Heterochromatin-Like Regions as Ecological Niches for Avirulence Genes in the *Leptosphaeria maculans* Genome: Map-Based Cloning of *AvrLm6*

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Map-based cloning of avirulence genes of the *AvrLm1-2-6* cluster was recently undertaken in *Leptosphaeria maculans* and led to the identification of *AvrLm1*. The ensuing chromosome walk toward *AvrLm6* resulted in the delineation of a 562-kb bacterial artificial chromosome (BAC) clone contig in an avirulent isolate. Following sequencing of the contig and sequence comparison with a virulent isolate, four *AvrLm6* candidate genes were identified. Complementation of the virulent isolate with the four candidates was performed and one gene was found to fully restore the avirulent phenotype on *Rlm6* oilseed rape genotypes. *AvrLm6* was found to be located in the same genome context as *AvrLm1*, because it is a solo gene surrounded by 85 and 48 kb of degenerated repeats on its 5' and 3' sides, respectively. *AvrLm6* is an orphan gene encoding a small, potentially secreted, cysteine-rich protein. Comparison of *AvrLm1* and *AvrLm6* expressions by quantitative reverse-transcription polymerase chain reaction revealed that both genes are highly overexpressed during primary leaf infection. Using RNA interference, decreasing expression of *AvrLm6* was shown to result in virulence toward *Rlm6* genotypes whenever the expression was reduced by more than 60% compared with the wild-type isolate.

Additional keywords: *Brassica napus*, stem canker.

Plants have developed specific defense mechanisms against microbial pathogens. These defense responses include a rapid localized cell death termed hypersensitive response (HR) that will stop pathogen infection (Hammond-Kosack and Parker 2003). These plant defense responses often are triggered by a specific recognition event that is controlled by a plant resistance gene (*R*) and a pathogen avirulence gene (*Avr*) as proposed in

the gene-for-gene model (Flor 1971). *Avr* genes encode either proteins that are recognized by resistant plants involving directly or indirectly the corresponding resistance gene product, or enzymes involved in production of molecules that are recognized by resistant plants (Keen 1990; Van Der Biezen and Jones 1998). Only a few *Avr* genes from fungal plant pathogens have been identified and they were restricted to seven fungal species: the Dothideomycetes *Cladosporium fulvum* (Joosten et al. 1994; Luderer et al. 2002; van Kan et al. 1991; Westerink et al. 2004) and *Leptosphaeria maculans* (Gout et al. 2006), the Leotiomyces *Rhynchosporium secalis* (Rohe et al. 1995) and *Blumeria graminis* f. sp. *hordei* (Ridout et al. 2006), the Sordariomycetes *Magnaporthe grisea* (Böhnert et al. 2004; Farman et al. 2002; Orbach et al. 2000) and *Fusarium oxysporum* (Rep et al. 2004), and the Basidiomycete *Melampsora lini* (Catanzariti et al. 2006; Dodds et al. 2004). The majority of the *Avr* proteins are small and cysteine rich, without homology to each other, and are known or expected to be secreted into the host plant tissues during infection (Rep 2005). Predicting intrinsic function for the proteins encoded by fungal *Avr* genes has proven difficult. *NIP1* from the barley pathogen *R. secalis* encodes a peptidic toxin that induces necrotic lesions (Rohe et al. 1995). *Avr4* from *C. fulvum* is a chitin-binding protein that protects fungal cell walls from degradation by plant chitinases, and *Avr2* is an inhibitor of the extracellular cysteine protease Rcr3 in tomato (Rivas and Thomas 2005). Last, in *Melampsora lini*, *AvrP123-A* contains a sequence characteristic of Kazal Ser protease inhibitors, suggesting a possible role in inhibition of host protease, and *AvrP4* contains a Cys-knot structure similar to that found in some inhibitors of receptors or proteases (Catanzariti et al. 2006). These data concur to suggest that fungal *Avr* genes are actually “effectors” (i.e., pathogen molecules that manipulate the host cells, thereby evading plant recognition or facilitating infection on susceptible genotypes, as shown for bacterial and oomycete *Avr* genes) (Chisholm et al. 2006; Kamoun 2006; Mudgett 2005).

The lack of homology of *Avr* genes at the amino-acid level, along with the development of whole-genome initiatives in plant pathogens, allows us to examine the role, acquisition, and evolution of these *Avr* determinants (Birch et al. 2006). To address these questions in filamentous fungi, there is a need to focus primarily on those species for which more than one *Avr* gene has been cloned or tightly mapped and for which whole-genome sequence is available or will shortly become available. In *M. grisea*, three *Avr* genes were located at different places in the genome, *AVR-Pita* being located close to a telomere (Orbach et al. 2000) while other genes identified had no par-

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ticular localization (Böhnert et al. 2004; Farman et al. 2002). Those genes were not clustered and they encoded proteins with different structures, from small secreted proteins to a large multifunctional enzyme (Böhnert et al. 2004). Four *Avr* genes were identified in *C. fulvum* and were found to encode small, cysteine-rich secreted proteins (Rivas and Thomas 2005). However, no information is available about their localization and possible clustering in the genome, because they were identified by reverse genetics after screening of apoplastic fluids or by functional screening using a binary PVX vector, and *C. fulvum* is not amenable to formal genetic studies. The four *Avr* genes recently identified in *Melampsora lini* were expressed in haustoria and encoded small secreted proteins, of which *AvrP123-A* and *AvrP4* are cysteine rich and probably act as effectors (Catanzariti et al. 2006; Dodds et al. 2004). No information is available about their clustering and localization in the genome. Finally, the two *Avr* genes recently identified in *B. graminis* f. sp. *hordei* (*AVR_{ail0}* and *AVR_{kl}*) encoded small proteins that lack secretion signal peptides and enhance infection success on susceptible host plant cells. *AVR_{ail0}* and *AVR_{kl}* belonged to a genetic cluster of avirulence genes linked by 1 to 2 cM and were separated by 7.5 kb. These genes were found to belong to a large family of more than 30 paralogs in the genome of *B. graminis* f. sp. *hordei* (Ridout et al. 2006). Overall, apart from *M. grisea*, *B. graminis* f. sp. *hordei*, and distantly related oomycete species, little is known about the genomic context in which *Avr* genes are located.

