number of isolates in each class.

^b Ratio of isolates displaying the presence or absence of the marker for each interaction phenotype; nd = not done. The marker Smex-2 was not analyzed in the Versailles and Châlons collections due to its rare occurrence in the other collections.

samples), because 88.0% of the isolates showed the J19-1.1:Vir1:J53-1.3 0:1:0 or 1:0:1 association of markers, corresponding to the same genome organization as that found in the parents of crosses #11 and #23. The very low frequency of alternative associations of markers (1:1:1, 1:0:0, 0:0:0, 0:0:1, 1:1:0, and 0:1:1), amounting to only 12.0% of the samples, with some combinations being totally absent, is surprising when considering the scale of the sampling, the life-cycle of the fungus, and the occurrence of recombination between AD1-1.53 and GTG5-0.4. The sampling represented a 10-year time scale and covered three French fields, separated by more than 200 km, as well as samplings performed throughout Europe. In spite of this wide sampling scale, the same combination of markers with the virulent allele is observed in isolate H5 (North of France, 1990), virulent isolates from Switzerland and Portugal (collected in 1998), 8 out of 11 virulent U.K. isolates (obtained between 1995 and 1997), most of the Versailles 1998 to 1999 virulent isolates, and all of the Châlons 2000 to 2001 virulent isolates. Extensive sexual recombination is an obligate part of the pathogen life and disease cycle (West et al. 2001), therefore suggesting that numerous crossovers should occur independently, also at the *AvrLm1* region. Finally, recombination occurred in lab crosses between the 14-kb-separated AD1-1.53 and GTG5-0.4 markers with a frequency that complies with the usual estimates of relationships between map distances and physical distances in filamentous fungi (Farman and Leong 1998). In contrast, the adjacent 121-kb region was highly conserved in the field isolates and corresponded to a genetic distance of only 1.6 cM in lab crosses. All these data are indicative of a strong protection from recombination in the whole 121-kb (or more) *AvrLm1* region, not only in our lab crosses but also in most of the EU populations of the fungus.

Such recombination-deficient regions already have been observed to occur at avirulence loci in another ascomycete, *M. grisea*, and even in the oomycete *P. infestans* (Farman and Leong 1998; van der Lee et al. 2001b). For instance, the *AVR1-CO39* region in *M. grisea* was characterized by recombination clusters surrounding a recombination-deficient area in the middle of the *AVR* gene locus (Farman and Leong 1998). In such a region, 1 cM could represent 14 to 220 kb (Farman and Leong 1998). Recombination-deficient regions, but also many *AVR* loci, often encompass diverse and com-

posite repeated elements (Dioh et al. 2000; Farman and Leong 1998; Sweigard et al. 1995; van der Lee et al. 2001b), as also was observed for the *AvrLm1* locus (Attard 2001; this study). One of the most common hypothesis to explain the occurrence of these recombination-deficient regions is the absence of the homologous region in the virulent parent or parents of the crosses (Farman and Leong 1998; Mandel et al. 1997). This was demonstrated for the *AVR1-CO39* region in *M. grisea* where a 20-kb deletion occurred in the virulent isolates (Farman and Leong 1998). Such was also the case for the *Avr3-Avr10-Avr11* region of *P. infestans* where all 15 amplified fragment length polymorphism (AFLP) markers tightly linked with the avirulence gene cluster were in coupling phase with avirulence (van der Lee et al. 2001a,b). One of the markers did not hybridize with the virulent parent and progeny, and was mostly present in avirulent field populations of *P. infestans* (van der Lee et al. 2001b). The putative absence of the *AvrLm1* homologous region in virulent isolates was not as easy to sort out in the case of *L. maculans*. The 5' bordering markers GTG5-0.4 and AD1-1.53 were present, with size polymorphism, in both the virulent and avirulent parents, as well as in all field isolates analyzed here, suggesting that the corresponding region was present in all isolates. In contrast, the cosegregant markers J19-1.1 and J53-1.3 never were amplified in the virulent parent or progeny of the in vitro crosses analyzed here. Unfortunately, extensive studies of these markers did not allow us to recover single-copy sequences among them (Attard 2001), needed to demonstrate the lack, or the presence, of the corresponding region in *avrLm1* isolates. Such was also the case for most *EcoRI* restriction fragments of clone BACJ53 that strongly hybridized with genomic DNA (Attard 2001). The recent availability of the complete sequence for the contig revealed that it contains up to 73% of dispersed repeats, either already or never described in *L. maculans* (A. Attard and T. Rouxel, unpublished data). Finally, the occurrence of one cosegregant marker, Vir1, that occurred in coupling phase with the virulence allele, contradicts the "deletion" hypothesis to explain "gain" of virulence on *Rlm1* genotypes, except if an unusual conformation of the repeated element LMR1, resulting from the deletion event, corresponds to the Vir1 marker (Attard 2001). Nonexclusive hypotheses to explain lack of recombination could involve two alternate idiomorph-type

