Chromosomal Deletion in Isolates of *Phytophthora infestans* Correlates with Virulence on *R3*, *R10*, and *R11* Potato Lines

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In *Phytophthora infestans*, a cluster of three dominant avirulence genes is located on the distal part of linkage group VIII. In a mapping population from a cross between two Dutch field isolates, probe M5.1, derived from an amplified fragment length polymorphism (AFLP) marker linked to the *Avr3-Avr10-Avr11* cluster, hybridized only to DNA from the parent and F1 progeny that is avirulent on potato lines carrying the *R3*, *R10*, and *R11* resistance gene. In the virulent parent and the virulent progeny, no M5.1 homologue was detected, demonstrating a deletion on that part of linkage group VIII. *P. infestans* is diploid, so the avirulent strains must be hemizygous for the region concerned. A similar situation was found in another mapping population from two Mexican strains. The deletion was also found to occur in many field isolates. In a large set of unique isolates collected in The Netherlands from 1980 to 1991, 37% had no M5.1 homologue and the deletion correlated strongly with gain of virulence on potato lines carrying *R3*, *R10*, and *R11*. Also, in some old isolates that belong to a single clonal lineage (US-1) and are thus highly homogenous, deletions at the M5.1 locus were detected, indicating that this region is unstable.

Additional keywords: late blight, oomycetes, plant pathogen.

Phytophthora infestans causes late blight on both potato and tomato and is one of the most devastating plant pathogens worldwide. Attempts to control late blight by introducing resistance into potato cultivars have been largely unsuccessful. The level of broad-spectrum or horizontal resistance in commercial cultivars is insufficient to control the pathogen, and the use of single major resistance genes (*R* genes) has been hampered by the complex race structure of most *P. infestans* populations (Drenth et al. 1994; Schöber 1975; Shattock et al. 1977). In the last 2 decades, late blight reemerged as a serious threat to potato production. In the late 1970s, a new *P. in-*

festans population migrated from Mexico, the center of diversity, to Europe. This resulted in a worldwide displacement of the existing population, which was dominated by a single clonal lineage of the A1 mating type, termed US-1. The new populations are genetically more diverse and consist of A1 and A2 mating-type isolates (Drenth et al. 1994; Fry et al. 1992). Since *P. infestans* is heterothallic, the occurrence of both mating types led to the establishment of sexual reproduction and recombination, and it is likely that this caused the evolution of more aggressive isolates now found in many populations (Day and Shattock 1997; Flier and Turkensteen 1999). In order to elucidate the complexity of race structure and the evolution and selection of new races of *P. infestans*, we study the genetics of *R* gene-specific virulence.

P. infestans belongs to the oomycetes; its somatic hyphae are diploid and meiosis occurs just before mating in well-differentiated oogonia and antheridia. Genetic analysis of *P. infestans* is hampered by the lack of well-defined laboratory strains and mutants (Judelson 1996a). Moreover, sexual progeny especially from back crosses and sibling crosses is difficult to generate. In recent years, various types of molecular markers have been exploited to study inheritance in *P. infestans* (Carter et al. 1999; Goodwin et al. 1992; van der Lee et al. 1997). Segregation of markers in sexual progeny appears to be largely Mendelian. Nevertheless, deviations are found, mainly due to variation in ploidy levels. The occurrence of triploid, trisomic, or aneuploid progeny and hemizygous regions is not uncommon (Carter et al. 1999; Judelson 1996b; van der Lee et al. 1997; T. van der Lee and F. Govers, *unpublished data*). This phenomenon is consistent with the variable amounts of nuclear DNA content often found in *P. infestans* isolates (Tooley and Therrien 1987).

The variation in ploidy levels hampers the analysis of inheritance of *R* gene-specific virulence in *P. infestans* without linked molecular markers. Previously, we identified markers linked to six avirulence genes in *P. infestans*. Interestingly, three avirulence genes, *Avr3*, *Avr10*, and *Avr11* cluster on a distal part of linkage group VIII (van der Lee et al. 2001). In order to isolate and characterize the *Avr3-Avr10-Avr11* gene cluster, we have cloned the linked amplified fragment length polymorphism (AFLP) markers and used these as probes on Southern blots containing DNA from the parental lines of a mapping population that was used for the construction of a molecular genetic linkage map (van der Lee et al. 1997). One cloned AFLP marker hybridized to DNA from the parent that

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is avirulent on potato lines carrying resistance gene *R3*, *R10*, or *R11*, but not to DNA from the parent that is virulent on these lines. In this paper, we study to what extent lack of this AFLP marker correlates with virulence. For this purpose, not only were two F1 mapping populations screened but also a large set of field isolates with known virulence patterns. The data show that the majority of the field isolates that lack the marker are indeed virulent on potato lines carrying resistance genes *R3*, *R10*, and *R11*. The significance of this finding for the evolution of virulent races of *P. infestans* will be discussed.

