Mapping of Avirulence Genes in *Phytophthora infestans* With Amplified Fragment Length Polymorphism Markers Selected by Bulked Segregant Analysis

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ABSTRACT

In this study we investigated the genetic control of avirulence in the diploid oomycete pathogen *Phytophthora infestans*, the causal agent of late blight on potato. The dominant avirulence (*Avr*) genes matched six race-specific resistance genes introgressed in potato from a wild Solanum species. AFLP markers linked to *Avr* genes were selected by bulked segregant analysis and used to construct two high-density linkage maps, one containing *Avr4* (located on linkage group A2-a) and the other containing a cluster of three tightly linked genes, *Avr3*, *Avr10*, and *Avr11* (located on linkage group VIII). Bulked segregant analysis also resulted in a marker linked to *Avr1* and this allowed positioning of *Avr1* on linkage group IV. No bulked segregant analysis was performed for *Avr2*, but linkage to a set of random markers placed *Avr2* on linkage group VI. Of the six *Avr* genes, five were located on the most distal part of the linkage group, possibly close to the telomere. The high-density mapping was initiated to facilitate future positional cloning of *P. infestans Avr* genes.

RESISTANCE of plants to pathogens often depends on the activation of defense responses after pathogen attack. A key factor in this type of resistance is the perception of the pathogen by the host, which triggers the appropriate defense responses. When defense responses completely block pathogen development, the interaction between pathogen and plant is called incompatible. Genes from the pathogen that mediate recognition and activation of host defense responses leading to incompatible interactions are called avirulence genes. Incompatible interactions are usually associated with a hypersensitive response in the host and a high degree of specificity between the pathogen genotype and the host genotype. This high specificity is also known as race-specific resistance of the host or race-specific virulence of the pathogen and had been observed by Flor in the early 1940s in the flax-flax rust pathosystem (Flor 1942; Ellis *et al.* 1997). In the last decade Flors's gene-for-gene model, which explains the high specificity, gained support as a general mechanism governing plant-pathogen interactions. Single dominant avirulence genes were identified and cloned from a wide range of pathogens (reviewed by Bonas and Van den Ackerveken 1999; Laugé and De Wit 1998), and dominant matching resistance genes were isolated from various plant species (reviewed by Ellis and Jones 1998).

The subject of our studies is *Phytophthora infestans*, the causal agent of potato late blight and one of the economically most important pathogens of potato worldwide. *P. infestans* and potato interact according to the gene-for-gene model. Eleven major resistance genes (R-genes) introgressed from *Solanum demissum* provide strong resistance against specific races of the pathogen (Black 1954; Eide *et al.* 1959; Malcolmson and Black 1966; Malcolmson 1969). These 11 R-genes suggest the presence of 11 corresponding virulence or avirulence factors in *P. infestans*, and genetic analyses on both host and pathogen have been performed to confirm the gene-for-gene model in this pathosystem.

In potato, the position on the genome of 5 out of 11 R-genes was determined by linkage analyses (R1, Meksem *et al.* 1995; R3, R6, and R7, El-Kharbotly *et al.* 1996; R2, Li *et al.* 1998), thereby demonstrating that a single locus in the host governs race-specific resistance. The inheritance of virulence and avirulence in *P. infestans* is less clearly defined. Spielman *et al.* (1989, 1990) analyzed segregation ratios of virulent and avirulent phenotypes in three F_1 progeny from matings involving five Mexican isolates and in some F_2 progeny and backcrosses. Their results were in favor of single locus control for (a)virulence against R1 and R2, but suggested involvement of more than one locus for (a)virulence against R3 and R4. A more extensive study by Al-Kherb *et al.*

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(1995) involving seven isolates, and segregation analyses in 10 F_1 crosses and in sib matings and backcrosses, revealed single locus control for (a)virulence against 10 of the 11 R-genes, while (a)virulence against R10 seemed to depend on two loci. However, a drawback of the studies by Spielman *et al.* (1989, 1990) and Al-Kherb *et al.* (1995) is the lack of molecular markers linked to virulent or avirulent phenotypes, which hampers drawing firm conclusions. For example, segregation ratios may be obscured by the fact that not all oospores of *P. infestans* are viable (Pittis and Shattock 1994; Al-Kherb *et al.* 1995). Also, loss of pathogenicity in F_1 progeny occurs frequently and that excludes part of the progeny from virulence tests. Furthermore, analysis of F_1 progeny of *P. infestans* with molecular markers revealed the occasional occurrence of trisomic individuals (Carter *et al.* 1999; T. van der Lee and F. Govers, unpublished results). Thus, predictions based on segregation ratios alone should be treated with caution.