L. maculans, the Dothideomycete causing stem canker of oilseed rape, is an emerging model to analyze genomic distribution and evolution of *Avr* genes within a genome. Indeed, formal genetic studies evidenced genetic clustering of *Avr* genes in the fungal genome (Balesdent et al. 2002), and the cloning of one gene of such a genetic cluster, *AvrLm1*, recently was performed following the sequencing of a large genomic region encompassing the gene (Gout et al. 2006). *AvrLm1* was found to be physically isolated from housekeeping genes in the middle of a large heterochromatin-like region. Although *AvrLm1* shared common features with other *Avr* proteins (presence of a cleavable signal peptide, small size, and lack of sequence homology in databases), it also had specific features, including a scarce number of cysteine residues, suggesting a short life duration in plant apoplast (Gout et al. 2006). The cloning of *AvrLm1* further questioned the genomic significance of genetic clustering of *Avr* genes in the *L. maculans* genome and whether the structural characteristics of the *AvrLm1* environment are specific to *AvrLm1* or the rule for *L. maculans* *Avr* genes.

To address these questions, we present here the cloning, functional characterization, and analysis of the genome environment of a second *Avr* gene of the *AvrLm1-2-6* cluster, *AvrLm6*. Following positional cloning of *AvrLm1*, detailed genetic and physical maps of the *AvrLm6* locus were constructed. Complete sequencing of the corresponding 520-kb contig allowed identification of several *AvrLm6* candidate genes, of which one was validated through complementation assays. *AvrLm6* structure, genomic location, and expression using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) were analyzed and compared with that of *AvrLm1*. The *AvrLm6* / *Rlm6* interaction was further analyzed by using RNAi in order to silence *AvrLm6* expression.

RESULTS

Construction of a BAC contig covering the *AvrLm6* locus of the v23.1.3 avirulent isolate.

Contig assembly started from *Vir2*, a *Pholy*-based PCR marker (Rep-PCR) (Attard et al. 2005; Gout et al. 2006), which cosegregated with *AvrLm6*. This Rep-PCR marker al-

lowed the identification of two bacterial artificial chromosome (BAC) clones, 56B7 and 6G9. BAC end sequences of 6G9 did not allow identification of a new marker, whereas end sequencing of *Bam*HI-digested subclones allowed us to design the *6G9Bam* marker (Fig. 1). The BAC clone 6H1 then was identified and marker *6H1F* was designed after BAC end sequencing. Finally, 115B5 was identified and found to contain a marker genetically bordering the *AvrLm6* locus on its 3' side (*micPKS*) (Fig. 1). In the 5' end of BAC clone 56B7, the *AvrLm6* contig was extended by the same method, generating markers after sequencing of BAC ends or after subcloning of the BAC clones. Two new BAC clones were identified: 33B11 and 20H11. During the map-based cloning of *AvrLm1*, Gout and associates (2006) defined a contig where the BAC located at the 3' end, 24A10, contained a marker that cosegregated with *AvrLm6*. The contig was extended from 24A10 with marker *24A10F* and allowed identification of 64D2. After the whole contig extension, a gap was still present between 64D2 and 20H11. This gap was filled after complete sequencing of 20H11 by Genoscope. From this sequence, a new marker, *20H11*, was designed and allowed identification of a BAC clone bridging the gap. The final *AvrLm6*-contig consisted of 10 overlapping clones covering a distance of 565,321 bp (Fig. 1). Full sequencing of the 10 BAC clones was performed. Additional markers were designed from the *AvrLm6*-contig sequence to saturate the genetic map of the *AvrLm6* locus (Fig. 1).

Genetic mapping of the *AvrLm6* locus and characterization of the *AvrLm6* contig.

The *AvrLm6* gene was mapped in a 14.8-centimorgan (cM) interval between the two flanking markers *mic2601* (6 cM on the 5' side) (Gout et al. 2006) and *micPKS* (8.8 cM on the 3' side) that delineated a 520-kb interval, giving an average physical-to-genetic-distance ratio of 35.1 kb/cM (Fig. 1). However, no recombination events were identified between 12 markers located along this genetic interval and corresponding to a physical distance of 458 kb (Fig. 1). This indicates that recombination was highly suppressed in the region encompassing *AvrLm6* and that equivalence between physical and genetic distance was variable in the region.

A total of 21 genes were predicted within the *AvrLm6* genetic interval (Fig. 1) (Supplementary Table S1). Based on homology to other genes or protein domains deposited in databases, 12 genes present in the *AvrLm6*-contig could be assigned putative functions and 7 genes showed homologies with conserved hypothetical fungal proteins (Supplementary Table S1). These genes mainly were distributed among three gene-rich regions, termed GC2 (20.7 kb), GC3 (21.7 kb), and GC4 (22.3 kb), characterized by an average 51.28% GC content and absence of retrotransposons. Sequences located between these gene-rich regions, namely REP3 (15 kb) and REP4 (453.3 kb), were characterized by a low GC content (average 34.91%). The REP regions mainly contained the four LTR retrotransposons *Pholy*, *Olly*, *Polly*, and *Rolly* (Gout et al. 2006; data not shown). Even if some complete copies of the four LTR retrotransposons were identified in the 520-kb contig, they generally appeared to be truncated at one or both termini or occurred as solo LTRs (data not shown). Sequences of the different retrotransposons identified were highly degenerated as a result of extensive repeat-induced point mutations (data not shown) as previously reported (Attard et al. 2005; Gout et al. 2006). The REP regions included six predicted genes, isolated in the region as solo genes or in couples (Fig. 1).

Identification of *AvrLm6* candidate genes.

Within the 520-kb interval, the best *AvrLm6* candidate among the 21 predicted genes was difficult to select due to

strong suppression of genetic recombination (Fig. 1). RT-PCR experiments on RNA extracted from plants infected with either the avirulent isolate v23.1.3 or the virulent isolate v29.3.1 showed expression of 19 of the predicted genes in both isolates, whereas two genes located in the REP4 region (*LmCys2* and *LmCys3* (Fig. 1) (Supplementary Table S1) were detected only in the avirulent isolate. In addition, analysis of genomic DNA in two *AvrLm6* isolates (v23.1.3 and Nz-T4) and one *avrLm6* isolate (v29.3.1) indicated that five genes were polymorphic between the avirulent and the virulent isolates. *LmPKS*, *LmCys1*, and *LmMFS* showed nucleotidic polymorphism among v23.1.3, Nz-T4, and v29.3.1, whereas *LmCys2* and *LmCys3* were absent in the virulent isolate v29.3.1. Those five genes were primarily considered as possible candidates for *AvrLm6*, but *LmPKS* finally was ruled out due to the presence of the *micPKS* marker in this gene. The four remaining *AvrLm6* candidate genes were located in the REP4 genomic region.

Identification of *AvrLm6* by functional complementation.

