Repeat-Induced Point Mutation (RIP) as an Alternative Mechanism of Evolution Toward Virulence in *Leptosphaeria maculans*

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Three avirulence genes, *AvrLm1*, *AvrLm6*, and *AvrLm4-7*, were recently identified in *Leptosphaeria maculans* and found to be localized as solo genes within large noncoding, heterochromatin-like regions mainly composed of retrotransposons, truncated and degenerated by repeat-induced point mutation (RIP). The *Rlm6* resistance gene has been overcome within 3 years in outdoor experiments in France and, here, we investigate the molecular basis of evolution toward virulence at the *AvrLm6* locus. A region of 235 kb was sequenced in a virulent isolate and showed the deletion of *AvrLm6* and three divergent mosaics of retrotransposons. *AvrLm6* was found to be absent from 66% of 70 virulent isolates, with multiple events of deletion. The sequencing of virulent alleles in 24 isolates revealed a few cases of point mutations that had created stop codons in the sequence. The most frequent mutation events, however, were RIP, leading to the modification of 4 to 9% of the bases compared with the avirulent allele and generating 2 to 4 stop codons. Thus, RIP is described for the first time as an efficient mechanism leading to virulence and the multiple patterns of mutation observed suggest that multiple RIP events could occur independently in a single field population during 1 year.

Plants have developed efficient defence systems to resist attacks by microbial pathogens. The plant immune response includes a basal level of pathogen recognition, the pathogen-associated molecular pattern (PAMP)-triggered immunity, which involves the recognition of common features of microbial pathogens (Zipfel and Felix 2005). Pathogens have evolved ways to escape this primary immune response through the delivery into the plant of pathogenicity effectors that suppress pathogen recognition (Chisholm et al. 2006). Then, plants developed a more specialized mechanism of pathogen detection that involves the recognition of effectors by resistance proteins directly or indirectly (Chisholm et al. 2006; Jones and Dangl 2006; Sacristan and Garcia-Arenal 2008). This specific recognition of the pathogen by its host plant is often controlled by a pathogen avirulence gene (*AVR*) and a plant resistance gene (*R*) as pro-

posed in the gene-for-gene model (Flor 1942), which usually triggers a rapid localized cell death, termed hypersensitive response (HR) that will stop pathogen infection (Hammond-Kosack and Parker 2003). Pathogens can escape avirulence-mediated host recognition by different molecular mechanisms, including nonsynonymous point mutations, frameshift mutations, deletion of the entire gene, or transposon insertion as exemplified for a few fungal plant pathogens (Catanzariti et al. 2006; Dodds et al. 2004; Farman et al. 2002; Gout et al. 2007; Joosten et al. 1997; Kang et al. 2001; Luderer et al. 2002; Orbach et al. 2000; Rep et al. 2004; Ridout et al. 2006; Schurch et al. 2004; Stergiopoulos et al. 2007; Westerink et al. 2004; Zhou et al. 2007). In addition, the genomic context can influence the potential of the avirulence genes to mutate. In the case of *AVR-Pita*, complete deletion of the gene was thought to be frequent as a consequence of its subtelomeric location (Orbach et al. 2000). Transposable elements can also alter avirulence-mediated host recognition by abolishing the *AVR* gene expression (e.g., Pot3 transposon insertion in the *AVR-Pita* promoter) (Kang et al. 2001) or by interrupting the avirulence gene (e.g., MINE retrotransposon insertion in the *ACE1* gene) (Fudal et al. 2005). Those mechanisms leading to virulence were mainly reported for laboratory mutants or collection isolates, and only a few large-scale molecular characterizations of virulent isolates were performed on natural populations of fungal plant pathogens in order to determine how virulent genotypes appear and disseminate (Gout et al. 2007; Schürch et al. 2004; Stergiopoulos et al. 2007).

Leptosphaeria maculans is responsible for one of the most devastating diseases of oilseed rape (OSR) (*Brassica napus*), called stem canker or blackleg. The fungus is present in most OSR-growing areas (Europe, North America, and Australia) (Fitt et al. 2006). Genetic studies demonstrated the occurrence of gene-for-gene interactions in the *L. maculans*–*B. napus* system where fungal avirulence (*AvrLm*) genes are the counterpart of plant resistance (*Rlm*) genes (Rouxel and Balesdent 2005). Currently, the use of resistant cultivars harboring single major resistance genes is the most cost-efficient method to control the disease when the corresponding avirulent allele (*AvrLm*) dominates in the pathogen population (Ansan-Melayah et al. 1997; Delourme et al. 2004). However, *L. maculans* displays a high evolutionary potential to overcome novel resistance sources, often only a few years after their commercial release. This was illustrated by the rapid “breakdown” of the commonly used *Rlm1* resistance in France in only 3 years concomitant to the drastic decrease of avirulent isolates in *L. maculans* populations, and the breakdown of the ‘Surpass 400’ resistance in Australia (Li and Cowling 2003; Rouxel et al.

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*The e-Xtra logo stands for “electronic extra” and indicates that a supplemental table and a supplemental figure are published online.

2003a). Similarly, the *Rlm6* resistance gene, currently absent from commercial cultivars, has been overcome within 3 years in France in experimental conditions (Brun et al. 2000). *L. maculans* is one of the few fungal pathogens for which avirulence genes have been cloned (Sacristan and Garcia-Arenal 2008), including *AvrLm1* and *AvrLm6*, located in the same genetic cluster (Fudal et al. 2007; Gout et al. 2006). The genomic region spanning these two genes was sequenced and showed a particular isochore-like structure consisting of alternation of small (20 to 70 kb) GC-equilibrated, open reading frame (ORF)-rich isochores intermingled with large (170 to 450 kb) AT-rich isochores mainly composed of mosaics of truncated retrotransposons degenerated following repeat-induced point mutation (RIP) (Fudal et al. 2007; Gout et al. 2006). *AvrLm1* and *AvrLm6* share common characteristics: i) they encode small potentially secreted proteins (205 and 144 amino acids long, respectively) with no homology in the databases or in the *L. maculans* genome; ii) they are highly overexpressed during the primary leaf infection; and iii) they are present as solo genes within noncoding, heterochromatin-like regions of 269 and 133 kb, respectively (Fudal et al. 2007; Gout et al. 2006). A large-scale study on the mechanisms leading to the inactivation of *AvrLm1* in 460 *L. maculans* field isolates from France, Australia, and Mexico was recently performed by Gout and associates (2007) and showed that a unique, large deletion (260 kb) of a chromosomal fragment containing *AvrLm1* was responsible for the switch to virulence in more than 90% of the cases. This major event was suggested to be influenced by the particular localization of *AvrLm1* as a solo gene in the middle of an AT-rich isochore (Gout et al. 2006).