RESULTS

AFLP markers linked to the *Avr3-Avr10-Avr11* cluster on linkage group VIII.

Fifteen AFLP markers tightly linked to the *Avr3-Avr10-Avr11* cluster were identified using bulked segregant analysis

(BSA) (van der Lee et al. 2001). All identified markers, i.e., 15 out of 25,000 fragments analyzed in the BSA, were in coupling phase with avirulence, while markers in repulsion phase in over 1,000 random markers were lacking. The deviation from the expected 1:1 ratio between markers in coupling and repulsion phases could be due to the absence of a homologous region on the other chromosome.

Twelve of the fifteen linked AFLP markers were cloned and used as probes on Southern blots containing genomic *P. infestans* DNA digested with *EcoRI*. Since the AFLP fragments were generated by polymerase chain reaction (PCR) amplification of *EcoRI-MseI* restriction fragments, they contain no internal *EcoRI* restriction site. Thus, fragments that are single copy in the genome should show one or, if the two homologous alleles are polymorph, two hybridizing fragments in the parental isolates. Nine of the twelve cloned AFLP markers, however, hybridized to several restriction fragments of differ-

Fig. 1. Segregation of marker M5.1 in the F1 progeny of *Phytophthora infestans* cross 71 (80029 × 88133). Autoradiographs of Southern blots containing total DNA from *P. infestans* isolates digested with *EcoRI* and hybridized with **A**, probe M5.1 and **B**, DNA fingerprint probe RG57. Virulence phenotypes on *R3*, *R10*, and *R11* potato plants are indicated by A for avirulent, V for virulent, or – for unknown. The sizes of the fragments (bp) are indicated on the left. The lanes contain DNA from the parental lines (80029 and 88133) and their F1 progeny (indicated by numbers with a prefix T). Two fragments in the avirulent parent, 80029, and the avirulent progeny hybridize to M5.1, but no homologous sequence is detected in the virulent parent, 88133, or the virulent progeny. Hybridization of the same blot with the multicopy probe RG-57 demonstrates that each lane contains DNA. **B**, Only one of the fragments (fragment 9) hybridizing to RG57 is shown.

Fig. 2. Segregation of marker M5.1 in the F1 progeny of *Phytophthora infestans* cross 68 (TV580 × TV618). Autoradiographs of Southern blots containing total DNA from *P. infestans* isolates digested with *BamHI* and **A**, hybridized with probe M5.1 and **B**, *calA*, a *P. infestans* gene encoding calmodulin. The sizes of the fragments (kb) are indicated on the left. The lanes contain DNA from the parental lines (TV580 and TV618) and their F1 progeny (indicated by numbers). M5.1 hybridizes to two fragments in parent TV580 that is avirulent on *R3* carrying potato lines and in part of the F1 progeny. In the other lanes, including the one containing DNA from the virulent parent TV618, no M5.1 homologue is detected. Hybridization of the same blot with a *calA* probe demonstrates that each lane contains DNA. This probe detects a polymorphism in the two parental lines that segregates in the F1 progeny.

ent lengths, suggesting that the cloned fragments contain repetitive DNA. Also, several of the cloned AFLP markers hybridized to more fragments in the DNA of the avirulent parent (strain 80029) as compared with the DNA of the virulent parent (strain 88133) (data not shown). Among these was marker M7.1, derived from the most distal AFLP marker on this linkage group, i.e., AE+CA/M+GGs826. M7.1 hybridized to up to seven fragments of different length. Several weaker hybridizing fragments were not polymorphic between the parental isolates and no intensity difference was observed, but two strongly hybridizing fragments were found in the DNA of the avirulent parent that were lacking in the virulent parent.

Two markers, M11.1 derived from marker AE+CT/M+ATs306 and M12.1 derived from marker AE+CT/M+TGs308, detected a single-copy *EcoRI* fragment of the same size (data not shown). Possibly, the two *EcoRI*-*MseI* markers are situated internally on the same *EcoRI*-*EcoRI* fragment. Neither M11.1

nor M12.1 revealed an *EcoRI* fragment length polymorphism between the two parental isolates.

One cloned AFLP fragment, designated M5.1 and derived from marker AE+AT/M+AGs400, hybridized to an 800- and a 1,200-bp *EcoRI* fragment of the avirulent parent 80029 but, even at low stringency hybridization conditions (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 55°C) and long exposure times, no hybridizing fragments were detected in the virulent parent 88133 (Fig. 1A). Lowering the hybridization and washing temperature from 65 to 55°C did not change the hybridization pattern. The hybridization signal of the 800-bp fragment was stronger than that of the 1,200-bp fragment, suggesting that M5.1 is derived from the 800-bp fragment and that the 1,200-bp fragment contains a homologue. When M4.1, one of the multicopy probes derived from AFLP marker AE+AG/M+AAs258, was used as probe for Southern blot hybridizations, an 800-bp fragment was de-

Table 1. Occurrence of the M5.1 homologue in field isolates of *Phytophthora infestans*^a