Fig. 3. Repartition of the alleles of the AD1-1.53 marker, in terms of number of repeats of the *MinLm1* core motif, in **A**, the pooled collections of isolates and **B**, the Versailles 2000 sampling of DNA directly extracted from leaf lesions in the field. **A**, The allele frequency is shown for *AvrLm1* (summing up 76 isolates) and *avrLm1* isolates (summing up 63 isolates). **B**, The allele frequency is shown for DNA samples displaying the following J19-1.1:Vir1:J53-1.3 association of markers = 0:1:0 (51 DNA samples) and 1:0:1 (11 DNA samples). Four samples displaying the J19-1.1:Vir1:J53-1.3 0:0:0 or 1:0:0 association of markers are not represented.

loci that would allow only a very limited amount of recombination, or a centromeric location of the *AvrLm1* locus.

The conservation of the organization of the markers indicated that a very similar event was quite systematically associated with loss of avirulence in EU-wide field isolates. However, alternative genomic events associated with the loss of avirulence seem to occur, mainly in the case of the virulent isolates of the IMAScore collection where only 61.5% of the 26 virulent isolates showed the expected J19-1.1:Vir1:J53-1.3 0:1:0 combination. Alternative associations of markers were mostly associations specific of the avirulent isolates (i.e., the 1:0:1 association) or similar combinations lacking either J19-1.1 or J53-1.3. These rarer associations of markers with the *avrLm1* allele mostly were observed for German isolates of the IMAScore collection and could represent less drastic events leading to virulence (i.e., point mutations in the *AVR* gene itself, or mutations in genes or genome regions involved in the regulation of expression of the *AVR* gene), in which the genomic region encompassing the genes is maintained with the association of markers characteristic of the avirulent isolates. This hypothesis would be consistent with the crop history in the different countries, mainly if comparing Germany and France. In Germany, a *Rlm1*-harboring cultivar, Quinta, was marketed in 1976 and was one of the prevalent cultivars for the following six years (G. Leiss, *personal communication*). In contrast, in France, the first *Rlm1*-harboring cultivar, Vivol, was released in 1991, and it is only with Capitol, released in 1995, that a *Rlm1*-harboring cultivar became a market leader (X. Pinochet, *personal communication*). The recent large-scale cropping of *Rlm1*-harboring cultivars in France probably exposed the populations of the pathogen, which mostly harbored *AvrLm1* in the 1990s (Ansan-Melayah et al. 1997), to a very strong selection pressure over a short time span. This would be in favor of the rapid dissemination of the most probable event leading to the loss of avirulence, corresponding to isolates harboring the 0:1:0 combination of markers, by either the simultaneous occurrence of a similar genomic event leading to facilitated loss of the avirulence function or favored dissemination of a preexisting, or recently selected, virulence locus. The linkage disequilibrium observed between *AvrLm1* and both GTG5-0.4 and AD1-1.53 (i.e., high frequency of both the 5× allele of the *MinLm1* minisatellite and the 0.42 GTG5-0.4 allele in virulent isolates compared with avirulent ones) would support the latter hypothesis. Assuming that the *AvrLm1* genomic region has positive functions favoring the competitiveness of the isolates, one can hypothesize that, in the longer term and even though the time scale considered corresponds to only approximately 20 years, isolates harboring minor modifications of the region have been selected by being more competitive and, in time, could have replaced the first generation of virulent isolates.