Here we present studies on the inheritance of race-specific virulence in *P. infestans*. Progeny of a cross in which six avirulence (*Avr*) genes segregate were investigated (Alfonso and Govers 1995). This F_1 progeny was previously used to construct a genetic linkage map and the parental lines were shown to behave genetically as diploid homokaryons (van der Lee *et al.* 1997). By means of *b*ulked *s*egregant *a*nalysis (BSA; Michelmore *et al.* 1991) amplified fragment length polymorphism (AFLP) markers tightly linked to five *Avr* genes were found. Linkage analysis showed that avirulence is dominant. By integrating the markers in the genetic linkage map (van der Lee *et al.* 1997), all six *Avr* genes could be positioned on the map, and high-density maps of two genomic regions containing *Avr* genes were constructed.

MATERIALS AND METHODS

Nomenclature of genes and phenotypes: Anticipating that the six avirulence genes analyzed in this study would be dominant, the nomenclature used for these genes (or gene loci) is *Avr* (with a capital A) for the avirulent genotypes and *avr* for the virulent genotypes. This is followed by a number indicating the corresponding host resistance gene (*e.g.*, *Avr1*). Consequently, the phenotypes are indicated by AVR and avr; *e.g.*, a strain with the AVR1 phenotype is avirulent on plants carrying the R1 resistance gene whereas an avr1 strain is virulent on R1 plants.

Avr3, *Avr10*, and *Avr11* were postulated to match the corresponding resistance genes R3, R10, and R11. Since there is no evidence that these three avirulence genes represent a single gene, they are treated as independent genes, and the locus in which the avirulence genes cluster is called *Avr3-Avr10-Avr11*.

***P. infestans* mapping population:** The mapping population consisted of 76 F_1 progeny from cross 71, a cross between two Dutch *P. infestans* isolates, 80029 (race 2.4.7; A1 mating type) and 88133 (race 1.3.7.10.11; A2 mating type). The progeny were derived from oospores generated in infected leaves (*in vivo*) and were recovered from sporulating lesions formed on leaves that were floated on water containing soil with oospores. The mapping population was previously characterized by Drenth *et al.* (1995) and van der Lee *et al.* (1997). Sixty-eight progeny of this cross were included in the virulence assays.

Virulence assays: *P. infestans* isolates, stored as spores or mycelium plugs in 15% DMSO in liquid nitrogen, were transferred to 9-cm petri dishes containing rye agar medium supplemented with 2% sucrose (Caten and Jinks 1968). When plates were fully covered with mycelium (after 1–2 weeks) small plugs were transferred to fresh medium. To isolate zoospores, sporulating cultures were flooded with 10 ml of demineralized water and incubated at 4° for 3 hr to allow formation and release of zoospores. The zoospore suspension was collected and kept on ice. The zoospore concentration was counted and the appropriate number was used for inoculation of potato leaves.

Potato lines of the differential set were obtained from Plant Research International and from the Laboratory of Plant Breeding of Wageningen University. We used the following lines (R-gene in parentheses): CEBECO43154-5 (R1), CEBECO44158-5 (R2), CEBECO4642-1 (R3), CEBECO4431-5 (R4), Black2182ef(7) (R7), Black3618ad(1) (R10), Black5008ab(6) (R11), CEBECO4739-58 (R1R3), and CEBECO5073-1 (R2R3). In addition we used the R-gene containing potato cultivars Ehud (R1), Saturna (R1), and Astarte (R1R3), and the cultivar Bintje, which has no R-genes (r0). Sterile plants were grown on Murashige and Skoog (MS) medium amended with vitamins and 2% sucrose at 20° and 16 hr light/8 hr dark. Top cuttings of plants were transferred to new medium and grown for 2 weeks under the regime mentioned above after which the plants were transferred to sterile soil in a climate chamber. In the first 2–3 days after transfer to soil, plants were covered with plastic. Plants were transferred to 5-liter pots 1–2 weeks after transfer to soil. Leaves were used for inoculation with *P. infestans* 8–10 weeks after transfer to soil.

Full-grown leaves were cut from the fourth to the seventh node and stuck in water-saturated flower foam. Leaves were transferred to trays with water-soaked filter paper at the bottom and transparent plastic lids to ensure high humidity in the tray. The lower side of the leaf was drop inoculated ($\sim$10 μl) with 10^3 or 2×10^3 zoospores. Per *P. infestans* strain and per R-gene differential at least eight inoculation spots with 10^3 and eight inoculation spots with $2 \cdot 10^3$ zoospores were analyzed in every test. Leaves were incubated for 4 days (16 hr at 18°/light and 8 hr at 15°/dark) before responses were scored. As explained in the results, five different classes of responses were distinguished. When different classes were observed at different inoculation sites the number in each class was counted.