Isolate ^b	Mating type	RG57 genotype ^c	M5.1	Virulence ^d				
				<i>R1</i>	<i>R3</i>	<i>R4</i>	<i>R10</i>	<i>R11</i>
80029	A1	01001000001011010001010101	+	A	A	V	A	A
81197	A2	11001010000011110011110101	–	V	V	V	V	V
84044	A1	11101011001011000011110101	+	V	V	V	V	V
85005	A1	10101110001011010101110101	+	V	A	V	A	A
85025	A2	1100101001011100001010101	–	V	V	V	V	V
85026	A2	11001101111011100001010101	+	V	V	V	V	V
85027	A2	10001101111011100001010101	+	V	V	V	V	V
85206	A1	11101111101011110011110101	+	V	V	V	V	–
86057	A2	10001000000011110011110101	–	A	A	V	A	V
87001	A2	01001000001011110001110101	+	A	A	V	A	A
87010	A2	10001100001011100011110101	–	V	A	V	V	V
87013	A1	11101111101011110001110101	+	V	V	V	–	–
87030	A2	11101010001011100011110101	–	A	A	V	V	V
87032	A2	11000100000011110001010101	–	A	V	A	A	V
87034	A2	10101010000011100001100101	+	V	–	V	V	V
87073	A2	10101111100011110000100101	+	A	A	V	A	A
87086	A2	11001000001011110011110101	+	V	A	V	A	A
87110	A2	10001000001011100001110101	+	A	V	V	A	V
87125	SF ^e	11101111001011010001110101	–	V	V	V	V	V
87134	A2	10001000001011110011111001	+	V	A	A	A	A
87148	A2	11001000001011101001010101	–	V	A	V	A	V
87174	SF ^e	10101110001011100001100101	+	A	V	A	V	V
87177	A1	10101110001011000011110101	–	V	V	V	V	A
88013	A2	10001000101011101001100101	+	V	V	A	V	V
88014	A2	10101010001011100001100101	+	V	V	A	A	V
88034	A2	10001001000011110011110101	+	V	A	V	A	A
88046	A1	11101110101011110011110101	–	V	V	V	V	V
88051	A2	11101111001011110011110101	–	V	V	V	V	V
88052	A2	11101111000011110001100101	–	A	V	V	V	V
88055	A2	01001100101011110011110101	+	A	V	V	V	V
88067	A1	10101010001011101011100101	+	A	V	V	–	–
88133	A2	10101011001011100111110101	–	V	V	A	V	V
88148	A2	10101111000011100001100101	–	V	V	V	V	V
88165-01	A1	10101111101011110011100101	+	V	V	V	V	V
88175	A2	10001000001011110001110101	–	V	V	V	V	V
88212	A2	10101010001011100101100101	–	V	V	A	A	V
89019	A2	11101110001011110001110101	+	A	A	V	A	A

(continued on next page)

^a The isolates were collected in The Netherlands from 1980 to 1991. Their mating type, RG57 DNA fingerprinting pattern, and virulence phenotype toward potato lines carrying resistance gene *R1*, *R3*, *R4*, *R10*, or *R11* are listed.

^b First two numbers refer to the year of isolation.

^c DNA fingerprinting pattern refers to bands 1, 2, 3, 4, 5, 6, 7, 8, 9, 9a, 10, 12, 13, 14, 14a, 16, 17, 18, 19, 20, 21, 22, 23, 24, 24a, and 25 hybridizing to probe RG57 as defined by Goodwin and associates (1992) and Drenth and associates (1993). The binary code 1 and 0 represents presence and absence, respectively.

^d Virulence on potato plants carrying the *R* gene listed. V = virulent, A = avirulent, and – = unknown

^e SF = self fertile.

tected in parent 80029 next to seven other hybridizing fragments (data not shown). Also, in this case, the 800-bp *EcoRI* fragment was absent in the virulent parent and it was the strongest hybridizing fragment in the avirulent parent, indicating that this is the *EcoRI-EcoRI* fragment from which the marker is derived. Since the markers M4.1 and M5.1 showed clear polymorphism between the parental isolates, they were used for further studies on the sexual progeny of the cross 80029 × 88133 (cross 71).

Segregation of marker M5.1 in mapping populations.

M5.1 hybridized to an 800- and a 1,200-bp *EcoRI* fragment of parent 80029, the avirulent parent from cross 71 in which the initial AFLP marker was identified. The two fragments cosegregated in the F1 progeny of cross 71, showing that the two homologues detected in the 80029 parent are not allelic but reside on the same chromosome (Fig. 1). The restriction fragment length polymorphism (RFLP) markers generated

with M5.1 cosegregated with the cluster of *Avr3-Avr10-Avr11* linked markers including marker AE+AT/M+AGs400. Only DNA from the avirulent progeny hybridized to M5.1, demonstrating that only F1 progeny avirulent on potato lines carrying *R3*, *R10*, and *R11* contain the homologues (Fig. 1A). The 800-bp fragment identified upon hybridization with M4.1 also cosegregated with markers tightly linked to the *Avr3-Avr10-Avr11* cluster in the progeny of cross 71 (data not shown).

