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Genetic variation among asexual progeny of *Phytophthora infestans* detected with RAPD and AFLP markers

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Genotypic variation among 32 single-zoospore isolates (SZI) of *Phytophthora infestans*, derived asexually from two hyphal-tip parental isolates (PI-105 and PI-1) of the US-8 genotype, was assessed with 80 random amplified polymorphic DNA (RAPD) primers and 18 amplified fragment length polymorphic DNA (AFLP) primer pairs. In previous investigations, the SZIs from parental isolate PI-105 showed high levels of virulence variability and were differentiated into 14 races, whereas the SZIs from PI-1 showed identical virulence to the parent. The purpose of this investigation was to determine if phenotypic variation observed among SZIs of *P. infestans* could be detected at the DNA level in these isolates. Polymorphism was detected with 51 RAPD primers and with all 18 AFLP primer pairs in PI-105 SZIs. In SZIs from PI-1, polymorphism was also detected with 25 RAPD primers and 17 AFLP primer pairs. Cluster analysis using the unweighted pair-group method with arithmetic averages (UPGMA) separated the SZIs from parent PI-105 into six virulence groups, 11 RAPD groups and three AFLP groups. Cluster analysis of PI-1 SZIs, which all belong to the same virulence group, differentiated them into four RAPD groups and six AFLP groups. No close correlation among RAPD, AFLP and virulence groups could be established within the two progenies of SZIs. Results of this study suggest that there is a considerable level of inherent genetic variability among SZIs derived asexually from the same parental isolate. The possible mechanisms and implications of this genetic variation are discussed.

Keywords: AFLP, *Phytophthora infestans*, potato late blight, RAPD, single-zoospore isolates, virulence

Introduction

Phytophthora infestans is a pathologically and genotypically heterogeneous species. In the past two decades, two major events have stimulated research to explore the genetic system in *P. infestans*. The first event was the appearance of A2 mating type in Europe and North America; the second was the development of new molecular markers that allowed the exploration of the genetic system beyond the visible phenotypes.

Recent studies have revealed that old *P. infestans* populations, occurring in most parts of the world before the 1980s (the US-1 clonal lineage) had very little genetic diversity based on the allozyme markers (*Gpi* and *Pep*) and DNA fingerprints with probe RG57 (Fry *et al*., 1992; Goodwin *et al*., 1994b). During the 1980s and early 1990s, the old *P. infestans* populations were displaced by a 'new population' characterized by the presence of two mating types, insensitivity to metalaxyl, new alleles at the *Gpi* locus, and multiple genotypes based on DNA fingerprinting (Fry *et al*., 1992; Goodwin *et al*., 1994a; Goodwin *et al*., 1998).

Variation among *P. infestans* populations in phenotypic characters such as growth rate, colony morphology, aggressiveness and virulence were recognized in the old populations of *P. infestans*, which were restricted to asexual reproduction (Graham, 1955; Wallin, 1957; Gallegly & Eichenmuller, 1959; Caten & Jinks, 1968; Upshall, 1969; Caten, 1970; Caten, 1971; Sujkowski, 1989; Zarzycka, 1996). The sources and mechanisms of this variability in the absence of sexual reproduction are not well understood. Understanding sources and mechanisms involved in this genetic variability during asexual reproduction was always hindered by the limited number of genetic markers available for population genetic studies.

The recent development of genetic markers based on the polymerase chain reaction (PCR), such as random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990) and amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995), provide an unlimited number of markers that can be used in population genetic studies. DNA polymorphism in these markers arises from differences

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in the DNA sequences caused by nucleotide pair substitutions, deletions, inversions and translocations (Waugh & Powell, 1992).

In a related study (Abu-El Samen *et al*., 2003), the extent of phenotypic variability among asexual progenies of *P. infestans* was investigated for virulence, mating type, allozyme banding patterns and response to metalaxyl. Considerable variation was found among some progenies derived asexually from the parental isolates. The objective of the study presented here was to use RAPD and AFLP markers to determine if phenotypic variation observed among single-zoospore isolates (SZIs) of *P. infestans* can be detected at the level of DNA in these isolates.

Materials and methods

Isolates

Thirty-two SZIs were produced asexually from two parental isolates of *P. infestans* using the procedures described by Caten & Jinks (1968). Twenty-four SZIs derived from the parental isolate PI-105P [hyphal-tip isolate, MN 1995; US-8 genotype, A2 mating type; American Type Culture Collection (ATCC) #MYA1114] were selected to study the genotypic variability among this phenotypically variable asexual progeny. These isolates demonstrated substantial differences in virulence phenotypes (Table 1) and response to metalaxyl (data not shown; Abu-El Samen *et al*., 2003). A set of eight SZIs derived from the parental isolate PI-1 (hyphal-tip isolate, MN 1994; US-8 genotype, A2 mating type; ATCC #MYA-1113) with the same virulence phenotype were also included in the analysis. One isolate of *P. infestans* US-940-480 (US-8 genotype, obtained from W. E. Fry, Department of Plant Pathology, Cornell University, Ithaca, NY, USA) was included in the RAPD analysis for comparative purposes. All isolates were maintained on rye B agar (Caten & Jinks, 1968) slopes at 15°C in the dark for the course of the study.

Virulence assessment

The virulence phenotypes of the SZIs derived from both parental isolates (PI-105 and PI-1) were evaluated on a single resistance gene (R1–R11) differential set (USDA-Potato Introduction Station, Sturgeon Bay, WI, USA). Inoculum preparation, inoculation, infection response scoring and race nomenclature were as previously described (Spielman *et al*., 1989; Goodwin *et al*., 1995; Abu-El Samen *et al*., 2003). The virulence phenotypes of these isolates had been characterized previously (Abu-El Samen *et al*., 2003) and were verified again by repeating the virulence test at least three or four times on different testing dates and using different sets of the potato differentials. Virulence data from PI-105 SZIs were converted into a binary matrix in which the reaction of each isolate towards each R gene was given a value of 0 for an incompatible interaction or 1 for a compatible interaction. This

Table 1 Virulence phenotypes of *Phytophthora infestans* SZIs derived from the parental isolate PI-105

