New Avirulence Genes in the Phytopathogenic Fungus *Leptosphaeria maculans*

M. H. Balesdent, A. Attard, M. L. Kühn, and T. Rouxel

Pathologie Végétale, INRA, Route de Saint Cyr, 78026 Versailles Cedex, France.
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ABSTRACT

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Leptosphaeria maculans, the causal agent of stem canker of oilseed rape (*Brassica napus*), develops gene-for-gene interactions with oilseed rape, and four *L. maculans* avirulence (AVR) genes (*AvrLm1*, *AvrLm2*, *AvrLm4*, and *alm1*) were previously genetically characterized. Based on the analysis of progeny of numerous in vitro crosses between *L. maculans* isolates showing either already characterized or new differential interactions, this work aims to provide an overview of the AVR genes that may specify incompatibility toward *B. napus* and the related species *B. juncea* and *B. rapa*. Two novel differential interactions were thus identified between *L. maculans* and *B. napus* genotypes, one of them corresponding to a complete resistance to European races of *L. maculans*. In both cases, a single gene control of avirulence was established (genes *AvrLm3* and *AvrLm7*). Similarly, a single gene control of avirulence toward a *B. rapa* genotype, also resistant to European *L. maculans* isolates, was demonstrated (gene *AvrLm8*). Finally, a digenic control of avirulence toward *B. juncea* was established (genes *AvrLm5* and *AvrLm6*). Linkage analyses demonstrated that at least four unlinked *L. maculans* genomic regions, including at least one AVR gene cluster (*AvrLm1-AvrLm2-AvrLm6*), are involved in host specificity. The *AvrLm3-AvrLm4-AvrLm7* region may correspond either to a second AVR gene cluster or to a multiallelic AVR gene.

Additional keywords: blackleg disease, *Phoma lingam.*

Dissecting the genetic control of specific interactions of both the pathogen and the host plant is a prerequisite for either developing molecular characterization of the genes involved in the interaction or for developing integrated and durable management strategies to control the disease. In the case of the ascomycete *Leptosphaeria maculans* (anamorph *Phoma lingam*), the causal agent of stem canker of oilseed rape (*Brassica napus* L. var. *oleifera*) and of dry rot of swede (*B. napus* var. *napobrassica*), genetic approaches toward the identification of both avirulence (AVR) genes in the pathogen and resistance (R) genes in the host plant have been developed only very recently. Following the description of a standardized cotyledon inoculation test (36), an initial *B. napus* differential set was described, comprising cvs. Westar (susceptible check, spring type) and Quinta and Glacier (winter types) (22). Using this differential set, *L. maculans* isolates were classified into three pathogenicity groups (PG), i.e., PG2 (avirulent on cvs. Quinta and Glacier), PG3 (avirulent on cv. Quinta but virulent on cv. Glacier), and PG4 (virulent on all three cultivars). Badawy et al. (6) replaced cv. Westar by winter type *B. napus* cv. Lirabon and added Jet Neuf as a differential, leading to the description of six PG that were termed A1 to A6 (6,23). PG4 isolates were thus further divided into A1 (virulent on Jet Neuf) and A5 (avirulent on Jet Neuf); PG3 into A2 (virulent on Jet Neuf) and A6 (avirulent on Jet Neuf); and PG2 into A4 and A3 (virulent and avirulent on Jet Neuf, respectively). The genetic control of these specific interactions was elucidated following genetic analyses performed both on the plant and the pathogen: (i) avirulence of PG3-A2 isolates on cv. Quinta was governed by the AVR/R gene pair *AvrLm1*/*Rlm1* (1,2); (ii) the AVR gene *AvrLm2*, identified in the PG2 isolate PHW1245, was found responsible for the induction of the hypersensitive response of cv. Glacier in which the corresponding R gene, *Rlm2*, was genetically identified (2). The *AvrLm2* locus was very closely linked to *AvrLm1*, also present in isolate PHW1245 (2); and (iii) incompatibility of A5 isolates toward Jet Neuf was governed by the interaction between the AVR gene *AvrLm4*, unlinked to *AvrLm1*, and the R gene *Rlm4* (7). Consequently, the specific interactions described using the Kuswinanti et al. (23) differential set could be clearly explained by gene-for-gene interactions, and A1 to A6 isolates could thus be considered as six races of the pathogen, differing in their AVR genes pattern. Independently, the PG2–*B. napus* cv. Major interaction was genetically characterized on both the plant and the pathogen: a single major locus (*LEM 1*) was identified in cv. Major (16) and the corresponding AVR gene, identified in the PG2 isolate PHW1245, was termed *alm1* (29).

Many additional specific interactions were described between *L. maculans* and *B. napus* but none of them were genetically analyzed. Specific interactions were described between *B. napus* cultivars or lines Chisaya, Rafal, RX3, and Australian *L. maculans* isolates (9). Kutcher et al. (24) described additional interactions using *B. napus* cvs. Karat, Global, Marnoo, and Regent, and the two breeding lines R83-14.DH47 and R83-14.DH26. Similarly, Kuswinanti et al. (23) subdivided European PG3 and PG4 isolates into seven subgroups following cotyledon inoculations on eight *B. napus* differentials (Lirabon, Quinta, Glacier, Jet Neuf, Doublol, Karat, R83-14.DH47, and R83-14.DH26). Using as differentials *B. napus* cultivars or lines (Westar, Glacier, Quinta, Quantum, Sentry, Sprint, Val-1, and Dac-1), *L. maculans* PG2 isolates were subdivided into 15 distinct PG and PG3 and PG4 were subdivided into eight PG (20). The Swede cv. Tina was first described as a new source of resistance to *L. maculans* (25), but *L. maculans* isolates virulent on cv. Tina were described later (19). All these interactions are probably due to genetically uncharacterized AVR/R gene pairs.

Corresponding author: T. Rouxel; E-mail address: rouxel@versailles.inra.fr

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Finally, specific interactions between *L. maculans* and its host plants can probably be expanded to the related *Brassica* species *B. rapa*, *B. oleracea*, *B. juncea*, and *B. nigra*. Different plant genotypes within *B. rapa* (6,22–24), *B. oleracea* (6), or *B. juncea* (21, 23,24) displayed differential reactions when challenged by different *L. maculans* isolates. Only one of these potential specific interactions was genetically analyzed in the pathogen, following in vitro crosses involving the *B. juncea* virulent isolate IBCN18 (or M1) (11,14). This interaction was found to be inherited as a single gene in the pathogen (11,14). Although clear evidence of hypersensitive response was reported in *B. juncea* when inoculated with the avirulent parent of the cross (10), this still unnamed locus was considered a virulence locus due to the lack of *B. juncea* genotypes displaying resistance toward IBCN18 (14).

Based on the analysis of progeny of numerous in vitro crosses between *L. maculans* isolates showing either already characterized or new differential interactions, the present study aims to provide an overview of the AVR genes that may specify cultivar and species-specific incompatibility toward *B. napus*, *B. juncea*, and *B. rapa*. Apart from identifying new *L. maculans* AVR genes, the present study suggests that cultivar and species-specific incompatibility may be of a similar genetic nature in this pathosystem and demonstrates that at least four unlinked *L. maculans* genomic regions, including one AVR gene cluster, are involved in host specificity.

MATERIALS AND METHODS

Fungal strains and culture maintenance. Isolates available at the beginning of this study, used as reference or for in vitro crosses, are listed in Table 1. The genotypes of isolates H5, 11.26.14, v11.5.1, v11.1.2, v23.1.2, v23.1.3, v23.1.11, and v23.2.1 at the three AVR loci *AvrLm1*, *AvrLm2*, and *AvrLm4* were previously determined following inoculations on *B. napus* cultivars or lines QuintaV (*Rlm1-Rlm4*), Columbus (*Rlm1*), Glacier (*Rlm2*), and Jet Neuf (*Rlm4*) (Table 1) (1,7). Isolates IBCN18, IBCN74, IBCN78, and IBCN79 belong to the International Blackleg of Crucifers Network (IBCN) collection (32,34). Isolate IBCN74 (PHW1245), analyzed in a previous study (crosses 7 and 14), possessed the two AVR alleles *AvrLm1* and *AvrLm2* (2). Isolate Nz-T, virulent on *B. napus* var. *napobrassica* cv. Tina, was further purified following single-conidia isolation as described previously (17). Eight single-conidia cultures were selected and their virulence on cv. Tina was controlled. Isolate Nz-T4 was selected for genetic studies. The two European PG4 isolates (i.e., possessing virulent alleles at all three AVR loci *AvrLm1*, *AvrLm2*, and *AvrLm4*) (7), namely UK1 from the United Kingdom and PT1 from Portugal, belong to the IMASCORE collection of European isolates (4,34). All fungal cultures were maintained and stored for long term as already reported (1,7).

In vitro crossing. Nine new crosses were performed for the present study (Table 2). In vitro crosses and tetrad or random ascospore progeny recovery were performed as previously established (8,18). All progeny from in vitro crosses were named as described by Balesdent et al. (7). Twin isolates within each tetrad were identified by their identical electrokaryotypes (7,26). Tetrads were considered complete when at least one isolate of each of the four genotypes of the tetrad was recovered. Progeny from crosses 7 and 14, which were obtained previously (2), were further characterized on *B. napus* cv. Jet Neuf.