DNA isolation and AFLP DNA fingerprinting: DNA isolation from *P. infestans* was performed as described previously by Drenth and Govers (1993) with some minor modifications as described in van der Lee *et al.* (1997). AFLP DNA fingerprinting was performed essentially as described by Vos *et al.* (1995) using the restriction enzyme combination *Eco*RI/*Mse*I with two selective bases on each side, which was shown to generate highly informative fingerprints for *P. infestans* (van der Lee *et al.* 1997). The nomenclature of the AFLP markers is as follows. The first letter, A, B, or H, indicates the origin of the marker (A for the A1 parent, B for the A2 parent, and H for fragments present in both parents). This is followed by E + XX/M + XX in which E and M refer to *Eco*RI and *Mse*I, respectively, and XX to the extensions of the selective bases used. The numbers at the end (preceded by s) refer to the approximate size of the fragment in base pairs. For example, the marker AE + AG/M + ATs400 is present only in the A1 parent; the fragment was generated using an *Eco*RI/*Mse*I restriction digest, amplified with a primer with an AG extension on the *Eco*RI site and a primer with an AT extension on the *Mse*I site, and has an estimated size of 400 bp.

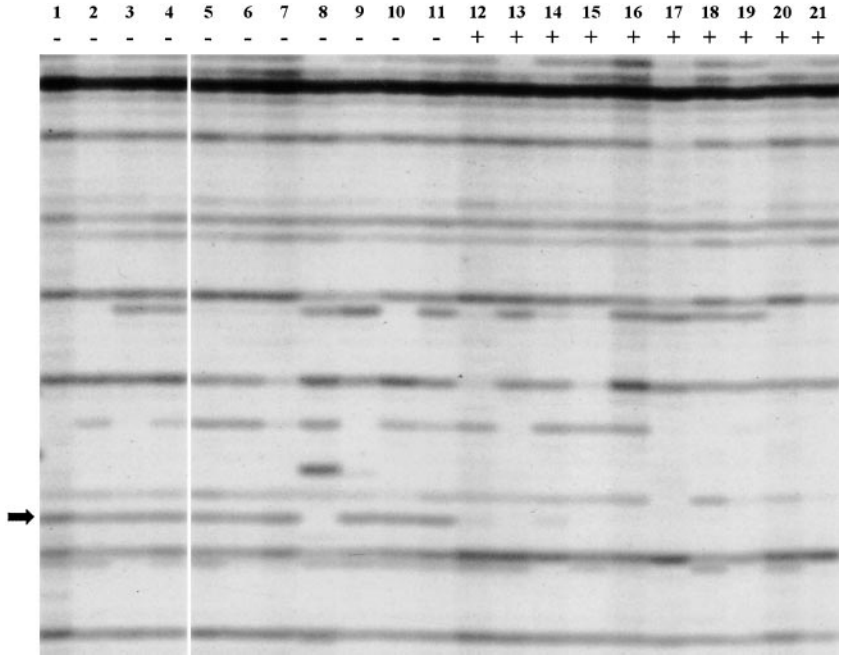

FIGURE 1.—Segregation of an *Avr4* linked marker in cross 71. Section of an autoradiograph showing AFLP DNA fingerprints generated using the primer combination E + AC/M + TT. The parental line 88133 (lane 6) and progeny T15-1 (lane 1), T30-2 (lane 2), T30-4 (lane 3), T30-5 (lane 5), RE11-8 (lane 8), RE11-12 (lane 9), and RE11-16 (lane 10) are avirulent on R4 plants. The parental line 80029 (lane 18) and progeny T15-2 (lane 12), T15-4 (lane 13), T30-7 (lane 14), RT15-3 (lane 16), RE11-9 (lane 17), T35-3 (lane 20), and RE11-14 (lane 21) are virulent on R4 plants. Lanes 4, 7, and 11, and lanes 15 and 19 are fingerprints from pooled avirulent and virulent progeny, respectively. The arrow on the left indicates an AFLP marker for *Avr4* (BE + AC/M + TTs165). Virulence and avirulence phenotypes are indicated by + and −, respectively. The absence of this marker in progeny E11-8 (lane 8) suggests a recombination between this marker and *Avr4*.

BSA: Two rounds of BSA were performed essentially according to the procedure described by MICHELMORE *et al.* (1991). One round was aimed at generating markers located in the *Avr3-Avr10-Avr11* region and the other at generating markers linked to *Avr1* and *Avr4*. In the screening for markers linked to *Avr3*, *Avr10*, and *Avr11* four pools of progeny with two different combinations of phenotypes were used. Pools 1 and 4 represented the phenotype avr3;avr10;avr11 and pools 2 and 3 the phenotype AVR3;AVR10;AVR11. The pools contained the following F_1 progeny: pool 1, D12-12, T15-1, T30-2, T35-3, and T35-4; pool 2, T15-2, T15-5, T15-7, and T15-9; pool 3, D12-9, D12-17, E12-3, E12-7, and T20-2; pool 4, D12-18, D12-21, D12-23, D12-25, E12-2, E12-15, and E12-22.

