Two different \( R \) gene loci co-evolved with \( Avr2 \) of \textit{Phytophthora infestans} and confer distinct resistance specificities in potato

C. Aguilera-Galvez\(^1\), N. Champoureut\(^1,3\), H. Rietman\(^1,4\), X. Lin\(^1\), D. Wouters\(^1\), Z. Chu\(^2,5\), J.D.G. Jones\(^2\), J.H. Vossen\(^1\), R.G.F. Visser\(^1\), P.J. Wolters\(^1\), and V.G.A.A. Vleeshouwers\(^1,7\)

\(^1\)Plant Breeding, Wageningen University and Research, Drouwendaalsesteeg 1, Wageningen, 6708 PB, The Netherlands; \(^2\)The Sainsbury Laboratory, Norwich Research Park, Norwich, NR4 7UH, UK

Abstract: Late blight, caused by the oomycete pathogen \textit{Phytophthora infestans}, is the most devastating disease in potato. For sustainable management of this economically important disease, resistance breeding relies on the availability of resistance (\( R \)) genes. Such \( R \) genes against \textit{P. infestans} have evolved in wild tuber-bearing \textit{Solanum} species from North, Central and South America, upon co-evolution with cognate avirulence (\( Avr \)) genes. Here, we report how effectormics screens with \( Avr2 \) of \textit{P. infestans} revealed defense responses in diverse \textit{Solanum} species that are native to Mexico and Peru. We found that the response to \( Avr2 \) in the Mexican \textit{Solanum} species is mediated by \( R \) genes of the \( R2 \) family that resides on a major late blight locus on chromosome \( IV \). In contrast, the response to \( Avr2 \) in Peruvian \textit{Solanum} species is mediated by \( Rpi-\text{mcq1} \), which resides on chromosome IX and does not belong to the \( R2 \) family. The data indicate that \( Avr2 \) recognition has evolved independently on two genetic loci in Mexican and Peruvian \textit{Solanum} species, respectively. Detached leaf tests on potato cultivar ‘Desirée’ transformed with \( R \) genes from either the \( R2 \) or the \( Rpi-\text{mcq1} \) locus revealed an overlapping, but distinct resistance profile to a panel of 18 diverse \textit{P. infestans} isolates. The achieved insights in the molecular \( R – Avr \) gene interaction can lead to more educated exploitation of \( R \) genes and maximize the potential of generating more broad-spectrum, and potentially more durable control of the late blight disease in potato.

Key words: \( Avr \) gene, Co-evolution, Late blight, \textit{Phytophthora infestans}, Potato, \( R \) gene, Resistance, \textit{Solanum}.

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INTRODUCTION

Potato (\textit{Solanum tuberosum} L.) is the most important non-cereal crop consumed worldwide and is affected by the destructive late blight disease. The oomycete pathogen \textit{Phytophthora infestans} is the causal agent of the disease, which destroys leaves, stems and tubers from growing potato plants (\textit{Fry} 2009). In Ireland, late blight destroyed a large portion of the crop and led to the Irish potato famine between 1845 and 1849, causing the death of over one million people and the emigration of one million more (\textit{Zadoks} 2008). Currently, late blight is the major threat to potato production, responsible for yield losses of around 16 \% of the global crop and representing an annual financial loss of approximately € 6 billion (\textit{Haverkort} et al. 2016).