With *AvrLm6* being localized in the same genomic context as *AvrLm1* (Fudal et al. 2007), the availability of collections of isolates experimentally submitted to the *Rlm6* selection pressure, followed by the setting up of novel experimental fields aiming to study the dynamics of *Rlm6* breakdown (this study), provides us here with material of choice to analyze whether *AvrLm6* would evolve to virulence through the same molecular mechanisms as *AvrLm1* or by other mechanisms. Thus, we analyzed the occurrence of deletions and their extent, along with nucleotide polymorphism at the *AvrLm6* locus, to identify the different molecular events leading to virulence under *Rlm6* selection pressure. In particular, evaluation of allelic variability of *AvrLm6* in avirulent and virulent isolates led us to identify RIP as a novel, still-undocumented mechanism of adaptation to resistance-gene selection pressure. The influence of the genomic context on the evolution of *AvrLm6* is discussed.

RESULTS

Identification of isolates virulent toward *Rlm6*.

A large-scale survey of *L. maculans* races in France, genotyping 1,797 isolates from 20 different locations for nine AVR loci (*AvrLm1* to *AvrLm9*), highlighted a very low frequency of *avrLm6* isolates (only one isolate virulent toward *Rlm6*) (Balesdent et al. 2006), consistent with the current absence of *Rlm6* in commercial cultivars. This current scarcity of virulent isolates in field populations made it necessary that we analyze isolates obtained during experiments aiming to apply an *Rlm6* selection pressure to field populations and to evaluate the speed of breakdown of *Rlm6* resistance (Table 1). All but two

Table 1. List and characteristics of the isolates used in this study

Isolates ^c	Short name	Year	Country	Host plant ^a		<i>n</i>	Phenotype ^b		Provider (ref.) ^d
				Species	Cultivar		<i>AvrLm6</i>	<i>avrLm6</i>	
Field									
Versailles	V03x	2003	France	<i>Brassica. napus</i>	Westar	4	4	0	L. Gout (7)
Oucques	Ox	2000	France	<i>B. napus</i>	Westar	7	7	0	M. H. Balesdent (6)
Mondonville	Bx	2000	France	<i>B. napus</i>	Westar	5	5	0	M. H. Balesdent (6)
Laxou	Lx	2000	France	<i>B. napus</i>	Westar	3	3	0	M. H. Balesdent (6)
Toury	Mx	2000	France	<i>B. napus</i>	Westar	9	8	1	M. H. Balesdent (6)
Experimental									
Rennes	EF03-x or ExF03-x	2003	France	<i>B. napus</i>	Eurol, Eurol MX	20	0	20	H. Brun
Rennes	Rx or MXx	1992–93	France	<i>B. napus</i>	MX line	10	0	10	H. Brun (1)
Rennes	SamR-Fx	1995	France	<i>B. napus</i>	Samourai MX	3	0	3	H. Brun (2)
Rennes	R1x	2001	France	<i>B. napus</i>	S MX, Maxol MX, Falcon MX, Eurol, Eurol MX	25	1	24	H. Brun
Saint Florent	C1x or C2x	2000–01	France	<i>B. napus</i>	Eurol MX	9	1	8	H. Brun
Reference									
v23.1.3	...	–	In vitro cross	–	...	...	1	...	M. H. Balesdent (3)
v29.3.1	...	–	In vitro cross	–	...	...	...	1	M. H. Balesdent (4)
BBA 62908	...	1966	Germany	–	...	...	1	...	
IBCN 16	...	1988	Australia	<i>B. napus</i>	...	...	...	1	P. Salisbury (5)
IBCN 18	...	1988	Australia	<i>B. napus</i>	...	...	...	1	P. Salisbury (5)
IBCN 42	...	1990	France	<i>B. napus</i>	...	...	1	...	C. Gall/M. H. Balesdent (5)
IBCN 44	...	1990	France	<i>B. napus</i>	...	...	1	...	C. Gall/M. H. Balesdent (5)
IBCN 73	...	–	Germany	–	...	...	1	...	P. H. Williams (5)
IBCN 74	...	1970s	France	<i>B. oleracea</i>	...	...	1	...	L. Lacoste (5)
UWA M3	...	2001	Australia	<i>B. napus</i>	...	...	...	1	M. Barbetti
Total	...	...	...	...	...	105	35	70	...

^a — = No data.

^b Interaction phenotype with *Rlm6* plants. *AvrLm6*: avirulent isolates. *avrLm6*: virulent isolates. The interaction phenotype (*AvrLm6* or *avrLm6*) of *L. maculans* isolates was assessed following inoculation on a line harboring the *Rlm6* resistance gene (Darmor-MX), as previously described (Balesdent et al. 2005).

^c Field populations, experimental isolates, and reference isolates.

^d References: 1, Somda et al. 1999; 2, Brun et al. 2000; 3, Balesdent et al. 2001; 4, Balesdent et al. 2002; 5, Mendes-Pereira et al. 2003; 6, Balesdent et al. 2006; 7, Gout et al. 2006

of these latter isolates (97%) were virulent on the *Rlm6* line Darmor-MX (Table 1). In addition to the reference *AvrLm6* and *avrLm6* isolates resulting from in vitro crosses, a total number of 103 isolates was analyzed. Of these, 69 were virulent toward *Rlm6* (Table 1).

Definition of race-specific primers.

Two sets of primers, termed *AvrLm6extU/L* and *AvrLm6intU/L*, were used in order to amplify *AvrLm6*: the external set of primers was specific to the promoter and the 3' untranslated region (UTR) of *AvrLm6* and the internal primer set was bound to the coding sequence of *AvrLm6* (140 and 490 bp after the ATG) (Fig. 1). As expected, *AvrLm6* was amplified by the two sets of primers in all the avirulent isolates. In contrast, *AvrLm6* was only amplified in 24 of 68 virulent isolates when using the internal primers and in 5 virulent isolates when using the external primers (also amplified by the internal primers) (Table 2). Thus, the external *AvrLm6* primers seem to preferentially amplify avirulent isolates with an overall diagnosis efficiency of 92.86% (Tables 2 and 3).