In the screening for markers linked to *Avr1* and *Avr4* six pools were used with the following combinations of phenotypes and F_1 progeny: pool 1, AVR1;AVR4 (F_1 progeny T15-1, T30-2, and T30-4); pool 2, avr1;AVR4 (F_1 progeny T20-2 and T80-3 and parent 88133); pool 3, AVR1;AVR4 (F_1 progeny RE11-8, RE11-12, and RE11-16); pool 4, avr1;avr4 (F_1 progeny T15-2, T15-4, and T30-7); pool 5, AVR1;avr4 (F_1 progeny RE11-09 and RT15-3 and parent 80029); pool 6, avr1;avr4 (F_1 progeny, RE11-14, RE11-15, and T35-03). Before starting the BSA all individuals were tested for contribution to the pools. AFLP fingerprinting was performed on pools and on individuals, and the contribution of each individual to the pool was balanced by adding more or less template. In both rounds of BSA, all 256 possible *Eco*RI + 2/*Mse*I + 2 primer extensions were used. Candidate markers were identified visually from the fingerprints obtained on the pooled DNA and were tested on the individual progeny of each pool. Markers showing good correlation were further tested on all individual progeny of cross 71. An example is shown in Figure 1.

Mapping of avirulence genes: Linkage analysis and mapping were performed using the mapping software JoinMap 2.0 (STAM 1993). Maps were constructed using an LOD linkage threshold value of 4.5. Mapping of dense clusters of markers is not always straightforward. Small inconsistencies in the data set result in strong friction as indicated by the χ^2 value. To reduce this friction JoinMap occasionally positioned markers outside this dense region to a region containing less markers. However, if the LOD values clearly indicated that the marker was located in the dense region, the χ^2 frictions were overruled and the markers were manually positioned in the dense regions.

RESULTS

Segregation of race-specific virulence in cross 71: F_1 progeny of a cross between two Dutch field isolates (cross 71: A1 mating-type parent 80029, race 2.4.7, and A2 mating-type parent 88133, race 1.3.7.10.11) were tested for virulence on a differential set of potato lines carrying the major R-genes R1, R2, R3, R4, R10, and R11, respectively. In the progeny virulence against these six R-genes segregates (ALFONSO and GOVERS 1995).

The scoring for virulence or avirulence is hampered by the fact that the differential set of potato lines is not genetically uniform and that the infection severity is not identical on all potato lines. Also, the *P. infestans* progeny appeared to be variable in aggressiveness on potato, and therefore in every virulence assay the aggressiveness on a potato cultivar without R-genes (r0) was analyzed. If the progeny was not able to infect r0 plants it was not included in the segregation analysis. Four days after inoculation, five different macroscopic responses were distinguished: (A) no symptoms, (B) dark localized necrosis, (C) spreading lesions without sporulation, (D) spreading lesions with some sporangiospores, and (E) spreading lesions with massive sporulation. On the R1 and R3 potato lines the interaction was rated compatible if the responses were of classes D and E. Class A or B responses were considered to be incompatible interactions and class C responses were rated unknown. These results were confirmed by virulence assays on the cultivars Ehud (R1), Saturna (R1), and Astarte (R1R3). The same rating was used for the virulence on R10 and

TABLE 1

Segregation of race-specific virulence in the progeny of *P. infestans* cross 71

	Phenotypes[a]						
	Parents		Progeny				
R-gene	80029	88133	Observed +:−	Expected +:−	N^b	$\chi^{2\,c}$	P^c
1	−	+	24:33	1:1	57 (63)	1.42	0.23
2	+	−	21.39	1:1	60 (62)	5.40	0.02
3	−	+	30:21	1:1	51 (62)	1.00	0.32
4	+	−	30:23	1:1	53 (63)	0.92	0.34
10	−	+	24:27	1:1	51 (62)	0.18	0.67
11	−	+	25:25	1:1	50 (62)	0.00	1.00

[a] +, virulent; −, avirulent.
[b] Number categorized; in parentheses, number tested.
[c] The χ^2 and the corresponding *P* value were calculated to test the probability that the data fit an expected ratio of 1:1 for segregation of a single gene conferring avirulence.

R11 lines but here symptoms were less severe when compared to those on R1 and R3 lines. Symptoms on the R2 potato line were also less severe; class E responses were not found; class C and D responses were rated compatible while class A and B responses were rated incompatible. Symptoms on R4 potato lines were more severe. Class E responses were rated compatible; class D responses were rated unknown; and class A, B, and C responses were rated incompatible. The scoring of virulence on the differential set was reproducible and in line with what was reported previously (Alfonso and Govers 1995).

As shown in Table 1, segregation ratios for virulence/avirulence on potato lines carrying the R1, R3, R4, R10, or R11 resistance gene did not differ significantly from 1:1. However, a significant deviation from the expected 1:1 ratio toward avirulence was found on R2 plants; here the ratio was close to 1:2.