Johanna Westerdijk believed that studying mechanisms that underlie plant immunity would help the breeding of resistant genotypes. In her inaugural lecture in 1917, when she became Professor of Phytopathology at Utrecht University, she described that diseases were most severe when pathogens or hosts are introduced in novel environments. She argued that co-evolution of hosts and pathogens is required for the evolution of resistance (\textit{Westerdijk} 1917). In the meantime, significant progress has been made in understanding plant immunity, and this knowledge has led to the development of resistant plants. Several \( R \) genes conferring resistance to \textit{Phytophthora infestans} (\( Rpi \)) have been introgressed into potato cultivars from \textit{Solanum} species native to Mexico (\textit{Malcolmson} & \textit{Black} 1966). The Toluca Valley in Mexico is a center of diversity for \textit{P. infestans} and suggested to be its center of origin (\textit{Goodwin} et al. 1992, \textit{Fry} et al. 1993, \textit{Grunwald} & \textit{Flier} 2005). The Mexican resistance (\( R \)) genes include \( R1-R11 \) from \textit{Solanum demissum}, \( Rpi-bib1 \), \( Rpi-bib2 \) and \( Rpi-bib3 \) from \textit{Solanum bulbocastanum}, \( Rpi-sto1 \) and \( Rpi-pta \) from \textit{Solanum stoloniferum}, \( Rpi-amr3 \) from \textit{Solanum americanum}, \( Rpi-mch1 \) from \textit{Solanum micranthum} and \( Rpi1 \) from \textit{Solanum pinnatisectum} (\textit{Kuhl} et al. 2001, \textit{Hein} et al. 2009, \textit{de Vetten} et al. 2011, \textit{Vleeshouwers} et al. 2011b, \textit{Jo} et al. 2015, \textit{Witek} et al. 2016, \textit{Sliwka} et al. 2012b). Some of these Mexican \( R \) genes belong to large gene families, such as \( R2 \) that occurs at a major late blight resistance locus (MLB) on chromosome \( IV \) (\textit{Park} et al. 2005a, \textit{Lokossou} et al. 2009). In the Andean region in South America, the other center of genetic diversity of tuber-bearing \textit{Solanum} (\textit{Hijmans} & \textit{Spooner} 2001, \textit{Spooner} et al. 2004) as well as \textit{P. infestans} (\textit{Abad} & \textit{Abad} 1997, \textit{Alpizar-Gomez} et al. 2007), additional \( R \) genes have been identified. These include \( Rpi-mcq1 \), \( Rpi-vnt1 \), \( Rpi-ber \), \( Rpi-chc1 \), \( Rpi-tar1 \) and \( Rpi-rzc1 \) from \textit{Solanum mochiquense}, \textit{Solanum venturii}, \textit{Solanum berthaultii},
**RESULTS**

**AVR2 induces cell death responses in *Solanum* species from Mexico and Peru**

To identify plants that recognize AVR2 of *P. infestans*, functional screens were performed on a highly diverse set of 80 wild *Solanum* genotypes that belong to nine different taxonomic series (Table 1) (Hawkes 1990, Vleeshouwers et al. 2011a). AVR2 was transiently expressed in leaves by agroinfiltration and responses were scored at 3–4 days post infiltration (dpi). Specific cell death responses to AVR2 were observed in twelve wild *Solanum* genotypes. These belong to *Solanum schenckii* (Snk) 213-1 and 212-5, *Solanum edinense* (Edn) 151-1 and 150-4, *Solanum hjertingii* (HjH) 349-3, 350-1 and 640-1 and *Solanum bulbocastanum* (Blb) 520-21 that all occur in the central highlands of Mexico (Champouret 2010), but also in *S. mochiquense* (Mqz) 717-3 and 186-2 and *Solanum huancabambense* (Hcb) 353-8 and 354-1, which originate from Peru (Table 1, Fig. 1A). These results indicate that AVR2 is specifically recognized in various wild *Solanum* species, which reside in two geographically distinct locations (Fig. 1B).

**Genetic diversity of Mexican and South American *Solanum* genotypes**

The *Solanum* species for which an AVR2 response was detected, belong to taxonomically separate series. The AVR2-responding Mexican genotypes belong to Demissia, *Longipedicellata* and *Bulbocastanum*, whereas the Peruvian genotypes belong to *Yungasensa* and *Tuberosa* (Table 1). To further determine the genetic relationship between the 12 AVR2-recognizing *Solanum* genotypes on the DNA level, we classified them using the division described by Bonierbale et al. (1990) and Spooner et al. (2014). Genomic DNA from all functionally screened *Solanum* genotypes (Table 1) was subjected to AFLP analysis according to the method described by Jacobs et al. (2008), and subsequently, a tree was constructed using Bayesian inference. The tree shows that the AVR2-responding *Solanum* genotypes from Mexico and Peru cluster in separate groups (Fig. 2), and suggests a different evolutionary origin of the Mexican vs. Peruvian AVR2-responding *Solanum* species.

**Two R gene clusters from Mexico and Peru mediate AVR2 recognition**

R proteins of the nucleotide-binding leucine-rich repeat (NLR) class have a conserved region ARC, which was found in *Apafl* in humans, *R* proteins in plants and CED4 in *Caenorhabditis elegans* (van der Biezen & Jones 1998). The nucleotide binding (NB) and ARC domains are contiguous and the combined domain is known as the NB-ARC, which activation triggers cell death (Raidan & Moffett 2006). To investigate the relationship between previously identified *R* genes against late blight (Vleeshouwers et al. 2011a), we aligned their full NB-ARC domains. In total, 27 NB-ARC domains of *Rpi* proteins were used in the alignment and a phylogenetic tree was constructed based on these data (Fig. 3). Additionally, all of the *Rpi* proteins contain a coil–coil domain in the N-terminus and belong to the CNL family. The *Rpi* proteins were classified in different CNL clades (Jupe et al. 2012) (Fig. 3, Supplemental Table 1).

