Genetic Characterization of *AvrLm1*, the First Avirulence Gene of *Leptosphaeria maculans*

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This research was supported by a grant from CETIOM. D. Ansan-Melayah was funded by a grant from the Ministère de l'Enseignement supérieur et de la Recherche.

We thank A. El Biari (Pathologie Végétale, INRA, Versailles, France) and A. M. Chèvre (Amélioration des Plantes, INRA, Le Rheu, France) for help in establishing the RAPD protocol, A. Lauth (Pathologie Végétale, INRA, Versailles, France) for her technical assistance, and B. Letarnec and L. Coudard (Pathologie Végétale, INRA, Versailles, France) for plant management. We also thank H. H. Hoppe (Institut für Pflanzenpathologie und Pflanzenschutz, Göttingen, Germany), G. Séguin-Swartz (Agriculture Canada, Saskatoon, Canada), and H. K. Love (Svalof Weibull Seed LTD, Lindsay, Canada) for supplying seeds of cvs. Quinta, Westar, and Glacier, respectively, and R. K. Khetarpal (Pathologie Végétale, INRA, Versailles, France) for review of the manuscript.

Accepted for publication 13 September 1995.

ABSTRACT

Ansan-Melayah, D., Balesdent, M. H., Buée, M., and Rouxel, T. 1995. Genetic characterization of *AvrLm1*, the first avirulence gene of *Leptosphaeria maculans*. Phytopathology 85:1525-1529.

Specific interactions of the fungal pathogen *Leptosphaeria maculans* with *Brassica napus* cultivars were observed when the cultivars were inoculated with isolates belonging to pathogenicity groups (PG) PG3 and PG4. PG3 isolates induced resistance responses on cotyledons or leaves of cv. Quinta, whereas PG4 isolates caused sporulating lesions on this cultivar. In contrast, both pathotypes caused disease symptoms on cvs. Westar and Glacier. The genetic basis of cultivar specificity was studied using tetrad analysis after in vitro crosses between one PG3 and one PG4 isolate. For the genetic study, the use of random amplified polymorphic DNA (RAPD) as genetic markers was assessed. Of 61 primers, 10 generated reproducible polymorphisms. Of these, 9 generated 18 RAPD markers displaying a 2:2 segregation ratio within the 10 analyzed tetrads. A 2:2 segregation ratio for avirulence/virulence to cv. Quinta also was observed in the progeny. Consequently, the single genetic locus controlling cultivar specificity on Quinta was considered the first avirulence gene described in *L. maculans* and was designated *AvrLm1*.

Additional keywords: blackleg disease, gene-for-gene relationship, *Phoma lingam*.

Plant-pathogen interactions are often determined by pairs of complementary genes of the host plant and pathogen. This gene-for-gene relationship states that for each dominant resistance gene conferring resistance in the host plant, there is a matching dominant avirulence gene in the pathogen (7). Combination of these alleles determines recognition events that lead to activation of the hypersensitive response (HR), which is the sum of a cascade of defense mechanisms (15). Avirulence gene characterization is, therefore, a powerful tool for better understanding of the molecular events associated with plant-pathogen interactions and for screening matching resistance genes in the host plant. For instance, transgenic tomato plants expressing the *Cladosporium fulvum* avirulence gene *Avr9* were used to clone the corresponding resistance gene *Cf-9* (11,13). In several plant-fungal pathogen systems, avirulence genes have been genetically characterized since the early 1940s (5). However, compared to bacterial pathogens in which more than 30 avirulence genes are already characterized at the molecular level, the cloning of only 5 avirulence genes from fungal pathogens has been reported (14,26).

The filamentous Ascomycete *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph *Phoma lingam* (Tode:Fr.) Desmaz.) is the causal agent of blackleg disease of oilseed rape (*Brassica napus* L.), a destructive disease with a worldwide distribution. Based on inoculation tests on cotyledons, specific interactions with *B. napus* cvs. Westar, Glacier, and Quinta have been described, allowing the discrimination of isolates into four pathogenicity groups (PG) (20). Under controlled conditions, PG4 isolates cause sporulating lesions on cvs. Westar, Quinta, and Glacier. PG3 isolates cause disease symptoms on Westar and Glacier, whereas they induce resistance reactions on cotyledons of Quinta. PG2 isolates are pathogenic on Westar, whereas they induce resistance responses on Glacier and Quinta. PG1 isolates, also termed Tox^0 isolates (4), are nonpathogenic on these differential hosts. However, PG1 isolates are now considered a distinct species (24).