Isolate[a]	Reaction to single R genes[b]												Virulence phenotype	Race[c]
	R0	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11		
PI-105	+	+	+	+	+	–	+	+	+	–	+	+	0,1,2,3,4,5,6,7,8,10,11	7766
PI-105C1	+	+	+	+	+	+	+	–	–	–	+	+	0,1,2,3,4,5,6,10,11	7702
PI-105C2	+	+	+	+	+	+	+	+	–	–	+	+	0,1,2,3,4,5,6,7,10,11	7746
PI-105C3	+	–	–	–	–	–	–	–	–	–	+	+	0,3,10,11	1006
PI-105C5	+	–	–	+	–	–	–	–	–	–	–	–	0,3	1000
PI-105C8	+	+	+	+	+	+	+	+	–	–	+	+	0,1,2,3,4,5,6,7,10,11	7746
PI-105C10	+	–	–	+	+	–	–	–	–	–	–	+	0,3,4,11	1402
PI-105C11	+	–	–	–	–	–	–	–	–	–	–	–	0	0000
PI-105C12	+	+	–	+	+	–	+	–	–	–	+	–	0,1,3,4,6,10	5504
PI-105C13	+	–	+	+	+	–	–	–	–	–	–	–	0,2,3,4	3400
PI-105C14	+	+	+	+	+	–	+	+	–	–	+	+	0,1,2,3,4,6,7,10,11	7546
PI-105C15	+	+	+	+	+	+	+	+	–	–	+	+	0,1,2,3,4,5,6,7,10,11	7746
PI-105C16	+	+	+	+	+	+	+	+	+	–	+	+	0,1,2,3,4,5,6,7,8,10,11	7766
PI-105C17	+	–	–	+	–	–	–	–	–	–	–	–	0,3	1000
PI-105C18	+	–	–	+	–	–	–	–	–	–	–	+	0,3,11	1002
PI-105C19	+	–	–	+	–	–	+	+	–	–	–	–	0,3,6,7	1140
PI-105C20	+	+	+	+	+	–	–	+	–	–	+	+	0,1,2,3,4,7,10,11	7446
PI-105C22	+	+	+	+	+	+	+	+	+	–	+	+	0,1,2,3,4,5,6,7,8,10,11	7766
PI-105C23	+	+	+	+	+	–	+	+	+	–	+	+	0,1,2,3,4,6,7,8,10,11	7566
PI-105C25	+	+	+	+	+	–	+	+	+	–	+	+	0,1,2,3,4,6,7,8,10,11	7566
PI-105C26	+	–	–	–	–	–	–	–	–	–	–	–	0	0000

[a]Virulence phenotypes of isolates PI-105C4, C9, C21 and C24 could not be determined because of inconsistency of virulence expression.
[b]Indicates R genes differential that allowed infection and sporulation by *P. infestans* isolate, where (+) represents virulent reaction and (–) represents avirulent reaction.
[c]Race designation using the reverse octal nomenclature system of Gilmour (Gilmour, 1973; Goodwin *et al*., 1995).

binary matrix was used to construct a similarity matrix between all pairs of isolates, based on simple matching coefficients (Romesburg, 1984). A dendrogram was obtained by cluster analysis of this similarity matrix, using the unweighted pair group method with the arithmetic means (UPGMA) clustering method. The cluster analysis was performed using the software NTSYS-PC Version 2·1 (Rohlf, 2000). To estimate the strength of the groups generated by cluster analysis, bootstrap analysis was performed with 2000 replications using the WINBOOT computer program (Immanuel & Nelson, 1996).

RAPD assay

Genomic DNA was extracted from *P. infestans* isolates using the procedure described by Drenth & Govers (1993). DNA from isolates PI-105, PI-1 and SZIs derived from them was amplified using 80 decanucleotide primers with arbitrary sequences obtained from Operon Technologies, Alameda, CA, USA (sets BA, BB, BC and BD; Table 2). The RAPD-PCR reactions were carried out in a volume of 12·5 μL and contained 25 ng genomic DNA, 100 μM each of dATP, dCTP, dGTP and dTTP (Eppendorf Scientific Inc., Westbury, NY, USA), 1·25 μL of 10× reaction buffer (500 mM KCl, 100 mM Tris–HCl pH 8·3, 15 mM $MgCl_2$, 1% Triton X100), 0·4 μM of each primer and 0·5 units of Taq DNA polymerase (Eppendorf Scientific). Amplification reactions were performed in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research Inc., Watertown, MA, USA). The amplification program consisted of three cycles of denaturation at 96°C for 2 min, annealing at 35°C for 1 min, and extension at 72°C for 2 min, followed by 32 cycles of 1 min at 94°C, 30 s at 35°C and 1 min at 72°C. A final extension step at 72°C for 4 min concluded the DNA amplification. Amplified products were kept at 4°C for a short period (24 h) or at –20°C for longer periods until electrophoresis. Amplification products were separated in 7·5% polyacrylamide gels using the Mini-PROTEAN 3 cell (Bio-Rad, Hercules, CA, USA). A standard size marker (1·0 kb DNA ladder; New England Biolabs, Beverly, MA, USA) was included in every gel, and gels were run at 200 V for 30 min in 1 × TBE running buffer. After electrophoresis, gels were stained with silver following the protocol described by Goldman & Merrill (1982). Reproducibility of RAPD analysis was tested by amplifying DNA from a randomly chosen set of 10 isolates (30% of total) with each primer, and the resulting banding patterns were compared. Primers that showed some inconsistency were excluded from the analysis.

AFLP analysis

The protocol employed for AFLP analysis was a modified version of the method described by Vos *et al.* (1995), which allowed the detection of AFLP bands without the use of radiolabelled nucleotides (Zhong & Steffenson, 2001). DNA fragments were preamplified with 17-base-pair primers, where 16 bases were complementary to the adapter and one base was selective at the 3′ end. The sequences of the primers used in the preamplification step were: 5′-GACTGCGTACCAATTCA-3′ (E + A), 5′-GATGAGTCCTGAGTAAC-3′ (M + C) and 5′-GATGAGTCCTGAGTAAA-3′ (M + A). Preselective amplification primer combinations were either (E + A) and (M + C), or (E + A) and (M + A), and amplification was performed in a 20 μL reaction volume using a PTC-100 Programmable Thermal controller, programmed for 20 cycles of 94°C for 30 s, 56°C for 60 s and 72°C for 60 s. The PCR reaction contained 200 μM dNTPs mix, 1 × reaction buffer (50 mM KCl, 10 mM Tris–HCl pH 8·3, 1·5 mM $MgCl_2$, 0·1% Triton X100), 1·0 unit of *Taq* DNA polymerase (Eppendorf Scientific), 36 ng of each preselective amplification primer, and 2·0 μL of the template DNA. Selective amplification was carried out with 18 primer-pair combinations (Table 3). The amplification reactions were performed in a 20 μL reaction volume, containing 1 × reaction buffer (50 mM KCl, 10 mM Tris–HCl pH 8·3, 1·5 mM $MgCl_2$, 0·1% Triton X100), 200 μM dNTPs mix, 1·0 unit of *Taq* DNA polymerase, 36 ng of each primer, and 2·0 μL of a 10-fold dilution of the preselectively amplified DNA template. The PCR reaction started with a cycle of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C, and was followed by 12 cycles with a stepwise lowering of the annealing temperature by 0·7°C in each cycle (touchdown profile), followed by 23 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min.