To test the ability of the four *AvrLm6* candidate genes to confer avirulence toward cultivars carrying *Rlm6*, complementation assays were carried out. Genomic fragments of v23.1.3 corresponding to the coding region and putative promoter of the four candidate genes were cloned in the binary vector pBHT2 and introduced into the virulent isolate v29.3.1 by *Agrobacterium*-mediated transformation. Overall, 17 independent transformants were isolated for pBHT2-*LmCys1*, 12 transformants for pBHT2-*LmCys2*, 16 transformants for pBHT2-*LmCys3*, and seven transformants for pBHT2-*LmMFS*. These transformants then were inoculated on two *Brassica napus* lines carrying *Rlm6*, Darmor-MX, and Falcon-MX (Table 1). V29.3.1 transformants carrying pBHT2-*LmCys1*, pBHT2-*LmCys2*, or pBHT2-*LmMFS* constructs exhibited the same phenotype as the wild-type recipient (Table 1). In contrast, transformation of pBHT2-*LmCys3* into v29.3.1 fully restored the avirulent interaction phenotype with Darmor-MX and Falcon-MX for 14 of

Fig. 1. Genetic and physical maps of the *AvrLm6* genomic region in the v23.1.3 (*AvrLm6*) isolate. Genetic and physical maps: dashed lines connect the physical location of markers to their location on the genetic map or connect markers to their location in the bacterial artificial chromosome (BAC) clone contig. Genes were predicted according to gene-prediction programs FGENESH and GENSCAN. The double-arrow corresponds to the *AvrLm6* genetic interval and shows the region spanning the *AvrLm6* locus. G+C content: sliding window (size 3,500 bp, step 100 bp) analysis of the G+C content in the 565 kb-contig. Regions REPx and GCx correspond to regions with homogeneous G+C content. *LmCys1*, *LmCys2*, *LmCys3*, and *LmMFS* are the genes identified as candidates for *AvrLm6* and used for complementation assays. PCR = polymerase chain reaction.

16 transformants (Table 1). The v29.3.1/pBHT2-*LmCys3* transformants showed a wild-type interaction phenotype with the susceptible cultivar (Table 1) and with the cultivars carrying either *Rlm1* or *Rlm2* (Table 1). Thus, *LmCys3*, a solo gene located in REP4, was suggested to be *AvrLm6*.

Sequence analysis and occurrence of the *AvrLm6* gene in *Leptosphaeria* spp.

The 3,405-bp PCR product amplified from v23.1.3 with primers 64D2-*sma*U and 64D2-*sma*L and used for functional complementation of the *AvrLm6* avirulence phenotype contained a single predicted open reading frame (ORF). *AvrLm6* cDNA was synthesized from v23.1.3 total RNA by RT-PCR and used to determine the position of the different introns. Three introns of 51, 47, and 48 bp were identified (Fig. 2). RACE PCRs (5' and 3') were performed on v23.1.3 cDNA to localize the transcription initiation and polyadenylation sites. Two independent protocols were used to perform 5' and 3' RACE PCRs, which gave the same results, a 5' untranslated region (UTR) of 58 bp and a 3' UTR of 178 bp (Fig. 2). The *AvrLm6* transcript was 817 bp in size and with a low GC content (43%), similar to the average GC content of the genomic

context (Fig. 1). A 435-bp ORF was predicted within the transcript and encoded a predicted preprotein of 144 amino acids (aa), which included a potential 20-aa cleavable signal peptide (signal peptide probability of 0.982 with SignalP 3.0 software), suggesting that it encodes a secreted protein of 124 aa and 13.4 kDa. The predicted *AvrLm6* protein contained six cysteine residues (Fig. 2) and two disulphide bonds were predicted using the SCRATCH software (amino acid positions 109 to 130 and 103 to 122). No particular structure, localization, or amino acid composition was found using PSortII and SAPS softwares.

No significant matches to the public DNA sequence databases and to the sequenced fungal genomes were detected and no homology with *AvrLm1* identified. Similarly, the predicted *AvrLm6* protein had no similarity to protein families deposited in the Pfam database. Moreover, no significant matches to sequences of the closely related species *Stagonospora nodorum* (*Phaeosphaeria nodorum*) were identified, whereas micro-synteny is observed between *L. maculans* and *S. nodorum* for other genes of the *AvrLm6* genomic region (Supplementary Table S1). Presence of *AvrLm6* homologs was investigated by PCR and by Southern blot hybridization using the *AvrLm6*

Table 1. Complementation assays with the *AvrLm6* candidate genes: pathogenicity of the v29.3.1 transformants

Isolate/transformant	Pathogenicity test on ^a				
	Westar (susceptible control)	Falcon-MX (<i>Rlm4 Rlm6</i>)	Darmor-MX (<i>Rlm6</i>)	Bristol (<i>Rlm2 Rlm9</i>)	Columbus (<i>Rlm1 Rlm3</i>)
v23.1.3 (<i>AvrLm1avrLm2AvrLm6</i>)	V	A	A	V	A
v29.3.1 (<i>avrLm1AvrLm2avrLm6</i>)	V	V	V	A	V
v29.3.1/pBHT2/ <i>LmCys1</i> ^b	V	V	V	A	V
v29.3.1/pBHT2/ <i>LmCys2</i> ^b	V	V	V	A	V
v29.3.1/pBHT2/ <i>LmCys3</i> ^b (transformants 1–6, 8, and 10–16)	V	A	A	A	V
v29.3.1/pBHT2/ <i>LmCys3</i> ^b (transformants 7 and 9)	V	V	V	A	V
v29.3.1/pBHT2/ <i>LmMFS</i> ^b	V	V	V	A	V

^a Conidia were inoculated onto cotyledons of 15-day-old plantlets of reference cultivars carrying known resistance genes (Balesdent *et al.*, 2005). The symptoms were scored 15 days postinfection as the isolate is avirulent (A) or virulent (V).

^b *LmCys1*, *LmCys2*, *LmCys3*, and *LmMFS* were amplified by polymerase chain reaction from v23.1.3 and cloned into the pBHT2 binary vector. Constructs were introduced into v29.3.1 by *Agrobacterium*-mediated transformation.

Fig. 2. Nucleotide sequence of the 1,440-nucleotide (nt) region encoding *AvrLm6* (accession number AM259336) and amino acid sequence of the corresponding predicted protein. 5' and 3' untranslated regions of the gene are underlined. Introns are indicated as italicized lowercase (intron 1: 51 nt; intron 2: 47 nt; and intron 3: 48 nt). The predicted signal peptide (20 amino acids) is indicated by dark gray shading and the six cysteine residues are indicated by light gray shading.

ORF as a probe in isolates from other species or subspecies of the *L. maculans*–*L. biglobosa* species complex (Table 2). The gene was absent from all subspecies of *L. biglobosa* and even of the closely related *L. maculans* subspecies ‘*lepidii*’, suggesting that it is extremely specific to the *L. maculans* subspecies infecting *Brassica* spp. (*L. maculans* ‘*brassicae*’).

