REVIEWS

Plant–Pathogen Molecular Dialogue: Evolution, Mechanisms and Agricultural Implementation¹

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Received June 23, 2020; revised June 24, 2020; accepted June 26, 2020

Abstract—Plant diseases persistently challenge sustainable crop production worldwide. The most economical and eco-friendly way to effectively deal with this problem is to breed new cultivars with stable and durable resistance. Current progress towards this goal has been reinforced by considerable advancements in the molecular studies of pathogens and host plants. These advancements have greatly benefited from recently developed methods to research into gene structure and activity, especially the “omics” technologies. These steps forward are vividly represented by the case of late blight, which is economically the most important disease of potato and tomato (*Solanum* L.). Late blight became a popular model of multidimensional plant-microbe interactions, and newly obtained molecular evidence has considerably reshaped both our vision of plant–pathogen molecular dialogue and our approach to mitigating this disease. Drawing on recent publications, this review will focus on genome of the causal agent of disease, the oomycete *Phytophthora infestans* (Mont.) de Bary, and its already characterized genes of virulence, with particular emphasis on their evolution, which underlines the exceptional genetic and phenotypic plasticity of this pathogen. Specially highlighted is the diversity of the immediate tools of virulence—effectors, which interact with potato target molecules, alter host physiology and facilitate plant colonization. Turning to plant defense barriers, the reviewer elaborates on the polymorphism and evolution of *Solanum* genes providing for plant resistance to *P. infestans*. The repertoire of *P. infestans* virulence genes in agroecosystems and the diversity of resistance genes in potato wild relatives are explored as regards the agriculture-oriented implementation of new molecular knowledge. The multifaceted approach to late blight combines the search for new resistance genes in genetic collections, the characterization of their function and stacking these genes in potato cultivars in order to breed new donors of long-lasting and durable resistance together with express assessment of pathogen virulence genes.

Keywords: *Phytophthora infestans*, *Solanum* species, potato, plant immunity, late blight, durable resistance, pathogen-host plant interaction, virulence genes, resistance genes, evolution, genome and transcriptome sequencing, phytophthora

DOI: 10.1134/S1021443721020072

INTRODUCTION

Plant diseases are a persistent and ruinous threat to sustainable crop production worldwide. The most economical and eco-friendly way to effectively deal with this problem is to breed new cultivars with stable

and durable resistance. Durable disease resistance is empirically defined as resistance efficient over long periods of widespread crop cultivation under conditions favorable to disease, a compromise between plant defense capacity and the evolutionary potential of the pathogen. The key to plant response to pathogen invasion is the ability to discriminate self from non-self. From a Darwinian perspective, the co-evolution of two actors of pathogen–host plant interaction under the natural selection in undisturbed environments is comparable to the processes observed when plants are bred in disrupted agricultural ecosystems. In both cases, we observe selection for virulence with its high fitness cost to the pathogen and consistent selection for effective resistance in the plant [1, 2]. Recent years have considerably strengthened our insight into the molecular interactions between plants and pathogens, which are instrumental for sustaining

Abbreviations: AFLP—amplified fragment length polymorphism; Avr—avirulence; CC-NB-LRR—coiled coil—nucleotide binding—leucine rich repeat; GM—genetically modified; dRenSeq—diagnostic resistance gene enrichment sequencing; ETI—effector-triggered immunity; HR—hypersensitive response; MAMP—microbe associated molecular pattern; NGS—next generation sequencing; PAMP—pathogen associated molecular pattern; PenSeq—pathogen target enrichment sequencing; PR proteins—pathogenesis-related proteins; PTI—PAMP-triggered immunity; QTL—quantitative trait locus; RenSeq—resistance gene enrichment sequencing; RGA—resistance gene analogue; RLK—receptor-like kinase; Rpi—resistance to *Phytophthora infestans*; RxLR—arginine-any amino acid-leucine-arginine motif; SNP—single nucleotide polymorphism.

¹ The article was translated by the author.

effective disease control. Therefore, the elucidation of the underlying genetic links and molecular machinery of virulence and resistance is critical for resourceful breeding of durably resistant crops.

Current progress towards stable and durable resistance has been reinforced by considerable advancements in the molecular studies of pathogen and host plant. These advancements have been greatly benefited from recently developed methods of research into gene structure and activity, especially the “omics” technologies. Due to these steps forward, late blight (LB), economically the most important disease of potato and tomato caused by oomycete *Phytophthora infestans* (Mont.) de Bary, became a popular model of multidimensional plant-microbe interactions. Newly obtained molecular evidence has considerably reshaped both our vision of plant-pathogen molecular dialogue and our approach to mitigating LB.

LB constantly endangers global food security and levies a permanent tax on potato growers: up to \$10 billion is lost annually as direct crop losses and costs of chemical protection [3–6]. Fungicide (oomycetide) applications currently deployed to control this unrelenting pathogen affect human health and damage biosphere. Apparently the most environmentally friendly and effective way to restrict LB is to breed new potato varieties with durable resistance to this disease [7–10]. Successful potato breeding for durable LB resistance is deeply rooted in the steadily expanding understanding of biology of pathogen *P. infestans* and host plant *Solanum* L., with the ever growing emphasis on recognizing the virulence agents of the former and the resistance tools of the latter [11–17].

Wherein potato breeders aim for stable and durable LB resistance, they focus on such primary aspects of virulence in *P. infestans* strains as functional diversity of pathogen virulent factors and incredible plasticity of pathogen genome resulting in rapid changes in the profile of these factors; these aspects are immediately reflected in the activities of pathogen populations [5, 6, 18–20]. Molecular studies play an ever increasing role in research into the multilayered plant defenses [1, 12, 21]. As a result, we witness the rapidly mounting awareness of complex *P. infestans* - potato molecular dialogue, which is dramatically manifested in LB outbreaks [5, 6, 22].