The selectively amplified PCR products were separated in a denaturing polyacrylamide gel. Gels were run on a 33 × 62 cm sequencing kit DASG-600-33 (CBS Scientific Co., Inc., Solana Beach, CA, USA). Electrophoresis was performed at constant power, 80 W for 1·5 h, using 0·5 × TBE as the buffer. Gels were stained with silver using the Silver Sequence DNA Sequencing System (Promega, Madison, WI, USA) according to the manufacturer's directions. Reproducibility of AFLP analysis was tested by amplifying DNA from a randomly chosen set of 10 isolates (30% of the total) with each primer pair, and the resulting banding patterns were compared.

RAPD and AFLP data analysis

The RAPD and AFLP bands were scored manually and analysed as binary data, with 1 representing the presence and 0 representing the absence of a band at a particular location in each lane. Polymorphic and monomorphic bands were determined for each RAPD primer or AFLP primer pair, but only polymorphic bands were included in the analysis. Bands were assumed to be independent, and those of identical size were assumed to have identical sequences. Data were analysed with the computer software NTSYS-PC Version 2·1. Similarity matrices were obtained based on the simple matching coefficients, using the SIMQUAL program in the software package. Cluster analysis of matrix values was performed by the unweighted pair-group method with arithmetic averages (UPGMA) using the SAHN algorithm of NTSYS-PC (Rohlf, 2000). Dendrograms showing the relationships among the RAPD and AFLP patterns

Table 2 Random amplified polymorphic DNA primers used for analysis of SZIs of *Phytophthora infestans* and the number of bands detected and scored with each primer

Primer[a]	Sequence	Total number of bands amplified[b]		Number of polymorphic markers[c]	
		PI-105	PI-1	PI-105	PI-1
OPBA-01	5′-TTCCCCACCC-3′	27	NP	26	–
OPBA-02	5′-TGCTCGGCTC-3′	25	NP	18	–
OPBA-03	5′-GTGCGAGAAC-3′	20	5	2	5
OPBA-04	5′-TCCTAGGCTC-3′	13	NP	12	–
OPBA-05	5′-TGCGTTCCAC-3′	20	NP	4	–
OPBA-06	5′-GGACGACCGT-3′	21	NP	2	–
OPBA-07	5′-GGGTCGCATC-3′	9	NP	1	–
OPBA-08	5′-CCACAGCCGA-3′	14	8	11	2
OPBA-09	5′-GGAACTCCAC-3′	24	NP	9	–
OPBA-10	5′-GGACGTTGAG-3′	18	7	6	2
OPBA-11	5′-CCACCTTCAG-3′	12	NP	12	–
OPBA-12	5′-TGTTGGGCAC-3′	22	10	9	1
OPBA-13	5′-AGGGCGAATG-3′	20	8	17	0
OPBA-14	5′-TCGGGAGTGG-3′	10	7	8	2
OPBA-15	5′-GAAGACCTGG-3′	30	7	29	5
OPBA-16	5′-CCACGCATCA-3′	NP	1	–	0
OPBA-20	5′-GAGCGCTACC-3′	NP	13	–	1
OPBB-01	5′-ACACTGGCTG-3′	25	15	24	3
OPBB-03	5′-TCACGTGGCT-3′	17	8	17	0
OPBB-04	5′-ACCAGGTCAC-3′	NP	5	–	4
OPBB-05	5′-GGGCCGAACA-3′	17	12	5	0
OPBB-06	5′-CTGAAGCTGG-3′	14	7	14	0
OPBB-07	5′-GAAGGCTGGG-3′	8	15	7	12
OPBB-09	5′-AGGCCGGTCA-3′	14	10	11	4
OPBB-11	5′-TGCGGGTTCC-3′	9	8	4	2
OPBB-12	5′-TTCGGCCGAC-3′	7	8	5	2
OPBB-13	5′-CTTCGGTGTG-3′	13	NP	11	–
OPBB-14	5′-GTGGGACCTG-3′	6	13	3	0
OPBB-15	5′-AAGTGCCCTG-3′	9	3	8	1
OPBB-16	5′-TCGGCACCGT-3′	NP	6	–	2
OPBB-17	5′-ACACCGTGCC-3′	NP	6	–	0
OPBB-19	5′-TTGCGGACAG-3′	12	9	8	0
OPBB-20	5′-CCAGGTGTAG-3′	13	4	8	3
OPBC-01	5′-CCTTCGGCTC-3′	21	NP	21	–
OPBC-02	5′-ACAGTAGCGG-3′	15	5	14	0
OPBC-04	5′-CCACGTGCCA-3′	18	13	9	0
OPBC-05	5′-GAGGCGATTG-3′	22	11	20	6
OPBC-06	5′-GAAGGCGAGA-3′	6	8	4	5
OPBC-09	5′-GTCATGCGAC-3′	3	9	3	2
OPBC-10	5′-AACGTCGAGG-3′	13	NP	13	–
OPBC-11	5′-TTTTGCCCCC-3′	NP	2	–	0
OPBC-12	5′-CCTCCACCAG-3′	10	NP	10	–
OPBC-13	5′-CCTGGCACAG-3′	9	NP	4	–
OPBC-14	5′-GGTCCGACGA-3′	16	13	14	0
OPBC-15	5′-CCAGACTCCA-3′	6	3	4	0
OPBC-16	5′-CTGGTGCTCA-3′	14	7	11	2
OPBC-18	5′-GTGAAGGAGG-3′	20	13	19	0
OPBC-19	5′-ACAAGCGCGA-3′	14	15	11	9
OPBC-20	5′-AGCACTGGGG-3′	10	12	5	2
OPBD-01	5′-GAGCCCCGAA-3′	NP	5	–	4
OPBD-04	5′-TCGGGTGTTG-3′	8	9	8	4
OPBD-05	5′-GTGCGGAGAG-3′	NP	8	–	0
OPBD-07	5′-GAGCTGGTCC-3′	NP	8	–	7
OPBD-09	5′-CCACGGTCAG-3′	NP	13	–	0
OPBD-10	5′-GACGCTATGG-3′	NP	1	–	0
OPBD-12	5′-GGGAACCGTC-3′	21	3	18	0
OPBD-13	5′-CCTGGAACGG-3′	14	NP	11	–
OPBD-15	5′-TGTCGTGGTC-3′	6	7	3	0
OPBD-16	5′-GAACTCCCAG-3′	10	NP	9	–
OPBD-17	5′-GTTCGCTCCC-3′	7	3	6	0
OPBD-18	5′-ACGCACACTC-3′	12	1	12	0
OPBD-20	5′-AGGCGGCACA-3′	10	12	8	0
Total		734	376	528	92

[a]Primer code (Operon Technologies, Alameda, CA, USA).