B. napus resistance to *L. maculans* PG is likely to be controlled by single dominant genes (23). Evidence for the involvement of a single dominant resistance locus to PG2 isolates in cv. Major was recently documented (6). These data suggest that gene-for-gene interactions could be involved in this plant-pathogen system. However, the genetic control of pathogenicity grouping has not been established.

In vitro crossing and tetrad analysis are well established for *L. maculans* (9,21). However, only three genetic markers (mating type, soluble protein profiles, and acid phosphatase electrophoretic types) are currently available (9). More markers are needed to develop genetic analysis of pathogenicity in *L. maculans*. Molecular markers, such as random amplified polymorphic DNA (RAPD), are attractive because they require no prior information about genome sequences and are less laborious to set up compared to restriction fragment length polymorphisms (28). RAPD have been successfully used as genetic markers in other plant fungal pathogens (2, 19).

The present investigations were undertaken to establish the inheritance of PG3 isolate avirulence to cv. Quinta using tetrad analysis after a cross between one PG3 and one PG4 isolate. As a

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prerequisite to the genetic analysis, we assessed the use of RAPD to generate additional genetic markers.

MATERIALS AND METHODS

Fungal strains and culture maintenance. The a.2 and H5 Tox$^+$ isolates of *L. maculans* were obtained from single ascospores isolated from diseased *B. napus* stubble (9). These isolates were previously characterized according to mating type (Mat) and soluble protein pattern (SPP) (9). Isolates PHW1223 (PG4), PHW1243 (PG3), and PHW1245 (PG2), provided by P. H. Williams (University of Wisconsin, Madison), were used as PG references in inoculation tests. All fungal cultures were maintained on V8 agar medium as previously described (25).

In vitro crossing. In vitro crosses were performed between a.2 (Mat$^+$, SPP$^+$, and PG3) and H5 (Mat$^-$, SPP$^-$, and PG4) according to Gall et al. (9). Tetrads were recovered using a micromanipulation method (9). Five tetrads were obtained previously (9), and six additional tetrads, designated 11.x.yz, were separated from three different pseudothecia in an independent experiment (Table 1).

TABLE 1. Characterization of the progeny of the cross between *Leptosphaeria maculans* isolates a.2 (Mat$^+$, SPP$^+$, and PG3) and H5 (Mat$^-$, SPP$^-$, and PG4)[x]

Isolate number	Mating type	Protein marker[y]	Pathogenicity group	Genotype[z]
v.1 and v.8	–	–	PG3	a (*)
v.2 and v.4	–	+	PG3	b
v.3 and v.5	+	+	PG4	c
v.6 and v.7	+	–	PG4	d
w.1 and w.8	–	–	PG4	a (**)
w.2 and w.7	+	+	PG3	b
w.3 and w.6	–	–	PG4	c
w.4 and w.5	+	+	PG3	d
s.1 and s.4	–	–	PG4	a (**)
s.2 and s.5	–	–	PG3	b
s.3	+	+	PG4	c
s.6	+	+	PG3	d
t.1	–	+	PG3	a (*)
t.2	+	–	PG4	b
t.4	–	–	PG4	c
t.5 and t.6	+	+	PG3	d
11.1.02	+	–	PG3	a (*)
11.1.03 and 11.1.04	+	+	PG4	b
11.1.05 and 11.1.07	–	–	PG4	c
11.1.06 and 11.1.08	–	+	PG3	d
11.1.11 and 11.1.13	–	+	PG3	a (*)
11.1.12 and 11.1.14	–	–	PG4	b
11.1.15 and 11.1.16	+	+	PG4	c
11.1.18	+	–	PG3	d
11.25.01 and 11.25.03	–	–	PG3	a (**)
11.25.02	+	+	PG4	b
11.25.04 and 11.25.07	+	+	PG4	c
11.25.05 and 11.25.06	–	–	PG3	d
11.26.01 and 11.26.04	–	–	PG4	a (**)
11.26.02 and 11.26.03	+	+	PG4	b
11.26.05	+	+	PG3	c
11.26.06 and 11.26.07	–	–	PG3	d
11.26.11 and 11.26.16	+	–	PG3	a (*)
11.26.12 and 11.26.13	–	–	PG3	b
11.26.14 and 11.26.18	+	+	PG4	c
11.26.15 and 11.26.17	–	+	PG4	d
11.26.21 and 11.26.23	–	–	PG4	a (*)
11.26.22	+	–	PG3	b
11.26.24 and 11.26.25	+	+	PG4	c
11.26.26 and 11.26.27	–	+	PG3	d