To investigate whether M5.1 segregates in a similar way in other crosses, we analyzed an unrelated cross between two Mexican isolates, TV580 × TV618 (cross 68, generated and kindly provided by L. J. Spielman and W. E. Fry, Cornell University; Goodwin et al. 1992). The same two *EcoRI* fragments were present in the parent that is avirulent on *R3* plants (TV580) and absent in the parent virulent on *R3* (TV618). Hybridization of M5.1 to *BamHI*-digested DNA again revealed two fragments in TV580, but no hybridization was detected in TV618 (Fig. 2). Also in cross 68, M5.1 segregates (Fig. 2).

Table 1. (continued from preceding page)

Isolate ^a	Mating type	RG57 genotype ^b	M5.1	Virulence ^c				
				<i>R1</i>	<i>R3</i>	<i>R4</i>	<i>R10</i>	<i>R11</i>
89038	A2	0100100000011110001010101	+	A	V	V	V	V
89056	A2	10101110101011110111110101	–	A	V	V	V	V
89061	A1	10101111101011110111110101	+	V	V	V	V	A
89070	A2	11001100101011110001010101	–	V	V	V	V	V
89075	A2	11101100101011111001010101	–	V	V	V	A	A
89076	A1	10101111101011110001110101	–	V	V	V	V	V
89081	A1	01101010101011111011110101	–	A	V	V	A	V
89140-01	A1	11111110101011110011110101	+	V	V	V	V	V
89140-02	A1	11111111101011110011110101	+	V	V	V	V	V
89140-07	A1	1011011001011110001100101	–	V	V	V	V	V
89140-09	A2	10011001001011100011110101	–	V	V	V	V	V
89140-15	A1	10111010001011110001100101	–	V	V	V	V	V
89141-02	A1	11111011001011110011110101	–	V	V	V	V	V
90041	A1	1110111001011110001110101	–	V	V	V	V	V
90056-02	A2	11101110011011010000110101	+	V	A	V	V	V
90056-03	A2	10001100001011000010100101	–	A	V	A	V	V
90062	A2	10101110001011010011100101	+	A	A	V	V	V
90080	A2	11101011001011110011110101	+	A	A	V	A	A
90083	A2	00101010101011110011100101	+	V	A	V	A	A
90101	A1	10101110001011110011101101	+	A	V	A	V	V
90111	A2	11001001101011000011110101	+	A	A	V	V	V
90121	A2	11001000100011000001110101	+	V	A	V	A	A
90124	A2	11101010001011010001010101	+	V	V	V	V	V
90125	A1	11101010001011110001110101	–	A	V	V	–	V
90127	A2	10101010001011000011100101	–	V	V	V	A	V
90128	A2	10001000101011100001100101	+	V	V	V	V	V
90137	A1	11000100001011100010110011	+	V	V	V	V	V
90149	A2	10001100100011110001100101	+	V	V	V	A	V
90190	A1	10001010000011100001100101	–	V	V	V	V	V
90196	A2	10101011001011110001100101	+	A	A	V	A	A
90208	A2	101010101010110001100101	+	V	V	V	A	V
90209	A1	11001000001011110001110101	–	V	V	V	V	V
90215	A1	10101111001011110011100101	+	V	V	V	A	A
91001	A2	10101011010011111001100101	+	A	A	A	A	A
91002	A2	10101011101011110001100111	+	A	A	A	A	A
91004	A2	10101011010011110001100101	+	V	V	A	V	V
91005	A2	11001000111011110001110101	+	V	A	V	A	A
91006	A2	11001000001011101000111011	+	V	A	V	A	A
91007	A2	10101010011011110011100101	+	V	V	V	A	V
91008	A1	1010111111011110011100101	+	V	V	V	V	V
91009	A1	10001001101011101011100101	+	V	V	A	V	V
91010	A2	11101010111011110001110101	+	V	V	V	A	A
91011	A2	10101010001011110011110011	+	A	V	V	A	V
91012	A2	10101010111011110011110101	+	A	V	V	V	–
91014	A1	10101110001011110011110101	+	V	V	V	V	V
91015	A1	11101011001011110101111111	+	V	A	V	A	A

The virulence of isolates TV580 and TV618 on *R10* and *R11* plants is not known, but in the progeny of cross 68, the two fragments generated with M5.1 cosegregated with AFLP markers that are mapped close to the telomere on linkage group VIII in cross 71, where the *Avr3-Avr10-Avr11* cluster is located (van der Lee et al. 2001).

Occurrence of marker M5.1 in field isolates.