The map-based cloning initiated here represents the first walk performed in *L. maculans*. As was the case for other phytopathogenic fungi (Farman and Leong 1998), obstacles encountered during the walk included arrays of repeated sequences and a large recombination-deficient area (Attard 2001; this study). The practical advantage of this specific genome situation was that the map-generated markers allowed us a diagnosis of race structure at the field scale, which is rarely the case for avirulence gene markers. The next steps will now involve a validation of the centromeric/deletion hypotheses and, ultimately, cloning of *AvrLm1* by combining additional chromosome walk steps (if needed), expression cDNA-AFLP-based studies on near-isogenic isolates differing only by the presence of *AvrLm1*, and functional identification of *AvrLm1* by transformation of a virulent strain with candidate open

reading frames present in the contig. Finally, when the gene is identified, the collections of virulent isolates described here will be the starting point to check our hypotheses on the events leading to the loss of avirulence.

MATERIALS AND METHODS

Collection of field isolates.

Four collections of isolates were analyzed in the current study. The Versailles collection contained 39 isolates sampled from three plants of *B. napus* cv. Capitol in November 1998 (leaf lesions), February 1999 (leaf lesions), and June 1999 (stem canker) in an INRA experimental field. Leaf lesion isolates originated from a single pycnidium, and all isolates of the collection were further purified by subculturing hyphal tips from young mycelia growing on agar media (Attard et al. 2001). Sixteen isolates were obtained from leaf lesions on cv. Drakkar (susceptible) in the autumn of 2000 in a field at Châlons-sur-Marne (eastern France, approximately 200 km from Versailles), and termed the "Châlons 2000" collection. The CETIOM collection comprised 63 isolates collected from leaves in one single experimental field at Saint Florent (central France, approximately 200 km south of Versailles, December 1997) or stem bases (June 1998) of cvs. Capitol and Lipton. The CETIOM isolates correspond to mass isolation of colonies growing out of plant tissues (Attard et al. 2001). The IMAScore collection contained 74 European isolates isolated between 1994 and 1998 in different EU countries (Volke 1999). Most of these isolates are single-ascospore or single-conidium isolates. The collection is described in detail by Volke (1999) and Attard and associates (2001). Each isolate used here corresponded to a single nonredundant haplotype, as shown by analysis of fingerprints generated with one RAPD and four ISSR primers (Attard et al. 2001; West et al., in press; L. Gout and J. Schmit, *unpublished data*).

For diagnostic purposes, 66 DNA samples directly extracted from leaf lesions in a Versailles experimental field in October 2000 (Attard et al. 2001) as well as 58 single-conidium isolates (termed the "Versailles 2000" collection) were also analyzed. All these isolates or DNA samples originated from independent leaf lesions naturally occurring on the susceptible cultivars Westar and Drakkar Goeland.

In vitro crosses.