Comparison of *AvrLm6* and *AvrLm1* expression.

AvrLm1 expression was investigated using RT-PCR (Gout et al. 2006) and found to be expressed at some level in all stages with a probable induced expression during primary leaf infection. Here, we developed a more precise analysis of the expression of *AvrLm1* and *AvrLm6* using qRT-PCR. Primer efficiency was calculated for *AvrLm1*, *AvrLm6*, β -tubulin, and *actin* and was found to range between 0.992 and 1. Gene expression levels of *AvrLm1* and *AvrLm6* were measured relative to β -tubulin expression (Fig. 3). Gene expression level of *actin* also was measured relative to β -tubulin and showed no significant variations among the different in planta and in vitro conditions (Fig. 3), indicating that β -tubulin and *actin* are adequate constitutive reference genes. *AvrLm6* expression was detected at a low level 3 days postinfection (dpi), then increased during the primary leaf infection and peaked at 7 dpi (13,190 times more expressed than in conidia germinating in rich medium). At this time, *AvrLm6* was even more expressed than β -tubulin (Fig. 3). Expression of *AvrLm6* slowly decreased after 7 dpi and, during stem necrosis, it was comparable to the expression exhibited 5 dpi. *AvrLm6* expression was also faintly detected in mycelium and at lower levels in conidia germinating in complete medium and under nitrogen starvation, suggesting that nitrogen starvation did not increase the level of *AvrLm6* expression. Expression kinetics of *AvrLm1* was very similar to that of *AvrLm6*: *AvrLm1* was also strongly expressed during primary leaf infection with a peak at 7 dpi (2,100 times higher than in conidia germinating in rich medium). Contrasting with *AvrLm6*, and as already shown by RT-PCR, the level of *AvrLm1* expression during mycelial growth was high (21.5 times higher than that of *AvrLm6*) (Fig. 3).

Characterization of the *AvrLm6*–*Rlm6* interaction following RNA silencing of *AvrLm6*.

RNAi had already been used successfully in fungi such as *Venturia inaequalis* and *M. grisea* (Fitzgerald et al. 2004; Nakayashiki et al. 2005) and was adapted for *L. maculans* (this study) (Fig. 4). We constructed a vector (pNAT1-*AvrLm6*) containing a fragment of *AvrLm6* cDNA in sense and antisense orientation, in order to allow hairpin formation when transcribed into mRNA. pNAT1-*AvrLm6* then was transformed into v23.1.3 (*AvrLm6*) by agrotransformation. Eighteen transformants were obtained and analyzed for their ability to infect Darmor-MX (Fig. 5) and for the level of *AvrLm6* expression, using qRT-PCR (Fig. 4). Four transformants showed an expression level as low as 3.5 to 13% of the wild-type control (Fig. 4, transformants 3, 13, 16, and 17). Statistical analyses showed that Westar developed similar symptoms when inoculated with v23.1.3 or all but one of the silenced transformants (transformant 1) (data not shown). The four nonsilenced transformants (10, 15, 18, and 19) never induced susceptibility symptoms on *Rlm6* and behaved like v23.1.3 (data not shown). In contrast, although v23.1.3 was avirulent, most of the silenced transformants induced susceptibility symptoms on the *Rlm6* line 21 dpi (Fig. 5B). The four silenced transformants exhibiting the lowest level of *AvrLm6* expression (less than 15%) were found to be fully virulent toward *Rlm6*, with a high percentage of susceptibility symptoms already detected 14 dpi (Fig. 5A). Transformants with intermediate expression of *AvrLm6* (transformants 1, 9, 11, 12 and 20) showed some susceptibility symptoms; however, those symptoms were delayed and mainly appeared 21 dpi. Correlation curves further showed linear relationships between symptom expression on *Rlm6* cultivars and *AvrLm6* expression level (Fig. 5).

DISCUSSION

In a recent article, Gout and associates (2006) reported the cloning of a first *Avr* gene from *L. maculans*, *AvrLm1*. *AvrLm1*, belonging to the *AvrLm1*–*AvrLm2*–*AvrLm6* genetic cluster

Table 2. Occurrence of *AvrLm6* in *Leptosphaeria maculans* ‘*brassicae*’ and related species and subspecies of the species complex

<i>Leptosphaeria</i> spp.	Geographical origin	Host plant sampled	Provider	Ref. ^b	Detection of <i>AvrLm6</i> ^a	
					PCR	Southern
<i>L. maculans</i> 'brassicae'						
V23.1.3	Laboratory isolate	...	M. H. Balesdent	1	+	+
V29.3.1	Laboratory isolate	...	M. H. Balesdent	2	–	–
Nz-T4	New Zealand	...	S. Gowers	2	+	+
IBCNI8	Australia	...	B. Howlett	3	–	–
<i>L. maculans</i> 'lepidii'						
IBCNI84	Canada	<i>Lepidium</i> sp.	G. Séguin-Swartz	3	–	–
Lepi1	Canada	<i>Lepidium</i> sp.	G. Séguin-Swartz	...	–	–
Lepi2	Canada	<i>Lepidium</i> sp.	G. Séguin-Swartz	4	–	–
<i>L. biglobosa</i> 'brassicae'						
B3.6	France	<i>Brassica juncea</i>	H. Brun	3	–	nd
IBCNI10	Germany	<i>B. napus</i>	H. H. Hoppe	3	–	–
IBCNI93	United States	<i>B. oleracea</i>	P. H. Williams	3	–	–
Gui2b3	China	<i>B. napus</i>	J. S. West	3	–	nd
<i>L. biglobosa</i> 'canadensis'						
IBCNI62	Canada	<i>Thlaspi arvense</i>	G. Séguin-Swartz	3	–	–
IBCNI81	Canada	<i>B. napus</i>	G. Séguin-Swartz	3	–	nd
IBCNI82	Canada	<i>B. juncea</i>	G. Séguin-Swartz	3	–	–
IBCNI92	Canada	<i>B. rapa</i>	P. H. Williams	3	–	nd
<i>L. biglobosa</i> 'australensis'						
IBCNI29	Australia	<i>B. napus</i>	B. Howlett	3	–	nd
IBCNI30	Australia	<i>B. napus</i>	B. Howlett	3	–	–
IBCNI91	United States	<i>B. oleracea</i>	P. H. Williams	3	–	–
<i>L. biglobosa</i> 'thlaspii'						
IBCNI64	Canada	<i>T. arvense</i>	G. Séguin-Swartz	3	–	–
IBCNI65	Canada	<i>T. arvense</i>	G. Séguin-Swartz	3	–	–

^a PCR = polymerase chain reaction, + = PCR amplification or Southern signal, – = lack of amplification or hybridization, and nd = experiment not done.

