The repertoire of Avr genes in two East European populations of *Phytophthora infestans*

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**SUMMARY**

Single-strand conformation polymorphism (SSCP) analysis was employed to assess avirulence genes (Avr genes): Avr1, Avr2, Avr2-like, Avr3a, Avr3b, Avr4, Avr8, Avr9, Avr-blb1, Avr-blb2 and Avr-vnt1 in 20 single-cell lines obtained from the isolates of *P. infestans* collected in the VIR potato genetic collection (Pushkin, Leningrad region), in 16 lines from the isolates collected in the potato stationary field plots of the Institute of Phytopathology (Ramenki, Moscow region), and in seven reference lines from the Western Europe and the USA. SSCP patterns of Avr genes were collated with the profiles of simple sequence repeats of these *P. infestans* lines and the complements of the genes for resistance to *P. infestans* in potato genotypes that were colonized by particular pathogen strains.

**KEY WORDS**

*Phytophthora infestans*, potato late blight, Avr genes, Rpi genes, allelic polymorphism, SSCP analysis, SSR genotyping

**INTRODUCTION**

Elucidating the repertoire of avirulence genes (Avr genes) that determine the pathogenicity of *P. infestans* strains would help better understand the mechanisms of rapid pathogen evolution that brings forth the epidemic development of late blight (LB). As an agronomic projection, the express assessment of the repertoire of Avr genes in particular agrocenoses would promote early discernment of the changes in pathogen populations crucial for diagnosing LB development and expansion.

Among the Avr genes of *P. infestans*, best known are those producing RXLR effectors (Raffaele and Kamoun, 2012). Potato resistance to LB evoked by these virulence genes is usually described by the Flor’s “gene-for-gene” paradigm (Flor, 1971) presuming that each RXLR effector is specifically recognized by the corresponding receptor kinase, the product of the race specific gene for resistance to *P. infestans* (Rpi gene). The virulence genes of *P. infestans* are
conventionally recognized using the Mastenbroek-Black set of differential plants (Kim et al., 2012) comprising the individual Rpi genes. The molecular genetic analysis of Avr genes has greatly expanded the scope of identified virulence genes in P. infestans and helped evaluate their polymorphisms immediately reflecting their evolution; however, in contrast with the differential set, the molecular studies do not always discriminate between the virulent and avirulent forms of the particular gene.

The SSCP analysis of Avr genes of P. infestans, when verified by cloning and sequencing individual DNA fragments that comprise SSCP patterns, turned out a robust method of comparing Avr profiles in several populations of P. infestans. Here we collated SSCP patterns in two East European populations of P. infestans, in Leningrad and Moscow regions of Russia, with the SSR profiles of these populations and the spectra of Rpi genes in potato plants colonized by these pathogen strains. Some data reported here were previously reported elsewhere (Martynov et al., 2019; Sokolova et al., 2017).

MATERIALS AND METHODS

LB-affected leaves were collected in 2015 in the potato stands of the VIR field genetic collection in Pushkin, St. Petersburg, and in 2018, in the potato stationary field plots of the Institute of Phytopathology (Ramenki, Moscow). P. infestans isolates were processed in the Institute of Phytopathology as described elsewhere (Sokolova et al., 2017). Following the SSR genotyping of P. infestans isolates collected in 2013 and 2014 from individual potato plants in Pushkin, we presumed that some individual plants in the VIR collection were colonized mutually by at least two different P. infestans genotypes. For this reason, single-cell lines were obtained from the 2015 Pushkin and 2018 Ramenki isolates, and further studies of Avr genes were carried out only with these lines. In addition, we investigated seven reference lines from the Western Europe and the USA provided as DNA samples by Dr David Cooke (The James Hutton Institute, Dundee, UK) and a highly virulent and aggressive line 161 (races 1.2.3.4.5.6.7.8.9.10.11, mating type A1) collected in the Moscow region (the State Collection of the Institute of Phytopathology). All P. infestans lines under study were genotyped at 12 SSR loci (Li et al., 2013).

