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Are simple *Phytophthora infestans* races really that simple?

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SUMMARY
Allelic diversity of the IpiO gene encoding the effector recognised by Rpi-blb1/RB gene from *S. bulbocastanum* and *S. stoloniferum* was assessed by PCR screening and sequencing in 15 races (1, 3, 4, 10, 11, 1.2, 1.3, 1.4, 2.4, 3.4, 1.11, 1.2.3, 1.2.4, 1.3.4, and 1.2.3.4) maintained in the collection of the Institute of Phytopathology. IpiO locus was present in all these isolates. Phylogenetic analysis demonstrated that IpiO alleles from races 3 and 4 belonged to the classes I and II recognised by R*pi-blb*1/RB. It follows that these "simple" races exhibit simple virulence patterns on potato comprising only R1-R11 genes from *S. demissum*. Growing interest in exploiting exotic *Solanum* germplasm in potato breeding is an incentive to reconsider and unambiguously redefine the terms "simple" and "complex" race.

KEYWORDS
Phytophthora infestans, *Solanum*, potato, late blight resistance, effectors, simple races

INTRODUCTION

Approaches to describe the diversity of P. infestans
Various methods and protocols were developed to identify, distinguish, and classify *P. infestans* isolates collected in the fields worldwide. The most prominent examples of such methods are pairing test (mating type A1, A2), response to metalaxyl (Sozzi *et al.*, 1992), polymorphism of isozymes (Goodwin *et al.*, 1995), RFLP markers mtDNA, marker RG57 (Goodwin *et al.*, 1991 and 1992), AFLP fingerprinting (Chen *et al.*, 2008), microsatellite analysis (Lees *et al.*, 2006), AVR profiling (Cárdenas *et al.*, 2011), and virulence spectra determined on potato differentials ("simple" and "complex" races). In our study, we critically analysed the concept of "simple" and "complex" races using current knowledge about the molecular basis of *P. infestans* - plant interactions.

Determinants of specificity in race-specific resistance
During attack on host plants, *P. infestans* secretes hundreds of molecules aimed at promoting infection by various mechanisms including reprogramming of plant immune system by interacting with proteins involved in the immune response. Such molecules are called effectors (Kamoun, 2006). The diversity of characterised effectors is classified according to the pathogen lifestyle, their allocation in plant tissues, and the presence of extremely conserved motifs, such as RXLR (Fig. 1).

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Inside a cell, effectors can be directly or indirectly recognised by the sentinel receptors encoded by R genes - determinants of race-specific resistance (Vanderplank, 1963). All intracellular effectors characterised as triggering R gene-mediated immune response belong to the class of RXLR, also known as Avr (avirulence) effectors. The genome of *P. infestans* strain T30-4 contains 563 genes encoding proteins with unique RXLR motifs (Haas *et al.*, 2009). Eight RXLR effectors were identified as factors triggering immune response following their recognition by well-characterised *Solanum* R proteins (reviewed in Vleeshouwers *et al.*, 2011; Table 1).
From the pathogen side, allelic diversity of Avr effectors apparently determines the specificity of their recognition by R proteins. As little as a single amino acid change in Avr3a could circumvent its recognition by R3a. Surprisingly, such mutations were always associated with the loss of the primary (pathogenic) function of Avr3a in suppressing the plant cell death response induced by INF1 elicitor protein (Bos *et al.*, 2009).

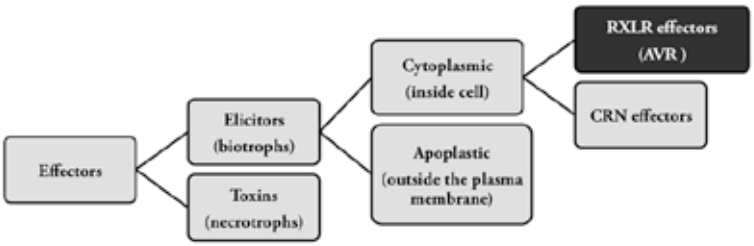

Figure 1. Diversity of effectors classified by the pathogen lifestyle, localisation, and family-specific conserved motifs. A family of RXLR Avr effectors cognate to the LB-related R gene products is highlighted in black.

Table 1. Functionally characterised R protein – Avr effector pairs (reviewed in Vleeshouwers et al., 2011).

Solanum species*	R protein	*P. infestans* effector
dms	R1	Avr1
blb/dms	Rpi-blb3/R2	Avr2
dms	R3a	Avr3a
dms	R3b	Avr3b
dms	R4	Avr4
vnt	Rpi-vnt1.1	Avr-vnt1
blb/sto	RB/Rpi-blb1/Rpi-sto1	IpiO1
blb	Rpi-blb2	Avr-blb2

*Abbreviations: dms – *S. demissum*, blb – *S. bulbocastanum*, sto – *S. stoloniferum*, vnt – *S. venturii*

MATERIAL AND METHODS

P. infestans races and DNA isolation

Genomic DNA was isolated from 15 races (1, 3, 4, 10, 11, 1.2, 1.3, 1.4, 2.4, 3.4, 1.11, 1.2.3, 1.2.4, 1.3.4, and 1.2.3.4) maintained in the Institute of Phytopathology using AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Biosciences) according to the manufacturer's recommendations. DNA concentration was measured using a UV/Vis NanoPhotometer P300 (IMPLEN), and DNA integrity was accessed using the agarose gel electrophoresis.