Potato resistance to *P. infestans* is best known as a cell death-associated defense reaction branded as the hypersensitive response (HR), which is preceded by pathogen invasion into plant cells and translocation of specific effectors acting as (a)virulence (*Avr*) factors. Regarding pathogen virulence, the current spotlight of molecular studies is on a special case of *Avr* genes and their products – effectors recognized by plant defense systems [16, 23–26]. No less significant are the mechanisms employed by plants to perceive and confront the pathogen. Best elucidated are the cases when *Avr* genes are recognized by matching

genes for resistance against *P. infestans* (*Rpi* genes) encoding immune receptor proteins. In potato, all already characterized receptors of *P. infestans* effectors belong to the coiled coil—nucleotide binding—leucine rich repeat (CC-NB-LRR) class of intracellular plant proteins. The knowledge of *Avr* and *Rpi* genes and their interactions rapidly fortifies the scientific basis of various breeding technologies, such as remote crosses and trans- and cis-genesis employed to introduce *Rpi* genes from wild *Solanum* species into susceptible potato varieties [7–9, 27–29].

Due to rapid genome evolution and arrival of new strains of *P. infestans*, however, severe LB outbreaks have been relentlessly overcoming plant defense barriers built up by breeders and negating, sometimes within a few days, their many years of effort [5, 6, 19]. Therefore it is an urgent task of plant biologists to constantly seek new sources of LB resistance, predominantly in wild *Solanum* germplasm, to expand the scope of *Rpi* genes thoroughly characterized and documented with molecular methods [17, 29–31] and to identify the best gene combinations (pyramids, stacks) for inclusion into prospective varieties [32].

In the particular case of potato, the studies involving new methods of analyzing the structure and activities of *Avr* genes of *P. infestans* and *Rpi* genes of wild and cultivated *Solanum* plants have greatly promoted molecular advancements toward successful dealing with the LB problem. Among the most notable breakthroughs in the field of methodology, we find so-called “omic” technologies (genomics, transcriptomics, proteomics, metabolomics and effectoromics), molecular cytogenetics, new types of DNA markers, mining for new *Rpi* alleles and, finally, next generation sequencing (NGS) of whole genomes and transcriptomes and selected target genes—together with the bioinformatics analysis of large sets of data on plant and pathogen genomes and transcriptomes. These breakthrough methodologies greatly promoted the molecular identification of the participants of gene-for-gene interactions already identified by classical genetic methods, and LB has become a popular model of pathogen-host plant communication and co-evolution [12, 15–17, 22, 28, 33–39]. At the same time, the current molecular framework and innovative genome toolbox help breed new and better performing potato cultivars.

In many aspects, this paper follows our previous publication [39]. Here, strong emphasis is made on the flow of new experimental data summoned by the recent research methodologies and the latest reviews integrating innovative ideas of plant and microbe evolution with new concepts of phytopathology and plant breeding. The bibliography is brought to June 2020.

PATHOGEN RECOGNITION AND HOST PLANT DEFENSE BARRIERS AT A CURSORY GLANCE

Pattern-triggered immunity. To perceive numerous pathogens and confine resulting invasion, infection, inhibition and damage, plants evolved a sophisticated immune system, which is usually envisaged as consisting of two barriers, layers, or tiers of defense [1, 12, 40, 41]. The first, so far largely unexploited, layer of plant innate immunity occurs at the cell surface and generally provides a broad spectrum of resistance. This apoplastic barrier for pathogen invasion at the extracellular host-microbe interface is described as immunity triggered by general elicitors called microbe- and pathogen-associated molecular patterns (MAMPs and PAMPs). The responses to these patterns are collectively termed pattern-triggered immunity (PTI). Plant cell surface-localized receptors recognizing PAMPs or MAMPs consist primarily of receptor-like kinases (RLKs) and receptor-like proteins (RLPs). Not all RLKs and RLPs are receptors; some act as co-receptors, scaffold proteins, or other components in the signaling pathways [40, 42].

PTI following pathogen invasion into apoplast leads to the production of antimicrobial substances, such as pathogenesis-related (PR) proteins. Most PR genes are induced by signaling compounds like salicylic and jasmonic acids or ethylene expressed in temporal waves during plant colonization; in addition to plant resistance to pathogen attack, PR proteins play specific role as the markers of the pathogen induced systemic acquired resistance [20, 43]. In its turn, *P. infestans* also secretes enzymes that may suppress such plant defenses; they include proteases and cell wall degrading pectate lyases, polygalacturonases, xylanases, and other enzymes.

The polygenic and partial LB resistance arising from host-nonadapted PTI is also called field, quantitative and race-nonspecific resistance. It is not durable [9, 10, 14]. More promising for PTI-based durable resistance appear the candidate genes elucidated by association genetics, genome wide association studies and comparative transcript profiling. These genes encode key enzymes involved in the synthesis of plant hormones that function in defense signaling: an allene oxide synthase and lipoxygenases from the jasmonate pathway, a 3-hydroxy-3-methylglutaryl coenzyme A reductase from the mevalonate pathway and a P450 protein participating in the terpene biosynthesis, as well as an anionic peroxidase associated with cell wall suberization [44, 45].

Effector-triggered immunity. In contrast to host-nonadapted PTI, host-adapted pathogens secrete into plant cells numerous effectors, proteins encoded by virulence genes and delivered into the host plant cells via specific translocation structures and mechanisms [22, 24, 46, 47]. To counter effector-mediated pathogenesis, plants have evolved the second barrier resid-

ing in plant cytoplasm. The effectors are sensed by intracellular RLKs. This process of effector-triggered immunity (ETI) specifically discerns pathogen races. Both PTI and ETI activate a complicated signal transduction network, including mitogen-activated protein kinase cascades and/or chemical signaling by plant hormones and transcriptional regulation via transcription factors, which culminate in a series of physiological changes in the plant, such as HR, reactive oxygen species production and cell wall reinforcement [12, 21, 23, 40, 43, 48]. An updated model describes plant-pathogen interactions as an integrated three-layered system, with a recognition layer, a signal-integration layer and a defense-action layer [22]. This continuum of pathogen-host plant interaction is presently a hotspot of molecular phytopathology.