The a.2 (Mat⁺, *AvrLm1*) and H5 (Mat⁻, *avrLm1*) isolates were obtained from single ascospores isolated from diseased *B. napus* stubble (Gall et al. 1994). Ten complete tetrads and 88 randomly isolated ascospores were obtained from the in vitro pairing of a.2 and H5 (cross #11) (Ansan-Melayah et al. 1995; Balesdent et al. 2001). From this progeny, the v11.3.6 isolate (Mat⁻, *AvrLm1*, *avrLm4*) was crossed with isolate 21.3.1 (Mat⁺, *avrLm1*, *AvrLm4*), which is itself a third-generation progeny obtained following an initial cross of an offspring of cross #11 with a German *avrLm1-AvrLm4* field isolate collected in 1992 (Fig. 1). Of this cross, 89 random ascospores were isolated (cross #23; Balesdent et al. 2001). One isolate in the progeny, v23.1.3 (Mat⁻, *AvrLm1*, *AvrLm4*), was used to construct a BAC genomic DNA library. Isolate v23.1.3 was further crossed with one sister isolate, v23.1.2 (Mat⁺, *avrLm1*, *avrLm4*) (cross #26) (Fig. 1). A series of three backcrosses finally was performed between v23.1.3 and one (Mat⁺, *avrLm1*) progeny of each cross (crosses #27, 28, and 36) (Fig. 1). In the present study, 26, 59, and 40 isolates were analyzed from crosses #26, #27, and #36, respectively.

All fungal isolates were maintained on V8 juice agar medium at 20°C with a 12-h photoperiod and stored as agar plugs in sterile mineral oil at 4°C.

Pathogenicity tests and plant material.

Highly sporulating cultures were obtained according to Ansan-Melayah and associates (1995). The interaction phenotypes (*AvrLm1* or *avrLm1*) were evaluated for each above-mentioned isolate by inoculation of 10^5 conidia on cotyledons of 15-day-old plantlets of the three *B. napus* cultivars Columbus (*Rlm1*), Quinta (*Rlm1*, *Rlm4*), and Westar (susceptible control), as previously described (Ansan-Melayah et al. 1995; Balesdent et al. 2001).

Genetic map of the *AvrLm1* linkage group and generation of SCAR markers.

The a.2 × H5 *L. maculans* map was mainly constructed with PCR-based markers including RAPD, ISSR (West et al., in press; Zietkiewicz et al. 1994), AFLP, and Rep-PCR (Jedryczka et al. 1999). Polymorphic bands were recorded as present or absent in the whole progeny. Monogenic segregation of each marker was first analyzed in tetrads, then confirmed with a χ^2 test ($P = 0.05$) performed on random isolate data. Monogenic markers were finally subjected to multipoint linkage analysis using the Mapmaker/Exp 3.0 software with a log of the likelihood ratio (LOD) value of 4.0 and a maximum distance of 35 cM. All PCR polymorphic bands linked to *AvrLm1* were cloned and sequenced as described before (Attard et al. 2001). Finally, sequence data were used to generate SCAR markers: J19-1.1 (primers J19F-J19R), Vir1 (primers LMR1F3-vR1), AD1-1.53 (primers AD1F-AD1R), Smex-2 (primers Ex2F-Ex2R), and GTG5-0.4 (primers GTG5F-GTG5R) (Table 1). All SCAR markers were checked for segregation pattern by comparison with the polymorphic band they originated from on the whole progeny of crosses #11 and #23. Primers were designed using the "Prime" program of the Wisconsin package (ver. 10.0; Genetics Computer Group, Madison, WI, U.S.A.) and synthesized by Invitrogen (Invitrogen Corp. Carlsbad, CA, U.S.A.). Primers to amplify marker J53-1.3 (J53F5-J53R5, Table 1) were designed from partial BAC sequence data obtained by the "Genoscope."

PCR conditions.

For PCR, DNA was extracted from freeze-dried mycelium or a pellet of conidia (approximately 10^8 to 10^9 conidia) with the DNeasy Plant Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendations. DNA extraction directly from leaf lesions in the field was performed as described by Attard and associates (2001). PCR was per-

formed in a 9600 Perkin-Elmer Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) with 1 U of Taq DNA polymerase (Appligene/Q-Biogene, Illkirch, France), 15 ng of template DNA, 2 μ M of each primer, 1.5 μ l of 10× buffer, and 1.25 μ M of each dNTP and milliQ sterilized water up to 15 μ l.