^b References: 1 = Balesdent et al. 2001, 2 = Balesdent et al. 2002, 3 = Mendes-Pereira et al. 2003, and 4 = Voigt et al. 2005.

(Balesdent et al. 2002), was cloned following a lengthy chromosome walk. It was shown to share characteristics common to other fungal *Avr* genes along with specific features. In common with most fungal *Avr* genes, *AvrLm1* was shown to encode a

Fig. 3. Comparison of *AvrLm1* and *AvrLm6* expression during *Leptosphaeria maculans* in vitro growth and oilseed rape infection. Expression of avirulence genes was analyzed in the avirulent isolate v23.1.3 by quantitative reverse-transcription polymerase chain reaction (qRT-PCR). Number 3 to 12 = RT-PCR products obtained from RNA isolated from oilseed rape cotyledons (Westar) 3 to 12 days postinfection; nec = RT-PCR product obtained from RNA isolated from stem necrosis (2 months after infection); myc = RT-PCR product obtained from RNA isolated from mycelial culture; sp = RT-PCR product obtained from RNA isolated from conidia germinating in rich medium (Fries) or under nitrogen starvation (B5). RNA extracted from uninfected cotyledons and water were used as negative controls. Gene expression levels are relative to β -tubulin. *Actin* is included to show that the calculated expression levels were not resulting from variations in the β -tubulin expression. Each data point is the average of two biological repeats (two extractions from different biological material) and two technical repeats (two RT-PCRs). Standard error of the mean normalized expression level is indicated by error bars.

small secreted protein with no match in the databases. More specific traits included i) a low number of cysteine residues similar to other genes obtained following the use of map-based cloning strategies, but dissimilar to the *Avr* genes cloned in the related fungal species *C. fulvum* (Rivas and Thomas 2005); ii) a very specific genome environment characterized by the lack of other coding sequences within 269 kb and a strong suppression of meiotic recombination; and iii) constitutive expression leading the authors to conclude that *AvrLm1* expression was not tightly regulated during plant infection (Gout et al. 2006).

In the present study, we undertook the cloning of a second gene of the *AvrLm1-AvrLm2-AvrLm6* genetic cluster, *AvrLm6*. This enabled us to further investigate structural features of *Avr* genes and their genome environment in *L. maculans*, the significance of the observed genetic clustering, and genome environment-dependent regulation of expression. In addition, RNAi analyses allowed us to perform preliminary investigations into dose-response effects of *AvrLm6* induction of the *Rlm6*-mediated resistance.

Structural traits and genome environment of *AvrLm6*.

Identification of fungal and oomycete *Avr* genes was in a few cases performed by “unbiased” methods, such as map-based cloning and screening of cDNA libraries (Böhnert et al. 2004; Catanzariti et al. 2006; Farman et al. 2002; Orbach et al. 2000; Ridout et al. 2006); whereas in other models, such as *F. oxysporum* and *C. fulvum*, reverse genetics strategies were used (Rep et al. 2004; Rivas and Thomas 2005). In these latter cases, all *Avr* genes recovered were small, cysteine-rich proteins. This is consistent with the fact that these proteins, secreted in the apoplast or the plant vessels, have to resist plant proteases via the formation of disulfide bridges. Here, map-based cloning of *AvrLm6* revealed structural features consistent with the *L. maculans* infection strategy and exemplary of *Avr* genes that appear to be common to most filamentous fungi analyzed to date (i.e., small-secreted proteins, containing an even number of cysteine residues involved in disulfide bridge formation, with no matches in the databases) (Rep 2005). In addition, *AvrLm6*, like *AvrLm1*, is specific to the *L. maculans* subspecies ‘brassicae’, suggesting, as already suspected for other fungal *Avr* genes, that *Avr* genes are highly variable and rapidly evolving genes (Rep 2005). However, the structural features of *AvrLm6* are quite different from those of the genetically closely linked *AvrLm1*. Actually, the putative secreted *AvrLm1* protein is unable to form disulfide bonds, due to the presence of only one cysteine residue (Gout et al. 2006). Cysteine-poor *Avr* proteins were suggested to be common in biotrophic fungal or oomycete species for which the host plant–pathogen interface primarily consists of the closely associated haustorial and plant cell membranes (Ellis et al. 2006). *Avr* proteins are then suggested to be translocated into the plant cell cytoplasm by an unknown mechanism (Birch et al. 2006; Dodds et al. 2004). Therefore, unlike *AvrLm6*, *AvrLm1* showed resemblance to *Avr* proteins produced at the haustorium–plant cell membrane interfaces, whereas *L. maculans* does not produce such specialized feeding structures. Thus, similarly to *Melampsora lini*, *L. maculans* is shown to be able to produce both cysteine-rich and cysteine-poor *Avr* proteins, whereas both genes are embedded in a similar, very specific genome environment.

The cloning of *AvrLm1* highlighted a particular, isochore-like structure of the genomic region encompassing the gene. Isochores consist of long stretches of DNA that are homogeneous in base composition and compositionally correlated with the coding sequences that they embed (Bernardi 2000). Thus, the *AvrLm1* genomic region showed a succession of small, GC-

equilibrated regions (20 to 30 kb) containing “housekeeping” genes and large AT-rich regions mainly containing noncoding sequences (LTR-retrotransposons) and *AvrLm1* as a solo gene. Surprisingly, *AvrLm6* was found here to mirror *AvrLm1*, with *AvrLm6* also occurring as a solo gene within a 133-kb noncod-

ing region mainly containing LTR-retrotransposons. Thus, the *AvrLm1-AvrLm6* genetic cluster actually corresponds to a large physical distance (230 kb separate *AvrLm1* from *AvrLm6*), but physical features of their genome environment condition a strong suppression of meiotic recombination around both

Fig. 4. Silencing of *AvrLm6* expression by RNA interference: real-time reverse-transcription polymerase chain reaction analysis of *AvrLm6* transcript accumulation in *Leptosphaeria maculans* *AvrLm6*-silenced transformants. Expression is relative to the β -tubulin expression level and to the expression of *AvrLm6* in the wild-type isolate v23.1.3 ($2^{-\Delta\Delta C_t}$ method). The dashed line corresponds to the expression level of the wild-type isolate v23.1.3.