The parents of cross 71 are field isolates that were collected in The Netherlands in 1980 (80029) and 1988 (88133). To investigate the occurrence of marker M5.1 in Dutch field populations, we selected a set of 83 field isolates collected between 1980 and 1991. The set is a mixture of A1 and A2 mating-type isolates and includes the parents of cross 71. As was determined previously (Drenth et al. 1994), each of these isolates shows a unique hybridization pattern with the multilocus DNA fingerprint probe RG57 (Table 1). Hybridization of *Eco*RI-digested DNA of these field isolates with probe M5.1 either yielded the 800- and 1,200-bp fragments (52 or 63% of the isolates) or no hybridization signal was seen (31 or 37% of the isolates) (Fig. 3A, Table 1). As the region is hemizygously present in the avirulent parents of cross 71 and cross 68, stronger intensities of hybridization to the 800- and 1,200-bp fragments might reveal homozygosity for the M5.1 locus in some of the field isolates. However, there were no indications for the occurrence of isolates homozygous at the M5.1 locus; differences in observed signal intensities were also seen with

other probes on the same blots, e.g., RG57, and were due in all cases to differences in the amount of DNA loaded on gel.

Isolates collected before the second worldwide migration of *P. infestans* (before 1980) are genetically highly similar (Drenth et al. 1993, 1994; Fry et al. 1992). When fingerprinted with the fingerprint probe RG57, most of these so-called 'old' isolates show an identical or nearly identical genotype that has been designated US-1. With AFLP DNA fingerprinting, some genotypic variation was found, albeit limited (Kamoun et al. 1998). We analyzed several Dutch representatives of the US-1 clonal lineage for the presence of M5.1 and found they showed a different hybridization pattern when compared with the 'new' isolates. The 800- and 1,200-bp fragments were absent, but a 2,100-bp or a 3,500-bp fragment, or both, was present (Fig. 3B). The hybridization signal was much lower when compared with the signals obtained with the 800- and 1,200-bp fragments, indicating that the 2,100- and 3,500-bp fragments are only distantly related to M5.1. In a Peruvian US-1 type isolate (PE-807, Fig. 3B), no hybridization was detectable. The 2,100- and 3,500-bp fragments were found in another isolate from Peru (PE821), but that isolate has an RG57 genotype other than US-1. In contrast, a non-US-1 type isolate from Mexico (TV525; Fig. 3B) has the 800- and 1,200-bp fragments similar to the new isolates from The Netherlands and to TV580, the A1 parent from cross 68. As in the Mexican isolates and the new isolates from The Netherlands, as well as in the US-1 isolates,

Fig. 3. Occurrence of marker M5.1 in field isolates of *Phytophthora infestans*. Autoradiographs of Southern blots containing total DNA from *P. infestans* field isolates digested with *Eco*RI and hybridized with probe M5.1. **A**, Dutch field isolates collected in 1990. Virulence phenotypes on *R3*, *R10*, and *R11* potato plants are indicated by A for avirulent, V for virulent, or - for unknown. Isolates 90056-3, 90056-7, and 90056-14 have identical RG57 genotypes (only 90056-3 is listed in Table 1). Similarly, isolates 90125 and 90126 are identical (only 90125 is listed in Table 1). The RG57 genotype of isolate 90206 is identical to that of isolate 88165-1 (listed in Table 1). **B**, Various field isolates from either unknown origin (46210 and 46211), The Netherlands (NL), Peru (PE), or Mexico (TV; Toluca Valley). The asterisk (*) indicates US-1 or US-1-related isolates. The sizes of the hybridizing fragments (bp) are indicated on the left.

polymorphism at the M5.1 locus seems to be due to deletions; in some cases, either the 2,100-bp fragment or the 3,500-bp fragment is lacking. In other cases, the M5.1 locus is completely absent. A complicating matter is isolate 46211. Isolates 46210 and 46211 were deposited in the American Type Culture Collection in 1946 but their origin is unknown. Isolate 46210 resembles the other US-1 type isolates, but in 46211, the 2,100-bp fragment is present in combination with the 800- and 1,200-bp fragments (Fig. 3B).

Correlation between absence of a M5.1 homologue and virulence on potato lines carrying resistance genes *R3*, *R10*, and *R11*.

Since the absence or presence of bands hybridizing to M5.1 matched the segregation of AFLP marker AE+AT/M+AGs400 in cross 71, and since this AFLP marker is tightly linked to the *Avr3-Avr10-Avr11* cluster (van der Lee et al. 2001), polymorphism of M5.1 in field isolates opens the possibility of studying its correlation with (a)virulence in field isolates toward potato lines carrying *R3*, *R10*, and *R11*.