Polymorphism of *AvrLm6* in avirulent isolates.

AvrLm6 avirulent alleles were amplified by polymerase chain reaction (PCR) using the external *AvrLm6* primers and sequenced in the 35 avirulent isolates (Table 1). The sequences obtained were compared with the *AvrLm6* sequence of the reference isolate v23.1.3 in which *AvrLm6* was first cloned (Fudal et al. 2007). A very limited nucleotide polymorphism was observed (eight polymorphic sites) and only six isolates exhibited polymorphism compared with v23.1.3. These isolates corresponded to the oldest isolates of our samples (IBCN 74 and BBA62908) and to some recent French isolates (C2E6-1 with three polymorphisms in the 5' UTR; V03-M4-305, B03-12, and B04-01 with one common polymorphic site). Of the eight polymorphic sites identified, five were located in the coding sequence: one polymorphism was shared by five isolates, while the four others were found in only one isolate each. The five polymorphisms located in the coding sequence all corresponded to C>T or G>A transitions (Table 4). These polymorphisms resulted in nonsynonymous mutations at the protein level (V² to M² in IBCN74, BBA62908, V03-M4-305, B03-12, and B04-01; F⁶⁸ to L⁶⁸ and K¹⁵⁶ to E¹⁵⁶ in IBCN 74; and S⁷⁰ to G⁷⁰ in BBA62908) (Fig. 2).

Fig. 1. Organization of the *AvrLm6* gene and locations of the primers used in this study. The 5' and 3' untranslated regions are indicated by gray boxes. Exons are indicated by open boxes. Introns are indicated by black boxes. Locations of the primers used in this study are indicated as arrows.

Table 2. Estimation of *AvrLm6* presence in avirulent and virulent isolates using a polymerase chain reaction (PCR) test

Phenotype ^b	No. of isolates tested	Percent PCR amplification (number) ^a		
		β -TubulinU/L	<i>AvrLm6extU/L</i>	<i>AvrLm6intU/L</i>
<i>AvrLm6</i>	35	100 (35)	100 (35)	100 (35)
<i>avrLm6</i>	70	100 (70)	7.14 (5)	34.29 (24)

^a Percentage and number of isolates amplified by PCR using external (*AvrLm6extU/L*) and internal *AvrLm6* primers (*AvrLm6intU/L*), and primers specific to β -tubulin as a control (β -tubulinU/L).
^b Interaction phenotype toward *Rlm6* plants. *AvrLm6*: avirulent isolates and *avrLm6*: virulent isolates.

Evaluation of the presence of *AvrLm6* in virulent isolates.

AvrLm6 was amplified in only 34% of the virulent isolates when using the internal primers. In order to determine whether the absence of amplification corresponded to an absence of the gene in the corresponding isolates, the presence of *AvrLm6* was analyzed by Southern blot analysis in a subset of isolates. Chromosomal DNA of 18 virulent isolates was digested by *SalI*, separated by CHEF (contour-clamped homogeneous electric fields) electrophoresis, and hybridized with an *AvrLm6* probe. The blots were also hybridized with an *LmCys1* probe, corresponding to a single-copy gene present on the same *SalI* fragment as *AvrLm6* in v23.1.3 (Fudal et al. 2007), in order to determine the size of the *SalI* fragment in the virulent isolates lacking *AvrLm6*. The *SalI* restriction fragment encompassing *AvrLm6* was 215 kb in v23.1.3, which is in accordance with the sequence data (Table 3). The *AvrLm6* probe gave a hybridization signal for 7 of 18 virulent isolates. These all corresponded to the isolates amplified using the internal *AvrLm6* primers and one isolate for which both external and internal primers allowed amplification of the gene (Table 3). The *AvrLm6* gene, or at least the part of the gene amplified by the internal primers, is thus extrapolated to be present in 34.78% of the virulent isolates (Table 2).

When using *LmCys1* as a probe, the size of the *SalI* fragment containing *AvrLm6* was shown to be variable, 145 to 314 kb (Table 3). Half of the virulent isolates, however, shared the same fragment size (215 kb) as that of the *AvrLm6* reference isolate. Interestingly, in nine virulent isolates, the *SalI* fragment was bigger (224 to 314 kb) than that of the *AvrLm6* reference isolate (Table 3). In virulent isolates lacking *AvrLm6*, the *SalI* fragment was very variable in size. No correlation could be found between the absence of the gene and the size of the *SalI* fragment, giving no evidence for a unique event of deletion and, thus, contrasting with the *AvrLm1* deletion case (Gout et al. 2007).

Organization of the *AvrLm6* genomic region in the virulent isolate v29.3.1.

Based on the known sequence of a bacterial artificial chromosome (BAC) contig spanning *AvrLm6* in the avirulent isolate v23.1.3 (Fudal et al. 2007), we identified the corresponding BAC clone contig for the virulent isolate v29.3.1. The 5' part of the region has already been described and BAC clones V29-38D1 and V29-41F7 were previously sequenced (Gout et al. 2007) (Fig. 3). The v29.3.1 BAC library was screened by PCR using BAC pools as described by Gout and associates (2006, 2007) and BAC clones V29-55E6 and V29-51C1 were identified by using markers generated from the sequence of the single-copy genes *LmCys1* and *LmMFS*, respectively (Fudal et al. 2007) (Fig. 3). The marker *55E6F* was designed after BAC end sequencing of 55E6 and a new BAC clone, V29-35C8, was identified. The three BAC clones V29-55E6, V29-51C1, and V29-35C8 were sequenced by Genoscope. The final contig had a size of 235,218 bp and included two gaps (Fig. 3). Sequence analysis confirmed the absence of sequences homologous to *AvrLm6* in the *AvrLm6*/v29.3.1 contig. Analysis of the GC content, gene density, and repeat distribution in the

AvrLm6/v29.3.1 revealed the same organization as in the *v23.1.3* contig (Fig. 3), which was already characterized to have highly conserved GC-equilibrated regions, gene-rich regions (Fudal et al. 2007), and large AT-rich regions comprising RIP-degenerated, truncated copies of the four long terminal repeat (LTR) retrotransposons *Pholy*, *Olly*, *Rolly*, and *Polly* (Gout et al. 2006) (Figs. 3 and 4). Although GC-equilibrated regions were highly conserved, large structural reorganizations were observed in AT-rich regions between *AvrLm6/v23.1.3* and *v29.3.1* contigs (Fig. 4). In contrast to what was observed for the *AvrLm1* region of the same isolates (Gout et al. 2007), the AT-rich region of the *AvrLm6* contig showed numerous divergent mosaics, of which some are likely to be due to events of insertion of retrotransposons or to deletions (only maintaining solo LTR). Three highly polymorphic regions were identified, one corresponding to the *AvrLm6* locus (Fig. 4). The presence of several sources of variations in the region explains why the size variations of the *SalI* fragment containing *AvrLm6* was not related to the absence of *AvrLm6* (Fig. 4).