RAPD-PCR was performed as described by Ansan-Melayah and associates (1995). The Rep-PCR amplification took place as described by Jedryczka and associates (1999). The ISSR-PCR program was as follows: 1 cycle of 4 min at 95°C and 37 cycles of 30 s at 95°C, 30 s at 64°C, and 2.30 min at 72°C followed by 7 min at 72°C. The SCAR-PCR programs were as follows: 1 cycle of 5 min at 95°C and 30 cycles of 1 min at 95°C, 1 min at 50, 62, or 65°C, depending on the primers, and 2 min at 72°C, followed by 10 min at 72°C.

PCR products were separated by agarose gel electrophoresis in 1× Tris-borate EDTA, viewed and analyzed as described previously (Balesdent et al. 1998).

Electrophoretic karyotyping, genomic DNA extraction and restriction, and Southern blotting.

Chromosomal DNA was obtained from conidia according to Plummer and Howlett (1993). Electrophoresis was conducted in a CHEF-DR III apparatus (BioRad, Hercules, CA, U.S.A.) as described by Leclair and associates (1996) and using the protocols designed by Morales and associates (1993b) for separation of *L. maculans* chromosomes.

Following growth in liquid Fries medium (Balesdent et al. 1998), DNA from freeze-dried mycelia was extracted according to Balesdent and associates (1998). A 5- μ g sample of DNA was digested by 40 U of restriction enzymes *HindIII* and *XhoI* in a final volume of 150 μ l. Following an EtOH precipitation, the restricted DNA was separated by electrophoresis in 0.8% agarose.

Chromosomal DNA and restricted genomic DNA were vacuum blotted onto Nylon N⁺ membranes (Appligene/Q-Biogene) according to the manufacturer's recommendations. Hybridization and visualization of hybridization patterns took place as previously described (Leclair et al. 1996), except that the NonaPrimer labelling kit I for DNA labelling (Appligene/Q-Biogene) was used.

BAC library construction.

The v23.1.3 genomic DNA BAC library was constructed as described by Brosch and associates (1998), with minor modifications. Conidia were embedded in low-melting agarose plugs

Table 3. Combination of markers J19-1.1:Vir1:J53-1.3 and their association with the *AvrLm1* allele displayed by field isolates as a function of their collection of origin^a

Origin	Ratio of isolates (%) ^b						
	1:0:1	0:1:0	1:1:1	1:1:0	1:0:0	0:0:0	0:0:1
<i>AvrLm1</i>							
CETIOM (36)	80.5 (29)	2.8 (1)	13.9 (5)	0	2.8 (1)	0	0
IMASCORE (48)	93.7 (45)	0	0	0	4.2 (2)	2.1 (1)	0
Châlons(8)	100 (8)	0	0	0	0	0	0
Total (92)	89.1 (82)	1.1 (1)	5.4 (5)	0	3.3 (3)	1.1 (1)	0
<i>avrLm1</i>							
Versailles (39)	0	87.2 (34)	0	5.1 (2)	2.6 (1)	5.1 (2)	0
CETIOM (27)	7.4 (2)	77.8 (21)	7.4 (2)	0	0	7.4 (2)	0
IMASCORE (26)	19.2 (5)	61.5 (16)	0	0	15.4 (4)	0	3.9 (1)
Châlons (8)	0	100 (8)	0	0	0	0	0
Total (100)	7.0 (7)	79.0 (79)	2.0 (2)	2.0 (2)	5.0 (5)	4.0 (4)	1.0 (1)
Field samples (66) ^c	16.7 (11)	77.3 (51)	0	0	1.5 (1)	4.5 (3)	0

^a The Vir1 marker is linked to the virulent phenotype, whereas the J19-1.1 and J53-1.3 markers are linked to the avirulent phenotype. As a consequence, as observed in a.2 and H5, the expected association of markers is 1:0:1 for avirulent isolates and 0:1:0 for virulent isolates. The 0:1:1 combination was never observed in the samples analyzed here. Numbers in parentheses are the total number of isolates in each class.