[x] Mat = mating type; SPP = soluble protein pattern; and PG = pathogenicity group.
[y] + = SPP$^+$ phenotype; – = SPP$^-$ phenotype.
[z] Genotypes within each tetrad were determined by combination of Mat/SPP (*) or random amplified polymorphic DNA markers (**).

Conidia production. A plug of agar medium was cut out of the periphery of an actively growing mycelial culture and was rubbed on the surface of V8 agar plates. Cultures were maintained 12 days at 28°C. Light (50 µE m^{-2} s^{-1}) was supplied by white and near-UV tubes (Sylvania, Puteaux, France), with a 12-h photoperiod. Conidia were collected as previously described (25).

RAPD protocol. *DNA extraction.* Conidia (10^8) were inoculated in 50 ml of filtered V8 medium (10%, vol/vol, V8 juice and 30 mM $CaCO_3$). Liquid cultures were incubated for 5 days at 20°C on a rotary shaker at 108 rpm. Mycelia were harvested by vacuum filtration on sterile muslin, frozen at –20°C, and freeze-dried. DNA was extracted from freshly freeze-dried mycelium ac-

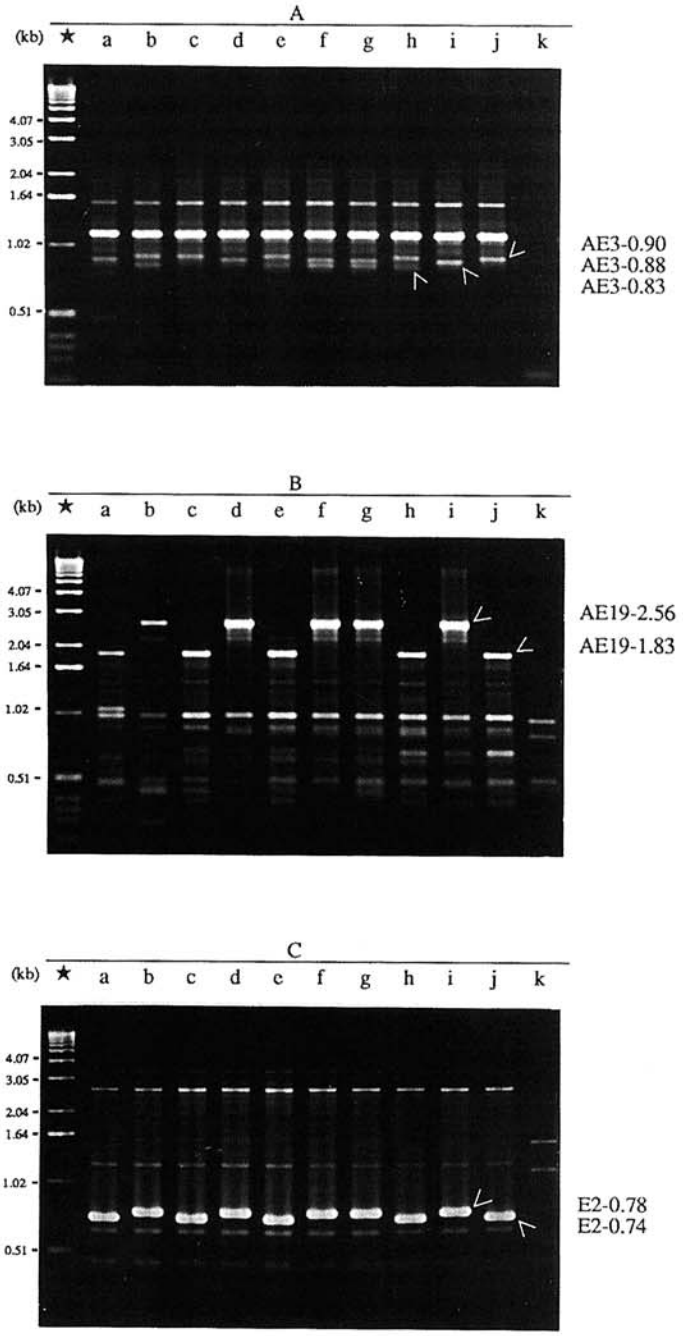

Fig. 1. Gel electrophoresis of polymerase chain reaction products generated by primers **A,** AE3, **B,** AE19, and **C,** E2. Parental isolates of *Leptosphaeria maculans*, lanes a, a.2 and b, H5, and one of their progeny, tetrad w: lanes c, w.1; d, w.2; e, w.3; f, w.4; g, w.5; h, w.6; i, w.7; and j, w.8. Twin isolates within the tetrad are w.1 and w.8, w.2 and w.7, w.3 and w.6, and w.4 and w.5, respectively. Lane k contains the control reaction. The arrows indicate the location of random amplified polymorphic DNA markers. Stars indicate the 1-kb ladder used as the molecular weight standard.

cording to Lee and Taylor (18) with minor modifications. Nucleic acids were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol, Sigma Chemical Company, St. Louis) and suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Concentrated DNA solutions were incubated with ribonucleases T1 (100 units ml^{-1}, Fluka, Buchs, Switzerland) and A (100 units ml^{-1}, Fluka) at 37°C for 1 h. Samples were diluted in TE to a final concentration of 10 ng μl^{-1}.