Plant material. Seeds of *B. napus* cvs. Westar and Glacier were provided by G. Séguin-Swartz (AAF, Saskatoon, Canada) and H. K. Love (Svalof Weibull Seed, Lindsay, Canada), respectively. Seeds of cvs. Symbol, Vivol, Capitol, Doublol, Bristol, and Columbus were obtained from Semences Cargill (Boissay, France). The Quinta S2-V95 line, possessing both *Rlm1* and *Rlm4* (7), was further multiplied by self-pollination of plants that were first checked for the presence of the two R genes following inoculation with reference isolates. Self-pollination took place in the greenhouse under pollen-proof bags to avoid pollen contamination. The resulting S3 line was termed QuintaV.

Seeds of *B. napus* cvs. Jet Neuf, Express, Falcon, and Darmor and *B. juncea* cvs. Aurea and Picra were obtained from M. Renard (INRA, Amélioration des Plantes, Le Rheu, France) and further multiplied by self-pollination. The *B. napus* line Falcon-MX, also provided by M. Renard, is a *B. napus–B. juncea* recombinant line containing the *Jlm1* resistance gene from *B. juncea* (12; A. M. Chèvre, *personal communication*).

Two individual *B. juncea* cv. Aurea plants were selected for their unusual behavior when inoculated with some progeny isolates from cross 22 and were self-pollinated. From the two S1 resulting lines, approximately 10 plants per line were selected following inoculation with the same isolates. Only plants showing the same interaction phenotype as the original plant were chosen for subsequent mass-pollination in the greenhouse under pollen-proof bags, resulting in S2 lines 150-2-1 and 151-2-1.

TABLE 1. List and characteristics of field or laboratory isolates used in this study

Isolate number	Country of origin	Origin	Source	Other name	Mating type	Available data on pathogenicity or avirulence (AVR) allele[a]	Reference
H5	France	F[b]	C. Gall		–	*avrLm1-avrLm2-avrLm4*	(1,7)
IBCN18	Australia	F	B. Howlett	M1	–	Virulent on *Brassica juncea*	(11,34)
IBCN78	Australia	F	P. H. Williams	PHW1321/WA43	+	PG2[c]	(34)
IBCN79	Australia	F	P. H. Williams	PHW1325/WA52	+	PG2	(34)
IBCN74	France	F	P. H. Williams	PHW1245	+	*AvrLm1-AvrLm2*	(2)
						alm1	(29)
						Avirulent on Jet Neuf	(6; this study)
Nz-T	New Zealand	F	S. Gowers	T	+	Virulent on *B. napus* cv. Tina	(19)
11.26.14	France	L	Cross 11[d]	…	+	*avrLm1-avrLm2-avrLm4*	(1,7)
v11.1.5	France	L	Cross 11	…	–	*AvrLm1-avrLm2-avrLm4*	(7)
v11.1.2	France	L	Cross 11	…	+	*AvrLm1-avrLm2-avrLm4*	(7)
v23.2.1	France	L	Cross 23	…	+	*avrLm1-avrLm2-AvrLm4*	(7)
v23.1.2	France	L	Cross 23	JN2	–	*avrLm1- avrLm2-avrLm4*	(7)
v23.1.3	France	L	Cross 23	JN3	+	*AvrLm1-avrLm2-AvrLm4*	(7)
v23.1.11	France	L	Cross 23	JN11	–	*AvrLm1-avrLm2-AvrLm4*	(7)
UK1	England	F	B. Fitt	95 A6	+	PG4-A1	(34)
PT1	Portugal	F	J. S. Dias	…	nd[e]	PG4-A1	(34)

[a] Genotypes at AVR loci deduced from genetic studies. *AvrLmi* and *avrLmi* represent the avirulent and the virulent genotypes, respectively, at the *AvrLmi* locus.
[b] F, field single-ascospore isolate; L, laboratory isolate originating from in vitro crosses.
[c] PG, pathogenicity groups according to the terminology of Koch et al. (22).
[d] Crosses 11 and 23 are described in Balesdent et al. (7).
[e] nd, not determined.

Six *B. napus*-derived lines and one *B. rapa*-derived line (156-2-1) were obtained following a preliminary screening of gene bank genotypes for resistance to isolates v11.1.1, v11.1.2, and v23.2.1 (T. Rouxel, E. Willner, and M. H. Balesdent, *unpublished data*) (Table 3). In all cases, only a few plants resistant to the three isolates were identified among the screened accessions. Depending on the lines, the selected resistant plants were either self pollinated individually (lines 149-2-1, 05-1-1, 04-1-2, 156-2-1, 23-1-1, and 22-1-1) or mass pollinated (line 148-1-1). When the S1 populations showed a heterogeneous behavior after inoculation with reference isolates (lines 05-1-1, 149-2-1, 23-1-1, 22-1-1, and 156-2-1) a second round of mass pollination took place with selected S1 plants (=S2 generation). In one case however, the S2 resulting line was still heterogeneous when inoculated with reference isolates v11.1.1, v11.1.2, and Nz-T4 with two main phenotypic classes, the first being resistant to v11.1.1 and v11.1.2 but susceptible to Nz-T4, the second being susceptible to the three isolates. Five plants of each phenotypic class were selected and mass pollinated. The two resulting S3 lines, 23-1-1 and 22-1-1, respectively, were 100% homogeneous and similar to the parental phenotypes. Lines 04-1-2, 22-1-1, and 23-1-1 were derived from *B. napus* var. *oleifera* cultivars, whereas lines 149-2-1, 148-1-1, and 05-1-1 were derived from *B. napus* var. *napobrassica*.

Pathogenicity tests. Isolates were inoculated on cotyledons of the brassica genotypes as previously described (1,7). Plants were incubated in a growth chamber at 16/24°C (night/day) with a 12 h photoperiod. Symptoms were scored 14 to 27 days after inoculation using the IMASCORE rating scale comprised of six infection classes (IC), in which IC1 to IC3 are resistance responses and IC4 to IC6 are susceptibility symptoms (7). Each isolate was inoculated on at least 10 different plants, and experiments were repeated twice. Finally, the result of each isolate–line interaction was recorded as compatible (more than 80% of susceptibility responses, the isolate is virulent [V]) or incompatible (more than 80% of resistance responses, the isolate is avirulent [A]). In a few cases, the isolate–line interaction was heterogeneous, with a majority of resistance responses (IC1 to IC3) but with a significant (more than 20%) percentage of susceptibility symptoms (IC4 to IC6), probably due to plant genotype heterogeneity (Fig. 1).

Genetic analyses. For each interaction, the ratio of A/V progeny was tested for deviation from Mendelian expectation by chi-square analysis. For random progeny in which more than two AVR genes segregated, linkage analyses among AVR loci was performed using the Mapmaker/EXP 3.0 software (available online; Whitehead/MIT Center for Genome Research, Cambridge,

TABLE 2. List of the in vitro crosses described in this study

	Parental isolates[a]		Progeny	
Cross	1	2	Recovered	Selected for subsequent crosses
19	IBCN79	H5	1 complete + 6 incomplete tetrads	...
20	IBCN18	11.26.14	13 ascospores from 5 asci	20.3.04 (Mat–)
22	20.3.04	11.26.14	10 complete tetrads	22.2.02 (Mat–); 22.2.03 (Mat+)
24	22.2.03	v11.1.5	70 random ascospores	…
29	22.2.02	22.2.03	67 random ascospores	v29.3.1 (Mat–)
34	v29.3.1	v23.1.3	62 random ascospores	...
30	Nz-T4	v23.1.2	66 random ascospores	...
33	Nz-T4	v23.1.11	48 random ascospores	...
35	IBCN78	v23.1.11	43 random ascospores	...

[a] Additional information on the isolates is provided in Table 1.

TABLE 3. Interaction phenotypes between selected *Brassica* cultivars or lines and *Leptosphaeria maculans* isolates used as reference or for genetic analyses

	Plant genotypes and known or putative resistance genes													
	Reference cultivars							Additional lines displaying new specific interactions						
	Westar	QuintaV *Rlm1* *Rlm4*	Columbus *Rlm1* (*Rlm3*)[a]	Glacier *Rlm2* (*Rlm3*)	Bristol (*Rlm2*)	Jet Neuf *Rlm4*	Falcon (*Rlm4*)	149-2-1 (*Rlm1*) (*Rlm7*)	148-1-1 05-1-1 04-1-2 23-1-1 (*Rlm7*)	22-1-1 (*Rlm3*)	*B. juncea* cvs. Picra and Aurea (*Rlm5*) (*Rlm6*)	*B. juncea* lines 150-2-1 and 151-2-1 (*Rlm5*)	Falcon-MX *Jlm1* (*Rlm4*) (*Rlm6=Jlm1*)	156-2-1 (*Rlm8*)
PHW1245 *AvrLm1 AvrLm2 AvrLm4*[b]	S[c]	R	R	R	R	R	R	R	R	S	R	R	R	R
H5, UK1, PT1, v23.1.2 *avrLm1 avrLm2 avrLm4*	S	S	S	S	S	S	S	R	R	S	R	R	R	R
v23.1.3, v23.1.11 *AvrLm1 avrLm2 AvrLm4*	S	R	R	S	S	R	R	R	R	S	R	R	R	R
v11.1.2 *AvrLm1 avrLm2 avrLm4*	S	R	R	S	S	S	S	R	R	S	R	R	R	R
v23.2.1 *avrLm1 avrLm2 AvrLm4*	S	R	S	S	S	R	R	R	R	S	R	R	R	R
IBCN18[d] *AvrLm1 AvrLm2 AvrLm4*	S	R	R	R	R	R	R	R	R	S	S	S	R	S
Nz-T4 *avrLm1 avrLm2 avrLm4*	S	S	S	S	S	S	S	S	S	S	R	R	R	R
IBCN78, IBCN79 *AvrLm1 avrLm2 avrLm4*	S	R	R	R	S	S	S	R	S	R	R	R	R	R

[a] Resistance genes between brackets are putative resistance genes deduced from the present study.
[b] *AvrLmi*, the isolate is avirulent, *avrLmi*, the isolate is virulent; the genotypes are given for the three avirulent (AVR) alleles *AvrLm1*, *AvrLm2*, and *AvrLm4*.
[c] R, the line is resistant (incompatible interaction); S, the line is susceptible (compatible interaction).
[d] For isolates IBCN18, Nz-T4, IBCN78, and IBCN79, the AVR alleles at *AvrLm1*, *AvrLm2*, and *AvrLm4* loci are deduced from the present study.

