Molecular analysis of wild Solanum clones in search for late blight resistance genes

Emil Khavkin\textsuperscript{1}, Maria Kuznetsova\textsuperscript{2}, Elena Rogozina\textsuperscript{3}, Kenneth Deahl\textsuperscript{4}, Richard Jones\textsuperscript{4}

\textsuperscript{1}Institute of Agricultural Biotechnology, Moscow, Russia (emil.khavkin@gmail.com)
\textsuperscript{2}Institute of Phytopathology, Bolshie Vyazemy, Moscow Region, Russia
\textsuperscript{3}N.I. Vavilov Institute of Plant Industry, St. Petersburg, Russia
\textsuperscript{4}Beltsville Agricultural Research Center, Beltsville, MD, USA

Abstract
Breeding for durable resistance to potato late blight (LB) heavily relies on expanding the resource of resistance genes (R genes). We developed clonal collections of wild Solanum species to study genetically identical plants with independent molecular and phytopathological methods. DNA markers for R genes were employed to screen about 300 Solanum accessions representing over twenty species from seven series of section Petota. By collating the distribution of the structural homologues of R genes with the indices of resistance to simple and complex races of Phytophthora infestans, we have searched for new alleles of the R genes already characterized in other Solanum species. Specific markers have been developed to elucidate genome constitution of Solanum species employed in breeding for LB resistance.

Key words: Solanum section Petota, Phytophthora infestans, R genes, SCAR markers, allele mining

Introduction
Late blight (LB) caused by Phytophthora infestans (Mont.) de Bary is among the pressing issues of potato industry, with average produce losses of 10-15\% in Russia and worldwide. LB resistance genes (R genes) previously introgressed from wild Solanum demissum have been defeated by rapidly evolving P. infestans races. Introducing new R genes or new alleles of already characterized R genes, especially by pyramiding broad-spectrum R genes of diverse specificity to pathogen races, would advance breeding potato for durable LB resistance (Haverkort et al., 2009). Searching for new R genes in wild tuber-bearing species of Solanum L. section Petota Dumort. has already proven effective (Vleeshouwers et al., 2011). In this context, Solanum species insufficiently researched by molecular methods invite special effort.

Wild Solanum accessions maintained in the world genebank collections (Hoekstra, 2009) are highly polymorphic as regards LB resistance (Douches et al., 2001). Following the initial screening for LB resistance, we have developed the clonal collections of the most promising genotypes and maintain them as microtubers. Such collections provide genetically consistent material for coupled studies by independent molecular and phytopathological methods (Rogozina et al., 2012).

Using the candidate gene approach (Gebhardt et al., 2007), we have developed and verified several SCAR markers based on the sequences of already characterized R genes and used these markers to mine the collections of wild Solanum species for new R-gene homologues. We have also developed SCAR markers of Solanum genomes A, B and D to help compare diploid and polyploid Solanum species as the sources of new R genes (Drobyazina and Khavkin, 2012).

Here we review in brief the outcome of these studies including an extended pattern of R-gene distribution in the section Petota and two cases of allelic polymorphism in these genes against the genome profiles of Solanum species under study.
Material and methods
Clonal collections of wild Solanum species were initiated on the basis of seed accessions obtained from The Centre for Genetic Resources, the Netherlands, the NRSP-6 Potato Genebank, USA, and The Vavilov Institute of Plant Industry, Russia. By selecting individual plants manifesting high LB resistance, we studied 92 clones of plants representing 15 wild tuber-bearing Solanum species in coupled phytopathological and molecular tests in the Institute of Phytopathology, Institute of Plant Industry and Institute of Agricultural Biotechnology. LB resistance was assessed by a detached leaf assay and by inoculating whole plants in a greenhouse (for Eucabligh protocols for whole plant and detached leaf LS resistance see www.euroblind.net). For molecular studies, genomic DNA was isolated by a modified CTAB protocol (Doyle and Doyle, 1987), or with the AxyPrep™ Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA) from young leaves of individual plants and amplified using the primers recognizing the specific regions of S. demissum genes R1, R2, R3a and R3b and S. bulbocastanum gene RB/Rpi-blb1. SCAR (sequence characterized amplified regions) markers R1-1205, R3-1380 (for R3a) and RB-629 were described previously (Pankin et al., 2010; Pankin et al., 2011; Sokolova et al., 2011). For SCAR markers R2-2500 and R3-378 (R3b), we additionally optimized the amplification protocols reported by Kim et al. (2012) The amplified genome fragments were cloned, and their sequences underwent the multiple alignment and phylogenetic analysis as described by Pankin et al. (2011). Solanum genome markers were developed by aligning 32 polymorphic sequences of intron 2 from the FLORICAULA/LEAFY gene (Flint2) representing nine Solanum species from five series of the section Petota. These markers were verified by screening 130 Solanum accessions representing 26 Solanum species (Drobyazina and Khavkin, 2012; Drobyazina and Khavkin, unpublished data).