Polymerase chain reaction (PCR) conditions. A 25-µl reaction was performed in a 0.5-ml polypropylene microtube containing 30 ng of DNA, 50 µM each of dNTP (Bioprobe Systems, Montreuil-sous-Bois, France), 0.4 µM primer (Bioprobe), and 0.5 units of *Taq* polymerase (Appligene, Pleasanton, CA) in Appligene dilution buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X100, and 0.2 mg of bovine serum albumin per ml). The mixture was overlaid with mineral oil to prevent evaporation during cycling. Amplifications were performed in a Perkin-Elmer Cetus DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, CT) under the following conditions: an initial denaturation cycle at 95°C for 5 min, followed by 45 cycles consisting of 1 min at 95°C, 1 min at 39°C, and 3 min at 72°C. A final extension step at 72°C for 10 min concluded the cycling, and the tubes were chilled to 4°C. The fastest available temperature transitions (1°C s^{-1}) were used. PCR products were analyzed by electrophoresis in 1.4% agarose gels with 1× TBE (45 mM Tris-borate and 1 mM EDTA, pH 8.0) as buffer. Gels were stained in 0.5 µg of ethidium bromide per ml for 1 h, destained in water for 15 min, and photographed on a UV transilluminator. Molecular weights were estimated using Bio-Profil software (Vilber Lourmat, Marne la Valleé, France) with the 1-kb ladder (Gibco-BRL, Life Technologies, Gaithersburg, MD) as reference. Sixty-one decamers of random nucleotide sequences from kits C (C5-C10, C13, and C18), E (E2-E7 and E10-E17), M (M1-M8, M10, M11-M13, and M15-M20), and AE (AE1 to AE20) (Bioprobe) were used as primers.

Nomenclature of RAPD markers. Polymorphic markers were named with reference to the primer that generated them, followed by the molecular weight (in kilobases) of the band. For instance, AE1-1.30 is the 1.30-kb polymorphic band generated by the AE1 primer.

Pathogenicity tests. Isolates were inoculated on cotyledons of *B. napus* cvs. Westar, Quinta, and Glacier as previously described (8). At least eight plantlets per isolate and two inoculation sites per cotyledon were used. Plantlets were incubated in a growth chamber at 16°C (night) and 24°C (day) with a 12-h photoperiod. Symptoms were scored 14 days after inoculation using the Williams and Delwiche (29) rating scale, comprising 9 infection classes (IC), ranging from 1 to 9, in which IC 1 is the HR and IC 7 to 9 represent a high level of susceptibility. Interaction phenotypes also were assessed at the leaf level by inoculating the first developed leaf at five inoculation sites with 10-µl droplets of conidia suspensions (10^6 conidia per ml). Inoculation conditions and rating scale were identical to those described above.