The set of 83 field isolates was previously tested for virulence (Drenth et al. 1994) on the same differential set of potato genotypes as was used for mapping the *Avr3-Avr10-Avr11* cluster in cross 71 (van der Lee et al. 2001). Combination of the virulence data and M5.1 hybridization data (Table 1) showed a correlation between virulence on *R3*, *R10*, or *R11* potato plants and absence of the M5.1 homologue (Table 2). Such a correlation is not found with virulence on *R1* and *R4* plants. The χ^2 distribution resulted in *P* values varying from *P* < 0.001 for virulence on *R11* to *P* < 0.02 for virulence on *R3* and *R10* (Table 2). Combining (a)virulence on all three differentials with the absence of marker M5.1 resulted in a corresponding *P* value of *P* < 0.0005. This correlation is mainly based on the complete absence of avirulent isolates that lack a homologue for marker M5.1. In contrast, the presence of marker M5.1 had no, or limited, predictive value for a virulent phenotype. Many isolates (nearly 50%) that have a homologue for M5.1 were still virulent on potato lines carrying *R3*, *R10*, and *R11*. The relation of the deletions in the US-1 genotypes with virulence toward potato lines with *R3*, *R10*, and *R11* is difficult to determine. The number of available US-1 isolates is limited and many of them are no longer pathogenic enough to perform reliable virulence tests on a differential set.

DISCUSSION

In the search for AFLP markers genetically linked to the *Avr3-Avr10-Avr11* cluster, we expected an even distribution of markers in coupling and repulsion phases with the *Avr* genes. Instead, all 15 tightly linked markers identified in cross 71 were in coupling phase with avirulence (van der Lee et al. 2001). We hypothesized that a homologous region for this avirulence locus might be lacking on one of the two homologous chromosomes. Indeed, we identified a region on the distal part of linkage group VIII that is only present on the chromosome that carries the dominant *Avr* genes. We cloned 12 linked AFLP markers and used them as RFLP probes for genomic Southern blots. This would enable identification of all alleles of the locus. Most of the cloned AFLP markers hybridized to multicopy fragments in genomic DNA of *P. infestans*. With some markers, including M7.1 representing the most distal marker on this linkage group, additional fragments were detected in the DNA of the avirulent parent of cross 71 as compared with DNA from the virulent parent. However, since these additional fragments might be allelic to other fragments at different locations in the genome, these data are inconclusive for proof of a deletion. Fortunately, three probes detected single-copy fragments and one, probe M5.1, hybridized only to DNA from the avirulent parent and the avirulent progeny of cross 71. This clearly showed that this chromosome contains a region for which no homologue is present, neither on the homologous chromosome in the avirulent parent nor on the homologous chromosomes of the virulent parent (Fig. 4). The same situation was found in the parents and progeny of a second unrelated cross, cross 68. Since a high proportion of this region contains repetitive DNA sequences, the size of the deletion is difficult to determine by Southern blot hybridization. However, an indication of the size of the deletion may be obtained from the number of AFLP markers that was generated. In the AFLP-based BSA, around 25,000 fragments were screened for linkage to the *Avr3-Avr10-Avr11* cluster (van der Lee et al. 2001). Given an expected genome size of 250 Mb, on average, one AFLP fragment was generated every 10 kb. Due to the deletion, all unique AFLP fragments in the region would be polymorphic and, therefore, the 15 tightly linked AFLP markers would represent around 150 kb. If all AFLP fragments in this region were polymorphic, this would also mean that the region should be highly saturated with markers.

Table 2. Correlation between virulence of 83 Dutch *Phytophthora infestans* field isolates toward potato lines carrying resistance genes *R3*, *R10*, and *R11*, either alone or together, and the presence of the M5.1 homologue^a

<i>R</i> gene	<i>N</i> ^b	M5.1+		M5.1–		<i>P</i> ^b
		VIR	AVR	VIR	AVR	
1	83	34	18	23	8	0.402
3	82	31	20	27	4	0.011
4	83	43	9	27	4	0.593
10	80	25	25	23	7	0.018
11	79	28	20	29	2	0.0007
3, 10, 11 ^c	55	18	17	20	0	0.00018

^a For the isolates containing (M5.1+) or lacking (M5.1–) M5.1, the numbers of isolates avirulent (AVR) and virulent (VIR) on plants carrying the indicated *R* gene are listed.

^b Number of isolates for which the virulence phenotype is known.

^c *P* value for the significance of deviation from the expected ratio using a χ^2 test (3 df) calculated using the probabilities for virulence and the presence of marker M5.1 over the total number of genotypes.

^d Combined virulence or avirulence on potato lines carrying *R3*, *R10*, and *R11*; only those isolates in which all three phenotypes are either avirulent or virulent are included.

Indeed, hybridization with the cloned AFLP markers suggests that by BSA in two independent complete genomic screens we have landed twice on the same *Eco*RI fragment. M4.1 and M5.1 hybridize to the same *Eco*RI fragment, and also M11.1 and M12.1 hybridize to *Eco*RI fragments of the same size and likely to be identical. In contrast to M5.1, M11.1 and M12.1 are present in both the virulent and the avirulent parent, which might imply that there are more deletions in the region or that the deletion is smaller than calculated above.

