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MOLECULAR ANALYSIS OF POLYMORPHISMS IN DIFFERENTIATING *Phytophthora infestans* RACES

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Abstract

Potato late blight caused by oomycete *Phytophthora infestans* (Mont.) de Bary is economically significant disease of worldwide importance. The traditional classification of specialized races of *P. infestans* is based on eleven resistance genes (*R* genes) introgressed from *Solanum demissum* to cultivated potato *S. tuberosum*. The selection of potato varieties, each comprising one of these *R* genes, is referred to as the Mastenbroek-Black set of differential plants. This set has been employed to establish the virulence genes (*r* genes) in isolates and strains of *P. infestans*, and collections of such individual strains have been maintained as tool sets of differential races for discerning *R* genes in cultivated and wild *Solanum* plants. While widely used in potato breeding for late blight resistance, these differential races have not been sufficiently explored by present-day molecular methods. We studied 11 differential races (1; 3; 4; 10; 11; 1.2; 1.3; 1.4; 1.2.3; 1.2.4; 1.2.3.4) maintained in the Institute of Phytopathology for over forty years and isolate 161 possessing all 11 genes of virulence. When the differential races of *P. infestans* were genotyped with the standard set of 12 microsatellite (simple sequence repeat, SSR) loci, these races were distinct from reference A1 strains and highly aggressive lines lately dominant in the Western and Central Europe. SSR clusters of differential races did not match their *r* gene profiles. To assess the profiles of virulence genes in the differential races of *P. infestans*, we cloned three avirulence genes (*Avr* genes): *ipiO* = *Avr-blb1*, which recognizes the *Rpi-blb1* = *Rpi-sto1* gene of *S. bulbocastanum* and *S. stoloniferum* characterized by broad resistance to *P. infestans* races, and also *Avr3a* and *Avr4* corresponding to *R3a* and *R4* of *S. demissum*. These *Avr* genes were found in all differential races under study, and each gene was represented by several alleles. The complex patterns of *Avr* genes are in sharp contrast with the conventional concept of «simple» monogenic races. In the case of *ipiO* (NCBI GenBank accession numbers KP308170-KP308174, KF154431-KF154433 and KF154434-KF154439) race 1 was represented by the alleles of classes I and II, whereas races 3 and 4 comprised only the class I genes. None of three races contained the most virulent class III *ipiO* gene. The virulent allele *Avr3a_EM* was found in all investigated races, while the avirulent allele *Avr3a_KI* was discerned only in races 1, 3 and 1.2.3 (KF154421-KF154426, KF154430, KP317568, KP317572, KP317580-KP317584, KP317588, KP317589). The *Avr4* gene was cloned from differential races 1, 3, 1.4 and 11 (KF188215-KF188223). All these races contained the virulent allele, and race 11 comprised both avirulent and virulent alleles. The molecular and phytopathological evidence for *Avr* and *r* genes, respectively, matched only in 30 % of races. Probably, these discrepancies are due to the accumulation of mutations in the *Avr* genes of differential races in the course of their long-term maintenance in the collection and more complex composition of *R* genes in plants which were initially to select the differentiating races.

Keywords: *Phytophthora infestans*, *Avr* genes, microsatellite (SSR) markers

Late blight caused by oomycete *Phytophthora infestans* (Mont.) de Bary

remains one of the most significant agronomic and economic problems of potato production [1-3]. Information about this disease obtained by phytopathological methods [1] have been greatly supplemented in the past two decades through the molecular studies of the pathogen and the host plant. The data about the structure of *P. infestans* and *Solanum* genomes have been obtained [4], transcriptome and proteomic analysis of late blight infected potato plants have been performed [5-8]. Great progress has been made in the study of virulence/avirulence genes (*Avr* genes) *P. infestans* and resistance genes (*R* genes) in wild and cultivated *Solanum* forms [9-11]. Cloning of these genes gives new possibilities for the study of pathogen-host plant interactions in individual plants and agroecosystems [9, 12, 13]. Development of high resolution microsatellite analysis methods made it possible to reliably distinguish *P. infestans* isolates and lines [14]. In general, the methods of molecular genetics and biotechnology have led to considerable progress in the study of pathogenesis and breeding potato varieties resistant to late blight [12, 15, 16].