Polymorphism of *AvrLm6* in virulent isolates.

The *AvrLm6*-virulent alleles were sequenced using the fragment generated by the internal primers (*AvrLm6intU/L*) for all

24 isolates in which the gene (or part of it) was present. Of these, additional sequencing was performed on the external fragment (*AvrLm6extU/L*) for the 5 isolates amplifying it and on an intermediate-sized fragment (*AvrLm6midU/AvrLm6intL*) for 15 of the remaining isolates (Fig. 1; Tables 2 and 3). Four of the five alleles amplified by the external primers only displayed a few point mutations compared with the reference avirulent allele of *v23.1.3*. *R1Max5-4*, *R1Max1-2*, and *R1Max1-3* (which were collected the same year in the same field and on the same plant for two of them) shared the same point mutation at the nucleotide level, which resulted in the generation of a premature stop codon at the protein level, leading to a dramatically truncated protein (Fig. 2). *EF03-24-1* exhibited three mutations compared with *v23.1.3*, only one mutation being nonsilent at the protein level with a tyrosine (aromatic) to a histidine (basic) change. In all the other isolates, the nucleotide diversity was high compared with *v23.1.3* (4 to 9%, corresponding to 83 polymorphic sites in which 64 were found in the coding sequence) (Tables 4 and 5), thus explaining some difficulties experienced in amplifying the virulent alleles. Forty (29.17%) polymorphic sites were identified over the 144 amino acids of *AvrLm6* and resulted in the generation of 2 to 4 stop codons in each sequence. Splicing sites of the introns were also mutated, which probably alters the

Table 3. Presence of *AvrLm6* in a sample of virulent isolates using Southern blot analysis and correlation between polymerase chain reaction (PCR) amplification of *AvrLm6* virulent alleles and nucleotide polymorphism

Isolate	Location ^c	Year	Avr ^d	Southern analysis ^a		PCR amplification ^b			Sequence compared with <i>v23.1.3</i>
				Hybrid	<i>SalI</i> (kb)	β -Tub	Ext	Int	
<i>v23.1.3</i>	In vitro cross	...	A6	+	215	+	+	+	...
<i>v29.3.1</i>	In vitro cross	...	a6	–	270	+	–	–	...
IBCN18	Australia	1988	a6	–	215	+	–	–	...
M3-10	Toury	2000	a6	–	145	+	–	–	...
UWA M3	Australia	2001	a6	–	215	+	–	–	...
R4	Rennes	1992	a6	–	215	+	–	–	...
R8	Rennes	1992	a6	–	215	+	–	–	...
R13	Rennes	1992	a6	–	215	+	–	–	...
MX7-19	Rennes	1993	a6	–	215	+	–	–	...
MX8-12	Rennes	1993	a6	+	224	+	–	+	Many point mutations
MX8-14	Rennes	1993	a6	+	314	+	–	+	Many point mutations
SamR F1M1	Rennes	1995	a6	+	224	+	–	+	Many point mutations
Sam R F3M2	Rennes	1995	a6	–	215	+	–	–	...
SamR F9M1	Rennes	1995	a6	–	215	+	–	–	...
ExF03-3-2	Rennes	2003	a6	+	259	+	–	+	Many point mutations
ExF03-5-2	Rennes	2003	a6	+	314	+	–	+	Many point mutations
ExF03-29-1	Rennes	2003	a6	–	259	+	–	–	...
EF03-3-2	Rennes	2003	a6	–	259	+	–	–	...
EF03-14-2	Rennes	2003	a6	+	259	+	–	+	Three point mutations
EF03-24-1	Rennes	2003	a6	+	215	+	+	+	Single point mutation
R1Max1-2	Rennes	2001	a6	nd	nd	+	+	+	Single point mutation
R1Max1-3	Rennes	2001	a6	nd	nd	+	+	+	Single point mutation
R1Max5-4	Rennes	2001	a6	nd	nd	+	+	+	Single point mutation
ExF03-7-2	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
ExF03-15-3	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
ExF03-32-3	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
ExF03-33-1	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
ExF03-37-3	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
ExF03-40-3	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
ExF03-41-1	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
EF03-22-3	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
EF03-31-3	Rennes	2003	a6	nd	nd	+	–	+	Many point mutations
C1E7-5	St. Florent	2000	a6	nd	nd	+	–	+	Many point mutations
R1F10-2	Rennes	2001	a6	nd	nd	+	–	+	Many point mutations
R1F11-8	Rennes	2001	a6	nd	nd	+	–	+	Many point mutations
R1F13-1	Rennes	2001	a6	nd	nd	+	+	+	Many point mutations
R1E4-2	Rennes	2001	a6	nd	nd	+	–	+	Many point mutations

^a Southern analysis using *AvrLm6* as probe to estimate the presence of the gene in a sample of virulent isolates and using *LmCys1* as probe to estimate the size of the *SalI* restriction fragment corresponding to the *AvrLm6* genomic region. Hybridization = *AvrLm6* hybridization, *SalI* = size of the *SalI* fragment, and nd = not determined.

^b β -tub = β -tubulinU/L, Ext = *AvrLm6* extU/L, and Int = *AvrLm6* intU/L.

^c Country or location in France.

^d Interaction phenotype toward *Rlm6* plants. A6 = avirulent isolates and a6 = virulent isolates.

splicing of intron 1 for five isolates and intron 3 for 12 isolates (Fig. 2).