MA) with a LOD score of 4.0 and a maximum recombination frequency of 20 centimorgans (cM).

RESULTS

AVR genes in the reference isolates and R genes in the reference cultivars. From previous genetic analyses, the genotypes of reference isolates were identified at the three loci *AvrLm1*, *AvrLm2*, and *AvrLm4* (Table 3) (7). PHW1245, previously described as possessing *AvrLm1* and *AvrLm2* (2), was avirulent on Jet Neuf (6) (Table 3). In order to better characterize the AVR genes present in PHW1245, the entire progeny of crosses 7 (six tetrads) and 14 (two tetrads) were further tested for virulence on Jet Neuf. The avirulence of PHW1245 on Jet Neuf segregated 1:1 in all tetrads from crosses 7 and 14. This result suggested that PHW1245 also possesses *AvrLm4*, recognizing *Rlm4* present in Jet Neuf and QuintaV (Table 3). In addition, three parental ditypes (PD), four nonparental ditypes, and one tetratype (TT) were observed between *AvrLm4* and *AvrLm1* (i.e., segregation of avirulence on *Rlm1* harboring cvs. Vivol, Doubol, Capitol, and Columbus [7]) in the eight tetrads from crosses 7 and 14, confirming the genetic independence between *AvrLm1* and *AvrLm4* (7).

Following inoculation of cv. Falcon with reference isolates, it was concluded that Falcon possesses *Rlm4*, because it was resistant to all three isolates possessing *AvrLm4* (Table 3). Line 22-1-1 was considered as possessing none of the R genes *Rlm1*, *Rlm2*, or *Rlm4* because it was susceptible to all reference isolates. Finally, because Bristol behaved similarly to Glacier, it was hypothesized that Bristol possesses *Rlm2* (Table 3). All other *B. napus*, *B. rapa*, and *B. juncea* cultivars or lines were resistant to all reference isolates, including the three European PG4 field isolates H5, UK1, and PT1 (Table 3).

Evidence for new specific interactions. Isolates IBCN18, IBCN78, IBCN79, and Nz-T4 presented novel interaction phenotype patterns compared with those involving the *AvrLm1*/*Rlm1*, *AvrLm2*/*Rlm2*, or *AvrLm4*/*Rlm4* gene pairs (Table 3). In accordance with Chen et al. (11), IBCN18 was virulent on *B. juncea* cvs. Picra and Aurea, as well as on the two *B. juncea*-derived lines 150-2-1 and 151-2-1. However, in the present study, this isolate was avirulent on Falcon-MX (Table 3). IBCN18 was also virulent on the *B. rapa*-derived line 156-2-1, whereas it was avirulent on all cultivars, except cv. Westar, from the differential set of Kuswinanti et al. (23). This suggested that isolate IBCN18 may possess AVR alleles at all three AVR loci *AvrLm1*, *AvrLm2*, and *AvrLm4*.

In contrast to all above-mentioned isolates, IBCN78, IBCN79, and Nz-T4 were virulent on the lines 148-1-1, 05-1-1, 04-1-2, and 23-1-1, which were independently selected for their resistance to European PG4 isolates. Finally, isolates IBCN78 and IBCN79, but not Nz-T4, were avirulent on Glacier (but not on Bristol), QuintaV, Columbus, and on lines 22-1-1 and 149-2-1 (Table 3). Although the QuintaV–IBCN78 or IBCN79 interactions could be simply attributed to the occurrence of *AvrLm1* in these isolates, all other interactions presented above suggested the occurrence of new gene-for-gene interactions.

***AvrLm1*, *AvrLm2*, and *AvrLm4* alleles are present in isolate IBCN18.** IBCN18 was crossed with the PG4 isolate 11.26.14 (cross 20; Tables 2 and 4). Very few progeny isolates, and no complete tetrads, could be recovered from this cross (Table 4). However, it was evident after analysis of this progeny that all differential interactions expressed by the parental isolates of cross 20 segregated in the progeny, because seven distinct phenotypic classes were recovered in the progeny (Table 4).

Cosegregation of avirulence on Columbus, QuintaV, Doublol, Bristol, and Glacier could be explained by the hypothesis that isolate IBCN18 possessed the two tightly linked AVR alleles *AvrLm1* and *AvrLm2*, whereas the independent segregation of avirulence on *Rlm4* cvs. Jet Neuf and Falcon is consistent with the hypothesis that AVR allele *AvrLm4* is present in isolate IBCN18 (Table 4). As expected by the occurrence of both *Rlm1* and *Rlm4* in QuintaV, all isolates in the progeny that were avirulent either on Columbus or on Jet Neuf were also avirulent on QuintaV (Table 4).

The whole progeny remained avirulent on lines 04-1-2 and 05-1-1, as were the two parents of the cross (data not shown). In contrast, 6 of 13 progeny isolates were virulent on Falcon-MX, although the two parent isolates induced a resistance reaction on this line (Table 4). This could suggest that two independent AVR genes, recognizing two R genes with epistatic effects present in Falcon-MX, are segregating in the progeny. It has to be noticed that all isolates that were avirulent on Jet Neuf and Falcon (i.e., possessing the *AvrLm4* allele) were avirulent on Falcon-MX. This indicated that *Rlm4*, present in cv. Falcon, was also present in the derived line Falcon-MX and could be one of the two Falcon-MX R genes. Due to the small number of progeny analyzed, the segregation of avirulence on the two *B. juncea* cvs. Picra and Aurea fits

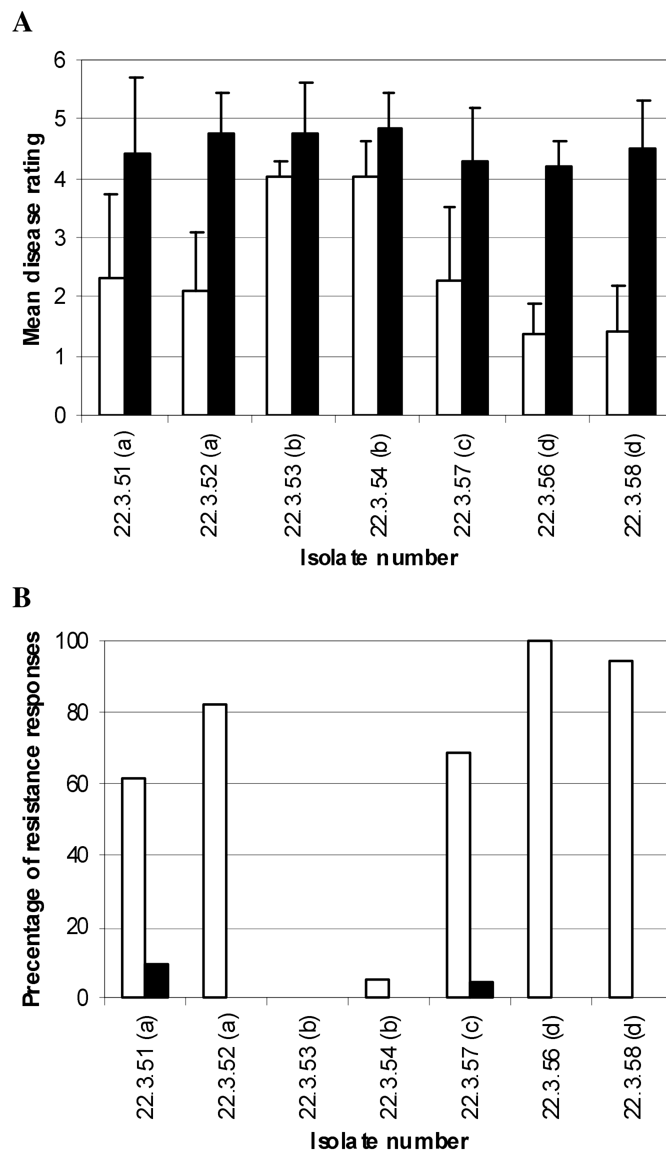

Fig. 1. An example of avirulence segregation of *Leptosphaeria maculans* on *Brassica juncea* cv. Picra in one complete tetrad (seven isolates, four genotypes) from cross 22. Symptoms were rated 15 days after inoculation using six infection classes (IC1 to IC6) for a rating scale (7). **A,** Mean disease rating (the vertical line is the standard deviation) and **B,** percentage of resistance responses (IC1 to IC3) of 16 to 23 inoculation sites for each isolate–plant genotype interaction. Isolate numbers are described in Table 5, and isolates with the same letter are twin isolates. White bars, *B. juncea* cv. Picra; and black bars, *B. napus* cv. Westar as a susceptible check.

both a 50:50 A/V ratio (chi-square = 0.692; $P = 0.405$) or a 75:25 ratio (chi-square = 1.256; $P = 0.262$). Finally, virulence of IBCN18 on the two *B. juncea*-derived lines 150-2-1 and 151-2-1 and on the *B. rapa*-derived line 156-2-1 segregated in cross 20 independently from all above-mentioned interactions (Table 4).