[b]Total number of bands scored for each primer in all isolates. NP, no amplification products.

[c]Number of polymorphic bands in all isolates.

Table 3 Primer combinations used for AFLP analysis of *Phytophthora infestans* isolates

Primer combination	Sequence	Total number of bands amplified		Number of polymorphic markers	
		PI-105	PI-1	PI-105	PI-1
E-AA + M-CT	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAACT-3′	103	60	31	0
E-AA + M-CA	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAACA-3′	72	43	6	36
E-AA + M-CC	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAACC-3′	64	30	18	19
E-AG + M-CT	5′-GACTGCGTACCAATTCAG-3′ 5′-GATGAGTCCTGAGTAACT-3′	60	55	39	39
E-AG + M-CC	5′-GACTGCGTACCAATTCAG-3′ 5′-GATGAGTCCTGAGTAACC-3′	62	44	7	23
E-AT + M-CA	5′-GACTGCGTACCAATTCAT-3′ 5′-GATGAGTCCTGAGTAACA-3′	80	49	18	30
E-AT + M-CC	5′-GACTGCGTACCAATTCAT-3′ 5′-GATGAGTCCTGAGTAACC-3′	63	27	5	14
E-AT + M-CT	5′-GACTGCGTACCAATTCAT-3′ 5′-GATGAGTCCTGAGTAACT-3′	63	35	9	19
E-AC + M-CC	5′-GACTGCGTACCAATTCAC-3′ 5′-GATGAGTCCTGAGTAACC-3′	93	35	18	17
E-AC + M-CA	5′-GACTGCGTACCAATTCAC-3′ 5′-GATGAGTCCTGAGTAACA-3′	84	30	9	14
E-AC + M-CT	5′-GACTGCGTACCAATTCAC-3′ 5′-GATGAGTCCTGAGTAACT-3′	72	50	13	31
E-AA + M-CG	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAACG-3′	28	30	0	17
E-AG + M-CG	5′-GACTGCGTACCAATTCAG-3′ 5′-GATGAGTCCTGAGTAACG-3′	58	43	6	29
E-AT + M-CG	5′-GACTGCGTACCAATTCAT-3′ 5′-GATGAGTCCTGAGTAACG-3′	70	39	16	22
E-AA + M-AC	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAAAC-3′	81	50	51	40
E-AA + M-AG	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAAAG-3′	70	52	16	49
E-AA + M-AA	5′-GACTGCGTACCAATTCAA-3′ 5′-GATGAGTCCTGAGTAAAA-3′	71	44	4	43
E-AG + M-AC	5′-GACTGCGTACCAATTCAG-3′ 5′-GATGAGTCCTGAGTAAAC-3′	83	27	23	26
Total		1277	743	289	468

were generated from these matrices using the TREE program from NTSYS-PC. To estimate the strength of the grouping generated by cluster analysis, bootstrap analysis was performed with 2000 replications using the WINBOOT computer program (Immanuel & Nelson, 1996).

Results

Virulence phenotypes

Twenty SZIs out of 24 isolates derived from the parental isolate PI-105 were previously tested for their virulence phenotypes (Abu-El Samen *et al*., 2003; Table 1). The virulence phenotypes of four SZIs from this group (PI-105: C4, C9, C21 and C24) could not be verified because of the low aggressiveness of these isolates or inconsistency in the expression of virulence. However, these isolates were included in both RAPD and AFLP analysis. All eight SZIs from parent PI-1 had the same virulence phenotype and were all designated as virulence phenotype 1,2,3,4,5,6,7,10,11 (race 7746, Table 1, data not shown).

Using the UPGMA method of cluster analysis of virulence data for SZIs from parent PI-105, the SZIs were differentiated into six virulence groups based on 80% similarity as a cut-off point (Fig. 1). However, this grouping was not supported by the bootstrap analysis because the bootstrap value for each group branch was low (15–38). Values of similarity coefficients of virulence phenotypes ranged between 0·09 and 1·0 (Fig. 1).

RAPD analysis

Genomic DNA was amplified from 32 SZIs, the parental isolates PI-105 and PI-1 and isolate US-940-480 (US-8

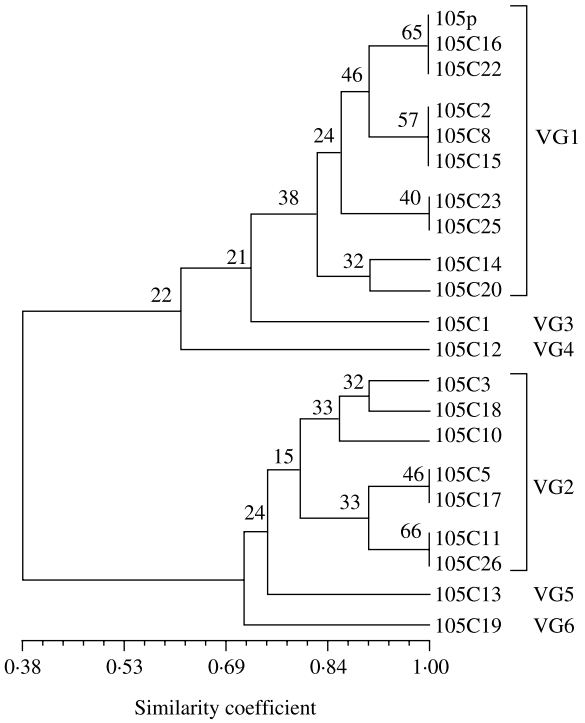

Figure 1 Dendrogram of single-zoospore isolates derived asexually from isolate PI-105. Genetic distances among these isolates were revealed by cluster analysis with the unweighted pair group method with arithmetic averages. The scale below the dendrogram is the genetic similarity coefficient (simple matching). Numbers at the nodes of clusters represent bootstrap values generated from 2000 replications. Clusters are based on virulence toward resistance genes R1–R11.