To detect and identify *R* genes in plants, breeders commonly use a standard set of simple and complex *P. infestans* differential races which differ in the composition of virulence factors. These factors match *R1-R11* resistance genes of *Solanum* species responsible for the hypersensitivity reaction to the pathogen. For traditional classification of specialized *P. infestans* races a set of differential plants is commonly used comprising the potato varieties with transferred *S. demissum* *R* genes [17-19]. Advances in molecular plant pathology made it possible to characterize *P. infestans* virulence/avirulence factors as AVR effectors, the *Avr* gene products. Their recognition by NB-LRR receptor kinases (products of *R* genes) induces plant protective reactions. With the lack of such recognition, effectors suppress plant immunity or change plant metabolism to provide their requirements [9, 20, 21].

To date, polymorphic *Avr* genes have been adequately described for their structure (including allelic polymorphism) and functional activity [9, 20, 22], but *Avr* gene and virulence factor matching validated in the tests with differential plants has been investigated insufficiently. According to the definition, Mastenbroek-Black differential races [18] recognize 11 *S. demissum* *R* genes only and do not identify other genes that may be present in *Solanum* species. However, we have demonstrated previously that simple *P. infestans* races contain several *Avr* classes of *ipiO* gene which recognizes *RB/Rpi-blb1* gene of *S. bulbocastanum* missed in *S. demissum* [23]. In addition, some differential plants contain more than one *R* gene [24]. Currently, the difficulties have been overcome by creating a new set of «monogenic» differential plants [25], but this set has not yet become available to the majority of research and breeding laboratories.

In this study, phytopathological characterization of *P. infestans* differential races was first supplemented by the results of genotyping using molecular markers and direct analysis of virulence genes.

The objective of this research was to study the polymorphism of *Phytophthora infestans* differential races of various geographical origins. For this purpose, the races were studied by microsatellite analysis for 12 loci, and a comparative structural analysis was performed for three *Avr* genes cloned after PCR amplification of *P. infestans* genomic DNA.

Technique. *Phytophthora infestans* samples from All-Russian Research Institute of Phytopathology (ARRIP) collection were used.

Genomic DNA was isolated from 11 simple and complex races (1, 3, 4, 10, 11, 1.2, 1.3, 1.4, 1.2.3, 1.2.4, 1.2.3.4) and isolate 161 which had all 11 virulence genes, using AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen

Biosciences, USA). DNA concentration was measured by an UV/Vis NanoPhotometer P300 (IMPLEN, Germany).

Primers for *Avr* amplification were synthesized by ZAO Synthol (Russia). PCR reaction mixture contained 1 µl of 10× PCR buffer, 100-150 ng of genomic DNA, 1 µl of 2.5 M dNTP, 10 pmol of each primer, 1 unit of Pfu DNA polymerase (Fermentas, Lithuania) for cloning or Taq DNA polymerases (ZAO Synthol, Russia) for screening, and sterile water to a volume of 10 µl. Amplification was performed in a MJ PTC-200 thermal cycler (Bio-Rad, USA) using the programs described above [23, 27-29].

PCR products were separated by electrophoresis in 1 % agarose gel with buffer supplemented with ethidium bromide. Amplified DNA fragments were excised from the gel under UV and purified using QIAquick Gel Extraction reagent kit (Qiagen N.V., Germany) according to the manufacturer's protocol. Cloning was performed in pGEM/T Vector (Pro-mega, CIIA). Isolated plasmids were sequenced using a NANOFOR 05 analyzer (Institute for Analytical Instrumentation RAS, Russia). Nucleotide sequences of the three *Avr* genes were deposited at GenBank NCBI (National Center for Biotechnology Information, USA) and compared with other sequences stored.

For *P. infestans* genotyping 12 microsatellite (simple sequence repeats, SSR) markers were used [14, 26]. The protocol of amplicon length analysis and standardization has been described previously [30].

Results. *P. infestans* differential races are shown in Table 1, and primers used for *Avr* amplification are shown in Table 2.

1. *Phytophthora infestans* differential races used in the study (collection of All-Russian Research Institute of Phytopathology, Moscow Province)

Isolate	Origin	Virulence factor	Matching type	Year of isolation
2K	Germany	1	A1	1970
4K	Germany	3	A1	1970
5K	Germany	4	A1	1970
6K	Georgia	10	A1	1978
7K	Georgia	11	A1	1978
8K	Germany	1.2	A1	1970
9K	Germany	1.3	A1	1970
10K	Germany	1.4	A1	1970
14K	Russia	1.2.3	A1	1974
15K	Russia	1.2.4	A1	1974
18K	Germany	1.2.3.4	A1	1970
161	Russia (Moscow Province)	1-11	A1	2010

Genes *Avr3a*, *Avr4*, and *ipiO* were found in all *P. infestans* isolates studied. These results confirm our previous data [23] and suggest a more complex composition of isolate *Avr* genes compared to the traditional ideas of «simple» races.

