Twelfth EuroBlight workshop
Arras (France), 3-6 May 2010

Potato resistance to late blight as related to the *R1* and *R3* genes introgressed from *Solanum demissum*

EMIL E. KHAVKIN[1], EKATERINA A. SOKOLOVA[1], MARIA P. BEKETOVA[1, 3], ARTEM A. PANKIN[1], MARIA A. KUZNETSOVA[2], IRINA N. KOZLOVSKAYA[2], SVETLANA YU. SPIGLAZOVA[2], NATALIA V. STATSYUK[2], ISOLDA M. YASHINA[3]

[1]Institute of Agricultural Biotechnology, Moscow, 127550 Russia
[2]Institute of Phytopathology, Bolshiye Vyazemy, 143050 Russia
[3]Institute of Potato Industry, Korenevo, 140052 Russia

SUMMARY
New races of *Phytophthora infestans* are known to rapidly defeat potato late blight (LB) resistance introgressed with the germplasm of *Solanum demissum*, the early source of race-specific resistance genes (*R*-genes). Nonetheless, the presence of the major *demissum* gene *R1* in potato cultivars was associated with higher field indices of LB resistance (Stewart *et al*., 2003; Gebhardt *et al*., 2004; Beketova *et al*., 2006) suggesting that these *demissum R*-genes in some way add to plant defense response. We developed and verified SCAR markers recognizing the race-specific genes *R1* and *R3* of *S. demissum* and *S. stoloniferum* and the germplasms of these species. By screening wild *Solanum* species and potato accessions reportedly free from wild *Solanum* germplasm (Chilotanum varieties and old cultivars), we established that these markers reliably discerned between germplasms of cultivated *Solanum tuberosum* ssp. *tuberosum* and wild sources of LB resistance. Screening 161 potato cultivars demonstrated that the presence of SCAR markers of *R1* and *R3* genes was significantly related to higher indices of LB resistance. Similar results were obtained when the presence of *demissum R*-genes was recognized using specific races of *Ph. infestans*. Such association presumes that both *R1* and *R3* genes contribute to overall LB resistance of potato cultivars.

KEYWORDS
Phytophthora infestans, *Solanum* species, race-specific genes, overall resistance

INTRODUCTION

For several decades, breeding for potato late blight (LB) resistance has heavily relied on germplasm introgression from *Solanum demissum*, the wild Mexican species comprising the race-specific resistance genes *R1-R11.* New pathogen races are known to rapidly overcome such resistance; nonetheless, many potato cultivars comprising the *R*-genes from *S. demissum* maintain higher field resistance than the genotypes lacking such genes (for review of the earlier evidence see Gebhardt *et al*., 2004; Stewart *et al*., 2003; Trognitz and Trognitz, 2007).

PPO-Special Report no. 14 (2010), 231 - 238

Stewart *et al*. (2003) reported that potato cultivars containing the *R1* gene identified with the specific races of *Ph. infestans* manifested considerably higher field LB resistance than the cultivars free of *R*-genes. Cloning *R*-genes (Hein *et al*., 2009) makes it feasible to detect these genes in potato and its wild relatives using the molecular markers developed from the particular gene sequences. Thus, Gebhardt *et al.* (2004) used the *R1* sequence to develop the marker R1-1400. This marker was found in *S. demissum* and *S. stoloniferum*, and by screening 415 potato cultivars, these authors established significant association between the presence of R1-1400 and the indices of LB resistance collected from cultivar passports (passport resistance). Similar relationship between the presence of R1-1400 marker and the passport resistance indices was reported from our laboratory for cultivars bred mostly in the former Soviet Union (Beketova *et al*., 2006). Trognitz and Trognitz (2007) demonstrated that the R1-1400 fragments cloned from differentials and cultivars were completely identical to the prototype gene *R1*.

In this study we employed both molecular and phytopathological methodologies for recognizing the *R1* and *R3* genes and both field and laboratory assays for LB resistance. The significant association between the presence of these genes and high LB resistance presumes that *R1* and *R3* someway contribute to LB resistance of potato cultivars.