genotype) using RAPD-PCR analysis. Out of 80 primers screened, 53 generated reproducible amplification products from PI-105 SZIs, and 51 produced polymorphism among the SZIs from this parent. A total of 734 bands were scored, of which 528 (71·9%) were polymorphic (Table 2). Among 528 polymorphic bands amplified from this progeny, 159 (30%) were amplified from some of the SZIs but were absent in the parental isolate. The SZIs from parent PI-1 produced amplification products with 47 RAPD primers, of which 25 primers demonstrated polymorphism among the eight SZIs. A total of 376 bands were scored, of which 92 bands (24·5%) were polymorphic (Table 2). Among the 92 polymorphic bands amplified from this progeny, 51 bands (55%) were amplified from some of the SZIs but were absent in the parental isolate. Among 62 primers included in the analysis, 26 primers did not amplify DNA from one or other progeny (Table 2).

The number of amplified products generated by each primer varied between one and 30 bands, and the size of the products was within the range 500 bp to 4·0 kbp. When fingerprints of these isolates were compared, some bands common to all isolates were observed, while others were unique to one or a few isolates. In most cases the polymorphism was characterized by the absence of certain amplification products from some isolates as exemplified by primers BB13, BC18 and BD12 with PI-105 SZIs (Fig. 2) and primers BB7 and BB9 with PI-1 SZIs (Fig. 3). Similarity matrices based on simple matching coefficients were calculated from the combined data of all the polymorphic bands for both progeny (SZIs from parents PI-105 and PI-1). Values of the similarity coefficients of RAPD data ranged between 0·60 and 0·88 for PI-105 SZIs, and between 0·29 and 0·95 for PI-1 SZIs. The dendrogram for PI-105 SZIs (Fig. 4a) shows that hierarchical clustering separated these isolates into three major RAPD groups (RGs) and seven minor groups at a similarity level of 80%. The RAPD groups RG4–RG10 each contained one isolate. These isolates did not cluster with any other isolate at this similarity level (80%) and were considered as minor groups. As expected, the US-8 genotype isolate US-940-480 was in a separate cluster (RG-8); however, it was more similar to some of the SZIs than to the parental isolate. If a 100% similarity level is considered to separate SZIs into distinct genotypes, then 23 genotypes were identified among the 24 SZIs analysed with RAPD (Fig. 4a). The grouping was not supported by the bootstrap analysis, as the bootstrap value for each branch was generally low except for RG11, which demonstrated 100% confidence as a separate group (Fig. 4a).

Cluster analysis of RAPD data of PI-1 SZIs at 80% similarity level differentiated these isolates into four RAPD groups (Fig. 4b). RG2 contained the majority of the isolates from this progeny. Bootstrap analysis demonstrated that this grouping was robust (90% confidence estimate). Similarly, bootstrap analysis revealed that the division between RG3 and RG4 was robust (100% confidence estimate); however, the cluster of RG1 was not supported by the bootstrap analysis (61% confidence estimate; Fig. 4b). If a 100% similarity level is considered to separate SZIs into distinct genotypes, then seven genotypes were identified among the eight SZIs analysed with RAPD (Fig. 4b).

AFLP analysis

All 18 primer pairs successfully amplified DNA from the 32 SZIs and the parental isolates PI-105 and PI-1. For each primer pair, 27–103 DNA fragments, ranging in size from 80 bp to 1·0 kb were detected. A total of 1277 amplification products were detected with PI-105 SZIs, of which 289 markers (22·6%) were polymorphic (Table 3). Among these 289 polymorphic bands, 113 bands (39%) were amplified from some of the SZIs but absent in the parental isolate. AFLP primer pairs produced 743 amplification products with PI-1 SZIs, of which 468 (62·9%) were polymorphic (Table 3). Among these 468 polymorphic bands, 142 bands (30%) were amplified from some of the SZIs but absent in the parental isolate.

As with RAPD primers, none of the primer pairs screened showed the ability to distinguish all 32 SZIs as distinct genotypes. When fingerprints of these isolates were compared, the majority of the bands were common to all isolates, while others were unique to one or a few