PCR amplification and cloning of IpiO
IpiO alleles were amplified from genomic DNA using the forward primer specific for the IpiO locus including the region of signal peptide (5'-CTTTCCGGCAATGCGTTCGC-3') and the reverse primer 5'-CTATACGATGTCATAGCATGACAC-3' described in Champouret *et al.* (2009). PCR primers were optimised using the Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools). The amplification reactions contained 1 µl of 10x PCR buffer, 100-150 ng of genomic DNA, 1 µl 2.5 mM dNTP, 10 pmol each of two primers, 1 U of either Pfu DNA polymerase (Fermentas) for cloning or Taq DNA polymerase (Syntol) for screening and sterile water to a volume of 10 µl. Reactions were run in an MJ PTC-200 thermocycler (Bio-Rad) using the following program: 3 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 65°C, 1 min at 72°C; 10 min at 72°C. PCR products were separated by electrophoresis in 1.5% w/v agarose and stained with ethidium bromide. Amplified fragments were eluted from the gel using QIAquick gel extraction kit (Qiagen), cloned using CloneJET™ PCR Cloning Kit (Fermentas), and sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit and ABI 3730 DNA Analyzer (Applied Biosystems).

Phylogenetic analysis of IpiO sequences
DNA sequences of IpiO effector genes characterised by Champouret *et al.* (2009) were extracted from NCBI Genbank (accession numbers GQ371190 - GQ371200, GQ371202). For multiple alignment, we employed MAFFT (Katoh *et al.*, 2002) algorithm implemented at GUIDANCE web server (http://guidance.tau.ac.il; Penn *et al.*, 2010). The alignment was manually curated and edited in BioEdit software (Hall, 1999). Maximum likelihood search was performed using RAxML algorithm with rapid bootstrap analysis (http://phylobench.vital-it.ch/raxml-bb; Stamatakis *et al.*, 2008) and the best scoring ML tree was analysed.

RESULTS AND DISCUSSION

What makes "simple" race simple?
The concept of "simple" and "complex" races was introduced by Black and associates who bred the set of 11 potato cultivars (so-called differentials) each presumably containing a single R gene introgressed from *S. demissum* (Black *et al.*, 1953). *P. infestans* isolates virulent only on one or two of these differentials were considered as "simple", isolates with wider virulence spectra as "complex". "Simple" races were widely used to map *S. demissum* R genes (Leonard-Schippers *et al.*, 1992) and to detect R genes in potato cultivars (Sokolova *et al.*, 2011). "Simple" races are frequently referred to as carrying one or two virulence factors (e.g. Ghimre *et al.*, 2001); but how does it fit the Avr-R gene model? We suggest that virulence factors as defined by classic phytopathology are products of mutated Avr genes evading recognition by R proteins. Therefore, we propose that "simple" races contain a single "broken" Avr gene, whereas other Avr effectors corresponding to *S. demissum* R1-R11 genes are intact.

The concept of "simple" race may need revising
We suggest revising the use of the term "simple" and "complex" races based on the following arguments.
According to the so-called "arms race" model, pathogens and plants are constantly co-evolving by the re-assortment of the virulence and defence factors (e.g. alleles of Avr and R genes; Bergelson *et al.*, 2001). Such co-evolution creates strong evolutionary forces that shape distribution of active and inactive alleles on both sides of the frontier. Recurrent selection of the "simple" races on the Black's differentials imposes constant selective constraint only on Avr effectors cognate to *S. demissum* R receptors, whereas allele frequencies of other effector genes (>500) apparently serve the best interest

of the pathogen. Recently, wild *Solanum* species other than *S. demissum* attracted close attention of potato breeders and the corresponding R genes were isolated and successfully introduced into potato cultivars (Vleeshouwers *et al.*, 2011). Selection on differentials will not affect virulence spectra of "simple" races on these cultivars; therefore, these spectra will not characterise a particular "simple" race. In other words, "simple" races are simple only in relation to the potato cultivars comprising *S. demissum* R1-R11 genes. While in potato cultivars R genotyping with "simple" races is in line with the presence of particular R genes, the evidence for wild *Solanum* species is apparently misleading.
The second argument is more philosophical. The term "simple" race would suggest that pathogen isolate characterised as "simple" may have less ability to infect or be less aggressive and vice versa for "complex" race isolates. Such presumption is not true. In fact, Montarry *et al.* (2010) showed that complexity of *P. infestans* race negatively correlated with pathogen fitness. Selection would favour less complex isolates with less virulent factors (and the higher numbers of intact Avr effectors). The trade-offs between acquiring more functional Avr effectors essential for fitness and the chance to be recognised by R protein would drive such selection. The evidence that mutation of Avr3a "hiding" this effector from R3a was also detrimental for the primary function of the effector in promoting infection fits this model (Bos *et al.*, 2006). Summing up, in contradiction with the semantics of their name, isolates with "simple" race are generally more fit and aggressive than "complex" race isolates.

