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SECOND LOCUS OF PEPTIDASE AS A MARKER FOR GENETIC INVESTIGATIONS OF *PHYTOPHTHORA INFESTANS*

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Abstract

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Second locus of peptidase (Pep-2) is useful, cheap, and technically quite a simple marker that can be used for comparative analysis of *Phytophtora infestans* strains and populations. This polymorphic locus is represented by two alleles 100 and 112; all their combinations commonly occur in the field populations. Genetic diversity for Pep-2 locus in the majority of populations is higher than for Pep-1. The use of Pep-2 in the complex with other markers such as mating type and Pep-1 has potential in the investigations of clonal structure of populations, the ways of spreading of the pathogen, and possible sources of infection. The complex of aforementioned features is promising for use in regional and interregional databases on late blight agent. The comparative analysis of mating type, Pep-1 and Pep-2 of Russian and Belorussian populations of *P. infestans* elucidated that the majority of investigated populations had the genotypes A1, 100/100, 100/100; A2, 100/100, 100/100, and A1, 100/100, 100/112. The genotypes A2, 100/100, 100/112 and A1, 100/100, 112/112 were rarer. Other possible *P. infestans* genotypes were found for a few isolates in different populations or were absent.

Keywords: *Phytophftora infestans*, late blight, analysis of populations, allozyme structure.

INTRODUCTION

Phytophthora infestans (Mont.) de Bary, the causal pathogen of late blight disease of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.), is the most damaging microbial pest of potato and tomato crops worldwide, especially in Russia and Eastern Europe. Average losses of potato yield in Russia caused by late blight including losses during tubers storage and early death of leaves exceed 15 %. Changes in management of potato production coincide with sharp increase of *Phytophtora infestans* aggressiveness. In many regions, the spreading of A2 mating type has been registered; the disease symptoms

occurred in unusually early stages of potato growth; potato tubers and stems were considerably more damaged; some resistant varieties became susceptible. High variability of the fungus and spore capability to migrate with air flows and within tubers result in frequent displacements of prevalent genotypes in populations, the attack on formerly resistant cultivars, and decrease of fungicide efficiency. This makes population monitoring extremely important.

Our preliminary investigations demonstrate that monoclonal populations of *P. infestans* often include more aggressive strains with higher number of virulence genes than panmictic or polyclonal populations. In panmictic and polyclonal populations the strains can mate and form hybrid oospores. Oospores can overwinter and cause early epidemies (SMIRNOV et al., 2001; VEDENYAPINA et al., 2002). The sexual reproduction leads to the increase of genetic diversity in a population and appearance of new genotypes. In general, in monoclonal populations the strains are more adaptive to environment; it allows them to spread in stable agrocenoses. The strains in polyclonal and panmictic populations have higher adaptability, which is advantageous in changeable environment. The need for information about population structure requires the creation of interregional (e.g., pan-European) databases on population structure of late blight agent (DUNKAN et al., 2002; HANSEN et al., 2002). For standardisation of the data, it is necessary to get cheap and informative markers easy to work with.

We propose to use second locus of peptidase (Pep-2) in comparison analysis of *P. infestans* strains and populations. This polymorphic locus is represented by two alleles 100 and 112; all their combinations commonly occur in field populations. In the article the application of Pep-2 together with first locus of peptidase (PEP-1) and mating type for the research of the structure of *P. infestans* populations is described.

MATERIAL AND METHODS

Isolate collection. Isolates of *P. infestans* were collected in commercial fields of potatoes and tomatoes during 1997–1998 at different places in Russia and Belarus. Only single, young, small lesions of late blight from tomato and potato were collected to reduce the possibility of isolating mixed genotypes. Isolates were taken from one field at a time, except Belarus ones, which represent 4 field populations, and strains from clones Sib 1 and Sib 2 (Table 1). In total, 174 isolates from 17 Russian field populations, 21 from clones Sib 1 and Sib 2, and 23 Belarus isolates were tested for allozyme structures of peptidase and mating type.

Mating type analysis. Mating type was determined by cultivating the isolates together with tester strains of known mating type in a Petri dish containing oatmeal agar (i.e., each sample isolate was paired with known mating types, A1 and A2). After 10–15 days oospore formation was checked in each pairing. Isolates forming oospores with the A1 mating type were registered as A2; isolates that formed oospores with the A2 mating type were registered as A1. Isolates forming oospores alone were registered as self-fertile.

Electrophoresis. Electrophoresis was performed on the equipment produced by Helena Laboratories Inc. according to their recommendations for cellulose-acetate gels (HEBERT & BEATON, 1993) with our modifications. The mycelium was grown in pea liquid medium for 10 days. Small piece of mycelium was placed to eppendorf tube (1.5 ml); 2 drops of distilled sterile water was added. The samples were homogenised during 30–40 seconds. Homogenated were centrifuged during 15–20 seconds at 10000 rpm. From each

Table 1.