Fig. 5. Relationship between the level of expression of *AvrLm6* and the symptoms expressed on Darmor-MX (*Rlm6*) after inoculation of silenced transformants (v23.1.3/pNAT1-*AvrLm6*), and of the v29.3.1 virulent isolate (relative gene expression = 0). Results are expressed **A**, as the percentage of susceptibility lesions observed 14 days postinoculation (dpi) or **B**, as the mean scoring using the IMAScore rating scale comprising six infection classes (IC), where IC1 to IC3 corresponded to resistance and IC4 to IC6 to susceptibility (Balesdent et al. 2006). Symptoms were scored 14 (diamond), 18 (square), and = 21 (triangle) dpi.

genes leading to this extreme distortion of genetic distance value. More surprisingly, whereas the average G+C content of housekeeping genes in *L. maculans* is approximately 52%, both *AvrLm1* and *AvrLm6*, which show no homology at the nucleotide or amino acid levels, share a low G+C content, mimicking that of their genome environment (Gout et al. 2006; this study). This feature is also uncommon for other fungal *Avr* genes, except for the telomeric *AVR-Pita* gene of *M. grisea* (Orbach et al. 2000) and for the *AVR_{kl}* paralogs of *B. graminis* f. sp. *hordei* that were located near retrotransposons (Ridout et al. 2006). However, comparisons with other fungal models are difficult to perform because there currently is no extensive information on the genome environment of *Avr* genes in other fungal species. The most documented example is that of *M. grisea*, for which most of the *Avr* genes show no specific bias toward insertion into noncoding regions.

Common regulation of *L. maculans* avirulence genes?

Fungal *Avr* genes are always expressed during plant infection (Dodds et al. 2004; Laugé and de Wit 1998; Rep et al. 2004). For example, *Avr2*, *Avr4*, and *Ecp2* from *C. fulvum* are expressed during the whole infection process (Laugé and de Wit 1998; Luderer et al. 2002), as are *NIP1* from *R. secalis* (Rohe et al. 1995) and *AVR-Pita* from *M. grisea* (Orbach et al. 2000). Indeed, the expression of these fungal *Avr* genes is not restricted to a specific stage of the infection process but is prevalent during colonization of host tissues. In contrast, the expression of some fungal *Avr* genes can be localized to fungal structures specialized in plant penetration, such as appressoria of *M. grisea*, or in feeding, such as haustoria of *Melampsora lini* (Böhnert et al. 2004; Catanzariti et al. 2006; Dodds et al. 2004). Consistent with the lack of specific feeding or penetration organs in *L. maculans*, both RT-PCR analysis of *AvrLm1* timecourse expression (Gout et al. 2006) and qRT-PCR analysis of both *AvrLm1* and *AvrLm6* timecourse expression suggested an early induced expression of these genes upon plant infection and the maintenance of a high level of expression at very late stages of the plant tissue colonization (i.e., at the stem necrosis stage, 2 months after inoculation). Expression of fungal *Avr* genes also can be induced in specific culture conditions, such as nitrogen starvation (e.g., with the *C. fulvum* *Avr9* expression regulated by the global nitrogen response factor NRF1) (Pérez-García et al. 2001). However, Thomma and associates (2006) showed that expression of most *C. fulvum* effectors was not affected by varying levels of nitrogen supplementation in vitro and that the NRF1 nitrogen response regulator controlled only the *Avr9* expression. Similarly here, although putative NIT2 binding sites were found in the promoter region of *AvrLm1* and *AvrLm6* (data not shown), there is no evidence that the expression of either gene is induced during nitrogen starvation.

Even though *AvrLm1* and *AvrLm6* are strongly expressed during plant infection, the RT-PCR data obtained by Gout and associates (2006) on *AvrLm1* expression also suggested a constitutive expression of this gene, leading the authors to postulate that the particular genome environment of *AvrLm1* prevented a tight regulation of gene expression. Here, qRT-PCR analyses showed a very similar pattern of expression for both *AvrLm1* and *AvrLm6*: a very low basal expression of both genes in germinating conidia; a rapid induction of expression 3 dpi, at a time when hyphae just begin to penetrate plant tissues; a strong increase of expression (with a level higher or similar to that of constitutively expressed *β -tubulin*) 7 dpi, at a time when a sheet of hyphae begin to colonize mesophyll tissues; and a moderate decrease of expression leading to maintenance of a high level of expression at very late stages of the disease. These data suggest a common

regulation of expression of both *Avr* genes, but they also are consistent with a tighter regulation of expression of *AvrLm6* as compared with *AvrLm1*, since *AvrLm1* is maintained at a significant level of expression during regular mycelial growth in culture media. *AvrLm1* and *AvrLm6* are located as solo genes within regions showing structural features of heterochromatic regions which are, in general, less accessible to DNA binding and transcription factors (Grewal and Moazed 2003); therefore, it is tempting to postulate that specific regulators may control coexpression of genes in the AT-rich regions of the genome.

RNA silencing, an efficient tool to study *Avr-R* interactions in *L. maculans*.

In contrast to the situation in plants and animals (Frantz 2003), RNA silencing has only recently been suggested as being a gene function exploratory tool in filamentous fungi (Nakayashiki 2005). RNA silencing has many advantages over gene disruption, including the generation of numerous transformants with different levels of target gene expression, the need to screen only a few transformants as compared with other targeting methods, and the efficiency to silence gene expression independently of the locus where the target gene is located. In *L. maculans*, knock-out strategies have been reported for a few genes (Gardiner and Howlett 2004; Idnurm et al. 2003; Wilson et al. 2002); however, they were found to be particularly inefficient, due to the need for large flanking homologous regions and for rare homologous recombination events. In the present study, *AvrLm6* was found to be a unique coding sequence with flanking regions uniquely consisting of retrotransposons. With these retrotransposons being present in multiple locations within the genome, it would have been unfeasible to use homologous recombination strategies necessitating large flanking regions. Moreover, the *AvrLm1-2-6* region was found to exhibit variable rates of recombination events, which were concentrated in the GC-equilibrated regions and highly suppressed in the AT-rich regions. Finally, the region containing *AvrLm6* shows structural features of a heterochromatin region, which generally are less accessible to targeted disruption than euchromatin regions (Grewal and Moazed 2003). RNA silencing was therefore the strategy of choice to study *AvrLm6*, and proved here to be efficient because the screening of only 18 transformants allowed us to recover 4 transformants showing a very low expression of *AvrLm6* and a virulent phenotype on *Rlm6* lines. This is, to our knowledge, the first report of RNA silencing of an *Avr* gene in fungi. RNAi analysis enabled us to evaluate the dose-response features of the *Rlm6*-mediated resistance, and showed that a level of *AvrLm6* expression corresponding to 40% of that of the wild-type isolate is needed to consistently induce a resistance response. In addition, a linear relationship was found between increased levels of silencing and increased expression of symptoms. Further investigations into the correlation of *AvrLm6* expression levels vs. quantity of *AvrLm6* protein produced would help us to better interpret the symptoms observed on resistant cultivars.