Once pioneering “gene-for-gene” paradigm [49] has implied that plant resistance is observed when a dominant gene for plant resistance is matched by a corresponding dominant *Avr* gene in the pathogen. The biochemical reflection of this concept is a receptor-ligand model wherein defending plants employ proteins that recognize pathogen-derived *Avr* gene products. However, this classic model is presently viewed as an oversimplification, even in the case of monogenic gene-for-gene interactions in plant cytoplasm. First, the direct steric interaction between the protein products of virulence and resistance genes has been observed infrequently, and the ligand-receptor model of direct interaction was supplemented with the advanced models of indirect interactions, such as guard and decoy concepts. Next, in addition to monogenic interaction, there are many cases of multiple resistance genes or clusters of tightly linked genes recognizing a single effector gene and vice versa; in addition, gene-for-gene interactions for diverse plant-pathogen combinations are environmentally affected [23, 41, 48, 50]. While two extreme concepts for resistance gene diversification and evolution, the Arms Race and the Trench Warfare, have been proposed, the resistance gene evolution most likely occurs in a mutualistic continuum between two defense systems, which share many mechanisms and molecular components of resistance to shape the pathogen invasive potential [12, 13, 46, 51, 52]. Presently the classic genetic studies of pathogen–host plant interactions are fortified with molecular research on their actors and integrate the diverse models of their co-evolution and functions; such integration will help better understand rapid changes in pathogen aggressiveness and disease progress [5, 6, 20, 22, 24, 53, 54].

Among the numerous agents of ETI in *Phytophthora*, best researched are RXLR effectors, the products of pathogen *Avr* genes, and CC-NB-LRR receptors, the products of potato *Rpi* genes [11, 13, 16, 17, 25]; these genes are dealt with in detail in the separate sections.

Phytophthora infestans: GENOME AND VIRULENCE GENE EVOLUTION

The present-day genomic landscape of *P. infestans* has been initially probed by sequencing genome of strain T30-4 [55]. The 240-Mb sequence of this genome greatly exceeds genomes of other *Phytophthora* species, primarily due to a proliferation of repetitive DNA accounting for approximately 74% of *P. infestans* genome. The most unusual characteristic of this genome crucial for etiology of potato LB is discontinuous distribution of gene density. The gene-dense regions with conserved protein-coding genes are interrupted by the expanded gene-sparse and repeat-rich genomic compartments; these compartments are populated with fast-evolving genes for pathogenicity effectors. The unusual genome structure intriguingly correlates with pathogen life style. The fast-evolving effector genes are confined within highly dynamic and expanded compartments of pathogen genome, and such localization of the effector genes dramatically increases the frequency of nucleotide substitutions, insertions/deletions, rearrangements and copy number variation, which occur at a higher rate than in the housekeeping genes. As a result, these chromosomal regions provide unique niches for the rapid evolutionary diversification that shapes the strain-specific repertoire of virulence genes [18, 53, 55, 56]. Haas et al. [55] postulated that such dynamic regions underlined the evolutionary plasticity of effector genes, generating the enhanced genetic variation required for defeating plant resistance so characteristic of LB epidemic.

These concepts were further expanded as sequencing of other *P. infestans* genomes and especially transcriptomes brought a rich crop of evidence consistent with the concept of repeat-driven expansion of the *P. infestans* genome [57, 58]. This concept of bipartite genome and two-speed (and even multi-speed) genome evolution expounds genome architecture that facilitates high rates of dynamic rearrangements and genetic diversification in virulence-associated regions and serves as a cradle for adaptive pathogen evolution [18, 59]. The accelerated adaptive evolution due to specific genome architecture is especially important in the case of artificial pathogen selection in agroecosystems [60].

Gene duplications, non-homologous recombinations and deletions in the transposon- and effector-rich regions of the *Phytophthora* genome are held responsible for most cases of pathogen adaptation. Nonetheless, other control mechanisms have been reported, such as large changes in gene copy numbers and selective gene loss, as well as point mutations, frameshift, defeated start and stop codons, and variations in gene expression patterns [53, 61]. Pathogen evolution is also regulated epigenetically [20, 62].

VIRULENCE TOOLS: *Avr* GENES AND EFFECTORS OF *P. INFESTANS*

New physiological and molecular methodologies have helped identify numerous cytoplasmic *Avr* genes and their products, effectors, produced by the pathogen to modulate multitier host immunity as well as the targets of these effectors in plant cells [16, 24, 25, 46, 55, 63–68].

Effector genes of *P. infestans*. In the completely sequenced genomes of *P. infestans*, two structure types, RXLR and CRN, were found to dominate numerous effector genes. The RXLR effectors are modular secreted proteins containing the N terminus with a conserved arginine-any amino acid-leucine-arginine (R-x-L-R) motif, which is usually followed by a short glutamic acid-glutamic acid-arginine (EER) domain required for delivery inside plant cells. The exploration of conserved sequence features in the model genome of strain T30-4 predicted 563 RXLR genes. Approximately half of them are lineage-specific, largely accounting for the expanded repertoire of effectors; this diversity is apparently driven by pathogen evolution. Much less is known of CRN (crinkling- and necrosis-inducing proteins) genes of unexpected complexity and diversity. Like RXLR effectors, CRNs are modular proteins; they are defined by a highly conserved N-terminal 50-amino-acid LFLAK domain, whereas their C-terminal regions are relatively diverse [26, 55].

Several cases illustrate the diverse manifestations of the RXLR *Avr* genes—other than the HR induction after the specific interaction with the corresponding *Rpi* genes of *Solanum* plants. AVR1 affects perihistrial accumulation of effectors [67]. Other effectors, including well-characterized AVR-BLB2, AVR2 and AVR3a, interfere with plant defense associated Ca²⁺-signaling in plants, enhance plant susceptibility and suppress cell death [69–71]. Different effectors target parallel steps in signal transduction pathways leading to the HR [72]. RXLR effectors show a range of localizations within plant cells, and co-expression of several RXLR effectors that target different immune pathways was shown to enhance pathogen colonization as compared to single effectors [67].

When introduced into wild and cultivated *Solanum* plants, effectors specifically recognize the matching receptor proteins encoded by *Rpi* genes. A powerful and high throughput technology for identification of *Avr* and *Rpi* genes based on such recognition is called effectoromics. The presence of the corresponding *Rpi* gene is established when the effectors are transiently expressed in *Solanum* leaves and plants response due to cell deaths is macroscopically recognized. To verify matching *Rpi-Avr* gene pairs, the candidate genes are co-expressed in leaves of tester plants, such as *Nicotiana benthamiana* Domin [15, 16, 65, 68].