Allelic diversity of IpiO effector gene in "simple" races
To verify our suggestions, we studied allelic diversity of the IpiO gene encoding the effector recognised by Rpi-blb1/RB gene from *S. bulbocastanum* and *S. stoloniferum* (Vleeshouwers *et al.*, 2008). To this end, we designed the forward primer specific for the IpiO locus and used the reverse primer described by Champouret *et al.* (2009). Screening 15 races (1, 3, 4, 10, 11, 1.2, 1.3, 1.4, 2.4, 3.4, 1.11, 1.2.3, 1.2.4, 1.3.4, and 1.2.3.4) maintained in the collection of the Institute of Phytopathology showed that IpiO was present in all these isolates. These results are in line with previous evidence that the diversity of IpiO locus results from allelic variation in nucleotide sequences rather than from the presence-absence polymorphism (Halterman *et al.*, 2010). Champouret *et al.* (2009) showed that the IpiO genes fell into three distinct classes. IpiO effectors of classes I and II were recognised by Rpi-blb1/RB, whereas IpiO class III did not trigger RB-mediated hypersensitive response. To classify IpiO alleles in "simple" races, we analysed ten clones of IpiO gene from races 3 and 4. Cluster analysis revealed that "simple" races 3 and 4 comprised alleles encoding IpiO variants avirulent on *Solanum* germplasm with active allele of Rpi-blb1/RB gene. Therefore, because virulence patterns of these "simple" races are not simple, they cannot be predicted by (in)compatibility reaction on potato differentials.

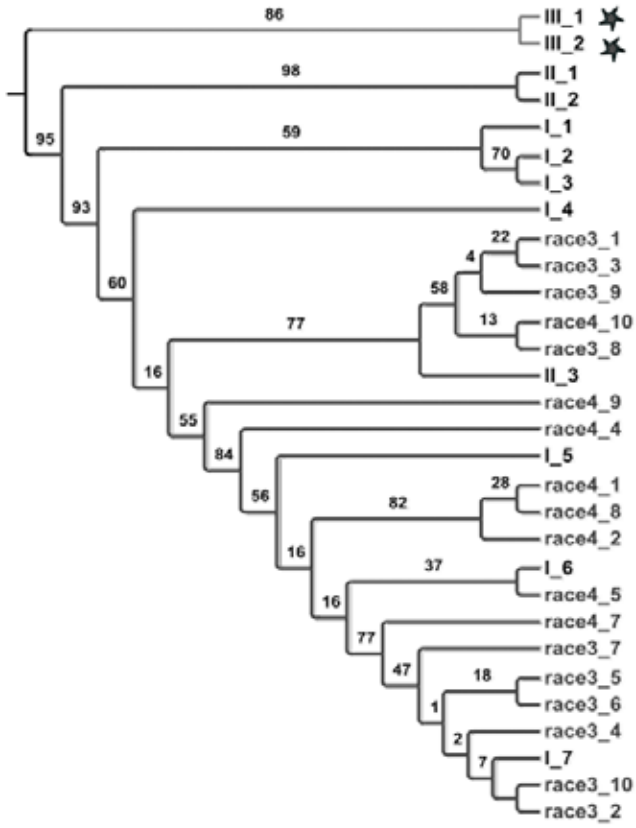

Fig. 2. Maximum likelihood cladogram of IpiO alleles from the "simple" and "complex" races of P. infestans. Star pictogram denotes the cluster of IpiO alleles of class III, which circumvents recognition by Rpi-blb1/RB. Alleles extracted from NCBI Genbank are I_1 – I_7, II_1 – II_3, and III_1, III_2 (accession numbers GQ371190 – GQ371200, GQ 371202). Other alleles are sequenced in this study. Bootstrap values are shown at the nodes.

CONCLUSIONS

We presume that the virulence factors described by classic phytopathologists are Avr effector genes, which avoided recognition by the corresponding R proteins due to the various mutations fixed by the evolutionary "arms race".

"Simple" races comprise active alleles of effectors cognate to the products of R genes other than R1-R11 initially recognised in *S. demissum*. Therefore, the "simple" virulence pattern of these races would emerge only in potato plants containing R1-R11 genes introgressed from *S. demissum*.

Apparently, the terms "simple" and "complex" race should be reconsidered and clearly redefined by the research community to avoid possible ambiguities.

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