To increase the progeny, a backcross (cross 22) was performed between isolate 20.3.04, displaying the same interaction phenotype as IBCN18, except that it was virulent on Jet Neuf and 11.26.14 (Tables 2 and 5). Cross 22 was much more fertile than cross 20, because 10 complete tetrads could be recovered. The whole progeny was virulent on Jet Neuf and Falcon (Table 5). As expected for a single gene control of avirulence on *Rlm1* and on *Rlm2* cultivars, a 2:2 A/V ratio was observed within each tetrad when inoculated on a range of cultivars possessing *Rlm1* (Capitol, Columbus, Doublol, and Vivol) on the one hand, or on Glacier, Bristol, and the two additional cvs. Express and Symbol, also possessing *Rlm2* (T. Rouxel and M. H. Balesdent, *unpublished data*), on the other hand (Table 5). In all cases, twin isolates displayed the same interaction phenotypes. For 9 of 10 tetrads, isolates that were avirulent on *Rlm1* cultivars were also avirulent on Bristol, Glacier, Express, and Symbol. For one tetrad however (tetrad 3), a tetratype was observed, with one twin pair avirulent on *Rlm1* genotypes but not on *Rlm2* genotypes, and one twin pair virulent on *Rlm1* genotypes but not on *Rlm2* genotypes (Table 5).

To further support the hypothesis that isolate IBCN18 possessed the *AvrLm1* AVR allele, rather than another independent AVR gene conferring an identical interaction spectrum, one additional cross (cross 24) was performed (Table 2). In this cross, isolate 22.2.03 (putatively *AvrLm1*-*avrLm2*) was crossed with isolate v11.1.5 possessing the *AvrLm1* allele. From the 70 progeny isolates recovered, 56 isolates were assessed for avirulence on Columbus. All of them were avirulent and no recombinant isolate (i.e., virulent on *Rlm1*) could be detected (Table 6).

Identification of three virulence alleles in IBCN18: *avrLm5*, *avrLm6*, and *avrLm8*. Crosses 22, 24, and 29 were used to analyze the genetic control of avirulence on *B. juncea* and *B. rapa* genotypes. For 9 of the 10 tetrads from cross 22, a 3:1 A/V ratio was observed on *B. juncea* cvs. Picra or Aurea, whereas a 2:2 A/V segregation was observed in one tetrad only (tetrad 6; Table 5). The predominantly observed 3:1 A/V ratio on *B. juncea* cultivars is most likely to correspond to the occurrence of two (or more) unlinked AVR genes present in isolate 11.26.14, each of them being able to induce the resistance response of *B. juncea*. For most tetrads however, the result of the interaction on *B. juncea* was not always easily scored as a compatible or incompatible interaction. One genotype within each tetrad was usually clearly virulent, another one (or two) clearly avirulent, the others were often scored as heterogeneous, mainly because of a high standard deviation of mean ratings due to heterogeneity of plant response (Fig. 1; Table 5). These heterogeneous responses were more frequently observed on cv. Aurea than on cv. Picra (data not shown). *B. juncea* lines 150-2-1 and 151-2-1 were obtained by selfing individual *B. juncea* cv. Aurea plants that were fully susceptible to isolates revealing such a heterogeneous interaction phenotype. Interaction phenotypes of cross 22 progeny were identical on 150-2-1 and 151-2-1 and segregated with a 2:2 ratio in all tetrads (Table 5). Similarly, a 2:2 A/V segregation was observed for each tetrad on Falcon-MX, and recombination between avirulence on Falcon-MX, and on the 150-2-1 and 151-2-1 lines, was very frequently observed (one PD and nine TT) (Table 5). From these data, the following hypothesis is proposed: avirulence on *B. juncea* cvs. Aurea and Picra is governed by two pairs of genes termed *AvrLm5*/*Rlm5* (*Rlm5* being present in the two *B. juncea* cultivars and in lines 150-2-1 and 151-2-1 but not in Falcon-MX) and *AvrLm6*/*Rlm6* (*Rlm6* being present in the two *B. juncea* cultivars and in Falcon-MX but not in lines 150-2-1 and 151-2-1). The genotypes of parent isolates of crosses 20 and 22 would be *AvrLm5*-*AvrLm6* (isolate 11.26.14) and *avrLm5*-*avrLm6* (isolates IBCN18 and 20.3.04). When one of these two AVR alleles is present, the isolate is avirulent on *B. juncea*, whereas virulence is only observed when the two virulent alleles are present. The characterization of cross 22 progeny on lines Falcon-MX, 150-2-1, and 151-2-1 allowed us to determine the putative genotype of each isolate at the two loci *AvrLm5* and *AvrLm6* (Table 5). The two clear interaction phenotypes observed within each tetrad corresponded either to the *avrlm5*-*avrlm6* or to the *AvrLm5*-*Avrlm6* genotypes, whereas the heterogeneous responses were observed when only one AVR allele was present (either *AvrLm5* or *AvrLm6*) (Table 5). This heterogeneous response, attributable to plant heterogeneity, would be more frequently observed when one R gene is lacking than if two R genes are lacking in one individual plant. Our hypothesis is further supported by segregation data from cross

TABLE 4. Interaction phenotypes of parental isolates and progeny of cross 20 (IBCN18 × 11.26.14) on a range of *Brassica napus*, *B. rapa*, and *B. juncea* cultivars or lines

	Brassica genotypes and known or putative resistance genes[a]						
	B. napus			*B. juncea*			*B. rapa*
	Jet Neuf, Falcon *Rlm4*	Columbus, Doublol *Rlm1* (*Rlm3*) Bristol *Rlm2* Glacier *Rlm2* (*Rlm3*)	QuintaV *Rlm1*, *Rlm4*	Picra, Aurea (*Rlm5*, *Rlm6*)	150-2-1 151-2-1 (*Rlm5*)	Falcon-MX[b] (*Rlm4*, *Rlm6*)	156-2-1 (*Rlm8*)
Parental isolates							
11.26.14	V[c]	V	V	A	A	A	A
IBCN18	A	A	A	V	V	A	V
Phenotypic classes in the progeny							
I (2 isolates)[d]	V	V	V	A	V	A	A
II (1 isolate)	V	V	V	A	V	A	V
III (1 isolate)	A	V	A	A	V	A	V
IV (1 isolate)	A	V	A	V	V	A	A
V (2 isolates)	A	V	A	A	A	A	V
VI (4 isolates)	V	A	A	V	V	V	V
VII (2 isolates)	V	A	A	A	A	V	V
Total							
Avirulent isolates	4	6	10	8	4	7	3
Virulent isolates	9	7	3	5	9	6	10

[a] Resistance genes between brackets are putative resistance genes deduced from the present study.
[b] Falcon-MX is a *B. napus* recombinant line with the *B. juncea* R gene *Jlm1* (12), termed *Rlm6* in the present study.
[c] A, the isolate is avirulent (incompatible interaction); V, the isolate is virulent (compatible interaction).
[d] Number between brackets is the number of recovered isolates for each phenotypic class.

24 in which only *AvrLm5* segregates (Tables 5 and 6). All tested progeny isolates from cross 24 were avirulent on *B. juncea* cvs. Picra and Aurea, whereas avirulence on lines 150-2-1 and 151-2-1 segregated as a single gene (Table 6).

Finally, a 2:2 A/V segregation was observed within each tetrad when inoculated on *B. rapa* line 156-2-1, suggesting again a single gene control of avirulence on this line. Because the segregation of this avirulence, in crosses 20 and 22, appeared independent from all other AVR genes previously characterized (Tables 4 and 5), it is suggested that a new AVR locus, termed *AvrLm8*, governs the interaction phenotype on line 156-2-1.