The nomenclature of *Avr* genes used below goes back to 11 *Rpi* genes recognized in the germplasm of

S. demissum Lindl. and employed in the conventional Mastenbroek-Black differential set [73, 74] for the assessment of *P. infestans* strains. The list of effectors has been expanding along with characterization of new *Rpi* genes and their deployment in effectoromics tests [13, 16, 65]. The discovery that many AVR proteins of *P. infestans* belong to the RXLR effector class provides an opportunity to predict new effector genes by the search in gene banks using the methods of bioinformatics. As for now, many targets of these effectors have been revealed; nonetheless, the functions have not been as yet assigned to numerous RXLR structures [13, 16, 22, 38, 47, 64, 67].

Genetic polymorphisms of the *Avr* genes. In the completely sequenced T30-4 genome and other isolates and strains of *P. infestans*, RXLR effectors were studied in several aspects. The effectoromics assays were used to test the effectors of *P. infestans* for their ability to specifically induce cell death in leaves of many wild *Solanum* species [65]. This study considerably expanded the range of established *Rpi* genes and indicated the broad functional diversity of already characterized RXLR *Avr* genes. Their structural variety is born from *Avr* gene duplication, recombination and allele selection by environment, primarily by infested plants themselves [54].

The structural vs. functional polymorphisms were, for the first time, reported in the case of *Avr3a*. Two alleles of this gene were found to encode proteins AVR3aKI and AVR3aEM, which differed in two amino acid residues immediately affecting host response: the former protein directly activated *Solanum* R3a kinase and triggered plant immunity, whereas the latter was virulent. AVR3a is essential for virulence during the biotrophic phase. Deletion of the C-terminal tyrosine from AVR3aKI, although not affecting recognition by R3a, abolished the ability of AVR3aKI to suppress cell death and in this way extend the biotrophic phase [75].

Our knowledge of RXLR *Avr* gene diversity was greatly complemented by the population studies. Cloning and sequencing a multigene family of *IpiO* effector genes from *P. infestans* isolates collected in Guatemala, Thailand and the United States revealed a broad range of alleles with varying structures and copy numbers. *IpiO* diversity correlated with pathogen aggressiveness. Potato *Rpi-blb1* gene recognized IPI-O1 effector; however, IPI-O4, another member of this family, could elude detection by *Rpi-blb1* and inhibit HR elicited by IPI-O1-*Rpi-blb1* interaction. This gain-of-function of IPI-O4 did not compromise its virulence effect: the inhibition of IPI-O1 recognition by IPI-O4 was associated with higher *P. infestans* aggressiveness [76].

The *Avr-blb2* family in T30-4 strain of *P. infestans* is another case of vast allelic polymorphism in effector genes. Here, variations were detected in 24 of the 279 examined nucleotide sequences. A total of 14 poly-

morphic amino acid sites were identified, ten of which localized to the C-terminal domain of the effector. The type of amino acid residue at position 69 determines the defense responses mediated by the *Rpi-blb2* gene: among four variants of AVR-BLB2 protein, Phe-69 compromises the activation of *Rpi-blb2*, implying that the virulent allele may have evolved to escape *Rpi* gene-mediated recognition [64]. The study of the *Avr-blb2* genetic structure in global metapopulation of *P. infestans* (352 isolates collected from 13 different hosts in 23 countries) suggests that *Avr-blb2* first emerged as a single-copy gene in a putative ancestral species and expanded its diversity in the *Phytophthora* lineage as it infected *Solanum* hosts worldwide. Surprisingly, all *Avr-blb2* variants are found in present-day *P. infestans* populations, suggesting a potential benefit for the pathogen to preserve duplicated and functionally different versions of *Avr-blb2* genes [77].

Noticeably larger structural differences were found in avirulent/virulent forms of two more *Avr* genes. The study of sequence variation across a series of *P. infestans* lines and isolates elucidated the difference of 13 amino acid residues between homologous classes of avirulent AVR2 and virulent AVR2-like effectors, whereas the sequence diversity within each class was not that significant; some isolates were homozygous and other heterozygous as regards the *Avr2* sequences [78]. Even larger structural differences of 38 amino acid residues were found between avirulent AVR1 and virulent AVR1-like effectors [79].

Genome analyses of *P. infestans* isolates obtained from more than 1100 outbreaks of potato LB across Great Britain revealed the extensive heterogeneity of the RXLR effector repertoire. In particular, the most aggressive 13_A2 isolate comprised six novel *Avr* alleles, including a virulent *Avr2* homologue, which were absent from the model strain T30-4 [5]. Sizeable allelic polymorphisms in the *Avr-vnt1* gene were reported for *P. infestans* populations from Europe and both Americas [80] and within Europe, in Polish vs. Norwegian populations [81]. When 96 *P. infestans* isolates, each with a distinct genotype determined previously by molecular and phenotypic markers, were collected from six locations representing a range of climate conditions and cropping systems in China, high genetic variation in the *Avr3a* gene, which resulted from diverse gene polymorphisms, included 51 nucleotide haplotypes encoding 38 amino acid isoforms [82].