Finally, one of the most intriguing questions raised during map-based cloning of both *AvrLm1* and *AvrLm6* concerns their isolation in the middle of large noncoding regions. The genome of *L. maculans* is currently considered as a mosaic of gene-rich, G+C-equilibrated regions and gene-poor, A+T-rich regions, which are present on all chromosomes. This genome is probably richer in repeats than most currently sequenced ascomycete genomes (Kuhn et al. 2006). On this basis, our current working hypothesis is that such gene-poor regions are specific “ecological niches” for effector genes in the genome of *L. maculans*. This will be evaluated in the

near future during the ongoing map-based cloning of another *Avr* gene cluster and through a systematic survey of gene-poor regions in the *L. maculans* whole-genome sequence (Genoscope website).

MATERIALS AND METHODS

Fungal isolates.

Isolates of the *L. maculans*/*L. biglobosa* species complex used in this study were either collected from naturally infected crucifers or obtained following in vitro crosses (Table 2). The v23.1.3 isolate of *L. maculans* (*AvrLm1-avrLm2-AvrLm6*) (Balesdent et al. 2002) was used for the positional cloning of *AvrLm6* (Attard et al. 2002) and the virulent isolate v29.3.1 (*avrLm1-AvrLm2-avrLm6*) (Balesdent et al. 2002) was used as a recipient isolate for genetic transformation and functional validation of the candidate genes. 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* wild-type isolates and mutants (Table 1) were assessed following inoculation with 10 µl of conidia suspensions (10^7 conidia/ml) on cotyledons of 15-day-old plantlets of *B. napus* cv. Westar (susceptible control) and lines harboring the *Rlm6* resistance gene (Darmor-MX and Falcon-MX). They also were inoculated onto cv. Columbus carrying *Rlm1* and cv. Bristol carrying *Rlm2*. These two resistance genes enable specific recognition of the *Avr* genes *AvrLm1* and *AvrLm2*, respectively, genetically clustered with *AvrLm6* (Balesdent et al. 2002). Plants were incubated in a growth chamber at 16 and 24°C (night and day, respectively) with a 12-h photoperiod. Symptoms were scored on 10 to 12 plants per interaction, 14 to 27 days after inoculation using the IMAScore rating scale comprising six infection classes (ICs), where IC1 to IC3 corresponded to avirulent isolates (*AvrLm*) and IC4 to IC6 to virulent isolates (*avrLm*) (Balesdent et al. 2006). Mean disease scorings were calculated for each isolate/cultivar/scoring date combination and were analyzed using analysis of variance. Isolates or conditions were compared using a Fisher test ($\alpha = 0.05$). Transformants or conditions were compared to a control by a Dunnett multiple comparison test ($\alpha = 0.05$). All statistical analyses were performed using XLStat 7.5 software.

For timecourse studies of in planta *Avr* gene expression, the second leaves of cv. Westar plantlets were inoculated using a conidial suspension of v23.1.3, as described for cotyledons, and sampling was performed at 3, 5, 7, 9, or 12 dpi. According to Balesdent and associates (1995), days 3 and 5 correspond to the initial colonization of leaf tissues with minute amounts of mycelium within the tissues, day 7 corresponds to the first detectable increase of the mycelial mass, and days 9 and 12 correspond to a dramatic increase of mycelium within the tissues, preceding the appearance of visible symptoms (100% of plants with symptoms at approximately day 15). Stem lesions were also analyzed for *Avr* gene expression. The junction of the second-leaf petiole and the stem of cv. Westar plantlets was punctured three times and inoculated with a 10-µl droplet of v23.1.3 conidial suspension (10^7 ml⁻¹). Plants were incubated in the same growth chamber conditions as for inoculated cotyledons, and necrotic stem tissue was collected two months after inoculation. For silencing experiments, RNA was isolated from oilseed rape leaves infected with the wild-type isolate v23.1.3 and the different transformants 13 dpi. Although *AvrLm6* expression does not

reach its highest level at this timepoint, this stage was chosen because it corresponds to a large amount of mycelium within the plant tissues.

DNA and RNA manipulations.

For PCR, genomic DNA was extracted from conidia, using the DNeasy 96 Plant Kit (Qiagen S.A., Courtaboeuf, France) as described previously (Attard et al. 2002). Primer sequences used for the chromosome walk are shown in Supplementary Table S2. PCR amplifications were 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 (Qbiogen, 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, electrophoretic karyotyping, Southern blots, and hybridizations have been reported (Attard et al. 2005) and were adapted from procedures described by Sambrook and Russell (2001).

Total RNA was extracted from mycelium grown for one week in Fries liquid medium, from germinating conidia grown for 36 h in Fries or B5 minimal medium, and from infected leaf or stem tissues, using the TRIzol reagent (Invitrogen, Cergy Pontoise, France) according to the manufacturer's protocol. Total RNA was treated with DNase I RNase-Free (New England Biolabs, Ipswich, MA, U.S.A.). All samples, either corresponding to 100% fungal material or to infected plant tissues, were adjusted to 4 µg of RNA and single-strand cDNA was generated using oligo-dT primed reverse transcription with PowerScript Reverse Transcriptase (Clontech, Palo Alto, CA, U.S.A.) according to the manufacturer's protocol.

qRT-PCR.

qRT-PCR was performed using model 7700 real-time PCR equipment (Applied Biosystems, Foster City, CA, U.S.A.) and ABsolute SYBR Green ROX dUTP Mix (ABgene, Courtaboeuf, France). For each condition tested, two RNA extractions from different biological samples and two reverse transcriptions for each biological repeat were performed. RNA extracted from uninfected leaves and water was used as negative controls. To determine the *AvrLm6* expression level in silenced transformants, primers were designed to amplify a region of the *AvrLm6* mRNA, *AvrLm6*-RTU being located within the *AvrLm6* region targeted by the pNAT1-*AvrLm6* vector and *AvrLm6*-RTL located in a region outside of the target sequence. A standard curve of amplification efficiency for β -tubulin, actin, *AvrLm1*, and *AvrLm6* primers (Supplementary Table S2) was generated from purified RT-PCR products corresponding to 12-dpi-infected plant tissue across a three-orders-of-magnitude dilution series (10^{-1} to 10^{-3} dilutions). Samples were analyzed from a 10-fold dilution of the original RT products. Diluted RT products (5 µl) were added to 12.5 µl of PCR mix and 7.5 µl of primers to a final concentration of 300 nM. The thermocycling profile consisted of an initial cycle of 50°C for 2 min, a second cycle of 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amplified product was detected every cycle at the end of the 60°C step. After the cycling, dissociation curve analysis confirmed the absence of nonspecific products in the reaction (temperature increase from 60 to 95°C in 20 min). The fluorescence threshold (Ct) value was determined at 0.1 of fluorescence intensity. Ct values were analyzed as described by Muller and associates (2002) for expression kinetic analysis or according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen

2001) for silencing experiments. *β -tubulin* was used as a constitutive reference gene. For silencing experiments, results were reported to the Ct value of the wild-type isolate v23.1.3.