Linkage analyses between the AVR loci. Segregation of avirulence or virulence of progeny from crosses 20 and 22 indicated genetic independence between the *AvrLm1-AvrLm2* region, *AvrLm4*, *AvrLm5*, and *AvrLm8*, whereas *AvrLm6* cosegregated with *AvrLm2*. However, too few meiotic events were analyzed with these two crosses (5 and 10 for crosses 20 and 22, respectively) to assess genetic distance between all these loci. In cross 29, the two sister isolates 22.2.02 (*avrLm1 AvrLm2 avrLm4 AvrLm5 avrLm6 avrLm8*) and 22.2.03 (*AvrLm1 avrLm2 avrLm4 avrLm5 AvrLm6 AvrLm8*) were crossed (Tables 2 and 5) and *AvrLm1*, *AvrLm2*, *AvrLm5*, *AvrLm6*, and *AvrLm8* were all segregating as simple traits in the resulting progeny (Table 6). When considering only *AvrLm1* and *AvrLm2*, three phenotypic classes were recovered, i.e., the two parental phenotypes *AvrLm1-avrLm2* (35 isolates) and *avrLm1-AvrLm2* (31 isolates), and the recombinant phenotype

TABLE 5. Tetrad analysis of avirulence segregation of *Leptosphaeria maculans* progeny in cross 22 (20.3.04 × 11.26.14) on a range of *Brassica napus*, *B. juncea*, and *B. rapa* lines

	Brassica lines and known or putative resistance genes[a]							
	B. napus			*B. juncea*			*B. rapa*	
Isolates	Jet Neuf Falcon *Rlm4*	Columbus, Vivol, Capitol, Doublol *Rlm1* (*Rlm3*)	Glacier, Bristol, Express, Symbol *Rlm2*	Picra, Aurea (*Rlm5*) (*Rlm6*)	150-2-1, 151-2-1 (*Rlm5*)	Falcon-MX[b] (*Rlm4*) (*Rlm6*)	156-2-1 (*Rlm8*)	Putative genotype of the isolates[c]
Parental isolates[d]								
IBCN18	A[e]	A	A	V	V	A	V	*A1A2A4a5a6a8*
20.3.04	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
11.26.14	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
Progeny isolates[f]								
(1) 22.1.01 & .04	V	A	A	A	A	V	A	*A1A2a4A5a6A8*
22.1.03 & .06	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
22.1.07 & .08	V	V	V	H	V	A	V	*a1a2a4a5A6a8*
22.1.02 & .05	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
(2) 22.1.11 & .14	V	A	A	V	V	V	A	*A1A2a4a5a6A8*
22.1.12 & .13	V	V	V	A	V	A	V	*a1a2a4a5A6a8*
22.1.15 & .16	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
22.1.17 & .18	V	A	A	A	A	V	V	*A1A2a4A5a6a8*
(3) 22.2.01	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
22.2.02 & .07	V	V	A	A	A	V	V	*a1A2a4A5a6a8*
22.2.03 & .05	V	A	V	A	V	A	A	*A1a2a4a5A6A8*
22.2.04 & .06	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
(4) 22.2.11 & .13	V	V	V	A	A	A	V	*a1a2a4A5A6a8*
22.2.12 & .14	V	V	V	H	V	A	A	*a1a2a4a5A6A8*
22.2.15 & .18	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
22.2.16 & .17	V	A	A	A	A	V	A	*A1A2a4A5a6A8*
(5) 22.3.01 & .03	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
22.3.02 & .04	V	A	A	A	A	V	V	*A1A2a4A5a6a8*
22.3.05 & .06	V	V	V	H	V	A	A	*a1a2a4a5A6A8*
22.3.07 & .08	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
(6) 22.3.11 & .14	V	V	V	A	A	A	V	*a1a2a4A5A6a8*
22.3.12 & .13	V	V	V	A	A	A	V	*a1a2a4A5A6a8*
22.3.15	V	A	A	V	V	V	A	*A1A2a4a5a6A8*
22.3.16 & .17	V	A	A	V	V	V	A	*A1A2a4a5a6A8*
(7) 22.3.21 & .22	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
22.3.23 & .24	V	V	V	H	V	A	A	*a1a2a4a5A6A8*
22.3.25 & .28	V	A	A	H	A	V	V	*A1A2a4A5a6a8*
22.3.26 & .27	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
(8) 22.3.31 & .33	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
22.3.32	V	A	A	V	V	V	A	*A1A2a4a5a6A8*
22.3.34 & .36	V	V	V	H	V	A	V	*a1a2a4a5A6a8*
22.3.35 & .37	V	A	A	H	A	V	V	*A1A2a4A5a6a8*
(9) 22.3.41 & .46	V	A	A	A	A	V	A	*A1A2a4A5a6A8*
22.3.42 & .44	V	V	V	H	V	A	V	*a1a2a4a5A6a8*
22.3.45	V	V	V	A	A	A	A	*a1a2a4A5A6A8*
22.3.47 & .48	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
(10) 22.3.51 & .52	V	A	A	H	A	V	A	*A1A2a4A5a6A8*
22.3.53 & .54	V	A	A	V	V	V	V	*A1A2a4a5a6a8*
22.3.55 & .57	V	V	V	H	V	A	V	*a1a2a4a5A6a8*
22.3.56 & .58	V	V	V	A	A	A	A	*a1a2a4A5A6A8*

[a] R genes between brackets are putative R genes deduced from the present study.

[b] Falcon-MX is a *B. napus* recombinant line with the *B. juncea* R gene *Jlm1* (12) termed *Rlm6* in the present study.

[c] *Ai*, presence of the avirulent allele at the *AvrLmi* locus; *ai*, presence of the virulent allele at the *AvrLmi* locus.

[d] Isolate 20.3.04 is a F1 progeny from cross IBCN18 × 11.26.14 (Table 2).

[e] A, the isolate is avirulent; V, the isolate is virulent; H, the interaction phenotype is heterogeneous, with a significant (more than 20%) percentage of susceptibility symptoms (infection class (IC)4 to IC6), but however a majority of resistance responses (IC1 to IC3).

[f] Each tetrad is identified by its number, indicated between brackets. Isolates on the same line are twin isolates, as identified following pulsed field gel electrophoresis.

AvrLm1-*AvrLm2* (one isolate). Thus, only 1 recombinant isolate out of 67 was identified between *AvrLm1* and *AvrLm2* in cross 29, confirming the tight linkage (1.5 cM) between these two loci. No recombinant isolates between *AvrLm2* and *AvrLm6* could be recovered in cross 29.

Cross 34 was set up to include *AvrLm4* in the linkage analysis. Segregation data confirmed the single gene control of *AvrLm1*, *AvrLm2*, *AvrLm4*, *AvrLm5*, and *AvrLm8* (Table 6). In contrast, a 75:25 A/V segregation ratio was observed on Falcon-MX, suggesting that in this cross two genes controlled avirulence on Falcon-MX (Table 6). As in cross 20, all *AvrLm4* isolates (i.e., avirulent on Jet Neuf) were also avirulent on Falcon-MX, but *avrLm4* isolates (i.e., virulent on Jet Neuf) were 50:50 A/V on Falcon-MX (16A:18V; chi-square = 0.118; $P = 0.732$). These data are consistent with the hypothesis that the digenic control of avirulence on Falcon-MX in cross 34 is due to the presence of both *Rlm4* and *Rlm6* in this line, as suggested from cross 20 segregation data. Consequently, the 50:50 A/V ratio of *avrLm4* isolates on Falcon-MX indicates that *AvrLm6* and *AvrLm4* are unlinked. Because of the presence of *Rlm4* in Falcon-MX, the genotype of *AvrLm4* isolates at the *AvrLm6* locus could not be inferred and, for these isolates, data at the *AvrLm6* locus were considered missing data for linkage analysis. In cross 34, five recombination events (out of 61) were detected between *AvrLm1* and *AvrLm2* (8.7 cM), whereas only one recombination event was observed between *AvrLm6* and *AvrLm1*, and two between *AvrLm6* and *AvrLm2*. Mapmaker analysis of cross 34 segregation data indicated that *AvrLm6* mapped between *AvrLm1* (2.9 cM) and *AvrLm2* (5.8 cM). Linkage analysis from crosses 29 and 34 further indicated that, conversely to the cluster formed by *AvrLm1*, *AvrLm2*, and *AvrLm6*, the AVR loci *AvrLm4*, *AvrLm5*, and *AvrLm8* were all independent from each other and from the *AvrLm1* region.

Novel specific interactions toward *B. napus*: *AvrLm3* specifies incompatibility between IBCN78 or IBCN79 and Glacier. Isolates IBCN78 and IBCN79 displayed a new specific interaction because they were avirulent on cv. Glacier but virulent on cv. Bristol (Table 3). In addition, these isolates were the only ones to induce a resistance response in *B. napus* line 22-1-1 (Table 3). We thus postulated the occurrence of a new interaction involving one AVR allele present in IBCN78 and IBCN79, termed *AvrLm3*, and one resistance gene, *Rlm3*, present in Glacier and 22-1-1, but not in Bristol (Tables 7 and 8). To check this hypothesis, two crosses were analyzed. In cross 35, isolate IBCN78 was crossed with v23.1.11 (*AvrLm1*-*AvrLm4*) (Tables 1 to 3 and 7). The entire progeny was virulent on Bristol, as expected under the hypothesis that none of the parental isolates possessed *AvrLm2* (Table 7). The progeny was 100% avirulent on Doublol and Columbus, suggesting that IBCN78 also possessed *AvrLm1*. A nonexclusive hypothesis, however, would be that Doublol and Columbus also possessed the putative R gene *Rlm3*, and that *AvrLm1* and the putative

TABLE 6. Segregation analysis of avirulence of *Leptosphaeria maculans* toward resistance sources present in *Brassica juncea* and *B. rapa*