Recently, a new highly efficient technology of Pathogen target enrichment Sequencing (PenSeq) was introduced to identify the effector genes; this technology facilitates the characterization of allelic diversity in pathogen effectors, enabling evolutionary and population genomic analyses of the pathogen. The massively parallel identification of presence/absence variations and sequence polymorphisms in key pathogen genes by PenSeq is a prerequisite for the efficient deployment of host resistance genes. The PenSeq

analysis of the genes for pathogenicity in several *P. infestans* lines, including 13_A2 and EC-1, identified 16 RXLR effector sequences absent from the reference T30-4 genome employed to produce baits for *Avr* gene enrichment. Comparison of six diverse isolates established the dissimilar profiles of presence/absence and allelic variation of already recognized *Avr* genes, such as *Avr1*, *Avr2*, *Avr3a*, *Avr3b*, *Avr4*, *Avr-Smira2=Avr8*, *Avr-Smira1=Avr9*, *Avr10*; *Avr-vnt1*, *Avr-blb1* and *Avr-blb2*. In addition, this PenSeq study expanded the list of *Avr* gene candidates beyond the model sequences selected as baits for target enrichment; such expansion was most evident in the case of complex gene families represented by numerous alleles [38]. Another case of *Avr* allelic diversification is vividly exemplified in the recent study of the non-host species *S. americanum* Mill. By using single-molecule real-time sequencing (SMRT RenSeq) and long-read and cDNA PenSeq to search for new effectors, 47 highly expressed effector genes were explored in four *P. infestans* isolates avirulent on potato plants carrying *Rpi-amr1*, such as EU13_A2, EC1_A1, EU6_A1 and US23. A new effector gene was identified as *Avr-amr1* by HR when it was transiently co-expressed in *N. benthamiana* with the matching *Rpi-amr1*. In the T30-4 genome, the newly characterized *Avr-amr1* locus was found physically close to two already known *Avr* effector genes, *Avr8* and *Avr-Smira1* [83].

Regarding the population biology and evolution in *P. infestans*, PenSeq analysis of *Avr* genes offers an important advantage by straight targeting virulence agents; in this way, genetic variation under positive (or balancing) selection can be studied directly, enabling the identification of causal genetic variants and revealing patterns of adaptive evolution much better than by whole-genome sequencing approaches. Transcriptome profiles of RXLR effector genes in the course of disease development demonstrated that these genes were highly upregulated during the early biotrophic phase of potato infection. These profiles were immediately related to specific resistance tools of plant hosts and in this way reflected pathogen-host interaction.

While the demographic studies indicate the potential effector armory of *P. infestans*, the profile of expressed *Avr* genes probably represents their actual repertoire. The unique capacity of 13_A2 line to defeat potato varieties was shown to result from the changes in gene coding sequences of *Avr* genes and also from the changes in gene expression. A distinct temporal pattern of in planta gene induction was shown in this line: twenty RXLR effector genes were specifically expressed in this genotype as compared to T30-4 strain. Most up-regulated genes in 13_A2 line, including *Avr-blb1*, *Avr-blb2* and *Avr-vnt1*, showed sustained induction over 2 and 3 days post infection, and such gene induction, which correlated with the pattern of LB progress, apparently contributed to the enhanced aggressiveness of 13_A2 [5].

The transcriptome deep sequencing strategy based on the conserved RXLR effector sequences was used to compare five diverse *P. infestans* strains from north-western and southern China and Europe, with different mating types, haplotypes, and pathotypes; this study identified potentially conserved core RXLR effector genes that contributed to virulence. *Avr2*, *Avr3a*, *Avr-blb1*, *Avr-vnt1*, and *Avr-Smira1* genes were all expressed in all five tested strains, while *Avr3b*, *Avr4*, *Avr-blb2* and *Avr-Smira2* were each expressed in two to four strains, and *Avr1* was found to be expressed only in one strain [58]. In the dominant Andean clone EC-1, numerous examples of structure and copy number variation were detected in *Avr* genes. Some of them were related to mitotic loss of heterozygosity. The most remarkable, however, was a considerable difference between isolates in expression of many *Avr* genes despite apparent absence of sequence polymorphisms. In this case, silencing of effector genes helped evade disease resistance conferred by the matching *Rpi* genes [60].

Solanum: GENES FOR PATHOGEN PERCEPTION AND CONFRONTATION

Mapping traits and putative genes for LB resistance.

Starting in the early 1990s, several reliable and highly efficient DNA markers, such as RFLP, Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats and later Single Nucleotide Polymorphism (SNP) markers and diversity array technology, have been employed in linkage disequilibrium mapping, or association analysis to locate candidate *Rpi* genes in cultivated and wild *Solanum* species. Most effective are markers residing within the resistance genes themselves or physically flanking these genes: in such cases the recombination between marker allele and resistance trait is absent or rare even after many generations of meiotic recombination. Such PCR-based Sequence Characterized Amplified Region (SCAR) and Cleaved Amplified Polymorphic Sequences markers have served to pinpoint and monitor candidate *Rpi* genes and Quantitative Trait Loci (QTLs) for LB resistance [17, 84–87]. The results of genome-wide or fine mapping helped choose different sets of degenerate primers recognizing resistance gene analogues (RGAs) or their clusters, and genome amplification with these primers combined with AFLP analysis considerably expanded the repertoire of putative *Rpi* genes. This versatile tool of allele mining and tagging called motif-directed profiling was successfully used to study new *Solanum* genomes and the diversity between individual genomes; the combination of such profiling with NGS offers enormous advantages over the classical gel-based profiling [17, 30].

To synthesize the mapping data for the loci for polygenic LB resistance, twenty-one maps of QTLs for LB resistance were put together with eight potato reference genetic maps obtained with various markers

and integrated into a consensus map comprising 2141 markers, on which QTLs were projected and clustered into meta-QTLs. Meta-QTLs for LB resistance were observed on every chromosome, and some meta-QTLs coincided with *Rpi* genes [88]. Other QTLs for LB resistance were not related to already established *Rpi* genes and RGAs; these loci are prospective targets to mine for new *Rpi* genes. The QTL regions established by standard mapping would cover several Mbs on physical maps and contain a large number of genes with diverse functions. This problem is resolved by sequencing extended genome fragments and whole genomes. Screening potato collections by the methods of effectoromics have also expanded the range of the *Rpi* genes on the basis of their functional activities [16, 17, 65, 66]; however, these data are often difficult to match to the QTL evidence.