Populations and number of tested isolates

Population	Host plant	Number of tested isolates
1997		
Sib 1[1]	Potato, leaves	14
Sib 2[2]	Potato, leaves	7
1999		
Moscow region, Plot 1 PL	Potato, leaves	16
Moscow region, Plot 2 PL	Potato, leaves	7
Moscow region, TL	Tomato, leaves	17
Moscow region, TF	Tomato, fruits	6
Belarus	Potato, leaves	6
2000		
Rjazan' region	Potato, leaves	13
Vologda region	Potato, leaves	8
Belarus	Potato, leaves	17
2001		
Leningrad region	Potato, leaves	9
Tula region	Potato, leaves	12
Brjansk region	Potato, leaves	4
Stavropol region	Potato, leaves	19
North Osetia	Potato, leaves	20
Mordovia, TL	Tomato, leaves	6
Mordovia, TF	Tomato, fruits	4
Moscow region, Šakhovskaja, TL	Tomato, leaves	9
Moscow region, Šakhovskaja, PL	Potato, leaves	7
2002		
Leningrad region	Potato, leaves	6
Ingushetia	Potato, leaves	11

[1] Sib 1 – one of the great clonal linages in Russian Siberia and Far East, isolates were collected in Čita, Omsk, Tomsk, Sakhalin, Irkutsk, Krasnojarsk, Ekaterinburg;

[2] Sib 2 – another great clonal linage mentioned in Khabarovsk and Birobijahn (Elansky et al, 2001).

sample 8 ml of supernatant were transferred into applicator die. Cellulose acetate gel was removed from the container with tris-glycine buffer (30 g of tris, 144 g of glycine adjusted to 1 l by distilled sterile water, pH 8.5. TG should be diluted with sterile water as 1 : 9 before use; the gel should be placed in the buffer slowly preventing fractures and exfoliation and incubated for at least 3 hours before use), dried between two filter papers, and placed on the plastic base with the cellulose acetate layer up. The investigated supernatants were transferred on the gel by an applicator. The gel was put in the electrophoresis chamber and marked at the margin by a control dye (bromphenol blue). The dye solution for the Peptidase included: TRIS HCl, 0.05M, pH 8.0 – 2 ml, Peroxidase, 1000 U/ml – 5 drops, o-dianisidine,

Table 2.

Phytophtora infestans genotypes found in Russia and Belarus

Number	Encoding	Mating type	Pep 1	Pep 2
1	111	A1	100 / 100	100 / 100
2	112	A1	100 / 100	100 / 112
3	113	A1	100 / 100	112 / 112
4	121	A1	92 / 100	100 / 100
5	123	A1	92 / 100	112 / 112
6	211	A2	100 / 100	100 / 100
7	212	A2	100 / 100	100 / 112
8	213	A2	100 / 100	112 / 112
9	221	A2	92 / 100	100 / 100
10	222	A2	92 / 100	100 / 112
11	311	SF[1]	100 / 100	100 / 100
12	312	SF	100 / 100	100 / 112
13	321	SF	92 / 100	100 / 100

[1] SF – self-fertile strains

4 mg/ml – 8 drops, $MgCl_2$, 20 mg/ml – 2 drops, glycine-leucyn, 15 mg/ml – 10 drops. All solutions were prepared in deionised water. The electrophoresis was performed at 200 V for 20 min. After termination of electrophoresis 2 drops of L-amino-acid oxidase (20 u/ml) were added to the mixture. The gel was removed from the chamber and put on the glass cellulose acetate layer up. 10 ml 1.6 % DIFCO Agar was melt in the microwave oven and cooled to 60 °C. 18 ml of dye solution were mixed with 2 ml of agar and carefully poured on the gel. The bands were detected in half an hour. Before estimating the results the dye mixture could be washed with distilled water.

RESULTS

Application of Pep-2 together with Pep-1 and mating type allowed determination of the population structure and comparison of populations of *P. infestans* in the European part of Russia and Belarus. The analysis of isolates indicated the majority of field populations being not monoclonal. They included the strains differing in the peptidase allozyme structure and mating types. In total, 13 different *P. infestans* genotypes were found in Russia and Belarus (Table 2). The most frequent were genotypes 111 (detected in 52 of tested strains from 13 populations, Table 3), 211 (49 strains from 14 populations), 112 (37 strains from 12 populations), and 212 (21 strains from 7 populations). Strains belonging to 113 genotype were found in 5 populations, genotypes 213 and 221 – in 4, 121 in 3, 222 in 2, genotypes 123, 311, 312, 321 – each in 1 population. Other genotypes were not detected. The strains from monoclonal populations (clones Sib 1 and Sib 2, monoclonality of which was preliminary proved by other neutral markers (ELANSKY et al., 2001) were the same according to the marker set presented in the current work. The tested populations were characterised by high genotypic diversity. Populations of *P. infestans* from Caucasus, Belarus, and Moscow region isolated from tomato leaves consisted of strains with maximum number of genotypes:

Table 3.