RESULTS

RAPD as genetic markers. Of the 61 primers, 7 did not amplify DNA in a.2 or H5 (C7: 5′GTCCCGACGA3′; E5: 5′TCAGG GAGGT3′; E8: 5′TCACCACGGT3′; E10: 5′CACCAGGTGA3′; E13: 5′CCCGATTCGG3′; E16: 5′GGTGACTGTG3′; and AE5: 5′CCTGTCAGTG3′). The remaining 54 primers yielded a total of 280 amplified DNA fragments. Depending on the primer, 1 to 11 bands per pattern were observed (Fig. 1).

Among the 54 efficient primers, only 10 generated reproducible polymorphisms between isolates a.2 and H5. Nineteen polymorphic markers, i.e., DNA segments present in one parent but absent in the other parent, were detected (Table 2), giving a 6.8% polymorphism rate between a.2 and H5. The molecular weight of the markers ranged from 0.26 to 2.56 kb. Primers AE1, AE3, AE7, AE13, AE14, and E2 generated at least two polymorphic markers of reduced molecular weight difference (Table 2). Finally, the RAPD markers were not primer artifacts (12) or amplified contaminant DNA because they had no correspondence in the control reactions in which DNA was omitted in the PCR mixture (Fig. 1).

The 0.26-kb band generated by AE18 in the parental isolate a.2 was absent in parent H5 but was present in all the progeny. The AE18-0.26 band, thus, behaved like a monoparental trait that may be due to a cytoplasmic origin of the target DNA. The other 18 polymorphic markers clearly exhibited a 2:2 segregation ratio within all the tetrads (Table 1; Fig. 1). Furthermore, within each tetrad, RAPD marker segregations were consistent with data regarding twin genotypes, as determined by combining Mat and SPP markers (9). This suggests that, apart from AE18-0.26, the RAPD markers described here are monogenic.

With the exception of RAPD markers generated by the same primer, combinations of RAPD markers in pairs generally led to tetratypes, allowing us to recognize twin isolates within tetrads when Mat and SPP markers only led to PD (parental ditypes) or NPD (nonparental ditypes) (Table 1; Fig. 1). These data confirmed that the 10 tetrads analyzed were complete tetrads, i.e., the four meiotic products were present in at least one copy number within each tetrad, even when only five (tetrad t) or six (tetrad s) ascospores were recovered after micromanipulation (Table 1).

Except for the pairs AE3-0.83/AE3-0.88, AE3-0.83/AE3-0.90, and AE19-1.83/AE19-2.56, only PD were observed when considering the pairs of RAPD markers generated by the same primer. According to Perkins (22), this 10:0 PD/NPD ratio is highly significant (1% confidence level) for a genetic linkage. This suggests that the two markers of each pair are the same allele or that they are linked genetically. Due to the limited number of tetrads analyzed, examination of the PD/NPD ratio indicated no significant evidence of genetic linkage between all the other markers (22).

Phenotypes of interaction with the differential set. Isolates a.2 and H5 induced typical gray-green tissue collapse (IC 7) on cotyledons of cvs. Westar and Glacier. Pycnidia usually developed on lesions (IC 8 and 9). Sporulating lesions also occurred on cotyledons of cv. Quinta when inoculated with H5. In contrast, a.2 induced dark necrotic reactions on Quinta. These lesions were either characterized by a dark necrosis restricted to the inocu-