Gene annotation.

Annotations of UTRs, transcription start and stop sites, and intron positions were performed following PCR amplification and sequencing of 3' and 5' ends of cDNA using the GeneRacer kit (Invitrogen) and the Creator SMART cDNA library construction kit (Clontech) according to manufacturer's recommendation and using *AvrLm6-1*, *AvrLm6-2*, *AvrLm6-nest1*, and *AvrLm6-nest2* (Supplementary Table S2) as specific primers.

BAC libraries screening and chromosome walk.

Construction of the v23.1.3 isolate genomic DNA *HindIII* and *NdeII* BAC libraries and screening strategies have been described previously (Attard et al. 2002, 2005; Gout et al. 2006). Markers were developed as described by Gout and associates (2006) and located on the v23.1.3 x v29.3.1 genetic cross (cross 34) (Balesdent et al. 2002; Gout et al. 2006) using the MapMaker/EXP version 3.0b software (available online; Whitehead/MIT Center for Genome Research, Cambridge, MA, U.S.A.) with a log of the likelihood ratio score of 4.0 and a maximum recombination frequency of 20 cM.

BAC DNA sequencing and gene identification.

End sequences of overlapping BAC clones or subclones identified in the course of the chromosome walk toward *AvrLm6* were obtained as previously described (Attard et al. 2005). The BAC clones were fully sequenced, finished, and assembled at Genoscope as described by Gout and associates (2006).

Sequences were analyzed using the BLAST programs against the public databases of nucleotide and protein sequences, including the National Center for Biotechnology Information nonredundant databases and the COGEME database of phytopathogenic fungi, the annotated fungal genomes of *Aspergillus nidulans*, *F. graminearum*, *M. grisea*, *Neurospora crassa*, *S. nodorum*, *Ustilago maydis*, and the available sequence releases of other fungal genomes (Fungal Genome Sequence Databases, Broad Institute Cambridge, MA, U.S.A.). Sequence analyses also were performed using the SignalP 3.0 program available online from the Center for Biological Sequence Analysis; 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; the pSORTII and SAPS softwares from the University of Tokyo and Stanford University (Palo Alto, CA, U.S.A.); the SCRATCH software available online from the School of Information and Computer Sciences; and by searches against the Pfam protein families database available online from Washington University in St. Louis. Two different gene-prediction programs also were used in combination to annotate the sequences of the *AvrLm6* contig obtained. GENSCAN, available online from the Massachusetts Institute of Technology, was used with vertebrate and *Arabidopsis* settings and FGENESH from Softberry was set with *N. crassa* as the parameter matrix. Identifications of putative genes were based on the comparison of the outputs of these two programs because they use different gene-finding algorithms to predict gene structure, including exons, introns, promoter, and poly-adenylation signals.

Vector constructions and transformation of fungal isolates.

The binary vector pBHt2, which carries as a selectable marker the bacterial hygromycin B phosphotransferase gene, under the control of the *Aspergillus nidulans trpC* promoter, was used for *Agrobacterium*-mediated complementation of *L. maculans*.

pBHt2-*LmCys1* was obtained by digesting the BAC clone 20H11 with *EcoRI* and by ligating the BAC fragment from 12,271 to 15,841 bp into pBHt2 linearized with *EcoRI*. pBHt2-*LmCys2* was obtained by digesting the BAC clone 6H1 with *KpnI* and *BamHI*. The *KpnI/BamHI* BAC fragment from 92,293 to 98,823 bp was introduced into pBHt2 digested with *KpnI/BamHI*. pBHt2-*LmCys3* resulted from the amplification of BAC clone 64D2 with primers 64D2-*SmaU* and L that added *SmaI* digestion sites at the 5' end of the PCR product. This product was digested with *SmaI* and was introduced into the *SmaI*-digested pBHt2. pBHt2-*LmMFS* resulted from a digestion of the BAC clone 33B11 with *SnaBI* and *Eco47III*. The BAC fragment from 28,128 to 37,327 bp was subcloned into pBluescript II, and the corresponding plasmid was digested with *SacI*. The *SacI* fragment corresponding to 31,015 to 37,327 bp from BAC clone 33B11 was introduced into the *SacI*-digested pBHt2.

Vector pNAT1-*AvrLm6* was constructed for RNA-mediated gene silencing of *AvrLm6*, using the method described by Fitzgerald and associates (2004) with some modifications. Vector pJK11 (a gift from K. Plummer, Horticulture and Food Research Institute of New Zealand) was used for the construction of inverted repeats of the target gene *AvrLm6*. pJK11 consists of a backbone of pBluescript II containing the *Glomerella cingulata gpdA* promoter fragment and the *Aspergillus nidulans trpC* terminator fragment, separated by unique *EcoRI* and *HindIII* restriction sites. The hairpin inverted repeat fragments were created from the coding region of *AvrLm6* by PCR, using primers described in Supplementary Table S2. The *AvrLm6* sense fragment of the inverted repeat was amplified from cDNA of v23.1.3 mycelium using primers *AvrLm6-Hind+* and *AvrLm6-BamHI-*. The sense product was digested with *HindIII* and *BamHI*. The antisense region was amplified with primers *AvrLm6-BamHI+* and *AvrLm6-EcoRI* and was digested with *BamHI* and *EcoRI*. Sense and antisense fragments were ligated into an *EcoRI-HindIII*-digested pJK11. This expression cassette was excised by digestion with *SpeI* and *XhoI*. In contrast with the strategy of Fitzgerald and associates (2004), we did not use pJK1 as final binary vector, because it was found to be unstable after transformation into *Agrobacterium tumefaciens* C58. The binary vector pNAT1, which carries the nourseothricin acetyltransferase gene (*NAT1*) conferring resistance to the antibiotic nourseothricin (a gift from B. Howlett, University of Melbourne, Australia), was used for *Agrobacterium*-mediated RNAi of *L. maculans* genes. The expression cassette was cloned into pNAT1 digested with *SpeI* and *XhoI*, creating the vector pNAT1-*AvrLm6*. The different constructs then were introduced into *A. tumefaciens* C58 by electroporation at 1.5 kV, 200 ohm, and 25 μ F.

Transformation of *L. maculans* was performed as described by de Groot and associates (1998) with minor modifications (Gout et al. 2006). Fungal transformants were selected on hygromycin B at 200 μ g/ml (Invitrogen) or nourseothricin at 50 μ g/ml (WERNER BioAgents, Jena, Germany).

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