Distribution of genotypes in the spatial populations in Russia and Belarus

Population	Genotypes (encoding according to Table 2), %										
	111	112	113	121	211	212	213	221	222	Other[1]	NG[2]
1997											
Sib 1	100	0	0	0	0	0	0	0	0	0	1
Sib 2	0	100	0	0	0	0	0	0	0	0	1
1999											
Moscow region Plot 1 PL	0	0	0	0	100	0	0	0	0	0	1
Moscow region Plot 2 PL	0	0	86	0	14	0	0	0	0	0	2
Moscow region TL	46	18	16	6	24	0	0	0	0	0	5
Moscow region TF	33	67	0	0	0	0	0	0	0	0	2
Belarus	33	17	0	33	17	0	0	0	0	0	4
2000											
Rjazan' region	76	0	16	0	8	0	0	0	0	0	3
Vologda region	38	24	0	0	38	0	0	0	0	0	3
Belarus	18	28	12	6	0	18	6	6	0	6	8
2001											
Leningrad region	89	0	0	0	11	0	0	0	0	0	2
Tula region	0	100	0	0	0	0	0	0	0	0	1
Brjansk region	25	25	0	0	0	50	0	0	0	0	3
Stavropol region	34	0	0	0	26	10	5	5	5	15	9
North Osetia	40	5	10	10	10	30	5	5	0	0	6
Mordovia TL	0	0	0	0	100	0	0	0	0	0	1
Mordovia TF	0	25	0	0	75	0	0	0	0	0	2
Moscow region, Šakhovskaja, TL	0	33	0	0	33	34	0	0	0	0	3
Moscow region, Šakhovskaja, PL	28	0	0	0	14	58	0	0	0	0	3
2002											
Leningrad region	17	49	0	0	34	0	0	0	0	0	3
Ingushetia	64	9	0	0	0	9	9	9	9	0	5
Number of populations [3]	13	12	5	3	14	7	4	4	2	1	19

[1] Include genotypes 123, 311, 312, 321.
[2] Number of genotypes in a population.
[3] Number of populations, in which genotype was found (excluding Sib 1 and Sib 2).

from 4 to 9. Other populations had 2–3 genotypes. Populations of Moscow region PC (Plot 1), Tula PL, Mordovia TL, Sib 1, and Sib 2 were monoclonal.

The mentioned markers were also applied for comparison of *P. infestans* populations on potato and tomato in one plot (Table 3). The investigation was done in 1999 in Zvenigorod

Table 4.

Genetic diversity calculated for Pep-1 and Pep-2 for some Russian populations

Population	D[1] Pep-1	D Pep-2	Average for two loci
Moscow region Plot 1 PL	0	0	0
Moscow region, Plot 2 PL	0.11	0.24	0.17
Moscow region, TL	0.06	0.26	0.16
Moscow region, TF	0	0.5	0.25
Brjansk region	0	0.46	0.23
North Osetia	0.04	0.38	0.21
Ingushetia	0.08	0.3	0.19
Stavropol region	0.13	0.26	0.2
Rjazan' region	0	0.26	0.13
Vologda region	0	0.2	0.1
Leningrad region	0	0	0
Belarus 1999	0.18	0.16	0.17
Belarus 2000	0.09	0.5	0.3

[1] D – genetic diversity, calculated as follows: $D_l = 1\text{-}\Sigma p_{lu}^2$, p_{lu} – frequency of allele *u* in locus *l* (WEIR, 1995).

Biological Station of Moscow State University on the plot 20 × 20 m further off other potato and tomato plots. Two potato groups (cultivars 'Sante' (Plot 1) and 'Sineglazka' (Plot 2)) and susceptible tomato cultivar 'Talalikhin' were planted in the experimental plot closely to each other. Fungi isolates from tomato leaves and fruits were collected separately. Genotypic analysis showed that *P. infestans* strains on different host-plants differed from each other. All strains isolated from 'Sineglazka' had genotypes 113 (86 % of strains) and 211 (all others). But all strains in neighbour plot from 'Sante' had only genotype 211. Isolates from tomato fruits had genotypes 111 and 112, not registered on potato, but present on tomato leaves. Population from tomato leaves has the most complicated genotype structure: genotypes 113 and 211 also occurring on potato, 111 and 112 also present on tomato fruits, and unique 121. Probably, all plots had their own sources of primary infection: possibly contaminated seed tubers for potato, infected seeds for tomato, and oospores from soil for all populations. The disease symptoms on potato leaves appeared earlier than on tomato. Fungal strains with genotypes 113 and 211 could come to tomato leaves from potato. Strains with genotypes 111 and 112 possibly originated from another source such as: soil oospores, infected tomato seeds, or those could be offsprings after mating between strains with genotypes 113 and 211. Our observations of oospores in many blighted tomato leaves from this plot support the last assumption.