Results and discussion
Validating markers for the R genes and their distribution within the section Petota
These markers were validated against large ranges of S. demissum and S. bulbocastanum accessions, and some amplified fragments were cloned. Their sequences were 99-100% similar to those of the prototype genes. Screening, with these markers, about 300 Solanum accessions representing over twenty species from seven Petota series produced the following patterns (Sokolova et al., 2011; Fadina et al., unpublished data). The R1 marker was predominantly found in the series Demissa (mostly in S. demissum and in some accessions of S. hougasii and S. iopetalum), Longipedicellata (S. stoloniferum and less frequently in S. polytrichon) and diploid Tuberosa species (S. bertaultii and S. microdontum). The R3a marker was observed in the series Bulbocastana (S. bulbocastanum), Cardiophylla (S. cardiophyllum and S. ehrenbergii), Demissa (S. demissum and S. hougasii), Longipedicellata (mostly S. stoloniferum and several accessions of S. papita and S. polytrichon), Polyadenia (S. polyadenium), Tuberosa (S. microdontum, S. verrucosum) and Yungasensia (S. huancabambens). The markers R2-2500 and R3-378 were recent additions to this screening, and up to now, only 53 Solanum clones have been assessed. The R2 marker has been found in S. bulbocastanum, S. cardiophyllum, S. hougasii, S. huancabambense and S. pinnatisectum, and the R3b marker was currently discerned in some clones of S. bulbocastanum and S. verrucosum; this marker was especially frequent in S. cardiophyllum, S. ehrenbergii and S. stoloniferum. When verified by cloning and sequencing, two markers, R1-1205 in S. polytrichon and S. stoloniferum and R3-1380 in S. bulbocastanum, S. cardiophyllum, S. hougasii, S. polytrichon and S. stoloniferum shared 98–99% identity with the corresponding regions in the prototype genes. A full-length homologue of R3a from S. stoloniferum shared 98%
identity with the prototype gene from *S. demissum* (Sokolova et al., 2011). This evidence is in line with the presence of functional *R3a* orthologue in *S. stoloniferum* as established in the experiments with the *Avr3a* gene (Vleeshouwers et al., 2011). The sequences of the marker R3-378 from *S. papita* and *S. ehrenbergii* were 96-98% similar to the corresponding fragment of the prototype *R3b*. The marker RB-629 was found in most *Solanum* series and in more than half of screened accessions; this marker recognized both the active R genes and their homologues with yet unknown function (Pankin et al., 2010). The allelic polymorphism of these genes is discussed below.

**Discerning R genes with SCAR markers and simple races of *P. Infestans***

The accessions of *S. demissum* and demissoid potato cultivars were screened with SCAR markers for race-specific R genes and with simple races of *P. infestans*, the isolates reportedly virulent each on one or two of Black’s differentials and widely used to recognize and map *R1-R11* genes introgressed from *S. demissum* (see Kim et al., 2012, for bibliography). In these cases, two independent sets of experimental data concurred quite satisfactorily. However, beyond *S. demissum*, the molecular and phytopathological evidence for wild *Solanum* species did not match persuasively. To clarify this contradiction, we investigated the pattern of avirulence (Avr) genes in simple *P. infestans* races 3 and 4 maintained in the Institute of Phytopathology. In addition to the expected Avr3 and Avr4, both these races comprised active alleles of Avr genes cognate to the products of R genes other than *R1-R11* initially recognized in *S. demissum*, such as *IpiO* alleles recognized by the RB/Rpi-bbl1 gene of *S. bulbocastanum* (Pankin et al., 2012).