The *R2* family from MLB locus show chromosome IV is present in various Mexican *Solanum* spp. including *S. demissum*, *S. bulbocastanum*, *S. edinense*, *S. schenckii* and *S. hjertingii*, which are, respectively, the donors of R2, *Rpi-bb3*, *Rpi-edn1.1* *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3* (Lokossou et al. 2009, Champouret 2010). Also, functional members of the *R* gene clusters on chromosome IV, V, VI, VII, VIII, IX, and XI, containing *Rpi-amr3*, R1, *Rpi-bb2*, *Rpi-mch1* and *Rpi1*, *Rpi-bb1*, R8 & R9a, (plus its allelic variants) and R3a/R3b, respectively, seem to be restricted to *Solanum* species of Mexican origin.

*R* genes from South American origin are *Rpi-vnt1* and its allelic variants from *S. venturi* from Argentina (Foster et al. 2009, Pel et al. 2009), *Rpi-chc1* from *S. chaacense*, *Rpi-bcr* from *S. berthaultii* and *Rpi-tar1* from *S. tarijense* from Bolivia, (Vossen et al. 2009), *Rpi-rrc1* from *Solanum sparsipilum* from Bolivia and Peru (Sliwka et al. 2012a) and *Rpi-mcq1* from *S. mochiquense*
Table 1. List of *Solanum* genotypes used in this study.