Fine mapping of *Avr3*, *Avr10*, and *Avr11* using bulked segregant analysis: Initial analysis of the progeny showed genetic linkage of virulence on potato lines carrying R3, R10, or R11. For BSA we constructed two pools of AFLP templates from progeny avirulent on R3, R10, and R11 plants and two pools of AFLP templates from progeny virulent on those plants. The number of progeny in the pools varied from four to seven. The pooled templates were fingerprinted by AFLP using all 256 combinations of *Eco*RI + 2/*Mse*I + 2 primer extensions. Over 25,000 AFLP fragments were analyzed and, on the basis of previous experiments (van der Lee *et al.* 1997), it is estimated that these include over 1250 markers segregating in the cross 71 progeny and originating from the A1 parent (Aa × aa). BSA yielded 20 fragments that were specific for the avirulent pools. These candidate markers were tested on the individuals of the bulks and on other progeny of cross 71. Fifteen of the 20 markers (75%) showed linkage to *Avr3*, *Avr10*, and *Avr11* and most were within 10 cM distance (Table 2). The direct distances between the markers and the avirulence loci are ~8 cM with LOD values ranging from 5 to 6. For this region no additional markers were found in a random set of 240 markers. All linked markers and *Avr3*, *Avr10*, and *Avr11* map on the distal part of linkage group VIII (Figure 2C). The calculated map distances between markers in the region and *Avr3*, *Avr10*, and *Avr11* were smaller than the direct distances. This difference between the direct distance and the map distance caused some friction in the map, but the LOD values and the direct distances clearly positioned *Avr3*, *Avr10*, and *Avr11* in the region.

Fine mapping of *Avr1* and *Avr4* using bulked segregant analysis: *Avr1* and *Avr4* segregate as independent loci in cross 71. Nevertheless, a BSA was set up that allowed identification of linked markers for both loci simultaneously. Six pools with AFLP templates from progeny with the following phenotypes were composed: AVR1;AVR4 (two pools with three individuals), AVR1;avr4 (one pool with three individuals), avr1;AVR4 (one pool with three individuals), and avr1;avr4 (two pools with three individuals). In this way markers linked to *Avr1* could be distinguished from markers linked to *Avr4*. Over 30,000 AFLP fragments were analyzed, resulting in 23 candidate markers for *Avr1* and 16 candidate markers for *Avr4* (Table 2). Only one of the *Avr1* markers, AE + CG/M + TGS317, appeared to be linked when tested on the individual progeny of the bulks and on additional progeny of cross 71. Linkage analysis with all markers currently identified in the cross 71 mapping population showed linkage of this marker with a marker distal on linkage group IV (Figure 2A). The direct distance between the marker and *Avr1* is 8 cM and the corresponding LOD value is 10.4.

Initially, 16 candidate markers were identified for *Avr4*, of which 7 appeared to be linked. Segregation of 1 linked marker is shown as an example (Figure 1). One additional marker was identified in a set of 240

TABLE 2

Number of candidate markers selected by BSA and number of markers linked to *Avr* genes

Avirulence gene	No. of AFLP fragments analyzed	Estimated no. of informative markers[a]	Candidate markers after BSA	Markers linked (<10 cM)
*Avr*1	>30,000	1,500	23	1
Avr2	>4,000	240[b]	NR[c]	7
Avr3-Avr10-Avr11	>25,000	1,250	20	15
Avr4	>30,000	1,500	16	7 + 1[d]

[a] Informative markers are markers that segregate in the cross 71 progeny.
[b] For *Avr2* no BSA was performed; consequently, the number of informative fragments is not an estimate but the real number.
[c] Not relevant.
[d] Seven from BSA and one from the set of random markers.

random markers. The *Avr4* locus could be mapped distally on A2-a, a linkage group containing only markers from the A2 parent (Figure 2D). The direct distance between *Avr4* and the closest marker is 2 cM with a LOD value of 12.4.

Mapping of *Avr2*: The *Avr2* gene was mapped with random markers. These markers were derived partly from the set generated to construct the first genetic map (VAN DER LEE *et al.* 1997) and partly from markers that were obtained when primer combinations used to test the candidate markers for the other five *Avr* genes were analyzed on all progeny of cross 71 individually. In the latter fingerprints, on average, 15 segregating markers could be scored in addition to the candidate marker. In total, 470 of these markers, of which 240 were derived from the avirulent 88133 parental line, were tested for linkage to virulence on the R2 potato line (Table 2). Seven markers derived from the A2 parent appeared tightly linked to *Avr2* and, as a result, *Avr2* could be positioned on linkage group VI (Figure 2B).