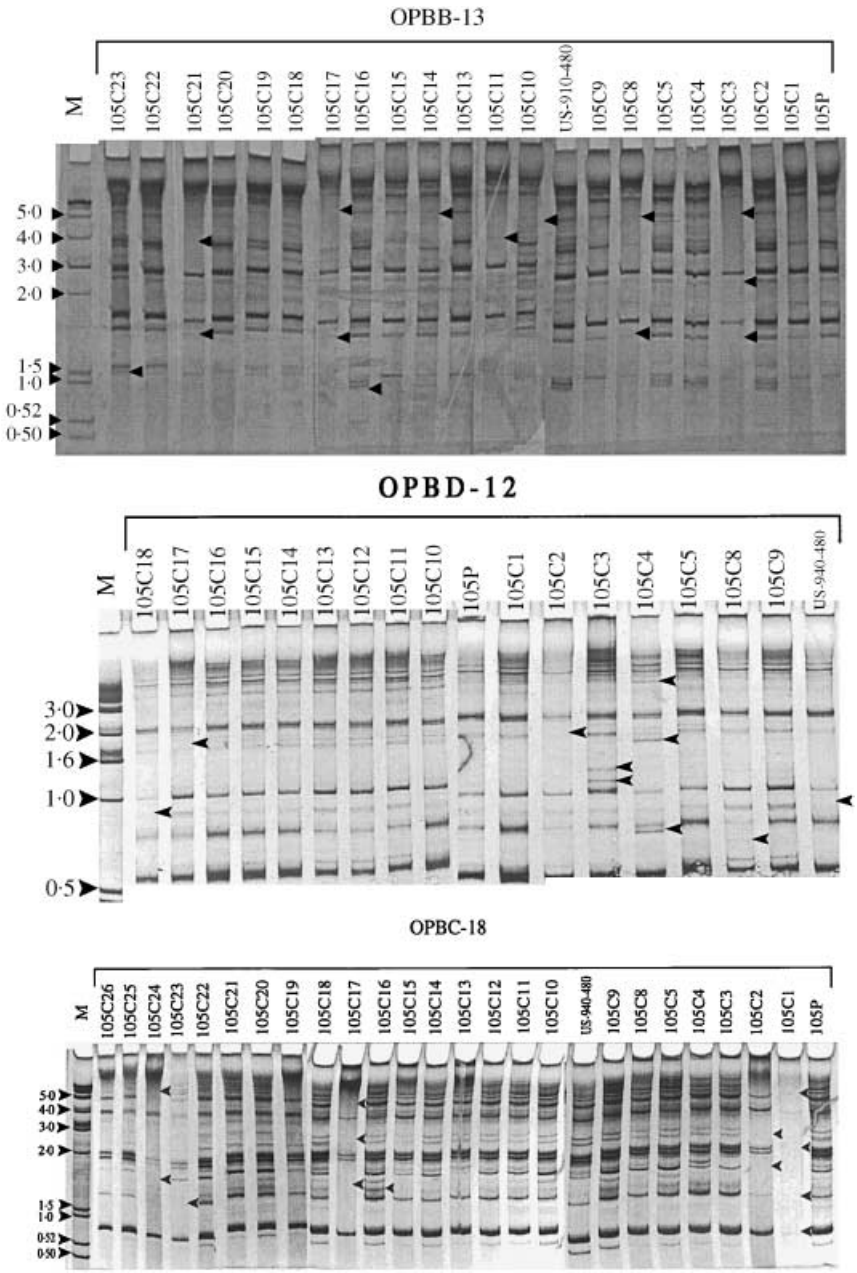

Figure 2 Random amplified polymorphic DNA banding patterns of single-zoospore isolates derived asexually from parental isolate PI-105 generated with primers OPBB13 (top), OPBD12 (middle) and OPBC18 (bottom). Each lane represents the banding pattern of a single-zoospore isolate. Amplification products were separated in 7·5% polyacrylamide gels and stained with silver. Some polymorphic bands are indicated with arrows.

isolates, as exemplified by the primer pair E-AT + M-CA (Fig. 5). Values of similarity coefficients of the AFLP data ranged from 0·48 to 0·96 for PI-105 SZIs, and from 0·27 to 0·95 for PI-1 SZIs. Hierarchical clustering at the 80% similarity level separated PI-105 SZIs into three AFLP groups (AFGs) (Fig. 6a). The majority of the isolates clustered in the first group (AFG1; 22 isolates). The second group (AFG2) contained two isolates (105C16 and 105C18). Isolate 105C3 formed a separate cluster (AFG3) with a bootstrap value of 100% confidence, whereas AFLP groups 1 and 2 were less robust, with 64 and 75% confidence levels, respectively. If a 100% similarity level is considered to separate SZIs into distinct genotypes, then 23 genotypes were identified among the 24 SZIs analysed with AFLP (Fig. 6a). At the 80% similarity level, the AFLP markers differentiated PI-1 SZIs into six groups. The first group enclosed five isolates, whereas the other five groups each consisted of a single isolate (Fig. 6b). Bootstrap analysis revealed that this grouping was relatively robust for AFLP groups 1, 4, 5 and 6 with

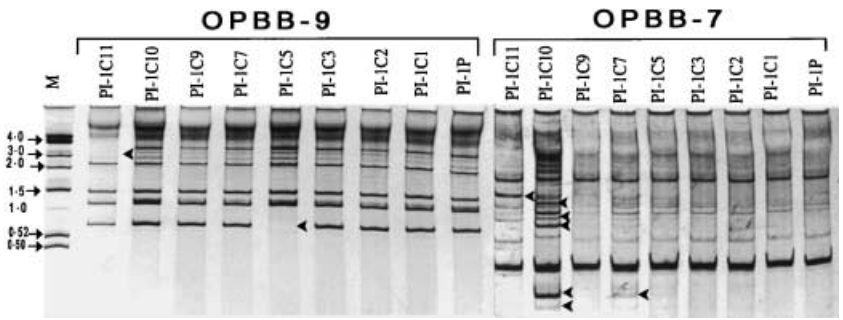

Figure 3 Random amplified polymorphic DNA banding patterns of single-zoospore isolates (SZIs) derived asexually from parental isolate PI-1 generated with primers OPBB-9 (left) and OPBB-7 (right). Each lane represents the banding pattern of a SZI. Amplification products were separated in 7·5% polyacrylamide gels and stained with silver. Some polymorphic bands are indicated with arrows.

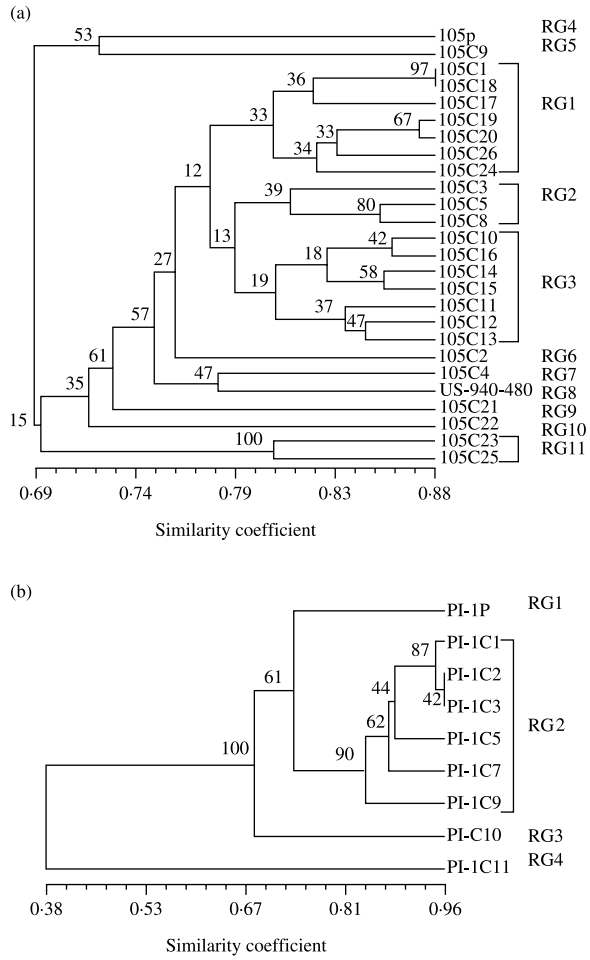

Figure 4 Dendrograms of single-zoospore isolates derived asexually from isolates PI-105 (a) and PI-1 (b) based on RAPD markers. Genetic distances among these isolates were revealed by cluster analysis with the unweighted pair group method with arithmetic averages. The scale shown below the dendrograms is the genetic distance calculated using the simple matching coefficients. Numbers at the nodes of clusters represent bootstrap values generated from 2000 replications.

confidence levels between 97 and 100%. However, the bootstrap values for groups 2 and 3 were relatively low (79 and 52% confidence estimates, respectively).