TABLE 2. Informative primers detected in this study

Primer	Nucleotide sequence	No. of bands[x]	RAPD markers[y]	Segregation ratio[z]
AE1	5′TGAGGGCCGT3′	9	AE1-1.26	2:2
			AE1-1.30	2:2
AE3	5′CATAGAGCGG3′	6	AE3-0.83	2:2
			AE3-0.88	2:2
			AE3-0.90	2:2
AE7	5′GTGTCAGTGG3′	9	AE7-1.51	2:2
			AE7-1.57	2:2
AE13	5′TGTGGACTGG3′	8	AE13-0.90	2:2
			AE13-0.95	2:2
AE14	5′GAGAGGCTCC3′	5	AE14-0.91	2:2
			AE14-0.95	2:2
AE18	5′CTGGTGCTGA3′	4	AE18-0.26	4:0
AE19	5′GACAGTCCCT3′	8	AE19-1.83	2:2
			AE19-2.56	2:2
E2	5′GGTGCGGGAA3′	7	E2-0.74	2:2
			E2-0.78	2:2
M5	5′GGGAACGTGT3′	5	M5-0.77	2:2
			M5-1.12	2:2
M20	5′AGGTCTTGGG3′	7	M20-0.37	2:2

[x] Total number of amplified and reproducible fragments.
[y] Random amplified polymorphic DNA (RAPD) marker nomenclature, according to molecular weight in kilobases.
[z] "Presence versus absence" segregation ratio of the RAPD markers, according to the 4 meiotic genotypes within each of the 10 tetrads analyzed.

lation point (IC 1) or by a larger dark reaction (IC 3). In a few cases, IC 4 (irregularly dispersed black necrotic reactions) and 6 (tissue collapse delimited by dark necrotic margins) were observed. Sporulation never occurred on these lesions. As a result, they were all considered resistance reactions. By comparison with the three reference isolates, isolates a.2 and H5 were classified as PG3 and PG4, respectively.

Symptoms observed on the first developed leaf were comparable to those described on cotyledons (Fig. 2). Large sporulating lesions were observed on cvs. Westar, Quinta, and Glacier when inoculated with PG4 isolates and on cvs. Westar and Glacier when inoculated with PG3 isolates. In contrast, resistance reaction restricted to the inoculation point (IC 1 to 3) was usually observed when PG3 isolates were inoculated on leaves of Quinta (Fig. 2).

Genetic control of the specificity of PG3-Quinta interaction. Within the progeny of the PG3 × PG4 cross, only PG3 and PG4 isolates were recovered. Twin genotypes within each tetrad always displayed the same PG (Table 1). The segregation ratio of PG3/PG4 isolates within all the tetrads was always 2:2, suggesting that interaction of PG3 isolates with cv. Quinta was controlled by a single genetic locus (Table 1). These data support the hypothesis that one functional avirulence gene is present in a.2 and, consequently, in the PG3 progeny. This avirulence gene was termed *AvrLm1*.

DISCUSSION

RAPD allowed us to characterize 18 monogenic markers. This is the first reported use of RAPD as genetic markers in the Tox^+ isolates of *L. maculans*. The reproducibility of the RAPD markers was excellent, because at least three independent amplifications resulted in identical profiles for parental isolates. Whenever variations were observed between independent amplifications, it was only in the intensity or presence/absence of a few minor bands. In contrast, major DNA segments, including the RAPD markers, were amplified consistently, as is generally observed with the RAPD technique (10,16). The reliability of the RAPD markers was supported further by the identity of profiles of twin isolates within a tetrad. Due to their Mendelian inheritance and reliability, RAPD markers are useful tools for genetic analysis in *L. maculans*. Using them in pairs allows us to clearly recognize the twin isolates within a tetrad, ensuring that the four genotypes resulting from meiosis are present in the cases of missing ascospores in the recovered tetrad.