	Brassica lines and known or putative resistance genes[a]							
	B. napus				*B. juncea*			*B. rapa*
	Westar	Bristol Glacier *Rlm2*	Jet Neuf *Rlm4*	Vivol Capitol Columbus *Rlm1* (*Rlm3*)	Picra (*Rlm5*) (*Rlm6*)	150-2-1 151-2-1 (*Rlm5*)	Falcon-MX (*Rlm4*) (*Rlm6*)	156-2-1(*Rlm8*)
Cross 24								
Parental isolates								
22.2.03	V[b]	V	V	A	A	V	A	A
v11.1.5	V	V	V	A	A	A	A	A
Number of progeny tested	33	20	0	56	28	43	0	0
A/V isolates in the progeny[c]	0:33	0:20	nd[d]	56:0	28:0	22:21	nd	nd
Chi-square (50:50)[e]	–	–	–	–	–	0.023	–	–
P value	–	–	–	–	–	0.879 NS[f]	–	–
Cross 29								
Parental isolates								
22.2.02	V	A	V	V	A	A	V	V
22.2.03	V	V	V	A	A	V	A	A
Number of progeny tested	67	67	0	67	51	62	67	67
A/V isolates in the progeny	0:67	32:35	nd	36:31	45:6	37:25	35:32	30:37
Chi-square (50:50)	–	0.134	–	0.373	29.8	2.323	0.134	0.731
P value	–	0.714 NS	–	0.541 NS	<0.001***	0.128 NS	0.714 NS	0.392 NS
Chi-square (75:25)	–	26.5	–	16.16	4.76	7.76	18.51	32.64
P value	–	<0.001***	–	<0.001***	0.029*	0.005**	<0.001***	<0.001***
Cross 34								
Parental isolates								
v29.3.1	V	A	V	V	V	V	V	V
v23.1.3	V	V	A	A	A	A	A	A
Number of progeny tested	61	61	61	61	7[g]	59	61	59
A/V isolates in the progeny	0:61	32:29	27:34	28:33	4:3	35:24	43:18	31:28
Chi-square (50:50)	–	0.148	0.803	0.410	0.143	2.051	10.24	0.153
P value	–	0.701 NS	0.370 NS	0.522 NS	0.705 NS	0.152 NS	0.001*	0.696 NS
Chi-square (75:25)	–	16.54	30.74	27.55	1.19	7.73	0.661	15.87
P value	–	<0.001***	<0.001***	<0.001***	0.27 NS	0.005**	0.416 NS	<0.001***

[a] R genes between brackets are putative R genes deduced from the present study.
[b] A, the isolate is avirulent; V, the isolate is virulent.
[c] The values given are number of isolates in each phenotypic class (A or V).
[d] nd, the progeny has not been characterized on this line.
[e] The expected A/V ratio for the tested hypothesis is indicated between brackets (50:50, single gene control; 75:25, digenic control with two unlinked genes).
[f] NS, the observed ratio is not significantly different from the tested hypothesis at a 5% level; *, **, and ***, the observed ratio is significantly different from the expected ratio under the chosen hypothesis at the 5, 1, and 0.1% level, respectively; –, the interaction phenotype on this line or cultivar is not segregating in this cross.
[g] Only isolates from cross 34 that were virulent on line 150-2-1 but avirulent on both Jet Neuf and Falcon-MX were tested on *B. juncea* cv. Picra.

AvrLm3 allele are linked enough to prevent the recovery of recombinant isolates within the limited number of progeny analyzed. The avirulent phenotype of IBCN78 on both Glacier and 22-1-1 was inherited with a ratio fitting the 50:50 expected ratio for a single gene control (Table 7). All avirulent progeny on Glacier were also avirulent on line 22-1-1. In addition, the avirulent phenotype of the parental isolate v23.1.11 on Jet Neuf was inherited as a single gene, as expected if only v23.1.11 possessed the AVR allele *AvrLm4* (Table 7). Finally, no recombinant isolates, displaying either avirulence on Glacier, Jet Neuf, and 22-1-1 or virulence on these three lines, could be recovered, because only the two parental phenotypic classes were detected among the progeny (data not shown). All these data indicated that one AVR locus is involved in the genetic control of avirulence of IBCN78 on Glacier and 22-1-1, but that this locus is tightly linked (or identical) to the *AvrLm4* locus. Because *AvrLm1* and *AvrLm4* are unlinked (7; this study), *AvrLm1* and *AvrLm3* must also be independent. Therefore, the lack of virulent isolates on Doublol and Columbus in cross 35 progeny strongly suggests that IBCN78 also possesses the *AvrLm1* allele.

Cross 19 (IBCN79 × H5) progeny was recovered as tetrads. Only one complete tetrad and six incomplete tetrads (i.e., in which

TABLE 7. Segregation analysis of the differential interaction of the *Leptosphaeria maculans* isolate IBCN78 toward the *Brassica napus* cvs. Bristol and Glacier

	Brassica lines and known or putative resistance genes[a]				
	Westar (*none*) Bristol *Rlm2*	Glacier *Rlm2* (*Rlm3*) 22-1-1 (*Rlm3*)	23-1-1 (*Rlm7*)	Jet Neuf *Rlm4*	Columbu, Doublol *Rlm1* (*Rlm3*)
Cross 35–parental isolates					
IBCN78	V[b]	A	V	V	A
v23.1.11	V	V	A	A	A
Number of progeny tested	39	39	39	39	39
A/V isolates in the progeny[c]	0:39	18:21	21:18	21:18	39:0
Chi-square[d]	–	0.231	0.231	0.231	–
P value	–	0.631 NS[e]	0.631 NS	0.631 NS	–

[a] R genes between brackets are putative R genes deduced from the present study.
[b] A, the isolate is avirulent; V, the isolate is virulent.
[c] The values given are number of isolates in each phenotypic class A (avirulent) or V (virulent).
[d] Chi-square value for the single gene control hypothesis (50:50 A/V ratio).
[e] NS, the observed ratio is not significantly different from the tested hypothesis at a 5% level; –, the interaction phenotype on this line or cultivar is not segregating in this cross.

TABLE 8. Tetrad analysis of avirulence segregation of *Leptosphaeria maculans* progeny from cross 19 (IBCN79 × H5) on a range of *Brassica napus* lines

	Brassica lines and known or putative resistance genes[a]					
	Jet Neuf, *Rlm4*, Bristol, *Rlm2*	QuintaV *Rlm1 Rlm4*	Doublol, Columbus *Rlm1* (*Rlm3*)	Glacier, *Rlm2* (*Rlm3*) 22-1-1 (*Rlm3*)	23-1-1 (*Rlm7*)	Putative genotype of the isolates[b]
Parent isolates						
IBCN79	V[c]	A	A	A	V	*A1A3a4a7*
H5	V	V	V	V	A	*a1a3a4A7*
Progeny[d]						
(1) 19.1.1 & 3	V	V	V	V	A	*a1a3a4A7*
19.1.2 & 5	V	A	A	V	A	*A1a3a4A7*
19.1.6 & 4	V	V	A	A	V	*a1A3a4a7*
Missing genotype						*A1A3a4a7*
(2) 19.2.1 & 6	V	V	A	A	V	*a1A3a4a7*
19.2.2 & 4	V	A	A	V	A	*A1a3a4A7*
19.2.3 & 5	V	A	A	V	A	*A1a3a4A7*
Missing genotype						*a1A3a4a7*
(3) 19.2.11 & 13	nd	A	A	A	V	*A1A3a4a7*
19.2.12 & 15	V	A	A	A	V	*A1A3a4a7*
19.2.14 & 16	V	V	V	V	A	*a1a3a4A7*
Missing genotype						*a1a3a4A7*
(4) 19.4.1	V	V	V	V	A	*a1a3a4A7*
19.4.2 & 3	V	A	A	A	V	*A1A3a4a7*
19.4.4 & 5	V	A	A	A	V	*A1A3a4a7*
19.4.7 & 6	V	V	V	V	A	*a1a3a4A7*
(5) 19.4.21 & 22	V	A	A	V	A	*A1a3a4A7*
19.4.23 & 24	V	V	A	A	V	*a1A3a4a7*
19.4.25 & 26	V	V	A	A	V	*a1A3a4a7*
Missing genotype						*A1a3a4A7*
(6) 19.4.31 & 33	V	A	A	V	A	*A1a3a4A7*
19.4.32 & 35	V	A	A	A	V	*A1A3a4a7*
19.4.34 & 36	V	V	V	V	A	*a1a3a4A7*
Missing genotype						*a1A3a4a7*
(7) 19.4.41 & 43	V	A	A	V	A	*A1a3a4A7*
19.4.42 & 44	V	V	V	V	A	*a1a3a4A7*
19.4.45 & 46	nd	V	A	A	V	*a1A3a4a7*
Missing genotype						*A1A3a4a7*

[a] R genes between brackets are putative R genes deduced from the present study.
[b] *Ai*, presence of the avirulent allele at the *AvrLmi* locus; *ai*, presence of the virulent allele at the *AvrLmi* locus.
[c] A, the isolate is avirulent; V, the isolate is virulent; nd, not determined.
[d] Each tetrad is identified by its number, indicated between brackets. Isolates on the same line are twin isolates, as identified following pulsed field gel electrophoresis.