**Allelic polymorphism of the R1 gene**

Based on the marker R1-1205, we developed SCAR markers discriminating between two putative *R1* alleles initially discerned in *S. demissum* and *S. stoloniferum* and therefore tentatively dubbed as the species-specific markers R1dms and R1sto. These markers never overlapped and were never present together when verified against 13 and 16 accessions of *S. demissum* and *S. stoloniferum*, respectively. It is noteworthy that the marker R1dms was also found in two more species from the series *Demissa*: *S. iopetalum* and *S. hougasii*; in contrast, R1sto was not found beyond *S. stoloniferum*, even in other species of the series *Longipedicellata*, including *S. papita* and *S. polytrichon*, the forms which are presently included into *S. stoloniferum* (Khavkin et al., unpublished data).

**Allelic polymorphism of the RB/Rpi-bbl1 gene.**

The phylogenetic analysis of RB-629 sequences from 12 *Solanum* species produced four distinct clusters, with LB-resistant *Solanum* accessions present mostly in cluster 1 of *S. bulbocastanum*-like RB/Rpi-bbl1 haplotypes (Pankin et al., 2010). We also employed another RB/Rpi-bbl1 marker, RB-1223, which corresponded to the whole length of CC domain in RB receptor kinase and included the RB-629 sequence. Screening 14 *Solanum* species with RB-1223 produced seven groups of RB homologues with several subgroups (Pankin et al., unpublished data). Only two of these groups included previously characterized haplotypes functionally active as LB resistance genes, such as the *Rpi-btl* group from *S. bulbocastanum* and the group comprising *Rpi-bbl1* from *S. bulbocastanum*, *Rpi-stol* from *S. stoloniferum* and *Rpi-pta1* from *S. papita*; these two groups comprised both diploid species with genome B and tetraploid species with genome AB. An interesting exception from this pattern is exemplified by the gene *RBver* from *S. verrucosum* (genome A1). Notably, *S. verrucosum* is the reported source of genome A in *S. stoloniferum* (see Cai et al., 2012).

**The association of R genes with particular Solanum genomes**

The RB/Rpi-bbl1 gene undoubtedly arrived to tetraploid *S. stoloniferum* as well as the synonymous species *S. papita* and *S. polytrichon* with genome B from diploid species *S. cardiophyllum/ehrenbergii* or *S. bulbocastanum* (Cai et al., 2012). Kuang et al. (2005)
demonstrated that in hexaploid *S. demissum*, the functional *R1* gene was associated only with one of three particular genomes (haplotypes). *R1* sequences in *S. demissum* (genome A1A2D) and *S. stoloniferum* (AB) are very similar, and we presume that in *S. demissum*, *R1* belongs to one of two genomes A, the one probably different from genome A1 in *S. tuberosum* devoid of functional R genes. The genes *R1*, *R2*, *R3a* and *R3b* initially identified in *S. demissum* were found in many taxonomically distant species. It is noteworthy that the markers of *R3a* and especially *R2* and *R3b* were discerned in many genome B species (series Bulbocastana and Pinnatisecta/Cardiophylla), whereas the close homologue of *R3a* was cloned from *S. bulbocastanum* (Sokolova et al., 2011). In opposite, we find homologues of the *RB*/Rpi-*blb1* gene, which was initially identified in *S. bulbocastanum*, in many genome A species. Such evidence suggests that the R-gene structures evolved before the divergence of genomes A and B and subsequent *Solanum* speciation. It is established that allelic *RB*-like sequences from genomes A and B in various *Solanum* species are not always associated with high LB resistance (Liu and Halterman, 2006; Pankin et al., 2010). This fact implies that early duplication and diversification of R-gene structural homologues are later followed by their neofunctionalization in the process of adaptive co-evolution of *Solanum* and *P. infestans* haplotypes.

**Conclusions**

The described clonal collections reflect wide ranges of genetic variation in wild tuber-bearing *Solanum* forms. Hopefully, such collections would become the basis for further research in comparative *Solanum* genomics, including the evolutionary study of R-gene sequences, mining for new R genes and establishing their functions. In this way, it is of prior importance to address the crucial question of assigning biological functions to newly found homologues of the prototype R genes for LB resistance. The characterized R genes and marker technologies for screening initial wild genotypes, segregants of crosses between wild and cultivated potato and backcrosses would certainly promote introgressive potato breeding for durable LB resistance.

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**References**