| Series | Genotype | GenBank accession | Agro infiltration | Accession origin |
|--------|----------|-------------------|-------------------|------------------|
|        |          |                   | pK7WG2:AVR2 | pK7WG2: empty | R3a/AVR3a | Country | Collection site |
| II. Bulbocastana |          |                   |              |              |           |         |
|        | S. bulbocastanum partitum | GLKS 35322 | 120-2 | - | - | + | Guatemala |
|        | S. bulbocastanum | CGN 23075 | 525-1 | - | - | + | Guatemala |
|        | S. bulbocastanum | CGN 23074 | 949-1 | - | - | + | Guatemala |
|        | S. bulbocastanum | CGN 23074 | 949-5 | - | - | + | Guatemala |
|        | S. bulbocastanum | CGN 22732 | 950-5 | - | - | + | Guatemala |
|        | S. bulbocastanum | CGN 17693 | 331-2 | - | - | + | Mexico |
|        | S. bulbocastanum | CGN 17689 | 945-2 | - | - | + | Mexico |
|        | S. bulbocastanum | CGN 22698 | 517-1 | - | - | + | Mexico |
|        | S. bulbocastanum | GLKS 31741 | 522-1 | - | - | + | Mexico |
|        | S. bulbocastanum | CGN 22367 | 946-1 | - | - | + | Mexico |
|        | S. bulbocastanum | PI 275199 | 541-2 | - | - | + | Mexico |
|        | S. bulbocastanum | GLKS 30099 | 539-2 | - | - | + | Mexico |
|        | S. bulbocastanum | CGN 18326 | 337-2 | - | - | + | Mexico |
|        | S. bulbocastanum | CGN 18326 | 337-1 | - | - | + | Mexico |
|        | S. bulbocastanum | CGN 18326 | 335-10 | - | - | + | USA |
|        | S. bulbocastanum | GLKS 31586 | 355-1 | - | - | + | USA |
|        | S. bulbocastanum | CGN 18346 | 355-1 | - | - | + | USA |
|        | S. bulbocastanum | CGN 18346 | 674-1 | - | - | + | USA |
| III. Pinnatisecta |          |                   |              |              |           |         |
|        | S. brachistotrichum | CGN 17681 | 325-3 | - | - | + | Mexico |
|        | S. brachistotrichum | GLKS 32714 | 118-22 | - | - | + | Mexico |
|        | S. cardiophyllum | CGN 18325 | 336-1 | - | - | + | Mexico |
|        | S. cardiophyllum | CGN 18326 | 337-2 | - | - | + | Mexico |
|        | S. cardiophyllum | GLKS 30099 | 124-1 | - | - | + | Mexico |
|        | S. cardiophyllum | GLKS 31741 | 522-1 | - | - | + | Mexico |
|        | S. cardiophyllum | GLKS 32714 | 539-2 | - | - | + | Mexico |
|        | S. cardiophyllum | PI 275199 | 775-1 | - | - | + | Mexico |
|        | S. cardiophyllum | GLKS 31586 | 204-1 | - | - | + | Mexico |
|        | S. cardiophyllum | GLKS 31586 | 882-4 | - | - | + | Mexico |
|        | S. cardiophyllum | PI 545742 | 229-2 | - | - | + | Mexico |
|        | S. cardiophyllum | PI 545742 | 229-3 | - | - | + | Mexico |
|        | S. jayesi | CGN 18349 | 355-10 | - | - | + | USA |
|        | S. jayesi | CGN 18349 | 355-1 | - | - | + | USA |
|        | S. jayesi | CGN 18346 | 674-1 | - | - | + | USA |
| IV. Polyadenia |          |                   |              |              |           |         |
|        | S. desteri | CGN 18337 | 358-2 | - | - | + | Mexico |
|        | S. desteri | CGN 18337 | 358-4 | - | - | + | Mexico |
|        | S. polyadenium | CGN 17749 | 376-4 | - | - | + | Mexico |
| VI. Circaeifolia |          |                   |              |              |           |         |
|        | S. capsicibaccatum | CGN 18254 | 335-10 | - | - | + | Bolivia |
|        | S. capsicibaccatum | CGN 22388 | 536-1 | - | - | + | Bolivia |
|        | S. circaeifolium | CGN 18133 | 564-2 | - | - | + | Bolivia |
|        | S. circaeifolium | CGN 18133 | 564-3 | - | - | + | Bolivia |
|        | S. circaeifolium | CGN 18158 | 567-1 | - | - | + | Bolivia |
| IX. Yungasensa |          |                   |              |              |           |         |
|        | S. chacoense | CGN 18365 | 544-5 | - | - | + | Bolivia |
|        | S. chacoense | CGN 23986 | 4-11 | - | - | + | Bolivia |
|        | S. huancambense | CGN 18306 | 353-8 | - | - | + | Peru |
|        | S. huancambense | CGN 18306 | 354-1 | - | - | + | Peru |
|        | S. huancambense | CGN 18306 | 354-2 | - | - | + | Peru |
|        | S. huancambense | CGN 17719 | 354-10 | - | - | + | Peru |
|        | X. Megistacroloba | S. astleyi | GLKS 32836 | 114-4 | - | - | + | Bolivia |
|        | X. Tuberosa | S. verrucosum | CGN 17768 | 393-10 | - | - | + | Mexico |
|        | S. verrucosum | CGN 17770 | 912-2 | - | - | + | Mexico |
|        | S. mochiquense | GLKS 32319 | 186-1 | - | - | + | Peru |
|        | S. mochiquense | CGN 18263 | 717-3 | - | - | + | Peru |
|        | S. mochiquense | GLKS 32319 | 186-2 | - | - | + | Peru |
|        | S. avilesii | CGN 18255 | 477-1 | - | - | + | Bolivia |
|        | S. avilesii | CGN 18256 | 478-2 | - | - | + | Bolivia |
|        | S. berthaultii | CGN 18190 | 481-3 | - | - | + | Bolivia |

(continued on next page)
**Table 1. (Continued).**

| Series | Solanum species | GenBank accession | Genotype | Agro infiltration | Accession origin |
|--------|-----------------|-------------------|----------|-------------------|-----------------|
|        |                 |                   | pK7WG2:AVR2 | pK7WG2: empty     | Country          |
|        |                 |                   | R3a/AVR3a |                   | Collection site  |

| S. gourlayi vidaurei | CGN 23045 | 626-2 | – | – | + | Argentina |
| S. microdontum gigantophyllum | CGN 18200 | 712-6 | – | – | + | Bolivia |
| S. microdontum gigantophyllum | CGN 23050 | 714-1 | – | – | + | Argentina |
| S. microdontum gigantophyllum | CGN 18295 | 956-1 | – | – | + | Argentina |
| S. microdontum gigantophyllum | CGN 18049 | 963-3 | – | – | + | Argentina |
| S. okade | PI 458368 | 283-1 | – | – | + | Argentina |
| S. okade | CGN 18109 | 366-1 | – | – | + | Argentina |
| S. okade | CGN 18108 | 367-1 | – | – | + | Argentina |
| S. okade | CGN 17998 | 368-6 | – | – | + | Argentina |
| S. okade | CGN 19279 | 741-1 | – | – | + | Argentina |