Discovering *Rpi* genes. Identification of the full complement of genes contributing to the resistance to *P. infestans* is crucial to understanding the molecular basis of diverse LB patterns. Within two last decades, over 20 *Rpi* genes were identified and cloned from wild *Solanum* species. Sequencing already characterized *Rpi* genes demonstrated that they all belong to the CC-NB-LRR class. The best characterized genes are *R1* and its RGAs in a gene cluster at chromosome 5 of *S. demissum*; *R2* from *S. demissum*, its orthologue *Rpi-blb3* from *S. bulbocastanum* Dun. and their orthologues from several other species in a large cluster at chromosome 4; *Rpi-blb2* from *S. bulbocastanum* at chromosome 6; *Rpi-blb1* and *Rpi-bt1* from the same species and *Rpi-sto1*=*Rpi-plt1*=*Rpi-pt1* from *S. stoloniferum* Schlecht. et Bché., *S. polytrichon* Rydb. and *S. papita* Rydb. at chromosome 8; *Rpi-vnt1* from *S. venturii* Hawkes et Hjerting and its orthologues from numerous South American *Solanum* species at chromosome 9 and *R8* and *R9a* from *S. demissum* at the same chromosome; *Rpi-chc1* from *S. chacoense* Bitt. at chromosome 10; *R3a* and *R3b* from *S. demissum* at chromosome 11 [10, 13, 14, 16, 17, 29, 37]. Several more candidate *Rpi* genes have been identified, but their sequences have not been yet published. Comparative sequencing of particular *Rpi* genes and whole genomes within tuber-bearing *Solanum* and beyond has identified functionally equivalent variants of *Rpi* genes and elucidated vast allelic polymorphisms to be further explored by breeders. As a whole, tuber-bearing or even non-tuber-bearing *Solanum* species, especially in South America, open a broad prospect for evolutionary studies of *Rpi* genes and their deployment by potato breeders [28, 29, 31, 89].

In the genome sequence of a doubled monolipoid *S. tuberosum* group *Phureja* clone DM [90], 361 CC-NB-LRR genes were predicted across all 12 potato chromosomes. The majority of these genes were physically organized within mostly homogeneous clusters, presuming that each of them evolved from a recent common ancestor. Remarkably, unlike RxLR effectors from *P. infestans* found in gene-sparse genomic

regions, CC-NB-LRR genes reside in the regions that are not specific regarding gene or repeat density [91]. By now, whole genome sequences have been established and published for several more cultivated species, polyploid *S. ×chaucha* Juz. et Buk., *S. curtilobum* Juz. et Buk., *S. juzepczukii* Buk. and *S. tuberosum* subsp. *andigena* Hawkes and subsp. *tuberosum* L. [92], and for two wild species, *S. commersonii* Dun. [93] and *S. chacoense* [94]. These sequences made it possible to assess the complexity of structures corresponding to CC-NB-LRR *Rpi* genes and their homologues and to establish cluster organization of *Rpi* genes.

Characteristically, the already known clusters of *Rpi* genes are similar between the diverse *Solanum* genomes. Some *Rpi* gene lineages seem to predate speciation in tuber-bearing *Solanum*. Apparently, following genome evolution, which established the present landscape of these species in two Americas, distinct *Rpi* genes independently evolved to adapt to local pathogen populations [95]. An enticing case is the contrasting gene distribution in wild potato species: while *Rpi-blb1*=*Rpi-sto1* are found exclusively in the Mexican Bulbocastana and Longipedicellata species, the *Rpi-vnt1* is characteristic for *Tuberosa* of South America [16]. Whole genome duplication, selective tandem duplication and gene retention as well as intragenic and intergenic recombination and conversion of CC-NB-LRR genes in the so-called “birth-and-death” model of resistance gene evolution have produced a widely diverse reservoir of structures, often clustered, used by natural and artificial diversifying selection to pick varying numbers of semi-independently evolving lineages of resistance genes of various pathogen specificity [1, 50]. Gene clustering may promote sequence polymorphism through unequal inter- and intragenic meiotic recombination of genes and in this way generate new specificities [13]. To illustrate, the evolution of almost completely identical potato structures resulted in two CC-NB-LRR genes of resistance to widely different infestants: the cyst nematode *Globodera pallida* and *Potato virus X* [96].

New sequencing technologies, such as NGS, generate data at a rate that is several orders of magnitude faster than by traditional technologies of preNGS era. RenSeq (Resistance gene enrichment Sequencing) technology includes bait design using known NB-LRR gene families followed by sequencing of the enriched samples of NB-LRR RGAs [34]. The method analyzes subsets of the genome by capturing particular gene families of interest. By substantial reduction in genome size and complexity prior to comparative sequencing, target enrichment enormously facilitates discovery of *Rpi* genes and their high-quality annotation. When applied to the genome of *S. tuberosum* clone DM, this method elucidated almost twice more NB-LRR genes as compared to the previous whole genome sequencing [90]. The technology also rapidly identified SNP markers that co-segregated with the loci of resistance to *P. infestans* in several *Solanum* spe-

cies and was able to reveal candidate *Rpi* genes from uncharacterized genomes of *Solanum* species. New sequencing technologies were enforced by rapid development of bioinformatics methods, with increasing power of discerning gene polymorphisms.

Yet how many candidate CC-NB-LRR *Rpi* genes established by whole genome sequencing and RenSeq are functional? Eliciting LB resistance in susceptible potato cultivars by stable transformation with putative *Rpi* genes is a reliable although very laborious and costly proof of their function. More accessible approach to such validation would integrate the evidence from the transient transformations in the effectoromics analysis [66] and meta-QTL analysis [88] with the data obtained by marker analysis and transcriptome analysis of plants infected by *P. infestans*.

NGS technologies [15, 17, 34, 35, 37] have dramatically accelerated high resolution mapping and gene discovery. They opened new possibilities for comprehensive studies of genomes (genotyping-by-sequencing, whole-genome re-sequencing, barcoding, etc.) and transcriptomes (RenSeq, Candidate gene-Sequencing, Bulk segregant RNA-Seq, QTL-Seq, dRenSeq, etc.). The DeepSAGE method of transcriptome analysis, a further development of serial analysis of gene expression (SAGE), uncovers novel candidate genes for plant host-pathogen interactions. When DeepSAGE was used to explore compatible and incompatible plant-pathogen interactions over the time course of infection, susceptible plants manifested more transcriptional changes in multigene families, mostly representing PTI defense responses [97]. Another development of SAGE technology, SuperSage, added extensive information on up- and down-regulation of numerous transcripts in response to pathogen attack, including the genes for LB resistance [33]. When transcriptomes of resistant and susceptible *Solanum* genotypes were compared by RNA-Seq, the resistant cv. Sarpö Mira had ca. 25% more expressed putative resistance genes than the susceptible cv. Desiree; however, none of these genes belonged to the already established *Rpi* set [98].