Two approaches were used to evaluate the repertoire of Avr genes and their polymorphisms. Full-length gene sequences were PCR-amplified from genomic DNA with the previously described primers, the amplicons were cloned, and ten randomly selected clones per line were sequenced; the sequences thus obtained were compared with the Avr genes identified in the fully sequenced genome of the P. infestans isolate T30-4 (prototype genes) and other Avr sequences of P. infestans deposited in the NCBI Genbank. In the case of SSCP analysis, amplicons obtained with primers known from the literature or developed by the authors were subjected to thermal and chemical denaturation and electrophoretic separation in a non-denaturing polyacrylamide gel. DNA fragments corresponding to the zones of different mobility were excised from gel, and DNA fragments were eluted, cloned and sequenced. The sequences thus obtained were compared with the Avr genes identified in the same P. infestans lines using the first approach as well as with the sequences deposited in the NCBI Genbank. SSCP patterns are well reproducible, and when the individual electrophoretic zones are validated by sequencing, these patterns can serve as reliable barcoding descriptors for deciphering the Avr polymorphisms. Our protocols for DNA isolation, SSR genotyping and SSCP analysis followed previously described standard procedures (Martynov et al., 2019; Sokolova et al., 2017).
RESULTS AND DISCUSSION

Distribution of allele variants of Avr genes in the geographically distant samples of P. infestans

We explored the polymorphisms of eleven Avr genes of P. infestans: Avr1, Avr2, Avr2-like, Avr3a, Avr3b, Avr4, Avr8=Avr-Smira2, Avr9=Avr-Smira1, Avr-blb1=ipiO, Avr-blb2, and Avr-vnt1 in 36 single-cell lines from Pushkin (Leningrad region) and Ramenki (Moscow region) and the reference lines with genotypes 4_A1, 8_A1, 5_A1, US8, EC-1, 13_A2 and 6_A1 (C1, C2, C4, C6, C7, 3928a and 4100a, respectively) from the Western Europe and the USA plus the line 161 from the collection of the Institute of Phytopathology. The Avr2-like gene is considerably different from Avr2 (Gilroy et al., 2011), and therefore we treat it separately.

The allelic profiles of the genes Avr3b, Avr4 and Avr8 as revealed by SSCP analysis were monomorphic, and all other profiles were very polymorphic (Figure 1). Three geographically diverse samples of P. infestans widely differed in their SSCP patterns and in the frequencies of these patterns. In the case of the Pushkin and Ramenki pathogen populations, such differences apparently stem from the fact that different potato varieties with dissimilar Rpi genes are grown in these two regions of the European Russia, under diverse agroclimatic conditions. The reference lines seem to reflect even more remote situations as regards climate and potato varieties colonized by P. infestans lines.

The Avr1 gene was present in all Pushkin line of P. infestans and 72% of the reference lines, whereas it was considerably less frequent in the Ramenki lines (29%). All Avr1 sequences reported in this study correspond to virulent alleles and differ in several single nucleotide substitutions, two of them apparently representing evolutionary hotspots. The absence of the avirulent Avr1 allele can probably reflect high frequency of the R1 gene in potato varieties grown in this area. Pattern 2 of the Avr2 gene dominated the Pushkin and Ramenki lines (80 and 88%, respectively) as compared to 57% in the reference lines. This allele corresponds to already described Avr2K gene recognized by the R2 gene (Saunders et al., 2012). With Avr2 effector recognized by R2 kinase, one would expect that plants comprising the R2 gene would manifest resistance to most Avr2 lines in this study. However, as reported recently, the presence of the intact Avr2 gene does not always imply that it is expressed (Stefańczyk et al., 2017). The virulent homologue Avr2-like corresponded to alleles Avr2-likeTV and Avr2-likeMI; this homologue dominated all pathogen populations under study and was most polymorphic.