Discussion

The primary aim of this research was to investigate the genotypic variation among SZIs of *P. infestans* derived asexually from two parental isolates using RAPD and AFLP markers. The phenotypic variability among the SZIs was the key stimulus to conducting this study. The SZIs derived from the parental isolate PI-105 were found to be highly variable in their virulence. In contrast, the SZIs derived from the parental isolate PI-1 were identical in their virulence phenotypes. Results from this study illustrate that a substantial level of genotypic variability exists among the asexual progenies of *P. infestans* studied, regardless of the level of phenotypic variation.

Virulence is usually considered the most important phenotypic marker in studying populations of plant pathogens. However, virulence/avirulence loci represent only a small portion of the total genetic variation that might exist among different races within a plant pathogen population. Molecular markers such as RAPD and AFLP are neutral markers, independent of host selection, and are usually used to assess total genetic variation that is not directly affected by hosts, or environmental factors that influence the expression of phenotypes.

The RAPD markers demonstrated a high level of genetic variability among the PI-105 SZIs compared to levels of genotypic variation detected by AFLP markers. However, the same AFLP markers revealed a higher level of genotypic variability among SZIs of PI-1 than did the RAPD markers. The high level of genotypic variability among the PI-105 SZIs was not surprising, as these isolates had also demonstrated high levels of phenotypic variability in virulence and metalaxyl sensitivity (Abu-El Samen *et al.*, 2003). Generally, the correlation among virulence groups and groups based on RAPD and AFLP analysis was low. These results suggest that these single-zoospore isolates have considerable levels of genetic variability, not only in their virulence phenotypes but also in other loci that are not linked to virulence/avirulence.

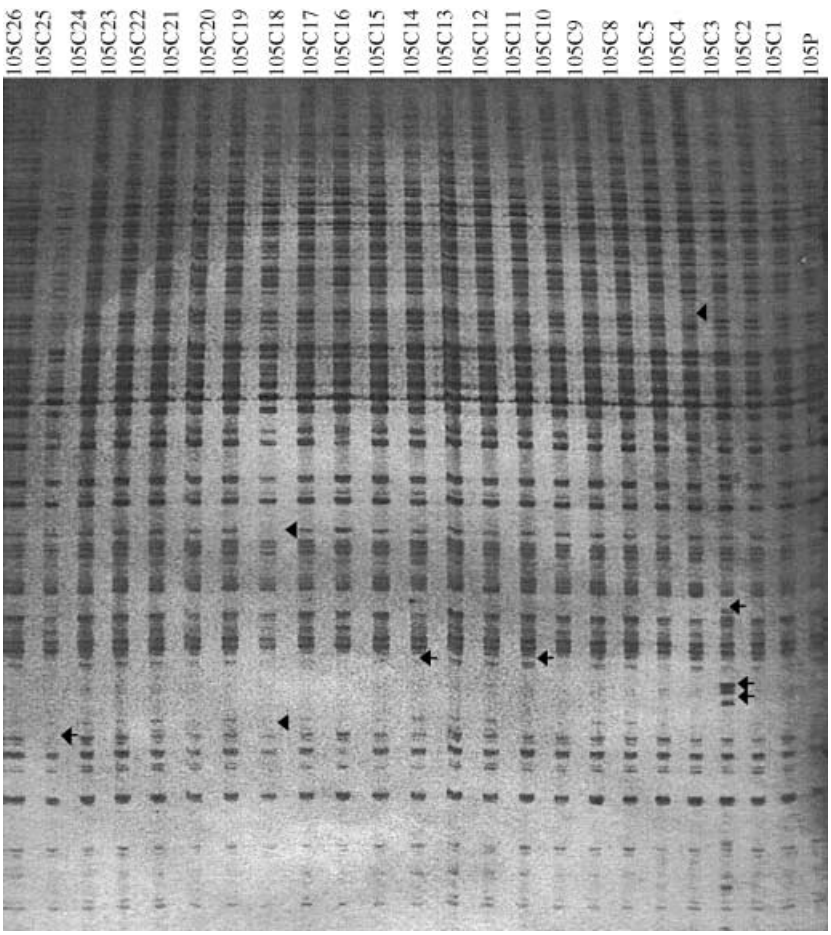

Figure 5 A partial silver-stained polyacrylamide gel of amplified fragment length polymorphism (AFLP) markers of single-zoospore isolates derived asexually from parental isolate PI-105 with primer pair E-AT + M-CA. Some polymorphic bands are indicated with arrows.

The lack of correspondence among virulence, RAPD and AFLP groups identified from the PI-105 SZIs was in agreement with the findings of Lebreton & Andrivon (1998), who did not find a clear correlation between phenotypes (races and mating types) and genotypes based on neutral markers (RFLP-RG57 fingerprints, mt-DNA haplotypes and allozyme genotypes). Similarly, Mahuku *et al*. (2000) found no correlation between groups defined by RAPD markers, allozymes, response to metalaxyl and mating type. Also, RAPD markers revealed some genotypic variability among SZIs of the oomycete pathogen *Aphanomyces euteiches*, but these genotypic variations were not correlated to pathogenic variability (Malvick & Percich, 1998).

The lack of association between groups based on DNA markers in this study was not surprising, as the two methods of analysis can reveal genetic variation in different regions of the genome. AFLP markers usually search for polymorphism in regions of the genome containing restriction sites for the restriction enzymes used in the analysis (*Eco*RI and *Mse*I sites). On the other hand, RAPD marker loci are distributed throughout the genome. The 10 bp primers can bind to any region in the genome that contain a matching sequence and generate polymorphism between two individuals, when the binding site of a primer is lost or modified in one individual but not in the other. This lack of association between different DNA markers was also observed by Purvis *et al*. (2001), who fingerprinted a collection of *P. infestans* isolates with AFLP markers and the RG57-RFLP marker. They found that some isolates with the same RG-57 genotype had remarkably dissimilar AFLP genotypes, and some isolates with dissimilar RG-57 fingerprints had similar or identical AFLP fingerprints. However, it is important to note that the aforementioned study did not use SZIs.