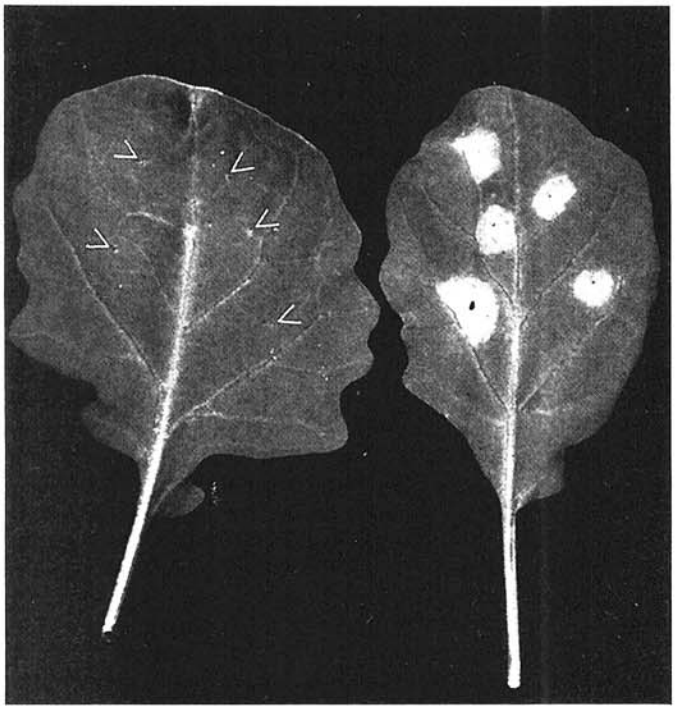

Fig. 2. Phenotypes of interaction on leaves of *Brassica napus* cv. Quinta observed 14 days after inoculation with *Leptosphaeria maculans*. Left, resistance responses (infection class 1) after inoculation with isolate a.2 (pathogenicity group [PG] 3). Right, susceptibility symptoms (infection class 8) after inoculation with isolate H5 (PG4). The arrows indicate the inoculation points displaying the hypersensitive responses.

Based on the cotyledon inoculation test, specific interactions were observed for the two single-ascospore field isolates a.2 and H5 on cvs. Westar, Quinta, and Glacier. Symptoms of susceptibility were characterized by tissue collapse with differentiation of pycnidia. Also, susceptibility was never accompanied by dark necrotic reactions. HR as well as intermediate reactions were observed on cv. Quinta when inoculated with PG3 isolates. As already noticed by Mengistu et al. (20), the PG3-Quinta interaction was characterized by dark necrotic reactions whose extent justified the intermediate rating sometimes observed. These intermediate responses were considered resistance responses due to the necrotic responses of the plant, which clearly differ from susceptibility, and the fact that further collapse of tissue and sporulation never occurred. This was substantiated by previous data that showed that the mycelium content detected by double-antibody sandwich enzyme-linked immunosorbent assay in infected cotyledons displaying IC 3 to 6 is often comparable to the mycelium content found in cotyledons displaying typical HR (3). These results also were supported by the evidence of the efficiency of Quinta resistance under field conditions (1).

The genetic analysis of the pathogen interaction with cv. Quinta revealed that a single genetic locus present in isolate a.2 controlled the interaction phenotypes with this cultivar. Interaction phenotypes were clearly cultivar-specific interactions, because all progeny developed disease symptoms on cvs. Westar and Glacier. Therefore, we concluded that the single gene responsible for specificity of interaction is an avirulence gene, termed *AvrLm1*. This terminology also can refer to the functional allele of the avirulence gene *AvrLm1*. However, *AvrLm1* may not be dominant. Even though dominant avirulence genes are prevalent in plant-fungal pathogen interaction models (5), dominance will be difficult to demonstrate in *L. maculans* because the infective stage of this Ascomycete is haploid. Compatible interactions observed on cv. Quinta may be explained by different modifications of the avirulence gene. In fungal plant pathogens, evasion of gene-for-gene recognition can result from point mutation, deletion (14,26), as well as complete absence of the avirulence gene (27). Consequently, the corresponding allele of *AvrLm1* in H5 cannot be determined yet without molecular characterization of the avirulence gene *AvrLm1*. More avirulence genes are probably present in *L. maculans*, because 19 PG have been described recently (17). Efforts must be made to characterize these avirulence genes to define races in *L. maculans*.

Identification of avirulence genes in *L. maculans* suggests that the discriminant *B. napus* cultivars have the corresponding disease resistance genes. Genetic and molecular characterization of these resistance genes will support the gene-for-gene concept in the *L. maculans*/*B. napus* plant-pathogen system. It also will advance our understanding of plant-parasite interaction mechanisms. Eventually, cloning new avirulence/resistance genes will allow improved blackleg disease control via genetic-engineering applications (5,15).

Increasing the number of RAPD markers can be expected by screening a larger set of primers. Consequently, it will be possible to establish linkage groups and develop a genetic map of the *L. maculans* genome. In the near future, RAPD markers will be starting points of map-based cloning strategies to initiate a chromosome walk to the avirulence gene *AvrLm1*.

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