All Pushkin and Ramenki lines of P. infestans contained only virulent variants of the Avr3a gene, whereas the reference lines also comprised the heterozygotes combining the virulent and avirulent alleles. Here we observed only random and synonymic substitutions. In contrast, the Avr3b gene was found only in some Pushkin and reference lines, whereas in the Ramenki lines its frequency was as high as 43%. Avr3b sequences described here differ by several SNPs from those previously reported. Characteristically, the Pushkin and Ramenki lines were similar in this aspect and differed from the reference lines. The Avr9 gene was found in all Pushkin and most Ramenki and reference lines of P. infestans and was represented by its virulent allele except in the Pushkin line 87/2-2 and the Ramenki lines 194, 3 and 5. The presence of the virulent Avr9 gene in P. infestans populations would decrease LB resistance in potato varieties comprising the R9=Rpi-Smira1 gene.
While the **R9** and **Rpi-vnt1** genes are structural homologues (Aguilera-Galvez *et al.*, 2018), we failed to recognize any extended homology between the **Avr9** and **Avr-vnt1** sequences, except in the conserved domains RXLR and s/dEER. The case of **Avr2** is directly opposite: the corresponding effector is recognized by two genes of diverse structures, **R2** and **Rpi-mcq1** on chromosomes IV and IX (Aguilera-Galvez *et al.*, 2018). Apparently, in these cases the structural evolution of gene predecessors was ahead of their functionalisation.
We found the *Avr-vnt1* gene in all Pushkin and reference lines and in many Ramenki lines. Earlier the gene was reported to exist in three allelic variants V1, V2 and V3 (Pel, 2010). Our samples were dominated by the V1/V3 combination and the homozygous form V2 was found in the lines 87/2-2 and 163. All three alleles combined only in the Ramenki lines.

The family of *Avr-blb1* genes is very polymorphic, and only distinct classes in this family differ in their functions. We therefore designed class-specific primers recognizing the conserved stretches of these genes. All lines contained one variant of the *Avr-blb1* gene, predominantly of class I, whereas line _5_A_1 comprised only class II gene. Class I alleles of the *Avr-blb1* gene are recognized by the *Rpi-blb1* gene and therefore are avirulent, while class II genes are virulent only in the absence of the class I genes (Champouret et al., 2009). We therefore conclude that all *P. infestans* lines under study will turn avirulent towards potato hybrids comprising the RB/Rpi-blb1 gene.

Avirulent and virulent alleles of the *Avr-blb2* gene (Oh et al., 2009) produce a contrasting pattern in the lines under study. The highly aggressive lines _13_A_2 and _6_A_1 comprise various avirulent alleles of this gene. It follows that the hybrids comprising the *Rpi-blb2* gene are prospective sources for potato breeding.

Most lines under study contained the *Avr8* gene with its sequence completely identical to the prototype gene in the isolate T30-4. High conservation of this gene was previously reported by Jo (Jo, 2013).

The phylogenetic analysis of *Avr* genes based on sscp patterns
The diversity of SSCP patterns that characterize the *Avr* genes in three samples of *P. infestans* lines fall into five clusters (Figure 2A).

![Figure 2. The phylogenetic analysis of SSCP patterns of *P. infestans* lines based on (A) seven polymorphic *Avr* genes and (B) 41 polymorphic SSR loci. The dendrogram is built with SplitsTree 4.10 using uncorrected P with gaps included in the analysis and a UPGMA network. Bootstrap support for main clades (1000 replicates) is indicated by the smaller numbers on specific branches. The Roman numbers and colour of clusters in Figure 2B follow Figure 2A](image-url)
Half of Pushkin lines share cluster I with all Ramenki lines plus line 161, also collected in the Moscow region; here we also find the reference line 4_A1. Besides, many Pushkin lines are found in cluster IV and some in cluster II. Single-cell lines produced from one and the same isolate are usually clustered together. The strikingly aggressive West European lines 6_A1 and 13_A2 (Cooke et al., 2012) form the isolated cluster V, whereas most other reference lines are in cluster III. The isolated position of 6_A1 and 13_A2 lines is apparently related to the fact that these lines are devoid of several Avr genes, such as Avr1, Avr4, Avr8 and Avr9; in addition, 13_A2 also lacks the Avr2 gene.

SSR genotyping produced less compact clusters (Figure 2B); nevertheless, the general arrangement of pathogen lines in clusters I, IV and V is compatible in two cases. The line 87/2-2 characterized by low aggressiveness and predominantly avirulent alleles holds a specific position in both SSCP and SSR distributions.