MATERIALS AND METHODS

Plant Material

Potato tubers for this study arrived from the collections of the Institute of Potato Husbandry (Korenevo, Moscow region, Russia), the Institute of Plant Industry (St. Petersburg, Russia), the Institute of Phytopathology (Bol'shiye Vyazemy, Moscow region, Russia), and the Research and Practical Centre for Potato, Fruit and Vegetable Growing, (Samokhvalovichi, Minsk region, Belarus). As a whole, we screened 161 potato accessions. The pedigree information, particularly on the presence of the germplasm of *S. demissum* and *S. stoloniferum*, was obtained from the already published evidence in the catalogs of the institutions mentioned above and the electronic catalogs of the European Cultivated Potato Database (www.europotato.org/varietyindex.php) and the Dutch-German Potato Collection (www.plantbreeding.wur.nl/potatopedigree). Seeds of *S. demissum* and *S. stoloniferum* accessions were obtained from the collections of the Institute of Plant Industry (St. Petersburg, Russia) and the United States Potato Genebank, NRSP-6 (Sturgeon Bay, WI).

Development of DNA markers

Standard protocols were employed for genomic DNA isolation from plant leaves, PCR analysis, and cloning and identifying genome fragments. Specific primers for sequence characterized amplified regions (SCAR markers) were designed following multiple alignment of the *S. demissum* and *S. stoloniferum* sequences (Table 1) with their structural homologs from the NCBI Genbank using the programs BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Vector NTI Suite 8 package (Invitrogen). The marker R1-1205 modified from R1-1400 (Gebhardt *et al*., 2004) provided more reliable scoring. Other markers were developed in this laboratory.

Table 1. *SCAR markers of the R1 and R3 genes of S. demissum and S. stoloniferum and the germplasms of these species.*

Markers and their size, bp	Prototype clone	Chromosome	Position in the prototype clone
R1-1205	AF447489	5	5126-6331
R3-1380	AY849382	11	1677-3056
Ssto-448	EU041625	5	100-548
Sdms-523	AY875783	5	59138-59530

Phytopathological assays
Identification of *R*-genes in potato plants was accomplished by the artificial inoculation of detached leaves with a set of *Ph. infestans* races (1; 3; 4; 10; 11; 1.2; 1.3; 1.4; 2.4; 3.4; 1.2.4; 1.3.4.; 1.2.3.4). The leaflets of the tested plants were placed into a wet chamber, representing a frame (30 × 40 × 4 cm) with the bottom made of metal gauze. The lower surface of each leaflet was inoculated, using a micropipette, with two drops of a pathogen spore suspension. The concentration of suspension corresponded to 10 spores in a microscopic field at 100x magnification. The frames were placed onto wet filter paper and covered with glass plates. After 24-h incubation, the drops were shaken off, and the leaves were turned upside down. The sporulation registered after 4-6 days of incubation testified a compatible reaction.

Field assays of potato LB resistance were carried out under natural infection conditions. Leaves were examined every 7-10 days, starting from the first blotches on a susceptible cultivar, until the complete leaf decay. The percentage of lesions on the leaf was recorded using a scale of the Britain Mycological Society (James, 1971), transformed into the area under the disease progress curve and ranked by the 1-9 score scale. For laboratory assays of LB resistance, we used the express method based on the combined laboratory and field tests following the artificial inoculation with *Ph. infestans* spores (Filippov *et al.,* 2004). The field indices of LB resistance for potato foliage and the laboratory indices for detached leaves were registered in 2008 and 2009. By the U-test of Mann-Whitney (1947), field indices for two years were highly consistent. The field and laboratory indices for a particular cultivar differed, on the average, by two scores; nonetheless they were closely correlated, and we further employed an integrated index calculated as the average resistance over two field and two laboratory scores for the particular potato cultivar.

Statistical methods
To link the presence of the *R*-genes to LB resistance indices in potato cultivars, we used the nonparametric U-test of Mann-Whitney (1947) implemented in SPSS Statistics 17.0 software (http://www.spss.com).

RESULTS AND DISCUSSION

Verification of SCAR markers
To verify marker specificity towards *R1* and *R3*, we screened *S. demissum* and *S. stoloniferum* plants, potato cultivars comprising the germplasm of *S. demissum* (demissoid cultivars), including the *R*-gene differentials, and potato cultivars reportedly free of wild *Solanum* germplasm. In addition, we cloned and sequenced genome fragments corresponding to markers R1-1400/1205 and R3-1380 from several potato cultivars and *S. stoloniferum*.

All four markers under study reliably discerned cultivated *S. tuberosum* ssp. *tuberosum* from two wild *Solanum* species and potato cultivars comprising the germplasm of these species (Table 2). However, only three markers passed through several crosses into the modern potato cultivars. The marker Sdms-523 recognizing *demissum*-specific polymorphisms of selectively neutral internal transcribed spacer (ITS) was lost as soon as after two meiotic generations.