The percentage of mutations in the coding and noncoding regions of the gene varied between 4 and 20%, with the higher percentage of mutations being found in exons 2 and 3 (Table 5). However, this result has to be considered cautiously because the number of sequences was variable, depending on the gene region: 25 sequences were obtained for exons 2, 3, and 4 and introns 2 and 3, but only 4 to 13 sequences were obtained for the promoter, exon 1, and intron 1, and part of exons 2 and 4. In spite of the different number of sequences available, no main differences in the mutation intensity could be observed between the coding and noncoding regions. The GC content in the corresponding virulent alleles was decreased from 43.6 to 33%, and the ratio of G>A and C>T mutations represented almost 100% of the mutations. The *avrLm6* alleles also showed a bias in the occurrence of dinucleotides, with an underrepresentation of the CpA and TpG dinucleotides and a strong overrepresentation of the TpA dinucleotide (Table 4). The TpA/ApT and CpA + TpG/ApC + GpT indices showed great differences between the *AvrLm6* allele of v23.1.3 and the 19 *avrLm6* alleles of the virulent isolates, indicating the presence of many point mutations (Table 4). All these data are indicative of sequences having undergone RIP (Attard et al. 2005; Galagan et al. 2003).

A great diversity of mutation patterns was observed, with 17 virulent alleles identified among the 24 isolates in which *AvrLm6* was sequenced. Except for some of the isolates that shared a common pattern of mutations (R1Max5-4, R1Max1-2

and R1Max1-3; ExF03-7-2 and ExF03-91-1; and ExF03-15-3, ExF03-33-1, ExF03-37-3, ExF03-5-2, and ExF03-30-3) (Fig. 2), a diversity of mutation patterns was even found for isolates that were collected the same year in the same field.

DISCUSSION

Similarly to *AvrLm1*, *AvrLm6* is located in a large genomic region devoid of active coding sequences and consisting of mosaics of truncated and degenerated retrotransposons (Fudal et al. 2007; Gout et al. 2006). In the case of *AvrLm1*, this had three main consequences: i) a strong suppression of meiotic recombination (Gout et al. 2006), as also observed for *AvrLm6* (Fudal et al. 2007); ii) a very low level of nucleotide polymorphism in the avirulent alleles of the gene (Gout et al. 2007); and iii) a large-size deletion event being the favored event leading to virulence (Gout et al. 2007). Here, we investigate the last two traits on the second “lost in the middle of nowhere” AVR gene of *L. maculans*, *AvrLm6*.

AvrLm1 avirulent alleles were extremely monomorphic: three polymorphic sites, of which only two were located in the coding region of the gene, and only one leading to a nonsynonymous mutation, were identified in 11 of 41 isolates (Gout et al. 2007). Even though the samples analyzed are not comparable with those analyzed by Gout and associates (2007), the analysis of *AvrLm6* alleles showed two specific features compared with those of *AvrLm1*: nucleotide polymorphism at the *AvrLm6* locus is found in fewer isolates than for *AvrLm1* (17 versus 27% for *AvrLm1*) (Gout et al. 2007) but more polymorphic sites are ob-

Table 4. Bases composition, structural features, number and type of mutations, and RIP indices of *AvrLm6* avirulent and virulent alleles^a

Isolate	Size (bp)	G+C content (%)	Identities with v23.1.3 allele of <i>AvrLm6</i> (%)	No. of stop codons	No. of mutations (%) ^b	Ratio of G to A or C to T mutations	CpA occurrence O/E	TpA occurrence O/E	TpG occurrence O/E	TpA/ApT ^c	CpA + TpG/ApC + GpT
v23.1.3	773	43.6	...	1	...	...	...	...	...	1.12	1.02
BBA 62908	773	43.7	99.5	1	4 (0.5)	25	0.98	1	1	1.11	1.02
IBC74	773	44	99.7	1	2 (0.3)	50	1.02	0.98	1	1.09	1.04
V03-M4-305	671	44.1	99.8	1	1 (0.2)	100	1	1	1	1.17	0.99
B03-12	652	44.2	99.8	1	1 (0.2)	100	1	1	1	1.15	0.99
B04-01	652	44.2	99.8	1	1 (0.2)	100	1	1	1	1.15	0.99
C2E6-1	524	43.7	99.6	1	3 (0.4)	0	1	1	1	1.29	0.89
R1Max1-2	524	43.5	99.8	2	1 (0.2)	0	0.94	0.96	1	1.29	0.89
R1Max1-3	527	43.3	99.8	2	1 (0.2)	0	0.94	0.96	1	1.31	0.89
R1Max5-4	623	44.8	99.8	2	1 (0.2)	0	1	1	1	1.22	0.97
EF03-24-1	773	44	99.6	1	3 (0.4)	0	1.04	0.97	1	1.11	1.02
R1F13-1	583	42	96.9	4	18 (3.1)	100	0.85	1.23	0.9	1.48	0.84
EF03-14-2	339	37.9	95	3	17 (5)	100	0.9	1.38	0.95	1.24	1.03
ExF03-7-2	327	37.2	95	3	16 (5)	100	0.75	1.44	0.81	1.62	0.80
ExF03-91-1	327	37.2	95	3	16 (5)	100	0.8	1.44	0.75	1.62	0.82
C1E7-5	529	38.4	94.6	2	28 (5.4)	100	0.9	1.31	0.81	1.58	0.83
R1E4-2	386	37.3	94	3	23 (6)	100	1	1.37	0.7	1.92	0.69
ExF03-3-2	533	37.1	93	3	36 (7)	94.4	0.97	1.4	0.62	1.68	0.80
SamRF1M1	608	35	92.6	4	39 (6.4)	100	0.92	1.47	0.63	1.53	0.82
MX8-12	517	37.3	92.4	4	34 (6.6)	97.1	1	1.43	0.65	1.74	0.76
MX8-14	608	35.9	92.4	3	40 (6.6)	90	0.82	1.45	0.67	1.6	0.86
R1F10-2	524	37	92.2	3	35 (6.8)	100	0.9	1.43	0.65	1.72	0.74
R1F11-8	469	35.8	92.1	3	37 (7.9)	100	1	1.62	0.54	1.91	0.71
EF03-22-3	348	34.1	91	4	30 (9)	100	0.85	1.71	0.63	1.66	0.91
EF03-31-3	324	33.5	91	4	28 (9)	100	0.8	1.73	0.62	1.61	0.87
ExF03-15-3	349	34.6	91	4	29 (9)	100	0.85	1.68	0.68	1.62	0.86
ExF03-32-3	326	33.6	91	3	27 (9)	100	0.85	1.67	0.625	1.61	0.90
ExF03-33-1	326	33	91	4	29 (9)	100	0.8	1.74	0.56	1.68	0.83
ExF03-37-3	326	33	91	4	29 (9)	100	0.8	1.74	0.56	1.68	0.83
ExF03-5-2	326	33	91	4	29 (9)	100	0.8	1.74	0.56	1.68	0.83
ExF03-90-3	326	33	91	4	29 (9)	100	0.8	1.74	0.56	1.68	0.83

^a Dinucleotide frequencies are expressed as the observed occurrence over the expected number (O/E).