The profiles of the Avr genes in P. infestans lines as compared to the complements of late blight resistance genes in colonised potato plants

The composition of Rpi genes in potato genotypes colonized by P. infestans lines under study has been established previously (Fadina et al., 2017). The profiles of Avr and Rpi genes and virulence factors registered with the differential set are presented in Table 1. Within the gene-for-gene paradigm, the profiles of Avr and Rpi genes match pretty well, except in the case of the Avr3b gene. This gene was found in the Pushkin line 11/2 and the Ramenki lines 155, 156, 157 and 196, which colonized potato hybrids comprising the R3b gene. However, the Avr3b sequences described in this study contain several non-synonymous substitutions, which would prevent its recognition by the corresponding kinase and render the gene non-functional. In hybrids 14/8-09 and 120 (118/6-2011) in var. Robijn, we registered marker R2-1137 and did not find marker R2-686, another marker of the same gene (Fadina et al., 2017) indicating some changes in the gene structure. In this case, we treated this gene as absent. In the pathogen lines derived from potato hybrids comprising the Rpi-blb2 gene, we found the virulent alleles of Avr-blb2 (Oh et al., 2009).

In four cases from the Pushkin population, 11/2-2 and 11/2-4, 43/1-1 and 43/1-2, 18/1-1, and 103b, the lines derived from the same isolates were dissimilar by one Avr gene presuming the possibility that an individual plant was simultaneous colonized by two independent although related pathogen lines.
Table 1. Repertoire of Avr genes as compared to the complement of virulence factors assessed with the Mastenbroeck-Black differentials and the profile of the Rpi genes

<table>
<thead>
<tr>
<th>Potato clones; Rpi genes</th>
<th>Lines</th>
<th>Avr genes</th>
<th>Virulence factors assessed with differential plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pushkin lines</td>
<td></td>
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<tr>
<td>Pushkin lines</td>
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<tr>
<td>139 (4/1-2012); R1, Rpi-blb1/Rpi-sto1, Rpi-blb2</td>
<td>120-1</td>
<td>avr1, Avr2_K, avr3a_EM, avr4, Avr8, Avr9 (I), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>12347891011</td>
</tr>
<tr>
<td>106 (171-3); R3b</td>
<td>11/2-1</td>
<td>avr1, Avr2-like_TV/MI, avr3a_EM, Avr3b, Avr8, Avr9 (I, II), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>134781011</td>
</tr>
<tr>
<td>113(50-1KWA); R1, Rpi-blb2</td>
<td>103-1, 103-2, 103-3, 103-5</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (I), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>123467891011</td>
</tr>
<tr>
<td>27; R1, Rpi-blb1, Rpi-blb2</td>
<td>107-1</td>
<td>avr1, Avr2_K, avr3a_EM, avr4, Avr9 (I), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>123478910</td>
</tr>
<tr>
<td>Robijn</td>
<td>87/2-2</td>
<td>avr1, Avr2_K, Avr2-like_TV/MI, avr3a_EM, avr4, Avr8, Avr9 (II), Avr-blb1 (I), Avr-blb2, Avr-vnt1 (V2)</td>
<td>12378</td>
</tr>
<tr>
<td>160-17; Rpi-blb2</td>
<td>43/1-1</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (I), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>12347891011</td>
</tr>
<tr>
<td>18/40-2000; Rpi-vnt1.3</td>
<td>18/1-1</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (I), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>1234567891011</td>
</tr>
<tr>
<td>18/40-2000; Rpi-vnt1.3</td>
<td>18/1-4</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (I), Avr-blb1 (I, II), Avr-blb2, Avr-vnt1 (V1, V3)</td>
<td>1234567891011</td>
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<tr>
<td>Ramenki lines</td>
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<td>Ramenki lines</td>
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<tr>
<td>2372-60; R1, R2, R3a, R3b</td>
<td>96</td>
<td>Avr2_K, Avr2-like_MI, avr3a_EM, Avr8, Avr-blb1, Avr-vnt1 (V1/V3)</td>
<td>nd</td>
</tr>
<tr>
<td>13/11-09; R3b, Rpi-blb1, Rpi-vnt1.3</td>
<td>155</td>
<td>Avr2_K, Avr2-like_MI, avr3a_EM, Avr3b, Avr-blb1, Avr-vnt1 (V1/V3)</td>
<td>nd</td>
</tr>
<tr>
<td>14/8-09; R3a, R3b, Rpi-vnt1.3</td>
<td>156</td>
<td>Avr2_K, Avr2-like_MI, avr3a_EM, Avr3b, Avr8, Avr9 (I, II), Avr-blb1 (I)</td>
<td>nd</td>
</tr>
<tr>
<td>16/27-09; R1, Rpi-blb1, Rpi-blb2</td>
<td>158</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, Avr3b, avr4, Avr8, Avr9 (I, II), Avr-blb1 (I), Avr-blb2, Avr-vnt1 (V1/V3)</td>
<td>nd</td>
</tr>
<tr>
<td>18/40-2000; Rpi-vnt1.3</td>
<td>18/1-3</td>
<td>Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (I), Avr-blb1 (I), Avr-blb2, Avr-vnt1 (V2)</td>
<td>nd</td>
</tr>
<tr>
<td>134-6-2006; Rpi-vnt1.3</td>
<td>194</td>
<td>avr1, Avr2_K, avr3a_EM, avr4, Avr8, Avr9 (II), Avr-blb1 (I)</td>
<td>nd</td>
</tr>
<tr>
<td>135-1-2006; R3a, R3b, Rpi-vnt1.3</td>
<td>196</td>
<td>Avr2_K, Avr2-like_MI, avr3a_EM, Avr3b, Avr8, Avr9 (I, II), Avr-blb1 (I), Avr-vnt1 (V1/V3)</td>
<td>nd</td>
</tr>
<tr>
<td>Easterling</td>
<td>3</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (II), Avr-blb1 (I, II), Avr-vnt1 (V1/V2/V3)</td>
<td>nd</td>
</tr>
<tr>
<td>Robijn</td>
<td>5</td>
<td>avr1, Avr2_K, Avr2-like_MI, avr3a_EM, avr4, Avr8, Avr9 (II), Avr-blb1 (I, II), Avr-vnt1 (V1/V2/V3)</td>
<td>nd</td>
</tr>
<tr>
<td>Gloria; R1, R3a, R3b</td>
<td>6</td>
<td>Avr-like_MI, avr3a_EM, Avr8, Avr9 (I, II), Avr-blb1 (I)</td>
<td>nd</td>
</tr>
</tbody>
</table>