A large number of *Avr* alleles complicates creating highly specific SCAR (sequence characterized amplified region) markers for screening *P. infestans* isolates

to characterize the pathogen in the early stages of infection. Full-length *Avr* sequences have to be amplified using conserved primers, and then clone the amplicons obtained. We found a significant polymorphism in the three *Avr* genes studied.

2. Sequences of primers used for *Avr* amplification in *Phytophthora infestans* differential races

Gene	5'→3' sequence of primers	Annealing temperature, °C	Amplicon length, bp	Reference
<i>ipiO</i>	F – CTTTCGGCAATGCGTTCGC R – CTATACGATGTCATAGCATGACAC	65	510	(23, 27)
<i>Avr3a</i>	F – CCATGCGTCTGGCAATTATGCT R – CTGAAACTAATATCCAGTGA	61	451	(28)
<i>Avr4</i>	F – ATGCGTTCGCTTCACATTTTGCTGG R – CTAAGATATGGGCCGTCTAGCTTGGAG	61	864	(29)

Gene *ipiO* = *Avr-blb1* had the most significant polymorphism recog-

nized by *S. bulbocastanum* *RB/Rpi-blb1* resistance gene and its ortholog *Rpi-sto1* in *S. stoloniferum* [9]. Frequent *Avr* occurrence in *P. infestans* gives *Solanum* and potato varieties carrying *RB/Rpi-blb1* and *Rpi-sto1* resistance to most pathogen races. Gene *ipiO* was represented by three polymorphic classes. In experiments on transient *ipiO* and *Rpi-blb1* co-expression in *Nicotiana benthamiana* plants, class I alleles *ipiO* were shown to be recognized by *Rpi-blb1* and, therefore, were avirulent. Genes from class II were also virulent, but in the absence of class I genes only. Alleles *ipiO* of the rarer class III did not interact with the *Rpi-blb1* and were virulent [9].

In our experiments, *ipiO* was cloned and sequenced from simple races 1, 3, and 4. In race 1, we found substitutions typical of class I and II *ipiO* genes. Races 3 and 4 had no class II *ipiO* genes. None of the isolates studied had *ipiO* genes of the most virulent class III (Table 3). Therefore, races 1, 3, and 4 should be avirulent on the plants carrying *RB/Rpi-blb1* and *Rpi-sto1*.

3. Virulence classes of *ipiO* identified in simple *Phytophthora infestans* differential races (collection of All-Russian Research Institute of Phytopathology, Moscow region)

Race	Class I							Class II	NCBI GenBank
	<i>ipiO-01</i>	<i>ipiO-06</i>	<i>ipiO-08</i>	<i>ipiO-09</i>	<i>ipiO-10</i>	<i>ipiO-11</i>	<i>ipiO-12</i>	<i>ipiO-03-13</i>	accession number
1	+	–	–	+	–	–	+	+	KP308170-KP308174
3	+	–	–	–	–	+	–	–	KF154431-KF154433
4	+	–	–	–	+	–	–	–	KF154434- KF154439

Note. «+» и «–» — variant presence or absence, respectively.

Avr3a was represented by two most common forms: *Avr3a_KI*, the avirulence allele recognized by R3a kinase, and *Avr3a_EM*, the virulence allele not recognized by this kinase. Effectors matching two alleles *Avr3a* differed in three amino acid residues. C19, K80, and I103 amino acid residues are typical of avirulence allele *Avr3a_KI*, while S19, E80, and M103 are typical of virulence allele *Avr3a_EM* [28].

Avr3a was cloned from races 1, 1.2, 1.3, 1.2.3, 1.2.4, 3, 4, 10, 11, and 1.2.3.4 and sequenced (Table 4). In this, we found virulence allele *Avr3a_EM* in all races studied, whereas the haplotype identical to avirulence allele *Avr3a_KI* was found in races 1, 3, and 1.2.3 only. In addition, inactive paralog *PEX147-2* was found in race 1. It is noteworthy that in three races only (1, 1.3, 1.2.3.4) allele composition matched the composition of virulence genes which was determined based on the earlier phytopathological analysis with differential plants (see Table 4). Several races contained not previously described *Avr3a* alleles.