^b Number and percentage of mutations compared with v23.1.3.

^c According to Galagan and associates (2003), TpA/ApT > 2.0 or CpA+TpG /ApC+GpT < 0.7 corresponds to regions predicted as repeat-induced point-mutated in *Neurospora crassa*. BBA, IBC74, V03-M4-305, B03-12, and B04-01 are avirulent toward *Rlm6* while all the other isolates are virulent. BBA, IBC74, M4305, B03-12, and B04-01 are the only avirulent isolates that showed polymorphism at *AvrLm6* locus compared with v23.1.3.

served in *AvrLm6* compared with *AvrLm1*, with eight polymorphic sites at the nucleotide level leading to four nonsynonymous mutations at the protein level, suggesting that *AvrLm6* could be submitted to a limited positive diversifying selection, leading to an accumulation of nonsynonymous point mutations allowing diversification of AVR proteins as observed in other plant pathogens such as *Melampsora lini* (Ellis et al. 2007) or *Hyaloperonospora parasitica* (Rehmany et al. 2005). More interestingly, five of these mutations are indicative of RIP. One of the common traits (and main characteristics compared with other fungal AVR genes) of the three AVR genes currently cloned in *L. maculans* is their much lower GC content compared with other coding sequences and available expressed sequence tag sequences of the genome (Parlange et al. 2009). In accordance with this low GC content, the existence of mutations due to RIP in functional *AvrLm6* alleles thus questions whether RIP is acting in this species as a positive diversification process as well as an inactivation process (see below) eventually leading to novel AVR-effector specificities.

The major event leading to virulence toward *Rlm1* is the deletion of the gene which was observed in 98.3% of the virulent isolates and which, in 90% of the cases, corresponded to the

deletion of a 260-kb repeat-rich chromosomal fragment comprising the unique gene *AvrLm1* but also to the insertion of a new genomic segment suggesting retrotransposon-mediated recombination (Gout et al. 2007). Compared with the evolution of *AvrLm1*, *AvrLm6* is shown here to be submitted to more diversified mechanisms toward gene inactivation: deletion, one to three point mutations (not attributable to RIP), and RIP inactivation. Even if *AvrLm6* was found to be present in much more virulent isolates than in the case of *AvrLm1* (35 instead of 1.7%) (Gout et al. 2007), the main event leading to virulence at the *AvrLm6* locus is the deletion of the gene. As in the case of *AvrLm1*, the deletion is accompanied by important modifications in the retrotransposon mosaics, with three polymorphic regions identified as potential sources of size variations. However, the deletions occurring at the *AvrLm6* locus do not reveal any correlation between the absence of *AvrLm6* and the size of the corresponding AT-rich region. Thus, they are much smaller than in the case of *AvrLm1* and suggest that multiple events of deletion or insertion occurred at this locus. As in the case of *AvrLm1*, the data provided here suggest that retrotransposons are key drivers in genome evolution and adaptation to novel selection pressure in *L. maculans*.

Fig. 2. GeneDoc-edited alignment of *AvrLm6* protein sequence of three avirulent and 22 virulent isolates compared with the sequence in the reference avirulent isolate v23.1.3. BBA, IBCN74, and V03-M4-305 are avirulent toward *Rlm6* while all the other isolates are virulent. B03-12 and B04-01 share the same sequence as V03-M4-305 and are not represented here. BBA, IBCN74, V03-M4-305, B03-12, and B04-01 are the only avirulent isolates that showed polymorphism at *AvrLm6* locus compared with v23.1.3. The virulent isolates R1Max1-2 and R1Max1-3 share the same sequence as R1Max5-4 and are not represented in the alignment. Stop codons are indicated as stars. Points represent the absence of sequence (e.g., region with a spliced intron). Dashes indicate data not available.

RIP, as defined in the model species *Neurospora crassa*, is a mechanism, specifically reported in fungi, which occurs during the sexual phase between fertilization and meiosis and which targets duplicated sequences whenever they are more than 400 bp in size and share at least 80% of sequence identity (Watters et al. 1999). RIP consists of C:G to T:A transitions, with a significant preference for the CpA and TpG contexts (Attard et al. 2005; Cambareri et al. 1989; Idnurm and Howlett 2003), resulting in an irreversible gene alteration or even inactivation. In *N. crassa*, in a single passage through the sexual cycle, up to 30% of the C and G nucleotides in duplicated sequences can be mutated (Cambareri et al. 1989). The RIP mechanism was originally described in *N. crassa* (Cambareri et al. 1989) but, since then, also observed to operate on *Podospira anserina* (Graia et al. 2001) and *L. maculans* (Idnurm and Howlett 2003). *L. maculans* is a haploid fungus in which sexual mating is the primary mode of reproduction (Rouxel and Balesdent 2005), and the current active RIP mechanism is con-

sistent with the key importance of sexual reproduction in its life cycle. In contrast, for fungi such as *Magnaporthe grisea* (Ikeda et al. 2002) and *Aspergillus oryzae* (Montiel et al. 2006), having lost the ability to cross in nature, RIP is not active and only signatures of RIP can be found. RIP is particularly extensive in *L. maculans*, as exemplified by the degeneration of the different copies of the retrotransposons located in the *AvrLm1*-*AvrLm6* genomic region and by the low GC content of the corresponding retrotransposon-rich isochores. In the case of *AvrLm1*, only five virulent isolates (1.7%) showed the presence of an *AvrLm1* fragment, of which only two could be sequenced and showed an extensive mutation pattern that was hypothesized by the authors to be due to RIP (Gout et al. 2007). In the case of *AvrLm6*, many more virulent isolates (i.e., 35%) carried a potentially incomplete copy of *AvrLm6*. In all except four isolates, this copy was highly mutated compared with the avirulent allele of v23.1.3, and a great variety of mutational patterns was observed. The GC content of these *AvrLm6* alleles decreased from 44 to 33%, and they showed all typical traits of sequences having undergone RIP mutations (overrepresentation of the TpA dinucleotides, mutations exclusively corresponding to C to T or G to A transitions, and so on). All these data are clearly indicative of a RIP-like effect acting on *AvrLm6* to inactivate the gene, as was the case for very rare virulent alleles of *AvrLm1*. The occurrence of extensive RIP degeneration at the *AvrLm6* locus, along with the retrospective reevaluation of effective occurrence of RIP mutations at the *AvrLm1* locus, question i) a possible duplication of *AvrLm6* and *AvrLm1* in the ancestor of the corresponding virulent isolates, because RIP is supposed to act exclusively on duplicated sequences (Galagan and Selker 2004), or ii) a loose control of the RIP process in *L. maculans* that can have effect on a single-copy sequence lost in the middle of repetitive elements.