one of the four products of meiosis was missing) were recovered (Table 8). The entire progeny was virulent on Jet Neuf and Bristol, which confirmed that both parents possess the virulent alleles *avrLm2* and *avrLm4* (Table 8). All interactions segregating in cross 19 segregated 2:2 in the complete tetrad (tetrad 4) (Table 8). Although incomplete, all other tetrads displayed segregation data that are consistent with a 2:2 A/V ratio on both QuintaV and the two putative *Rlm3* genotypes. In contrast, such a 2:2 segregation was not observed on Columbus and Doublol, in at least two of the six incomplete tetrads (i.e., tetrads 2 and 5) (Table 8), because the three recovered genotypes of each of these two tetrads were all avirulent on these lines. In addition, recombinant isolates, i.e., virulent on QuintaV but avirulent on Columbus and Doublol, were recovered in tetrads 1, 2, 5, and 7 (Table 8). Finally, all progeny isolates from cross 19 that were avirulent on either QuintaV or on the two putative *Rlm3* lines were also avirulent on Columbus. All these data support the hypothesis that *AvrLm3* is present in IBCN79 and matches *Rlm3* present in 22-1-1 and Glacier, but also that (i) *AvrLm1* is present in IBCN79 and (ii) *Rlm3* is present in Columbus and Doublol, but not in QuintaV. Under these hypotheses, the recovery of the recombinant genotypes *AvrLm1-avrLm3* and *avrLm1-AvrLm3* in four of seven tetrads confirms the genetic independence between *AvrLm1* and *AvrLm3*.

Novel specific interactions toward *B. napus*: virulence of isolates Nz-T4, IBCN78, and IBCN79 on *B. napus* resistance sources is under a single gene control. Screening of *B. napus* gene banks for resistance to European PG4 isolates, i.e., having virulent alleles at the *AvrLm1*, *AvrLm2*, and *AvrLm4* loci, allowed us to select five plant genotypes, from diverse geographic origin, that displayed an identical interaction phenotype, i.e., resistance to PG4 isolates and susceptibility to Nz-T or its single-conidia progeny Nz-T4 (lines 149-2-1, 148-1-1, 05-1-1, 04-1-2, and 23-1-1) (Table 3). Most of these lines were also susceptible to IBCN78 and IBCN79 (Table 3), suggesting both that avirulence on these resistance sources may be under a simple genetic control and that all these lines may possess the same R gene. The progeny of cross 30 (Nz-T4 × v23.1.2) was analyzed for avirulence on selected genotypes 148-1-1, 149-2-1, and 23-1-1. All progeny analyzed displayed the same interaction phenotype on each of these lines, with an A/V ratio fitting the 50:50 ratio expected for a single gene control of avirulence (Table 9). It was thus postulated that one single locus governs avirulence on these new resistant lines. The corresponding putative gene pair was termed *AvrLm7/Rlm7*. To further analyze the genetic linkage between this locus and *AvrLm1* and *AvrLm4*, cross 33 was performed between isolate Nz-T4 and the *AvrLm1-AvrLm4* isolate, v23.1.11 (Tables 1 to 3). Avirulence toward Jet Neuf, Columbus, and the *B. napus* lines 148-1-1 and 23-1-1 all segregated in cross 33 as monogenic traits (Table 9). Four phenotypic classes were observed among cross 33 progeny, i.e., the two parental phenotypic classes, and two phenotypic classes showing recombination between avirulence toward Columbus (due to the interaction between *AvrLm1* present in isolate v23.1.11 and *Rlm1* in Columbus) and avirulence toward both Jet Neuf and lines 148-1-1 and 23-1-1 (Table 10). These four phenotypic classes were recovered in proportions fitting the 25:25:25:25 ratio (chi-square = 2.17; df = 3; P = 0.538 NS). No recombinant isolates were recovered between avirulence toward Jet Neuf, and virulence on 148-1-1 and 23-1-1 (Table 10). Segregation data from cross 33 thus confirmed the single gene control of avirulence on lines 148-1-1 and 23-1-1, but also indicated that the corresponding locus is independent from *AvrLm1*, but tightly linked (or allelic) to *AvrLm4*.

In independent crosses 19 and 35, the parental isolates IBCN78 and IBCN79 displayed virulence on resistant lines 23-1-1, 148-1-1, and 149-2-1 (Table 3). As in cross 33, specific interaction on 23-1-1 was under a single gene control (Tables 7 and 8; data not shown). In cross 35, the corresponding locus also appeared to be tightly linked, or identical, to the *AvrLm4* locus because no recombinant isolates could be recovered in the progeny. Avirulence on line 23-1-1 also segregated in cross 19 (Table 8) with a 2:2 ratio in the complete tetrad, and with a 1:2 or 2:1 ratio in incomplete tetrads, i.e., still fitting a single gene control. In cross 19, *AvrLm7* was tightly linked, or identical, to the *AvrLm3* locus (Table 8).

DISCUSSION

In this paper, we report on the genetic identification of five novel avirulence genes or alleles: (i) *AvrLm3*, specifying incompatibility on cv. Glacier but genetically independent from *AvrLm2*; (ii) two independent loci, *AvrLm5* and *AvrLm6*, inducing resistance on *B. juncea*; (iii) *AvrLm7*, specifying incompatibility on a novel *B. napus* resistance source to PG4-A1 (i.e., *avrLm1-*

TABLE 9. Segregation analysis of the differential interaction of the *Leptosphaeria maculans* isolate Nz-T4 toward a range of *Brassica napus* cultivars or lines

	Brassica lines and known or putative resistance genes[a]					
	Westar	Bristol *Rlm2*	149-2-1 (*Rlm1*) (*Rlm7*)	148-1-1, 23-1-1 (*Rlm7*)	Jet Neuf *Rlm4*	Columbus *Rlm1* (*Rlm3*)
Cross 30 parental isolates						
Nz-T4	V[b]	V	V	V	V	V
v23.1.2	V	V	A	A	V	V
Number of progeny tested	66	nd[c]	66	64	nd	nd
A/V isolates in the progeny[d]	0:66	–	36:30	34:30	–	–
Chi-square value (50:50 ratio)[e]	–	–	0.545	0.250	–	–
P value	–	–	0.46 NS[f]	0.617 NS	–	–
Cross 33 parental isolates						
Nz-T4	V	V	V	V	V	V
v23.1.11	V	V	A	A	A	A
Number of progeny tested	32	33	nd	48	48	48
A/V isolates in the progeny	0:32	0:33	–	27:21	27:21	28:20
Chi-square value (50:50 ratio)	–	–	–	0.75	0.75	1.333
P value	–	–	–	0.386 NS	0.386 NS	0.248 NS
Chi-square value (75:25 ratio)	–	–	–	8.999	8.999	7.111
P value	–	–	–	0.0027 **	0.0027 **	0.007 **

[a] R genes between brackets are putative R genes deduced from the present study.
[b] A, the isolate is avirulent; V, the isolate is virulent.
[c] nd, segregation analyses not performed on this *Brassica* line.
[d] The values given are number of isolates in each phenotypic class (A or V).
[e] The expected A/V ratio for the tested hypothesis is indicated between brackets (50:50, single gene control; 75:25, digenic control with two unlinked genes).
[f] NS, the observed segregation is not significantly different from the expected one; **, the observed segregation is significantly different from the expected one (1% level).

avrLm2-avrLm4) isolates; and (iv) *AvrLm8*, recognizing a resistance source to PG4-A1 isolates present in *B. rapa*.

Although numerous interactions were described between *L. maculans* and *B. napus* cultivars or lines (9,19,20,23,24), only a few of them were subjected to genetic analyses (1,2,7,16). One main difficulty that probably slowed down such analyses was the frequent occurrence of numerous AVR alleles in field *L. maculans* isolates and numerous R genes, with epistatic action, in *B. napus* cultivars. The presence of both *Rlm1* and *Rlm4* in some, but not all, *B. napus* cv. Quinta-derived lines was reported previously (7). Similarly, it is suggested from the present study that numerous commercial cultivars possess both *Rlm1* and *Rlm3*, whereas the differential cv. Glacier possesses *Rlm2* and *Rlm3*. On the fungal side, most of the field isolates subjected to genetic analyses possess more than one AVR allele. For instance, PHW1245 possesses at least *AvrLm1*, *AvrLm2*, *AvrLm4*, and *AvrLm5-AvrLm8*. The four AVR alleles *AvrLm1*, *AvrLm2*, *AvrLm4*, and *AvrLm7* were identified in isolate IBCN18. Similarly, European PG4 isolates typically possess four AVR alleles (*AvrLm5* to *AvrLm8*). One of the main deliverables of the present study is the obtainment, following in vitro crosses, of isolates with a limited number of AVR alleles. This was the case for isolate 22.2.02, which possesses only the AVR alleles *AvrLm2*, *AvrLm5*, and *AvrLm7*. To our knowledge, this is the first record of genetic characterization of one isolate being avirulent on cv. Glacier (and Bristol and other cultivars possessing *Rlm2*) but virulent on cv. Quinta, which constitutes a very useful tool to identify the presence of *Rlm2* in *B. napus* cultivars or segregating populations. Similarly, we genetically characterized a few *avrLm1-avrlm2-AvrLm3-avrlm4* isolates, in progeny of cross 19, which will help us identify the presence of *Rlm3* in *B. napus* lines, and which will be used to map this gene. However, additional in vitro crosses are still needed to obtain *L. maculans* isolates possessing only one AVR allele for all AVR loci described here, in order to further improve the *L. maculans* differential set.