The detection of genetic variation revealed by RAPD and AFLP markers among a relatively small number of SZIs belonging to the same virulence phenotype (PI-1 SZIs) suggests that genetic changes occur in *P. infestans* during asexual reproduction, and these changes may or may not affect the phenotypes of the asexual progeny. The significant differences in sensitivity to metalaxyl among these isolates (Abu-El Samen *et al*., 2003) support the conclusion from the current results that these isolates have undergone some genetic changes that did not affect their virulence phenotypes.

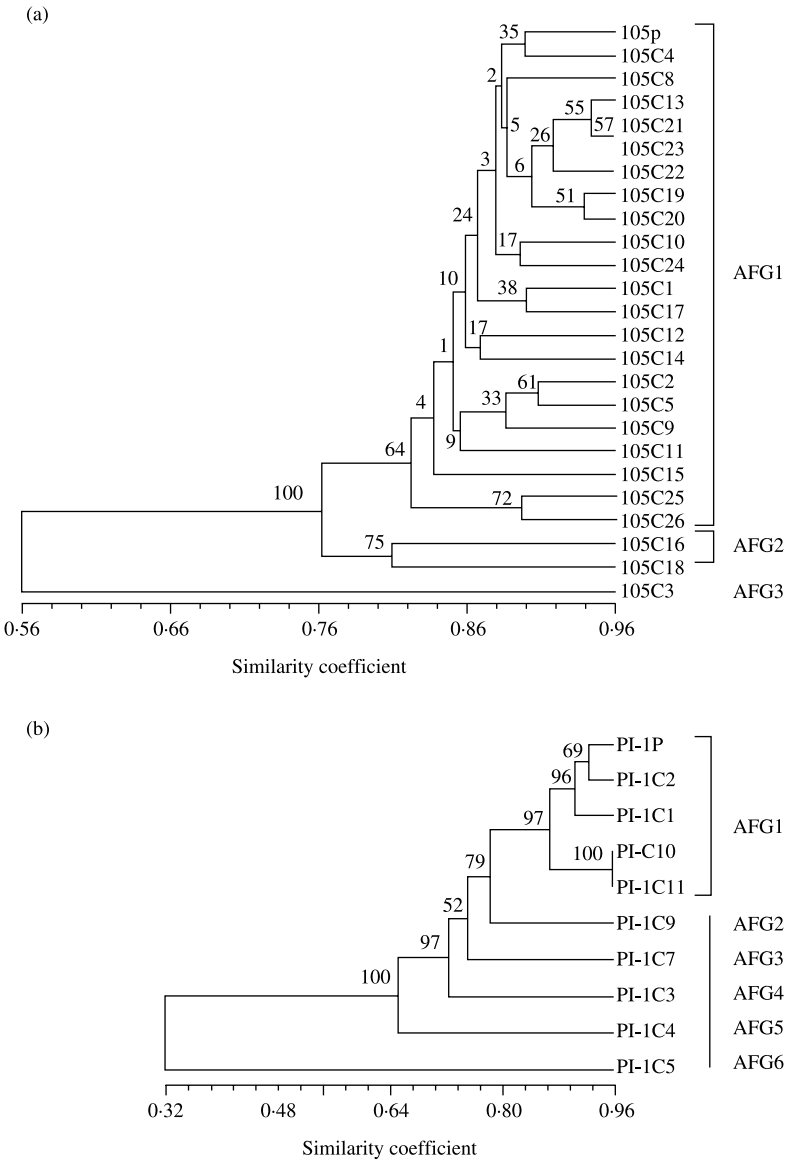

Figure 6 Dendrograms of single-zoospore isolates derived asexually from isolates PI-105 (a) and PI-1 (b) based on AFLP markers. Genetic distances among these isolates were revealed by cluster analysis with the unweighted pair group method with arithmetic averages. The scale shown below the dendrograms is the genetic distance calculated using the simple matching coefficients. Numbers at the nodes of clusters represent bootstrap values generated from 2000 replications.

The underlying mechanisms of genotypic variability during asexual reproduction observed in this study can be predicted if the sources of polymorphisms in RAPD or AFLP markers are considered. Mutations that cause slight changes in DNA sequences throughout the genome can account for the high levels of polymorphism observed among the SZIs as revealed by RAPD and AFLP markers. These mutations, in most cases, can be neutral and cause no observable changes in phenotypes. However, the high levels of virulence variability observed in PI-105 progeny cannot be explained solely by the mutation hypothesis, especially when considering the rate of spontaneous mutations in plant pathogenic fungi, estimated to be 1×10^{-6} (Fincham *et al*., 1979). Thus it is safe to hypothesize that at least part of the genotypic variation observed with these markers can be attributed to spontaneous mutations. However, it is premature to extend this assumption to account for the high levels of virulence variability observed with the PI-105 SZIs, based on the results of the current study. It seems likely that the virulence variation observed among the PI-105 SZIs was caused by mechanisms other than spontaneous mutation.

Other possible mechanisms of genetic variability during asexual reproduction in *Phytophthora* spp. include mitotic crossing over, gene conversion and extra-chromosomal elements (Goodwin, 1997; Abu-El Samen *et al*., 2003). Mitotic recombination probably plays an important role in generating genotypic variability. However, because of the dominant nature of the DNA markers used in this study, it was not possible to obtain concrete evidence that mitotic recombination was contributing to the genotypic variation observed. A set of well defined codominant markers such as RFLPs (Carter *et al*., 1999) or microsatellites (Knapova & Gisi, 2002; Knapova *et al*.,

2002) that differentiate homozygous from heterozygous loci would be necessary to establish the role of mitotic recombination in generating genotypic variability during asexual reproduction.

In conclusion, the results of this study suggest that genetic changes during asexual reproduction are possible, and these changes might affect any phenotype in the asexual progeny, including virulence and metalaxyl sensitivity (Abu-El Samen *et al*., 2003). Previous studies (see Abu-El Samen *et al*., 2003) on phenotypic variation among asexual progenies of *P. infestans* presented limited conclusions and often yielded contradictory findings. In contrast, the present study employed DNA markers and provides some evidence that the phenotypic variation among asexual progeny of *P. infestans* is part of the total genetic change that can occur during asexual reproduction. The potential of *P. infestans* to change its virulence phenotype in the absence of the sexual cycle needs to be considered when conducting any study aimed at predicting the significance of the sexual cycle in generating new races. Additional studies are required to better understand the genetic mechanisms involved in this variability.

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