The marker R1-1400/R1-1205 was present in the differentials R1, R5, R6 and R9, thus corroborating data by Trognitz and Trognitz (2007), whereas the marker R3-1380 was found in Black's differentials R3, R7, R8 and R9 (Black *et al*., 1953). To explain such discrepancies we would suggest three possible reasons. First, Huang (2005) reported that functional *R5-R11* genes are structurally similar

to *R3* gene located in the same gene cluster with *R5-R11* on *demissum* chromosome 11; therefore the marker R3-1380 probably did not discern between these loci. Second, the differentials R5-R9 apart from the corresponding *R5–R9* genes may contain non-functional homologues of *R1* and *R3* genes corrupted by the nucleotide changes, which were nevertheless tagged by the *R1-* and *R3-*specific markers. Finally, the differentials initially selected for the presence of single *demissum R* gene could be in fact not monogenic and need further genetic improvement. The sequences of genome fragments of *S. stoloniferum*, differentials and potato cultivars corresponding to the markers R1-1400/R1-1205 and R3-1380 were 98-100% identical to the prototype *S. demissum R1* and *R3a* genes (Ballvora *et al.*, 2002; Huang, 2005).

Table 2. *Frequencies of the markers of R-genes and germplasms of Solanum species in potato and its wild relatives*

Genotypes (number of accessions)	SCAR markers			
	R1- 1400/1205	R3-1380	Ssto-448	Sdms-523
S. demissum (35)	0.40	0.11	0.80	1.0
S. stoloniferum (51)	0.18	0.16	0.80	0.0
S. tuberosum ssp. *tuberosum*: Chilotanum forms (6) and old cultivars free from *dms/sto* germplasm (11)	0.0	0	0	0.0
Demissoid potato cultivars (144)	0.40	0.38	0.84	0.0

Comparison of two methods for discerning R-genes

When *R*-genes were assessed in potato cultivars using simple *Ph. infestans* races, the data obtained by phytopathological *R*-genotyping in most cases matched the evidence for the presence of *R*-genes recognized with the molecular markers (Table 3). The agreement was higher for *R1* than for *R3* probably because the marker R3-1380 does not discriminate between *R3* and the structurally related *R5-R11* genes (see above).

Table 3. *Agreement of the data obtained by molecular and phytopathological methods of R-genotyping*

	Recognized genes		
	R1	*R3*	*R1* and *R3* together
The number of matches between two methods	53	44	33
Per cent	75	62	47

Association of LB resistance with the presence of R-genes

We investigated association between the presence of *R*-genes discerned by molecular and phytopathological genotyping and the indices of LB resistance assessed in the field and laboratory trials.

Potato cultivars with well-established pedigrees were divided into several subpopulations by their marker patterns (Table 4). The initial three subpopulations comprised the cultivars with the marker R1-1205, the marker R3-1380, and two markers present together. The fourth subpopulation is a batch of three previous sets. The control 1 subpopulation lacking the markers R1-1205 and R3-1380 is a heterogeneous group combining the genotypes with the germplasm of *S. demissum* and *S. stoloniferum* (control 2) and the genotypes free of such germplasm (control 3). The percentage of highly resistant potato accessions (scores 7-9) in genotypes comprising the marker R1-1205 or both R1-1205 and R3-1380 was notably higher than in the genotypes devoid of these markers (Table 4). The statistical analysis using the Mann-Whitney U-test demonstrated highly significant association between the presence of both markers R1-1205 and R3-1380 and high LB resistance. However, the

association of high LB with the presence of the markers R1-1205 and R3-1380 separately was not evident. LB resistance in the control subpopulation 2 devoid of the markers R1-1205 and R3-1380 exceeded that in the control 3 free of the *S. demissum* and *S. stoloniferum* germplasm; these data suggest that *S. demissum R*-genes other than *R1* and *R3* are present in some genotypes comprising the control 2. Indeed, *R*-genotyping with *Ph. infestans* simple races discerned the genes *R2* and *R4* in some of these cultivars.

LB resistance indices in the subpopulation conferring the *R1-R4* genes, as discerned by the phytopathological method, and in the subpopulation devoid of these genes differed by two scores of resistance revealing highly significant effect of the *R*-genes (Table 5).