Five *B. napus* lines were independently selected as new sources of resistance to European PG4-A1 isolates. Three isolates from diverse geographic origin (Nz-T, IBCN78, and IBCN79) were however virulent on these lines. Genetic studies demonstrated that (i) the virulence of all three isolates on these lines was under a simple genetic control; and (ii) the corresponding loci mapped identically in the three isolates (i.e., cosegregating with *AvrLm4* and/or *AvrLm3*). Although allelic tests were not performed between these three isolates, it can thus be hypothesized that Nz-T, IBCN78, and IBCN79 all possess the same virulence allele, termed *avrLm7*, interacting with the putative R gene, *Rlm7*, present in all these lines. These results support the assumption that the Nz-T isolate belongs to a new race of *L. maculans*, as proposed by Gowers and Armstrong (19).

Although differential interactions between *B. rapa* lines and *L. maculans* isolates were described previously (6,22–24), the genetic control of these interactions was not elucidated. Mainly, screening of worldwide collections of *B. rapa* for resistance to PG3 and PG4 isolates indicated a continuous variation in *L. maculans* resistance, with no plant genotypes being completely susceptible or completely resistant to either pathotypes (27). However, the same authors demonstrated that after four generations of selection for resistance to a PG4 isolate, using the cotyledon inoculation test, the mean disease severity shifted from 8.11 to 2.42, on a 0-to-9 scale, with a clear bimodal distribution of IC after the third selection generation (27). These results clearly indicate that major resistance genes to *L. maculans* could be present, although at low frequencies, in *B. rapa* lines. The *B. rapa* line 156-2-1 described in the present study was obtained similarly, because 1 resistant plant out of 12 was initially selected from a *B. rapa* commercial cultivar. After two selfing/selection generations, the resulting line contained more than 95% of resistant plants (data not shown). The genetic control of avirulence to *B. rapa* was clearly monogenic and the corresponding locus, termed *AvrLm8*, was genetically unlinked to any other *L. maculans* AVR gene.

To date, the *L. maculans*–*B. juncea* interaction was mainly genetically analyzed on the plant side, and depending on the authors, a single, digenic, or trigenic control was reported (13,21,31,37). The occurrence of two independent R genes in *B. juncea* was supported by genetic analysis of resistance to *L. maculans* either from segregating populations, including DH lines, or from addition or recombination lines (12,21,31). Analysis of F1, F2, and F3 from three independent crosses between resistant and susceptible *B. juncea* accessions demonstrated the occurrence of two nuclear genes, one dominant and one recessive with epistatic action, that control the *B. juncea* resistance to *L. maculans* (21). It was thus expected to find at least two AVR genes from the pathogen, each one recognizing one of these R genes, especially when the parent isolate used was virulent on many unrelated *B. juncea* cultivars, such as Picra, Aurea (this study), Stoke, and Zaria (11) along with 25 additional *B. juncea* accessions from diverse geographic origin (30). The genetic analyses presented here indicate that the *L. maculans*–*B. juncea* interaction is controlled by two pairs of AVR/R genes, i.e., *AvrLm5*/*Rlm5* and *AvrLm6*/*Rlm6*, with epistatic action between the two R genes. Our results suggest that *AvrLm6* interacts with the *B. juncea* locus *Jlm1* (termed *Rlm6* in the present study), introgressed into the Falcon-MX *B. napus* line (12). In contrast, we cannot yet establish whether *AvrLm5* interacts with the resistance source described in *B. nigra* addition lines and located on chromosome B4 (12,13) or with another R gene from *B. juncea*.

The virulence of isolate IBCN18 on *B. juncea* was previously genetically analyzed and a single gene control of virulence was described (11,14). Our results therefore could appear to contradict those from Chen et al. (11), because we found three independent virulent alleles in IBNC18, two of them at least interacting with R genes present in *B. juncea* cultivars. Two hypotheses could however explain this apparent discrepancy. The first one would be that only one of the two putative *B. juncea* R genes, *Rlm5* or *Rlm6*, was present in the *B. juncea* cv. Stoke used by Chen et al. (11) to characterize their progeny. In the present study, we obtained progeny isolates possessing either the virulent or the avirulent allele for each locus *AvrLm5* and *AvrLm6*, which can now be used to test this hypothesis. The second hypothesis would be that the three alleles *AvrLm5*, *AvrLm6*, and *AvrLm8* were not all segregating in the IBCN17 × IBCN18 cross studied by Chen et al. (11). We demonstrated that *AvrLm5* and *AvrLm8* were indeed segregating in the IBCN17 × IBCN18 cross, as revealed by the incompatible interaction obtained between IBCN17 and lines 150-2-1, 151-2-1, and 156-2-1 (data not shown). However, we could not conclude this as regards *AvrLm6*, because isolate IBCN17 was avirulent on Falcon-MX, but also on Jet Neuf and Falcon (data not shown),

TABLE 10. Interaction phenotypes of *Leptosphaeria maculans* progeny isolates from cross 33 (Nz-T4 × v23.1.11) on a range of *Brassica napus* cultivars or lines

	B. napus lines and known or putative resistance genes[a]			
	148-1-1, 23-1-1 (*Rlm7*)	Jet Neuf *Rlm4*	Columbus *Rlm1* (*Rlm3*)	No. of isolates
Parental isolates				
Nz-T4	V[b]	V	V	
v23.1.11	A	A	A	
Phenotypic class				
I	A	A	A	15
II	A	A	V	12
III	V	V	A	13
IV	V	V	V	8

[a] R genes between brackets are putative R genes deduced from the present study.
[b] A, the isolate is avirulent; V, the isolate is virulent.

indicating that IBCN17 possesses *AvrLm4*. This prevented us from characterizing IBCN17 genotype at the *AvrLm6* locus due to the presence of both *Rlm4* and *Rlm6* in Falcon-MX (Table 4). If IBCN17 actually possesses the *avrLm6* allele, then only *AvrLm5* (and *AvrLm8*) segregates in the cross-analyzed by Chen et al. (11), and consequently the virulence locus mapped by Cozjinsen et al. (14) probably corresponds to *AvrLm5*. This is further supported by the physical mapping of this virulence locus on a chromosome whose size ranged from 1.80 to 1.85 Mb (14), whereas *AvrLm6* was found in the present study to be closely linked to *AvrLm1*, which is located on a chromosome whose size ranged from 2.48 to 3.10 Mb, depending on the isolate (3).

Linkage between *AvrLm1* and *AvrLm2* (2) was confirmed in the present study, and the genetic distance between the two loci was estimated to range from 1.5 cM (cross 29) to 8.7 cM (cross 34). Linkage analyzes demonstrated that *AvrLm6* is tightly linked to, but not an allele of, *AvrLm1* and *AvrLm2*, and is localized between *AvrLm1* and *AvrLm2*. The *AvrLm1* region has thus to be considered as a cluster of AVR genes, with three AVR genes in less than 10 cM. Importantly, the *AvrLm1-AvrLm2-AvrLm6* cluster contains AVR genes corresponding to R genes originating not only from the *B. napus* species, but also from the related species *B. juncea.* AVR gene clusters have been described for only a few phytopathogenic fungi, such as *Magnaporthe grisea*, *Phytophthora infestans*, and *P. sojae* (15,33,35), but clusters of AVR genes are reported in *L. maculans* for the first time. An identical situation may also exist at the *AvrLm4* locus because we were not able, in the present study, to establish whether *AvrLm3*, *AvrLm4*, and *AvrLm7* were three different allelic forms of one single locus or whether they are truly distinct, but clustered, AVR genes. The lack of recombination between *AvrLm3*, *AvrLm4*, and *AvrLm7* has to be interpreted carefully, because for all crosses performed to analyze linkage between them, a relatively small number of progeny isolates was analyzed. In addition, crosses 19, 30, 33, and 35 in which *AvrLm3*, *AvrLm4*, and/or *AvrLm7* were segregating, were all F1 crosses. In contrast, crosses 22, 24, and 29, for which recombination between loci *AvrLm1*, *AvrLm2*, and *AvrLm6* was observed, were BC1 or F2. Due to the well-known chromosome size polymorphism in *L. maculans* (26,28), it can be hypothesized that in F1 progeny from crosses between very genetically distant isolates (such as crosses 19, 30, 33, and 35 which all involved two isolates from different continents), the genome organization of the two parental isolates is so different that recombination cannot occur as frequently as in more inbred isolates (5). Additional BC are underway to further analyze the linkage between the putative genes *AvrLm3*, *Avrlm4*, and *AvrLm7*.

Finally, linkage analyses demonstrated that at least four independent regions of the *L. maculans* genome, i.e., the *AvrLm1-AvrLm2-AvrLm6* cluster, the *AvrLm3-AvrLm4-AvrLm7* region, *AvrLm5*, and *AvrLm8*, are involved in host specificity toward *B. napus*, *B. rapa*, or *B. juncea*. Molecular mapping of these AVR genes using the segregating populations described in the present study is in progress and will allow us to identify, among the 18 linkage groups identified to date in the *L. maculans* maps (5), which ones are bearing genes involved in host specificity.

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