Estimation of genetic diversity of fungal alleles in loci Pep-1 and Pep-2 showed that in most populations genetic diversity of locus Pep-2 had been higher than of Pep-1 (Table 4). Almost all *P. infestans* populations, except monoclonal, have high value for Pep-2 – from 0.2 to 0.5 (maximal value). In comparison, genetic diversity for Pep-1 in all populations is less than 0.18. This makes Pep-2 a good marker for genetic investigations of *P. infestans*.

DISCUSSION

For comparative analysis of *P. infestans* strains and populations in the majority of investigations such markers as mating type, resistance to fungicides, virulence genes, allozyme structure of peptidase (locus Pep-1) and glucose-6-phosphate isomerase (locus GPI-1), as well as genome structure (haplotypes of mytochondrial DNA, hybridization probes RG57 for total DNA) are used (DYAKOV et al., 1994; ELANSKY et al., 1999 a, b; ELANSKY et al., 2001; GOODWIN et al., 1994; PETERS et al., 1999; SUJKOVSKI et al., 1994). Application of these markers for genotypic analyses in Russia has some limitation. Mating type is a useful marker in case of high procreation, but the presence of only two mating types makes this marker low informative. Self-fertile strains of *P. infestans* are very rare. Resistance to fungicides is important only in the applied aspect. For the investigation of *P. infestans* population structure this marker is not recommended as the same clone can have different levels of resistance to fungicides (ELANSKY et al., 2001). Virulence to potato is also hardly informative because in the majority of field populations highly virulent strains and those characterised by high intraclonal diversity prevail (ELANSKY et al., 2001). Virulences of *P. infestans* to tomato applied as a marker in population research enable to conduct additional investigation of its possible intraclonal variation. Until now in many (but not in all) *P. infestans* populations from Moscow region and probably from Europe the T0 race predominates on potato and T1 – on tomato (DYAKOV et al., 1994; ELANSKY et al., 1999 b). Allozyme structures of glucose-6-phosphate isomerase (GPI-1) and the first locus of peptidase (Pep-1) were promising for testing *P. infestans* populations from North and Central America (PETERS et al., 1999; TOOLEY et al., 1985). The investigation of these markers for Russian populations was not productive. All isolates investigated during last years were monomorphic in GPI-1 and low polymorphic in Pep-1 (ELANSKY et al., 1999 a, b). Haplotype of mytochondrial DNA is also not informative enough. In Russia only two haplotypes are known – Ia and IIa, at strong predominance of Ia (MALEEVA et al., 1999; ELANSKY et al., 2001). Hybridization probes for genome structure is one of the best markers for investigation of population structure and strain comparison. However it is expensive, time-consuming method demanding high qualification of the personnel; therefore, it is possible to use this method only for investigation of limited number of isolates.

So, from a wide range of typically used markers only mating type, spectrum of isozymes of Pep-1, and haplotype of mitochondrial DNA can be recommended for researches of population structure of late blight agent in Russia and Belarus. But this set of markers is not informative enough for investigations of genotypic structure of populations. The obtained results allow to conclude that Pep-2 together with Pep-1 and mating type can be used in *P. infestans* population researches.

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ANTRASIS PEPTIDAZĖS LOKUSAS KAIP ŽYMIKLIS GENETINIUOSE *PHYTOPHTHORA INFESTANS* TYRIMUOSE

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Santrauka

Antrasis peptidazės lokusas (Pep-2) yra patogus, nebrangus ir techniškai gana paprastas žymiklis, kurį galima naudoti lyginamajai *Phytophthora infestans* kamienų ir populiacijų analizei. Šį polimorfinį lokusą sudaro du aleliai: 100 ir 112; gamtinėse populiacijose paprastai randama įvairių jų derinių. Genetinė Pep-2 lokuso įvairovė daugumoje *P. infestans* populiacijų yra didesnė nei Pep-1. Naudojant Pep-2 drauge su kitais žymikliais, tokiais kaip poravimosi tipas bei Pep-1, galima tyrinėti populiacijų klonų struktūrą, ligų sukėlėjų plitimo būdus ir galimus infekcijos šaltinius.

Lyginamoji Rusijos ir Baltarusijos *P. infestans* populiacijų poravimosi tipų, Pep-1 ir Pep-2 analizė parodė, kad dauguma tyrinėtų populiacijų pasižymėjo šiais genotipais: 1) A1, 100/100, 100/100; 2) A2, 100/100, 100/100 ir 3) A1, 100/100, 100/112. Genotipai A2, 100/100, 100/112 ir A1, 100/100, 112/112 buvo retesni.

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