In most potato accessions under study, *R1* and *R3* were transferred from *S. demissum*; however, many of these cultivars reportedly comprise the germplasm of *S. stoloniferum*. The presence of several *R*-genes in *S. stoloniferum* was first attested by phytopathological methods (McKee, 1962; Toxopeus, 1964; Grünwald and Flier, 2005). Gebhardt *et al*. (2004) reported the marker R1-1400 in *S. stoloniferum*, and recently several functionally active *R3a* sequences were revealed in this genome (Champouret, 2010). It is therefore of considerable interest to discover whether introgression of *stoloniferum R1* and *R3* genes into potato cultivars has ever occurred and to evaluate the relative inputs of these genes introgressed from each species.

Table 4. *Association of potato LB resistance with the presence of markers of the R1 and R3 genes (The number of potato accessions in each subpopulation is given in parentheses)*

Subpopulations of potato cultivars	Percentage of highly resistant cultivars (scores 7-9)	Mean scores by the 9-score scale	Standard deviation
comprising only R1-1205 (22)	27	5.3[c]	1.30
comprising only R3-1380 (23)	13	5.4[c]	1.24
comprising both R1-1205 and R3-1380 (20)	45	5.9[ac]	1.58
all accessions comprising the *R*-gene markers (65)	28	5.5[bc]	1.38
control 1: accessions devoid of R1-1205 and R3-1380 markers (44)	16	4.76[c]	1.61
control 2: cultivars comprising the germplasm of *S. demissum* and *S. stoloniferum* but devoid of R1-1205 and R3-1380 markers (36)	19	5.1[c]	1.56
control 3: *tuberosum* accessions free of the germplasm of *S. demissum* and *S. stoloniferum* (8)	0	3.2[ac]	0.46

[a]*Significantly different from the control 1 at 1% confidence level*
[b]*Significantly different from the control 1 at 5% confidence level*
[c]*Significantly different from the control 3 at 1% confidence level*
[d]*Significantly different from the control 3 at 5% confidence level*
[e]*Significantly different from the control 2 at 5% confidence level*

Table 5. *Association of potato LB resistance with the presence of R-genes discerned with Ph. infestans races (The number of potato accessions in each subpopulation is given in parentheses)*

Subpopulations of potato cultivars	Mean resistance scores by 9-score scale	Standard deviation
comprising the *R1-R4* genes (54)	5.8*	1.37
free of the *R1-R4* genes (18)	3.86	1.42

Difference significant at 0.1% confidence level by the Mann-Whitney U-test

Thus, using two independent methodologies for recognizing *demissum R*-genes, we established highly significant association between the presence of the *R1* and *R3* genes and high LB resistance. Further experiments are required to prove that such statistical association is derived from the activity of functional *R*-genes.

CONCLUSIONS

The highly significant correlation between the presence of the *demissum R* genes and high LB resistance described in this paper corroborates the earlier evidence from several authors (Stewart *et al*., 2003; Gebhardt *et al*., 2004; Beketova *et al*., 2006). The attested effect of both *R1* and *R3* genes from *S. demissum* and probably *S. stoloniferum* presumes that these genes contribute to overall LB resistance of potato cultivars.

Tan *et al.* (2008) discussed several models explaining the participation of *R*-genes in overall potato LB resistance, including (1) non-durable race-specific resistance rapidly defeated by new races of *Ph. infestans*, (2) durable broad-spectrum resistance conferred by several *R*-genes from *S. bulbocastanum* and other wild *Solanum* species, (3) QTLs involved in resistance and (4) residual resistance with poorly understood mechanisms. Recent studies of race specificity of *S. bulbocastanum* genes (Champouret e*t al*., 2010; Halterman *et al*., 2010) suggest that they are also prone to defeat, and the difference between the models (1) and (2) is inconsiderable and transient. Meanwhile, rapid advance in physical mapping reveals new *R*-genes behind earlier established QTLs for LB resistance (Hein *et al*., 2009). The current progress in elucidating the specific interactions between R-receptor kinases and pathogen effectors (Jones and Dangl, 2006; Vleeshowers *et al*., 2008) will undoubtedly help better understand the role of defeated *R*-genes in overall LB resistance.

ACKNOWLEDGMENTS

The authors thank all colleagues who kindly provided *Solanum* accessions and the relevant genetic and pedigree information. This study was supported by the ISTC-USDA-ARS project 3714p.

REFERENCES

Ballvora, A., M.R. Ercolano, J. Weiss, K. Meksem, C.A. Bormann, P. Oberhagemann, F. Salamini and C. Gebhardt, 2002. The *R1* gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant Journal 30, 361-371.