Avirulence is a dominant trait: Analysis of the diploid F_1 progeny of an outbreeding cross allows discrimination between dominant and recessive traits if phenotypes can be mapped and if the phenotypes of the parents are known. Linkage can only be found between markers or traits from the same parent. In the progeny of cross 71 we found clear linkage between avirulence on R1, R3, R10, or R11 potato lines and markers from parent 80029. This indicates that the gamete of this parent determines whether the progeny will be virulent or avirulent on R1 or on R3, R10, and R11 potato lines. Since parent 80029 itself is avirulent on these lines, avirulence is dominant. Similarly, from the linkage of avirulence on R2 or R4 potato lines with markers from

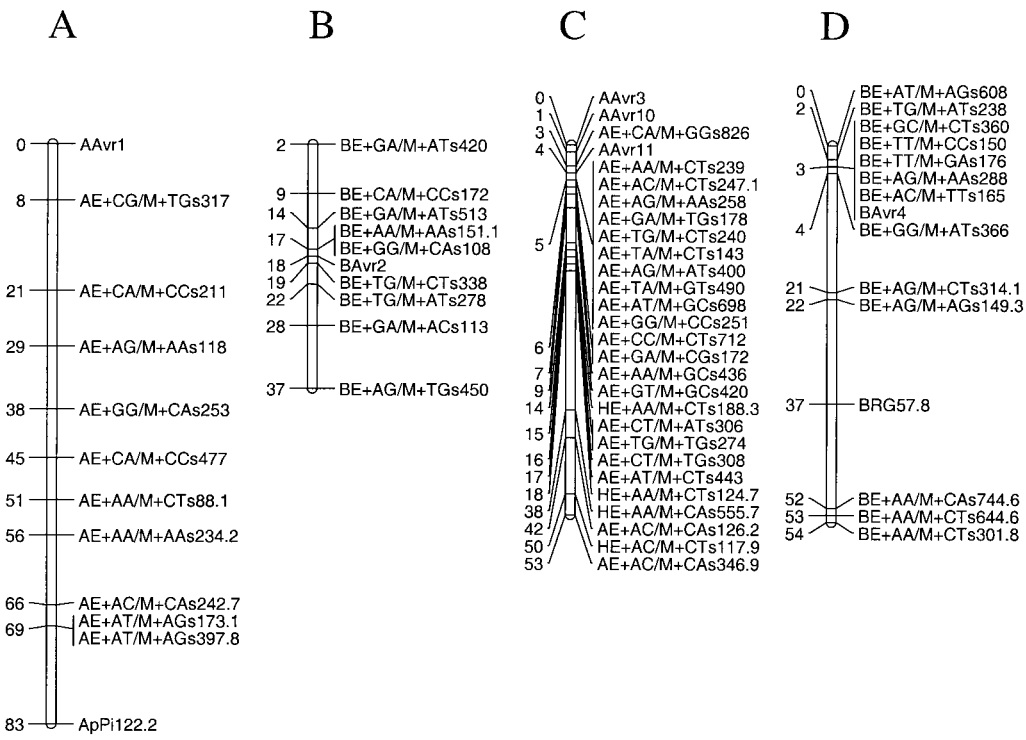

FIGURE 2.—Genetic maps of linkage groups containing avirulence genes. Maps were constructed using JoinMap 2.0 (STAM 1993) with a link LOD threshold of 4.5 and a map LOD threshold of 0.01. Markers are indicated on the right, cumulative map distances (in centimorgans) on the left. (A) Linkage group IV containing *Avr1*. (B) Linkage group VI containing *Avr2*. (C) Linkage group VIII containing *Avr3*, *Avr10*, and *Avr11*. (D) Linkage group A2-a containing *Avr4*.

parent 88133, we conclude that the gamete of 88133 determines the phenotypes on R2 and R4. Parent 88133 is avirulent on potato plants carrying the R2 and R4 gene, and consequently *Avr2* and *Avr4* are dominant.

DISCUSSION

P. infestans populations are notorious for the appearance of new virulent races that are able to overcome monogenic resistances introduced in potato. To gain insight into the genetic mechanisms underlying race specificity, the inheritance of avirulence genes in *P. infestans* was studied. Here we report the mapping of six race-specific avirulence genes in the sexual progeny of two Dutch field isolates. AFLP DNA fingerprinting was used in combination with BSA to identify markers tightly linked to these avirulence genes, and the relevant chromosomal regions were saturated with DNA markers, a prerequisite for positional cloning of avirulence genes.