In the late 1970s to early 1980s, Europe was invaded by a new population of *P. infestans* (Drenth et al. 1994). The new population shows a large genetic variation but no distinctive genetic clusters (Kamoun et al. 1998; Zwankhuizen et al. 2000). It is assumed that sexual reproduction plays an important role in the continuous appearance of new genotypes in *P. infestans* populations in The Netherlands (Drenth et al. 1994; Zwankhuizen et al. 2000). Despite the large genetic variation, virulence toward particular *R* gene combinations is observed more frequently than expected on the basis of random distribution. This is especially true for virulence on *R10* and *R11* plants, and this might point to a common origin for these virulence phenotypes. The clustering of *Avr3*, *Avr10*, and *Avr11* on linkage group VIII suggests a single region determining avirulence toward a number of *R* genes. We observed a strong correlation between the absence of marker M5.1 and the virulence of *P. infestans* field isolates on potato lines carrying *R3*, *R10*, and *R11*. This correlation was not observed with virulence on potato lines carrying *R1* and *R4* and it suggests a similar organization or regulation, or both, of the *Avr3-Avr10-Avr11* cluster in many field isolates. The strong correlation is due to the low number of isolates that lack M5.1 and are avirulent (two for *R11*, four for *R3*, and seven for *R10*). In fact, these numbers are close to the error rates that occur in the virulence testing on the standard differential set. Isogenic *R* gene-containing potato lines are not available, so the standard set is composed of potato lines with the various *R* genes in different genetic backgrounds. Especially *R10* is difficult to score.

Fig. 4. Hypothetical model of the distal part of the two homologues of linkage group VIII of *Phytophthora infestans* in the parents of two crosses and in field isolates. Virulence phenotypes on *R3*, *R10*, and *R11* potato plants are indicated by A for avirulent and V for virulent. In both crosses, the A1 parent is avirulent (80029 in cross 71 and TV580 in cross 68) and the A2 parent is virulent (88133 in cross 71 and TV618 in cross 68). The open vertical bars represent deletions and the small black horizontal bars represent mutations.

Whereas absence of M5.1 shows a clear correlation with gain of virulence on *R3*, *R10*, and *R11* potato plants, presence of M5.1 in field isolates is much less predictive for the virulence phenotype (Table 2; Fig. 4). Around 50% of the *P. infestans* genotypes that contain M5.1 are still virulent on potato lines with *R3*. In the genotypes that have M5.1, there is still, albeit less pronounced, a correlation between virulence on potato lines carrying the *R3*, *R10*, and *R11* resistance genes. In these cases, the size of the deletion may be smaller, or additional or different mutations either at the locus or elsewhere can result in the acquirement of *R* gene-specific virulence toward more than one *R* gene. Still, the high number of isolates that lack this genomic region (38% of the total number of genotypes and 53% of the isolates virulent on *R11*) suggests that this deletion is associated with an important mutation in Dutch field isolates to gain virulence on potato plants carrying *R3*, *R10*, and *R11*. The importance of the deletion is further strengthened by the fact that this region is also deleted in Mexican and Peruvian isolates.

R gene-specific virulence of *P. infestans* field isolates has been the subject of many studies (Andrison 1994; Drenth et al. 1994; Mooi 1970; Schöber 1972; Shattock et al. 1977). Even in the old clonal lineage, new races frequently appeared that could infect cultivars with new *R* genes (Mooi 1970). Stepwise increases in the number of *R* genes used in commercial potato cultivars were postulated to lead to stepwise increases in the complexity of races in the *P. infestans* population. However, *R* gene-specific virulence in *P. infestans* populations to *R* genes to which it was not exposed has been reported (Andrison 1994; Drenth et al. 1994). Also, selection pressure on the Dutch *P. infestans* populations to adapt to the new *R* genes has been very limited. The most popular potato cultivars in The Netherlands contain no known *R* genes and some less important cultivars contain mostly *R1*, *R3*, or *R10*, either alone or in combination.

How then can we explain the presence of virulent isolates in these populations? In situations in which *Avr* genes do not contribute much to the fitness of *P. infestans* on potato lines not carrying the corresponding *R* gene, spontaneous mutations can easily get fixed in the population or population frequencies caused by selection in the past might not alter in a new situation. Alternatively, some mutations may effect virulence toward more than one *R* gene. A deletion overspanning several linked *Avr* genes or a mutation in a regulatory gene that acts dominant on several *Avr* genes might have such an effect and could explain our findings with respect to *Avr3*, *Avr10*, and *Avr11*. Virulence toward potato plants carrying *R11*, an *R* gene that has not been used in commercial cultivars, could have hitchhiked along with the selection for virulence toward potato cultivars containing *R3* and *R10*.