Fig. 3. Organization of the *AvrLm6* genomic region in an avirulent (v23.1.3) and a virulent (v29.3.1) *Leptosphaeria maculans* isolate. GC content: analysis of the GC content in the two contigs (sliding window: size 1,000 bp, step 100 bp). Dashed lines indicate sequence gaps in the v29.3.1 contig sequence. Markers used for the chromosome walk and contig assembly are shown. The stars represent the probes used for Southern analysis.

Table 5. Distribution of point mutations in the different coding and noncoding regions of *AvrLm6* in virulent isolates compared with the reference avirulent isolate v23.1.3

Region	Size (bp)	No. of polymorphic sites (%)	No. of sequences available ^a
Promoter + 5' UTR	118	8 (6.78)	11
exon 1	44	3 (6.82)	13
intron 1	51	4 (7.84)	13
exon 2	119	23 (19.33)	13/24
intron 2	47	2 (4.26)	24
exon 3	50	10 (20.00)	24
intron 3	48	5 (10.42)	24
exon 4	222	28 (12.61)	24/4

^a *AvrLm6* virulent alleles were amplified by external primers (located in the promoter and 3' untranslated region [UTR] of the gene) in 4 isolates, by internal primers (located in the exon 2 and exon 4 of the gene) in 24 isolates, and by intermediate primers (located in the 5' UTR and exon 4 of the gene) in 13 isolates.

Fig. 4. Schematic organization of the *AvrLm6* genomic region in v23.1.3 and v29.3.1. The plain black arrows indicate predicted genes, including *AvrLm6*. Boxes represent different long terminal repeat (LTR) retrotransposons flanked or not by LTR represented by small internal triangles. Small boxes with an internal triangle represent solo LTR. Absence of LTR at the 5' or 3' ends of the retrotransposon indicates a truncated retrotransposon. Lines connecting two boxes indicate the presence of a unique retrotransposon interrupted by the insertion of another element. Gray areas connecting the two contigs represent regions with the same organization. Within these homologous regions, dotted lines represent the same alternation of retrotransposons, not represented here. Dashed lines indicate sequence gaps in the v29.3.1 sequence.

No second copy of *AvrLm6* and *AvrLm1* and no closely related paralog are present in the genome of the reference avirulent isolate v23.1.3 following a whole genome search (I. Fudal and J. Grandaubert, *unpublished data*), and no duplication of these genes was detected by CHEF hybridization or by subcloning and sequencing after PCR amplification in virulent isolates. Finally, no mixed electrophoregram, indicative of the presence of more than one sequence, was found when we sequenced *AvrLm6*-virulent copies (data not shown). One last possibility would be that two copies of *AvrLm6* and *AvrLm1* are located in tandem in the genome, thus rendering it difficult to identify. The more likely explanation, however, is that, as observed by Irelan and associates (1994) for *N. crassa*, RIP extend beyond the boundaries of duplicated sequences and have mutational effects on neighboring unique sequences. However, in contrast to what was observed by Irelan and associates (1994), no gradient of mutations could be identified from the repeated elements to the *AvrLm6* locus and almost the same rate of mutations was found in the different coding and noncoding parts of the gene. This is probably fully attributable to the peculiar genomic context into which both *AvrLm1* and *AvrLm6* are located because, being surrounded by repeats and being small-sized genes, RIP would act on both sides of the gene and, thus, cause no gradient of degeneration.

Finally, one important point to address in durable management of resistance is not only to understand how new virulences appear but also how (and how fast) they disseminate in natural populations. Here, the data obtained show the occurrence of multiple events leading to virulence, even for a single year of sampling at the same place. For example, for the isolates sampled in Rennes in 2003, deletions, point mutations not attributable to RIP, and diverse RIP mutations were observed. Those results suggest a fast and independent generation of mutations (in particular the ones due to RIP), instead of the diffusion of a particular molecular event in the populations.

Similar events are shown here to contribute to evolution of *AvrLm6* and *AvrLm1* under *Rlm* selection pressure. The main difference between the two genes, however, is the diversity of events, including the diversity of RIP-mutated alleles observed for *AvrLm6* compared with *AvrLm1*. The level of polymorphism at the *AvrLm6* locus was analyzed at the time when resistance was in the process of being overcome whereas, in the case of *AvrLm1*, isolates were sampled several years after the resistance was overcome (Gout et al. 2007; Rouxel et al. 2003a). On these bases, it can be hypothesized that the low diversity of events at the *AvrLm1* locus reflects diffusion in population of the most favorable event leading to virulence then reaching, 10 years after the breakdown of *AvrLm1*, a relative homogeneity at this locus, whereas we are experiencing at the *AvrLm6* locus an ongoing process toward the overcoming of the corresponding resistance gene by numerous molecular events leading to virulence. In the coming years, this hypothesis will be evaluated using another recently cloned avirulence gene of *L. maculans*, *AvrLm4-7* (Parlange et al. 2009), which is localized in a genomic context similar to that of *AvrLm1* and *AvrLm6*, and for which the corresponding resistance gene *Rlm7* has been released very recently (2005) in commercial cultivars. This will give us a unique possibility to identify the different molecular events leading to virulence when overcoming a resistance gene in agrosystems, and to study the dynamics of the different virulent alleles in the pathogen populations.

MATERIALS AND METHODS

Fungal isolates and culture conditions.