For each of the six tested R-genes, a single corresponding avirulence locus was identified that could be positioned on the genetic linkage map. Our analyses, however, indicate that, in addition to these major factors, minor factors influence the responses on the potato lines that comprise the differential set. The genetic background of the lines is not uniform and different levels of "basal" resistance were observed. The potato lines carrying resistance genes R1, R3, or R4 were easily infected by virulent *P. infestans* isolates. Under high disease pressure, for example, resistance in the R4 differential was occasionally lost. On the other hand, the lines with R10, R11, and certainly R2 appeared to be less susceptible. All the progeny in which no correlation was found between the closest linked markers and *Avr2* are avirulent, indicating that this differential may contain other resistance genes. This is supported by the aberrant segregation ratio showing a significantly higher number of avirulent progeny than expected (Table 1). To reduce the variability caused by environmental factors and by genetic differences in the differential set other than the R-genes, the plants were grown in controlled conditions in climate chambers, high concentrations of zoospores were used, and scoring was done at early time points in disease development. In cases where the response was doubtful, it was rated as unknown, and this contributed to the reliability of the linkage analysis.

Since no avirulence genes of *P. infestans* were mapped previously, the pools for the BSA experiments were carefully designed. Approximately 20 progeny were divided over at least four pools. This allowed us to screen in different genetic windows and enabled us to neutralize an incorrectly scored individual, once identified, in one of the pools. Initial analysis showed that the use of at least four relatively small pools allowed reliable identification of markers with two recombinants in the pools, providing a genetic window of 10 cM (2 out of 20). Because two or three phenotypes were screened simultaneously, the number of progeny in the pools was limited to minimize incorrect scorings of individuals in the pools for one of the traits. It was estimated previously that the genome size of *P. infestans* in cross 71 is ~1200 cM (van der Lee *et al.* 1997). With a genetic window of 10 cM and the screening of 30,000 AFLP markers, of which 5% would be a segregating marker for either the 80029 or 88133 parent, ~10 markers were expected for a target gene at the end of a linkage group and 20 linked markers for a gene in the middle of a linkage group. Screening for markers linked to *Avr3, Avr10*, and *Avr11* yielded more markers than expected (*i.e.*, 15), whereas the BSA for *Avr1* linked markers resulted in a remarkably low number of markers, *i.e.*, only 1. For *Avr4*, 7 linked markers were found, which is a bit less than expected. For *Avr2*, no BSA was carried out. Yet, by analyzing linkage of *Avr2* with random markers we identified more markers linked to *Avr2* than to *Avr1* (7 *vs.* 1).

The BSA for *Avr3*, *Avr10*, and *Avr11*, as well as for *Avr4*, was efficient; 75% of the candidate markers were indeed linked to the *Avr3-Avr10-Avr11* cluster, and nearly 45% of the candidate markers for *Avr4* were truly linked. This was different in the screening for *Avr1*. A large number of candidate markers were identified, but only one appeared to be linked. It is not very likely that this is caused by the involvement of a second locus or by less reliable virulence data. Avirulence on R1 potato lines segregated in a 1:1 ratio, and R1 is the most reliable differential in the set. Moreover, the virulence data were confirmed using potato cultivars Ehud (R1), Saturna (R1), and Astarte (R1R3), and the fact that one tightly linked marker was identified contradicts incorrect scorings in the virulence assays. In the same BSA round we successfully selected a number of *Avr4* linked markers, eliminating the chance that technical problems are the cause. The reason for the relatively low number of markers in the *Avr1* region may be low polymorphism of the homologous chromosomes in the *Avr1* region in the 80029 parent or a high recombination frequency. Likewise, the relatively high number of markers linked to *Avr2* and the *Avr3-Avr10-Avr11* cluster might be explained by high rates of polymorphism or recombination suppression in these chromosomal regions. Even though marker densities differ in different regions of the cross 71 map constructed by linkage analysis (van der Lee *et al.* 1997), the large differences observed in this study in the various regions surrounding avirulence genes are exceptional.

All 256 *Eco*RI + 2/*Mse*I + 2 primer combinations were tested in the two rounds of BSA, one for *Avr1* and *Avr4* and the other one for *Avr3*, *Avr10*, and *Avr11*. If no markers were overlooked in the BSA, no additional markers would be identified in the random set of markers generated by the 50 primer combinations tested on all progeny. For *Avr1* and the *Avr3-Avr10-Avr11* cluster, this was indeed the case but for *Avr4* one additional

marker was found. This demonstrated that the BSAs were sufficiently systematic.

Our analyses clearly show that avirulence is a single dominant trait for all six segregating *Avr* genes tested in cross 71. Most fungal and bacterial *Avr* genes studied so far act in a dominant fashion (reviewed by Laugé and De Wit 1998) and indeed if avirulence genes encode proteins with elicitor function, dominance is to be expected. However, unlike the reverse genetics approach that is often used for cloning fungal avirulence genes (Joosten *et al.* 1994), genetic analysis might also reveal genes that do not themselves encode elicitors but, instead, mediate the synthesis of elicitors such as transcriptional regulators, enzymes, or transporters. These factors may act in a dominant or recessive fashion. Therefore, the conclusion by Spielman *et al.* (1989) that virulence in *P. infestans* on R4 plants is dominant does not necessarily contradict our findings. Since they studied segregation in other crosses, they may have analyzed another locus determining the AVR4 phenotype but, obviously, this locus does not segregate in cross 71.