Particularly interesting is the finding that in *P. infestans* isolates 80029 and TV580 the chromosomal region containing the *Avr* gene cluster is present in a hemizygous state. Based on hybridization intensities, this is likely to be true for many more isolates, but for definite proof of hemizygosity, sexual offspring must be analyzed. Another hemizygous region in *P. infestans* was found at the mating-type locus (Judelson 1996b). Moreover, the high density of markers in coupling phase found on other linkages groups suggests that hemizygosity also occurs at other loci in the *P. infestans* genome (T. van der Lee and F. Govers, unpublished data). Hemizygous

regions increase variability and may account for spontaneous loss of particular phenotypic characters. For example, Al Kerb and associates (1995) described a sudden change in virulence from race 1.4 to 1.3.4.7.11 in one of their parental *P. infestans* lines upon storage in liquid nitrogen. This may be the result of a spontaneous deletion of a region carrying the relevant *Avr* genes. In addition, in isolates of the clonal lineage US-1, deletions at the M5.1 locus were found, thus supporting the hypothesis that this region is unstable. The telomeric position, the hemizygous state, and the high number of repeats might result in a higher mutation rate for genes located in this region, as was found for *Pi-ta* from the rice blast fungus *Magnaporthe grisea* (Orbach et al. 2000). We are in the process of cloning the *Avr3-Avr10-Avr11* cluster, enabling us to test whether virulence results from loss of *Avr* genes as has been reported, e.g., for *Avr9* from *Cladosporium fulvum* (Van Kan et al. 1991) and *Pi-ta* from *M. grisea* (Orbach et al. 2000).

MATERIALS AND METHODS

Cloning of AFLP markers.

The AFLP markers were generated as previously described (van der Lee et al. 1997, 2001) and were separated on 6% denaturing polyacrylamide gels. The gels were dried on gel blotting paper GB002 (Schleicher & Schuell, Dassel, Germany) and labeled fragments were visualized on an X-ray film. Markers were cut from the gel. Accuracy was verified by reexposure of a film to the gel. The tiny pieces of acrylamide were washed twice with 500 µl of 10 mM Tris-HCl, 1 mM EDTA for 20 min. Subsequently, the acrylamide pieces were crushed and kept for at least 48 h in 100 µl of 1 mM Tris-HCl, 0.1 mM EDTA to allow the DNA to elute from the acrylamide. Five microliters of the eluted DNA was used to reamplify the fragment in 30 PCR cycles (30 s at 94°C, 30 s at 56°C, and 1 min at 72°C) using AFLP primers without the two selective 3' nucleotides. The amplified fragments were run on 1% agarose gels and the fragments were purified from agarose, ligated in pGEM-T-easy vector (Promega Benelux, Leiden, The Netherlands), and transformed to *Escherichia coli* strain JM109 according to the instructions of the suppliers.

Southern blot hybridizations.

The cloned AFLP markers were used as probes for genomic Southern blot hybridizations. Plasmid DNA was isolated from *E. coli* and digested with *EcoRI*. By agarose gel electrophoresis, the insert was separated from the vector, gel-purified, and labeled with ³²P-α-dATP by random prime labeling. Genomic DNA of *P. infestans* was isolated as described previously (Drenth and Govers 1993). From each isolate, 5 to 10 µg of DNA was digested with *EcoRI* or *BamHI*, separated on a 0.8% agarose gel, blotted onto Hybond N, and hybridized overnight in modified Church buffer (Church 1984) at 55 or 65°C, followed by three 20-min washes at the same temperature in, subsequently: 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0 (2× SSC)/0.1% SDS (twice) and 0.5× SSC/0.1% SDS (once). Autoradiographs of the blots were made by exposure of KODAK-Xomat AR film (Eastman Kodak Company, Rochester, NY, U. S. A.) at -70°C with an intensifying screen. Hybridizing fragments were scored visually. The ethidium bromide-stained gels and hybridization patterns obtained with the DNA fingerprint probe RG57 (Goodwin et al. 1992) and

with the single-copy probe *calA* encoding calmodulin (Pieterse et al. 1993) were used as loading controls.

Characterization of *P. infestans* field isolates.

RG57 DNA fingerprinting patterns, mating type, and virulence phenotypes were compiled from databases from our laboratory and were determined according to methods described by Drenth and associates (1994). Data on the virulence phenotypes of the old US-1 isolates have not been included because they are incomplete. Attempts to repeat the virulence tests and to obtain complete and reliable data were unsuccessful. We, and others, found that determining virulence phenotypes of *P. infestans* isolates that have been in storage for a long time is difficult and unreliable. Over time the isolates tend to lose pathogenicity.

Data handling and computation.

RG57 fingerprint patterns were used to identify unique genotypes. Isolates with the same RG57 fingerprint were grouped, and only one isolate of each RG57 genotype was used for further analysis. If isolates with identical RG57 genotypes had different virulence scorings on a particular *R* gene differential, the virulence on this particular *R* differential was rated unknown. Statistical analyses were performed using Microsoft Excel.

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NOTE ADDED IN PROOF

More precise estimations of the length of the M5.1 hybridizing *EcoRI* fragments in the genomic DNA of the avirulent strains revealed that both fragments are slightly larger. What we have indicated here as 'the 800-bp fragment' is more in the range of 1,000 bp, whereas 'the 1,200-bp fragment' is approximately 1,500 bp.

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