Next, RenSeq technology was considerably refined: the enrichment probes specifically designed to capture and sequence fragments were shortened to the average length of a candidate *Rpi* gene, and SMRT RenSeq was employed to clone individual *Rpi* genes. Such technology has enabled de novo assembly of *Rpi* genes, their regulatory elements and complex loci from uncharacterized germplasm and has helped rapidly clone multiple new *Rpi* genes [15, 35].

Expanding bait range for capturing candidate genes led to another robust and cost-effective application of this technology as a diagnostic tool for already known *Rpi* genes. Diagnostic RenSeq (dRenSeq) enables the high-confidence identification and complete sequence validation of already known functional *Rpi* genes in genetic collections and breeding programs. Depend-

ing on stringency conditions (mismatch rates) during capturing *Rpi* genes and RGAs, dRenSeq could also identify hitherto unknown polymorphisms prospective for mining new *Rpi* genes and new alleles of already characterized *Rpi* genes in such insufficiently investigated species, as *S. americanum*, *S. andigena*, *S. pinnatisectum* Dun. and *S. verrucosum* Schlecht. [37, 38, 57, 99, 100]. In the study of *S. andigena* transcriptomes, dRenSeq successfully discerned up- and down-regulated genes: the compatible interaction caused higher induction of susceptibility genes when compared with the incompatible interaction. To relate the resistance of *S. andigena* genotype 03112-233 to *P. infestans* to known or novel resistance genes, the method was validated against a panel of 21 known functional *Rpi* genes. None of characterized genes was elucidated by the dRenSeq analysis; apparently, resistance of this accession to *P. infestans* was derived from an unknown gene [57].

SUMMING UP: AGRONOMIC IMPLEMENTATIONS OF MOLECULAR ADVANCES IN LB RESEARCH

Recent years have witnessed rapid harnessing of potato genetic and genomic resources to deploy them in new cultivar development. The experimental data obtained with the latest molecular technologies and novel notions on pathogen virulence and plant resistance that grew therefrom have greatly promoted potato breeding for stable and durable LB resistance and helped inform integrated disease management.

GM differential plants. For almost a century, pathotypes (races) of *P. infestans* have been conventionally discerned by the HR response of the Mastenbroeck-Black differential plants [73, 74]. These potato cultivars were presumed to comprise individual *Rpi* genes introgressed from *S. demissum* [9]; however, some Mastenbroeck-Black “monogenic” differentials actually contain more than one *Rpi* gene thus distorting diagnostic evidence [101]. Besides, the conventional differential sets are devoid of genes that presently are increasingly involved in breeding for durable LB resistance, such as *Rpi-blb1*, *Rpi-sto1*, *Rpi-blb2*, *Rpi-vnt1*, etc. [8]. This situation was radically improved by transforming cv. Desiree plants each with one of ten *Rpi* genes [101]. Such genuinely monogenic differential set is more accurate for virulence typing than the conventional one; however, because of its genetically modified (GM) origin, its deployment is presently limited by legislative restrictions.

Effectoromics. Direct molecular studies of *Avr* genes immediately related to pathogenicity of *P. infestans* populations open new vistas for monitoring pathogen populations, tracking pathogen migration in agroecosystems and early warning of arrival of new potentially aggressive pathotypes. Among most promising diagnostic applications of *Avr* gene research, we find effectoromics as a ready available sensitive and power-

ful tool for analyzing *Avr* allelic diversity and searching for new *Rpi* genes by screening germplasm collections and characterizing the functions of newly found genes as regards breeding for durable LB resistance [66, 102]. Effectoromics provides unique evidence on *Avr* gene profiles, which can be exploited as part of integrated disease management, such as alternation of resistance genes by rotating cultivars between particular fields or changing the schedule of fungicide applications [103].

Direct assessment of *Avr* gene repertoires and deployment of the effector arsenal. A better understanding of evolution and diversification of pathogen *Avr* genes might allow designing for agroecosystems more complex strategies of *Rpi* gene deployment resembling those that evolved in wild plant populations, which rarely experience epidemics [32]. While effectoromics discerns avirulent effector genes by their functions, the ever-accelerating progress in the technologies of genome sequencing provides wider possibilities to early detect the changes in *Avr* gene structures and in this way inform of new pathotype arrival. Effector gene repertoires determined by genomics-based technologies can monitor spatial pathogen dispersion in agroecosystems and promote breeding for LB resistance by identifying *Avr* genes in pathogen populations and *Rpi* and susceptibility genes in affected potato stands [68]. The PenSeq technology addresses many biological questions and limitations of current plant pathogen studies by the substantial parallel identification of presence/absence variations and sequence and allelic polymorphisms in key *Avr* genes of *P. infestans*, as well as their positions on genome and territory maps [38]. PenSeq reveals the effector genes crucial for the potential durability of deployed potato resistance genes. Moreover, this technology facilitates re-annotation of effector candidates across the *P. infestans* genome thus expanding the field of future development of *Avr* genes as a breeding tool.

Mining for *Rpi* genes. Two last decades evidenced the great expansion of sequence diversity panels in cultivated and wild potatoes related to their speciation and geographic origin. While potato genetic diversity at the whole-genome level remained largely unexplored in the preNGS period, various allele mining technologies offered to potato breeders many prospective *Rpi* genes [10, 13, 14, 17]. Recently, postNGS genomics approaches have provided new and deeper insight into the genomic diversity of germplasm collections, revealed such evolutionary processes, as historic introgressions and hybridization events, and identified genes targeted during potato domestication and, most recently, breeding [28, 31]. The novel dRenSeq technology was successfully deployed to look into allelic polymorphisms of *Rpi* genes and RGAs, corroborate the presence of several *Rpi* genes in elite potato varieties and identify new alleles of these genes in wild *Solanum* species [37, 57, 99, 100, 104].