High-density mapping confirmed the tight clustering of *Avr3*, *Avr10*, and *Avr11* that was noted at the phenotype level in the virulence assays. Linkage of *Avr* genes in *P. infestans* was reported before (Al-Kherb *et al.* 1995; Carter *et al.* 1999) but the tight clustering of *Avr3*, *Avr10*, and *Avr11* was not observed, even though the three avirulence phenotypes segregated in the crosses examined by Al-Kherb *et al.* (1995). This not only raises the question of whether we have been analyzing the same or different factors as Al-Kherb *et al.* (1995), but also whether we are really dealing with three different independent genes at the same locus. Tight clustering of *Avr* genes in plant pathogens is not uncommon. Cosegregation of avirulence genes was reported for *Avr4* and *Avr6* as well as for *Avr1b* and *Avr1k* in *P. sojae* (Whisson *et al.* 1995; Gijzen *et al.* 1996). The rice blast fungus *Magnaporthe grisea* contains one cluster with three and another with two *Avr* genes (Dioh *et al.* 2000). Also, in the leaf blotch pathogen *Mycosphaerella graminicola*, avirulence phenotypes on genetically distinct cultivars of wheat appear to be linked at the genetic level (G. H. J. Kema, personal communication). In none of these cases have the corresponding R-genes been cloned, so their primary structure is still unknown. It may well be that R-genes with different names or numbers represent the same R-gene in a different genetic background or are just slightly different but have the same *Avr* specificity. In both cases, a supposed *Avr* gene cluster might just be a single gene encoding an elicitor that is recognized by the highly homologous R-genes. The *Avr3-Avr10-Avr11* cluster can also represent a single regulatory gene encoding, *e.g.*, a positive regulator or modifier, and, as such, controlling other loci involved in avirulence against R3, R10, and R11. Presumably these loci are homozygous in the parental lines of cross 71 but heterozygous in some of the crosses used by Al-Kherb *et al.* (1995). This is consistent with their explanation that the observed aberrant segregation ratios with regard to virulence on R10 potato plants may be caused by a dominantly acting gene. The involvement of regulatory loci in determining race-specific virulence has been postulated for other pathogens. In *Melampsora lini*, the causal agent of flax rust, two dominant inhibitor loci were found. One compromises resistance provided by the resistance alleles L1, L7, L8, L10, and M1 whereas the other locus inhibits resistance provided by M1 (Jones 1988; Ellis *et al.* 1997). The gene involved might be a suppresser interfering with the race-specific resistance of the host, but it might also be a suppressor of avirulence genes. Alternatively, an *Avr* gene cluster may contain distinct genes that belong to a gene family and encode structurally related proteins. Such gene clusters do exist in *P. infestans*. One example is the *ipiB* gene family with three distinct members located on a 5-kb fragment (Pieterse *et al.* 1994). Another example is the *elicitin* gene family that consists of two gene clusters (R. Y. H. Jiang and F. Govers, unpublished results) and whose members encode species-specific avirulence factors (Kamoun *et al.* 1998).

Molecular cloning of the *Avr3-Avr10-Avr11* cluster and identification of the encoded protein(s) will reveal the true nature of the avirulence factors involved in R3, R10, and R11 resistance and may explain why these genes are clustered. Whatever the explanation is, stacking of R-genes to obtain broader resistance, but without knowledge of the genetic and molecular basis of avirulence, may not give the desired result. Complete loss of resistance may be caused by a single mutation if the supposed gene cluster appears to be just one gene or by a single deletion of the *Avr* gene cluster itself. Five out of six *Avr* genes in this study (*i.e.*, *Avr1*, *Avr3*, *Avr4*, *Avr10*, and *Avr11*) map on the most distal part of the linkage groups, probably close to the telomere. Taking into consideration that in eukaryotes, telomeric regions are among the most flexible regions in the genome, there may be a reasonable chance that avirulence is lost because of a deletion. In *M. grisea* a relatively large number of avirulence genes map near telomeres (Smith and Leong 1994; Valent and Chumley 1994; Mandel *et al.* 1997; Dioh *et al.* 2000) and in one case, indeed a deletion causes a phenotypic change from avirulent to virulent (Mandel *et al.* 1997). Mapping of telomeres might thus be instrumental in positioning avirulence genes. A telomeric repeat of *P. infestans* was cloned (Pipe and Shaw 1997), and it will be interesting to test its linkage to the *Avr* genes studied here and to other avirulence genes in *P. infestans*. Overall, identification of unstable genomic regions might contribute to the assessment of the flexibility of pathogens, particularly with regard to pathogenicity genes and avirulence genes (Stringer 1996; Freitas-Junior *et al.* 2000), and therefore to the assessment of the durability of the corresponding resistance genes.

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