^b Ratio of isolates displaying the corresponding association of markers for one given interaction phenotype/origin combination.

^c DNA samples extracted from leaf lesions in the field. DNA was directly extracted from infected leaves of susceptible cultivars Goeland and Westar, grown in a Versailles experimental field, October 2000, interaction phenotype of individual isolates was not determined (Attard et al. 2001).

to a final concentration of 6×10^3 conidia per ml. Following *Hind*III partial digestion (15 U of *Hind*III, 20 min, 37°C), DNA fragments whose size ranged between 75 and 100 kb, or between 100 and 125 kb, were ligated into pBeloBAC11 and electroporated into *Escherichia coli* DH10B using an Easyject Plus electroporator (Equibio Ltd, Ashford, U.K.). The recombinant colonies were inoculated into 160 µl of liquid Luria broth (LB) containing chloramphenicol at 12.5 µg ml⁻¹ and 10% glycerol in 96-well plates and were incubated overnight at 37°C with shaking at 240 rpm. The library (6,720 clones) was stored at -80°C.

BAC DNA miniprep and digestions.

Each clone was incubated in 3 ml of LB-chloramphenicol and grown overnight at 37°C with shaking. The cells were harvested by 5 min of centrifugation at $6,000 \times g$ and resuspended in 150 µl of Qiagen P1 buffer (Tris-HCl, 50 mM; pH 8; EDTA, 10 mM; RNase A, 100 µg ml⁻¹). The suspension was maintained on ice for 5 min and cell lysing took place at room temperature by adding 150 µl of Qiagen P2 buffer (NaOH, 0.2 N; sodium dodecyl sulfate, 1%). Lysis was stopped by adding 150 µl of P3 neutralization buffer (C₂H₃KO₂, 3 M, pH 5.5) and maintaining the suspension on ice for 10 min. Following centrifugation at $12,000 \times g$ for 15 min, DNA was precipitated from the supernatant with 0.7 volume of isopropanol. Following centrifugation at $12,000 \times g$ for 10 min, the DNA pellet was rinsed with EtOH (70%), air dried, and resuspended in 40 µl of Tris-EDTA buffer. Insert size was determined following cleavage of 10 µl of the plasmid solution with 10 units of *Not*I in 20 µl of total reaction volume. The restricted solution then was loaded onto a 1% agarose gel and was submitted to CHEF DRIII pulsed-field gel electrophoresis using a 1- to 6-s pulse, 6 V/cm, and a 120° angle at 15°C for 14 h. Midrange (BioRad) and 1-kb Plus DNA ladders were used as size standards.

Screening of the BAC library and organization into contigs.

Clones from 32 plates, corresponding to a genome coverage of seven genome-equivalent (Attard 2001), were transferred on two HD nylon filters using the Biomek 2000 workstation (Beckman Coulter, Inc., Fullerton, CA, U.S.A.). The filters were incubated overnight on LB-chloramphenicol (12 µg ml⁻¹) agar at 37°C, then transferred twice to lysing buffer (0.5 M NaCl, 0.5 M NaOH) for 7 min, then to neutralization buffer (1.5 M NaCl, 0.5 M Tris HCl, 1 mM EDTA). After drying, the membranes were hybridized with ³²P-labeled DNA probes as described previously. Positive clones were checked by PCR, either directly using the bacteria as a PCR template or on plasmids extracted following overnight growth of 48 pooled clones in LB.

BAC clones identified as containing the markers linked to the target gene were primarily organized into contigs following restriction enzyme digestion with *Eco*RI, *Xho*I, and *Eco*RI + *Xho*I.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Genoscope Project: www.cns.fr.
 Mapmaker/Exp 3.0 software: waldo.wi.mit.edu/genome_software
 Qiagen Operon website: www.operon.com