Avr9 (I) and Avr9 (II), virulent and avirulent alleles, respectively; nd, no data
In the history of introgression breeding, some \( Rpi \) genes, such as \( R1 \) and \( R2 \), were early arrivals to potato varieties, as compared to \( R9, Rpi-blb1, Rpi-blb2 \), and \( Rpi-vnt1 \), which are met in a small number of new varieties and hybrids. Meanwhile many \( P. infestans \) lines described in this communication comprise the \( Avr-blb2 \) and \( Avr-vnt1 \) genes corresponding to the \( Rpi \) genes as yet uncommon in potato varieties. Similarly, the lines 6_A1 and especially 13-A2, which are devoid of the \( Avr \) genes corresponding to the typical profiles of \( Rpi \) genes in widespread potato varieties, rapidly expand, displace the already present pathotypes and inflame most dramatic LB epidemics (Cooke et al., 2012; Knaus et al., 2019). Of special interest is the line 87/2-2, which differs from all other lines in both the SSCP and SSR patterns (Figure 2). This line colonised the susceptible var. Robijn, with the single \( R2 \) gene; this line comprises mostly avirulent alleles of the \( Avr \) genes.

CONCLUSIONS
Screening, by SSCP analysis, \( P. infestans \) lines from the Leningrad and Moscow regions of the European Russia as compared to the reference lines from the Western Europe and the USA revealed the wealth of \( Avr \) allelic variants, with their frequencies different as regards to the agroclimatic characteristics of the territories.

The evidence from the SSCP screening doe not completely match the results of the SSR genotyping apparently because the genome loci assessed by two methods evolve at contrasting speeds (Raffaele and Kamoun, 2012). It is also essential that, in contrast to SSR loci, the \( Avr \) genes assayed by the SSCP analysis are immediately related to pathogenicity of \( P. infestans \) lines under study.

The repertoire of the \( Avr \) genes revealed by the SSCP analysis does not completely match the profile of virulence genes (factors) examined with the Mastenbroek-Black differential set. The latter provides indirect evidence bases on plant response to infection; in addition the currently employed differential set is devoid of many important \( Rpi \) genes recognizing such \( Avr \) genes as \( Avr-blb1, Avr-blb2 \) and \( Avr-vnt1 \).

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