4. *Avr3a* allelic diversity in *Phytophthora infestans* simple differential races (collection of All-Russian Research Institute of Phytopathology, Moscow region)

Race	Expected <i>AVR3a</i> genotype	Allele <i>Avr3a</i> (molecular data)		NCBI GenBank accession number
		<i>Avr3a_KI</i> (avirulence)	<i>Avr3a_EM</i> (virulence)	
1 ^e	<i>Avr3a_KI</i>	+	+	KF154421, KP317568- KP317572
3	<i>Avr3a_EM</i>	+	+	
4	<i>Avr3a_KI</i>	–	+	
1.2	<i>Avr3a_KI</i>	–	+	KF154422, KF154423
1.3 ^e	<i>Avr3a_EM</i>	–	+	KF154424, KF154425
1.2.3	<i>Avr3a_EM</i>	+	+	KF154426-KF154429
1.2.4	<i>Avr3a_KI</i>	–	+	KF154430
10	<i>Avr3a_KI</i>	–	+	KP317580-KP317584
11	<i>Avr3a_KI</i>	–	+	
1.2.3.4 ^e	<i>Avr3a_EM</i>	–	+	KP317588, KP317589

Note. «+» и «–» — variant presence or absence, respectively; e — races for which the results were consistent with the expected ones, based on phytopathological analysis.

Avr4 was cloned from races 1, 3, 1.4, and 11 (GenBank KF188215-KF188223 accession numbers) and sequenced. For *Avr4*, virulence allele was different from avirulence one in the reading frame failure and the presence of stop codons

$\Delta T12$, $\Delta T196$, and G376T [29].

Fig. 1. Dendrogram based on differential race and reference isolate *Phytophthora infestans* genotyping for 12 microsatellite loci [14, 26]: cluster I — differential races, clusters II-V — reference strains (Neighbor Joining method; bootstrap values for 1000 repetitions exceeding 0.70 are shown).

A full length protein can not be translated from virulence *Avr4* nucleotide sequence. We found this allele in all races studied. Both *Avr4* alleles were found in race 11. The results for the races 11 and 1.4 only were consistent with the phytopathologically based hypothesis of the presence of avirulence allele *Avr4* in the genomes of the races in the names of which figure 4 is absent, and vice versa.

In potato varieties' genotyping by phytopathological methods to

determine *R1-R11* composition in *S. demissum*, the variety infected by a certain race is assumed to contain *R* gene (or genes) matching the race number strictly. Races for genotyping (simple races) are selected on differential plants (plants with a known set of *R* genes). A discrepancy between the molecular and phytopathological data can be due to a change in the set of active *Avr* genes in *P. infestans* simple races during their long-term maintenance in the collection and to a more complex composition of *R* genes in differential plants than previously thought [24].

We performed phylogenetic analysis of our results (Fig. 1) comparing previously published data on the allelic composition of the same loci in standard genotypes *US1-A1*, *EC1-A1*, and *US8-A2*, several Western European reference A1 lines and recently emerging highly aggressive clones *13_A2* and *6_A1* [14, 26] (Fig. 1).

Fig. 2. Dendrogram based on *Phytophthora infestans* differential race genotyping for 12 microsatellite loci [14, 26] reflecting SSR profile association with their geographical origin (Neighbor Joining method).

Genotyping for 12 microsatellite loci identified

notable differences between differential races and all lines from the Western Europe [14, 26]. Interestingly, differential race subclusters matched the geographical origin of these races partially (Fig. 2).

Thus, we were the first to define the profile of virulence genes in *Phytophthora infestans* differential races using molecular techniques. These races appeared to have more complex *Avr* allelic composition than previously thought. They all contained active *ipiO* genes, and *Avr3a* and *Avr4* virulence alleles. The presence of avirulence alleles *Avr* that match *r* genes identified by the phytopathological method (with differential plants) not always was in line with expected results. Probably, the discrepancies in the molecular and phytopathological data on the composition of virulence genes were due to the accumulation of mutations in *Avr* genes in the course of long-term maintenance of differential races in the collection, and more complex composition of *R* genes in differential plants initially used to select these races. Genotyping for 12 microsatellite loci identified notable differences between differential races and reference isolates from Western Europe. This may be due to the fact that differential races have been maintained in the collection since 1970s and may be significantly distinct from the modern pathogen isolates. Microsatellite profiles of differential races were not associated with the profiles of virulence/avirulence genes.

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