Isolates of *L. maculans* used in this study were either collected from naturally or experimentally infected plants or ob-

tained following in vitro crosses (Table 1). The field populations analyzed were sampled in five locations from France (Versailles, Oucques, Mondonville, Laxou, and Toury) and described by Balesdent and associates (2006) and Gout and associates (2006). Additional reference field isolates were obtained from the IBCN collection maintained at INRA Versailles or from the University of Western Australia collection (UWA isolates). They included the reference isolate BBA62908, one of the oldest European *L. maculans* isolates available (Rouxel et al. 2003b). They were collected from *B. napus* or *B. oleracea* in France, Germany, or Australia. Field isolates enriched in *avrLm6* isolates were obtained from different experiments in Rennes or Saint-Florent (France) using a protocol described by Brun and associates (2000), in which the natural inoculum in the fields was increased in *avrLm6* isolates by incorporating infected stem residues of *Rlm6* genotypes (hereafter, collectively named MX lines) from the previous year's culture, thus mimicking culture conditions with insufficient rotation. A first experiment was performed between 1992 and 1996 in Rennes (Brun et al. 2000; Somda et al. 1999). Isolates termed Rx and MXx were obtained as single ascospores from pseudothecia on MX lines in 1992 or 1993, respectively (Somda et al. 1999). Isolates termed SamRFx were collected from Samourai MX in 1995, the year when *Rlm6* was overcome (Brun et al. 2000). A second experiment was performed between 1997 and 2003 in Rennes and Saint-Florent on Eurol MX, Falcon MX, Maxol MX, and S MX. Isolates R1x or C1-2x were collected in 2000 or 2001 in Rennes and Saint Florent from these MX lines. A third experiment was performed from 2002 to 2006 in Rennes using infected residues of Eurol MX. Isolates termed ExF03-x or EF03-x were collected in 2003 on Eurol MX or on the corresponding line lacking *Rlm6*, Eurol, respectively. The v23.1.3 isolate is a progeny from a cross between two isolates derived from a.2 and H5, two field isolates collected in France in 1990 and 1989, respectively. The v29.3.1 (*avrLm6*) isolate is a fourth-generation progeny originating from the same cross, a.2 × H5 (Balesdent et al. 2001, 2002). The v23.1.3 isolate of *L. maculans* (*AvrLm6*) (Balesdent et al. 2001, 2002) has been used for the map-based cloning of *AvrLm6* (Attard et al. 2002) followed by a complete sequencing of the corresponding genomic region (Fudal et al. 2007). All fungal cultures were maintained on V8 juice agar medium and highly sporulating cultures were obtained on V8 juice as previously described (Ansan-Melayah et al. 1995).

Plant inoculations.

The interaction phenotypes (*AvrLm6* or *avrLm6*) of *L. maculans* isolates were assessed following inoculation with 10^5 conidia on cotyledons of 15-day-old plantlets of *B. napus* cv. Westar (susceptible control) and one line harboring the *Rlm6* resistance gene (Darmor-MX), as previously described (Balesdent et al. 2005).

DNA manipulations.

For PCR, genomic DNA was extracted from conidia with the DNeasy 96 plant kit (Qiagen S.A., Courtaboeuf, France) as described previously (Attard et al. 2002). Sequences of the primers used to amplify PCR markers during the chromosome walk and for polymorphism studies are available in Supplementary Table S1. PCR amplification was done in a total volume of 15 µl containing 0.2 µM each dNTP, 1.2 µM each primer, 0.6 U of *Taq* DNA polymerase (Qiagen, Illkirch, France), 1.5 µl of 10× reaction buffer, and 10 to 30 ng of genomic DNA. PCR amplifications were performed using an Eppendorf Mastercycler EP Gradient thermocycler (Eppendorf, Le Pecq, France). Sequencing was performed using a Beckman Coulter CEQ 8000 automated sequencer (Beckman Coulter,

Fullerton, CA, U.S.A.) according to the manufacturer's instructions. Procedures for gel electrophoresis, Southern blots, and hybridizations have been reported (Attard et al. 2005).

BAC libraries coverage and screening.

Construction of the v29.3.1 genomic DNA *Hind*III BAC library and screening has been described previously (Gout et al. 2006). To allow screening of the BAC libraries by PCR, BAC DNA pools were prepared according to a three-dimensional pooling scheme and BAC DNA was extracted from pooled BAC clones by a boiling-freezing method (Sambrook and Russell 2001). The BAC clones were fully sequenced, finished, and assembled at Genoscope (Evry, France) as described by Gout and associates (2006). Sequence analyses were performed using the Isochore program to compute GC content (available online from the Institut Pasteur), which was set to a sliding window size of 3,500 bp and a shift increment of 100 bp.

Sequence comparisons.

AvrLm6 virulent and avirulent alleles were aligned using the CLUSTALX program and these aligned sequences were edited using the Genedoc program.

Electrophoretic karyotyping and DNA hybridization.

Chromosomal DNA was prepared as described by Plummer and Howlett (1993). For *Sal*I digests, agarose plugs of chromosomal DNA were washed four times for 20 min in distilled water and then incubated in *Sal*I buffer for 30 min at 4°C. Plugs were then transferred into fresh *Sal*I buffer containing 20 units of *Sal*I (Invitrogen, Carlsbad, CA, U.S.A.), equilibrated for 30 min at 4°C, and then incubated at 37°C for 16 h. Plugs were submitted to CHEF electrophoresis on a CHEF DRIII pulsed-field electrophoresis system (Bio-Rad, Hercules, CA, U.S.A.) using 0.5× Tris-borate EDTA buffer (Sambrook and Russell 2001), with buffer circulation at 10°C in 1% agarose gel (Seakem LE, FMC, Rockland, ME, U.S.A.), with the following conditions: pulse time ramping from 60 to 120 s for 24 h, 6 V cm⁻¹, pulse angle 120°C. Digested fragment sizes were estimated from digital images by comparison with the lambda ladder PFG marker (New England Biolabs, Beverly, MA, U.S.A.). Genomic restricted DNA was vacuum blotted on nylon N+ membranes (Qbiogene, Illkirch, France). Two probes corresponding to the *AvrLm6* gene (a 775-bp fragment amplified using primers *AvrLm6extU* and *AvrLm6extL*) (Fig. 1) or to *LmCys1*, a gene present in the same *Sal*I fragment as *AvrLm6* and always present in field isolates of *L. maculans* (a 957-bp fragment amplified using primers *LmCys1U* and *LmCys1L*), were used. These DNA fragments were PCR amplified, labeled using the NonaPrimer kit (Qbiogen), and hybridized on the membrane.

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