Deploying *Rpi* genes for broad-spectrum and durable LB resistance. Wild *Solanum* populations typically maintain many allelic variants of *Rpi* genes in their polyploid genomes. A better understanding of *Rpi* gene diversity and evolution helps develop breeding strategies of pyramiding *Rpi* genes, which closely resemble the processes evolved in wild plant populations that rarely experience epidemics. Cultivar mixtures and hybrids combining different *Rpi* genes with gradually increasing diversity within potato stands maintain higher disease resistance.

In natural ecosystems, such as the Toluca Valley in Mexico [105], spatially confined potatoes and *P. infestans* co-evolve to establish plant and microbial populations with only sporadic disease outbreaks. In contrast, in agroecosystems, especially with rapid introduction of new increasingly virulent strains, LB epidemics cause significant yield losses [5, 6]. One of the effective strategies to combat LB is breeding for long-lasting durable resistance by pyramiding *Rpi* genes that recognize different *Avr* genes. Wild potatoes are a readily accessible source of such germplasm, and multiple *Rpi* genes can be introgressed into marketable cultivars by marker-assisted crosses or by genetic engineering.

Such gene pyramids will remain effective as long as at least one *Rpi* component of the pyramid can recognize the corresponding *Avr* gene of the pathogen and trigger defense response. The principle underlying stacking several resistance genes into a single cultivar to create more durable disease resistance is that a pathogen is unlikely to simultaneously mutate the series of *Avr* genes from avirulent into virulent, with the probability of mutations “all at once” decreasing as the number of resistance genes in the pyramid increases. Theoretically, a pyramid of four resistance genes would withstand pathogen invasion - on condition that both the resistance gene pyramids and the colonizing pathogen population(s) would simultaneously fulfill several criteria. First, the stacked resistance genes should be highly effective and not leaky, so that every pathogen strain carrying the particular *Avr* allele would not infect and colonize the plant genotype carrying the corresponding resistance gene. Second, not all resistance genes contribute equally to pyramids, the best resistance genes and their combinations are those truly novel for the infecting pathogen population. Third, the pathogen should only rarely recombine its genome, a criterion easily met only in a primarily asexual population; to illustrate, pathogen recombination in the *P. infestans* population comprising two mating types will bring together independent virulence mutations much more rapidly. Fourth, the resistance will stay durable at a low level of gene flow amongst field pathogen populations [1, 32, 106].

In the case of potato, the most evident way to achieve long-lasting and durable resistance against *P. infestans* is to recruit new *Rpi* genes into breeding and to stack as many *Rpi* genes as possible into a single

cultivar. Within last two decades, combining multiple resistance genes into a single plant genotype has heavily relied on identification and cloning of *Rpi* genes of interest, especially from the rich pool of wild *Solanum* species. Particularly inviting are insufficiently explored South American wild potatoes, which have not been earlier involved in practical breeding [28, 31, 36, 107, 108]. Development of breeding sources of durable resistance engages many “omics” tools described above for rapid identification, cloning and characterization of *Rpi* and *Avr* genes. Germplasm enhancement focused on identifying and introgressing new *Rpi* genes and new alleles of already characterized *Rpi* genes must also include careful study of the gene pools currently exploited by breeders in order to reduce the chance to wastefully deploy *Rpi* genes that have already been broken by local pathogen strains [9, 27–29, 31, 87, 108].

Many breeders have focused on developing potato genotypes with durable LB resistance by genetic engineering methods. At present, the stacks transferred to the established cultivars are up to three *Rpi* genes with broad range of race specificity, such as *Rpi-sto1:Rpi-vnt1.1:Rpi-blb3* or *Rpi-blb1:Rpi-blb2:Rpi-vnt1.1*. While these genes are sometimes individually defeated by *P. infestans* isolates, their stacks provide durable resistance [109]. An important advantage of these GM strategies, as compared to crosses, is the absence of linkage drag. However, genetic engineering of crops is an expensive process; in addition, it lacks appreciation by many consumers and is heavily restricted by GM regulations in several countries, especially in Europe [13, 110]. To overcome the problems with clearance of transgenic plants, the Dutch geneticists and breeders have put forward a concept of cis-genic plants obtained by transferring *Rpi* genes only from wild *Solanum* species that are crossable with cultivated potato varieties [8, 27, 111, 112].

A traditional stacking of the *Rpi* genes based on sexual and somatic hybridization requires numerous crosses and progeny selections [9] and therefore is a slow and laborious process, even when greatly assisted with molecular markers. However, in such way more *Rpi* genes are stacked than with today GM technologies. To illustrate, the Russian geneticists and breeders used remote crosses to introgress germplasm from a dozen of wild *Solanum* species into numerous multiparental hybrids; these hybrids comprise SCAR markers of up to five *Rpi* genes per genotype. Many of advanced lines bred from these multiparental hybrids have manifested for many years durable LB resistance and are prospective breeding donors containing pyramids of such broad specificity genes, as *Rpi-blb1=Rpi-sto1*, *Rpi-blb2*, *Rpi-vnt1*, *R2=Rpi-blb3*, etc. [113]. An important advantage of such breeding donors is that they maintain the genetic environment of the introgressed race-specific *Rpi* genes inherited from parental forms, including race-nonspecific resistance genes [84]. Besides, rather than single genes, the remote crosses transfer whole clusters of genes providing for resistance simultane-

ously to several pests. These characteristics of multiparental hybrids ensure stability of future varieties and slow down onset of more adapted pathogen forms in potato stands [107, 113]. And yet relying only on *Rpi* gene pyramids is unlikely to safeguard durable control with large *P. infestans* populations, mixed A1 and A2 mating types and substantial *Avr* gene flow due to pathogen migration. Therefore, the control strategies and management practices, such as crop rotations and fungicide application, which lower the pathogen effective population size, will promote durable resistance [106].

ACKNOWLEDGMENTS

The author is grateful to V.I. V. Kuznetsov and E.V. Rogozins for constructive criticisms and suggestions.

FUNDING

The study was supported by the State Task 0574-2019-0001.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflicts of interest.

Statement on the welfare of humans or animals. This article does not contain any studies involving animals performed by any of the authors.

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