Beketova, M.P., P.E. Drobyazina, E.E. Khavkin, 2006. The *R1* gene for late blight resistance in early and late maturing potato cultivars. Russian Journal of Plant Physiology. 53, 384-389.

Black, W., C. Mastenbroek, W.R. Mills, L.C. Peterson, 1953. A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivatives. Euphytica 2,173-179.

Champouret, N., K. Bouwmeester, H. Rietman, T. van der Lee, C. Maliepaard, A. Heupink, P.J. van de Vondervoort, E. Jacobsen, R.G. Visser, E.A. van der Vossen, F. Govers, V.G. Vleeshouwers, 2009. *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato. Molecular plant-microbe interactions 22, 1535-1545.

Filippov A.V., B.I. Gurevich, B.E. Kozlovsky, M.A. Kuznetsova, A.N. Rogozhin, S.Y. Spiglazova, T.I. Smetanina and A.N. Smirnov, 2004. A rapid method for evaluation of partial potato resistance to late blight and of aggressiveness of *Phytophthora infestans* isolates originating

from different regions. Plant Breeding and Seed Science 50, 29-41.

Gebhardt, C., A. Ballvora, B. Walkemeier, P. Oberhagemann and K. Schüler, 2004. Assessing genetic potential in germplasm collections of crop plants by marker-trait association: a case study for potatoes with quantitative variation of resistance to late blight and maturity type. Molecular Breeding 13, 93-102.

Grünwald, N. and W.G. Flier, 2005. The biology of *Phytophthora infestans* at its center of origin. Annual Review of Phytopathology 43, 171-190.

Halterman, D.A., Y. Chen, J. Sopee, J. Berduo-Sandoval and A. Sánchez-Pérez, 2010. Competition between *Phytophthora infestans* effectors leads to increased aggressiveness on plants containing broad-spectrum late blight resistance. PLoS ONE 5, e10536.

Hein, I., P.R.J. Birch, S. Danan, V. Lefebvre, D.A. Odeny, C. Gebhardt, F. Trognitz and G.J. Bryan, 2009. Progress in mapping and cloning qualitative and quantitative resistance against *Phytophthora infestans* in potato and its wild relatives. Potato Research 52, 215-227.

Huang, S, 2005. The discovery and characterization of the major late blight resistance complex in potato genomic structure, functional diversity, and implications. PhD Thesis, Wageningen University, Wageningen, The Netherlands, 134 pp.

James, C., 1971. A manual of assessment keys for plant diseases. Canada Department of Agriculture Publication No. 1458.

Jones, J.D. and J.L. Dangl, 2006. The plant immune system. Nature 444, 323-329.

McKee, R.K., 1962. Identification of R-genes in *Solanum stoloniferum*. Euphytica 11, 42-46.

Mann, H. B. and D.R. Whitney, 1947. On a test of whether one of two random variables is stochastically larger than the other. Annals of Mathematical Statistics 18, 50-60.

Stewart, H.E., J. E. Bradshaw and B. Pande, 2003. The effect of the presence of *R*-genes for resistance to late blight (*Phytophthora infestans*) of potato (*Solanum tuberosum*) on the underlying level of field resistance. Plant Pathol., 2003, 52: 193-198.

Tan, M.Y.A., R.C.B. Hutten, C. Celis, T.H. Park, R.E. Niks, R.G.F. Visser and H.J. van Eck, 2008. The *R* (*Pi-mcd1*) locus *from Solanum microdontum* involved in resistance to *Phytophthora infestans*, causing a delay in infection, maps on potato chromosome 4 in a cluster of NBS-LRR genes. Molecular Plant - Microbe Interactions 21, 909-918.

Toxopeus, H.J., 1964. Treasure digging for blight resistance in potatoes. Euphytica 13, 206-222.

Trognitz, B.R. and F.Ch. Trognitz, 2007. Occurrence of the *R1* allele conferring resistance to late blight in potato *R*-gene differentials and commercial cultivars. Plant Pathology 56, 150-155.

Vleeshouwers, V.G.A.A., H. Rietman, P. Krenek, N. Champouret, C. Young, S.-K. Oh, M. Wang, K. Bouwmeester, B. Vosman, R.G.F. Visser, E. Jacobsen, F. Govers, S. Kamoun and E.A.G. Van der Vossen, 2008. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. PLoS ONE 3, e2875.