**XVIII. Longipedicellata**

| Genotype Agro infiltration | Accession origin |
|---------------------------|-----------------|
| pK7WG2:AVR2 | pK7WG2: empty |
| R3a/AVR3a |                   |
|                   | Collection site  |
| S. fendleri | CGN 18116 | 596-2 | – | – | + | USA |
| S. papita | CGN 17830 | 369-7 | – | – | + | Mexico |
| S. papita | CGN 18303 | 765-1 | – | – | + | Mexico |
| S. papita | CGN 18732 | 370-5 | – | – | + | Mexico |
| S. stoloniferum | CGN 18333 | 842-9 | – | – | + | Mexico |
| S. stoloniferum | CGN 17606 | 837-2 | – | – | + | Mexico |
| S. stoloniferum | CGN 18333 | 842-6 | – | – | + | Mexico |
| S. stoloniferum | CGN 18348 | 832-5 | – | – | + | Peru |
| S. hjertingii | CGN 22370 | 640-1 | + | – | + | Mexico | 5 |
| S. hjertingii | CGN 17718 | 350-1 | + | – | + | Mexico | 6 |
| S. hjertingii | CGN 17717 | 349-3 | + | – | + | Mexico | 7 |
| S. polytrichon | CGN 17750 | 378-2 | – | – | + | Mexico |

**XIX. Demissa**

| Genotype Agro infiltration | Accession origin |
|---------------------------|-----------------|
| pK7WG2:AVR2 | pK7WG2: empty |
| R3a/AVR3a |                   |
|                   | Collection site  |
| S. edinense | PI 611104 | 150-4 | + | – | + | Mexico | 1 |
| S. edinense | PI 607474 | 151-1 | + | – | + | Mexico | 2 |
| S. schenkii | GLKS 30659 | 213-1 | + | – | + | Mexico | 3 |
| S. schenkii | GLKS 30658 | 212-5 | + | – | + | Mexico | 4 |
| S. hougasii | CGN 21361 | 655-1 | – | – | + | Mexico |

The 80 genotypes are derived from wild Solanum accessions native to diverse geographic locations and belong to 9 taxonomic series of Solanum section Petota (Hawkes 1990). Plants were subjected agro-infiltration and occurrence of cell death responses (+) or no responses (−) is indicated. The pK7WG2 empty vector and agro-co-infiltration with R3a/AVR3a were included as negative and positive controls, respectively. Collection sites 1–12 correspond to Figs 1 and 2.

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from Peru (Smilde et al. 2005, Jones et al. 2014a), the same Solanum species as was found to respond to AVR2 (Fig. 1, Table 1). To test whether Rpi-mcq1 can recognize AVR2 (Fig. 4). Specific cell death responses occurred in leaf panels co-infiltrated with AVR2 and the R2 homolog Rpi-blb3 or Rpi-mcq1, respectively. This indicates that AVR2 recognition can be mediated by both Rpi-blb3 and Rpi-mcq1. These R genes are localized at different chromosomes (Supplemental Table 1) and different phylogenetic clades (Fig. 3), which supports the theory of different evolutionary origin between R2/Rpi-blb3 and Rpi-mcq1 genes.

**Transgenic Désirée-Rpi-blb3 and Désirée-Rpi-mcq1** display a different resistance spectrum to P. infestans isolates

Transgenic potato cv. ‘Désirée’ were generated that express Rpi-blb3 and Rpi-mcq1, respectively, under the control of their native promoters. To functionally analyze the R gene activity, leaves of Désirée-Rpi-blb3 and Désirée-Rpi-mcq1 were agroinfiltrated with Agrobacterium tumefaciens carrying the pK7WG2 vector harboring AVR2. Infiltrations using pK7WG2: empty vector and co-infiltration of R3a/AVR3a were included as negative and positive controls, respectively. In both transformants, cell death responses were observed in AVR2 infiltration sites and with the positive control at 4 dpi (Supplemental Fig. 1), confirming that Rpi-mcq1 and Rpi-blb3 are functional in these plants and lead to the recognition of AVR2.

The resistance spectrum of Désirée-Rpi-blb3, Désirée-Rpi-mcq1 and wild type ‘Désirée’ control was investigated by performing detached leaf assays with 18 P. infestans isolates (Supplemental Table 2). Macroscopic observations were carried out at 6 dpi. The susceptible ‘Désirée’ control was infected by all tested isolates, but three distinct resistance patterns (I−III) were observed on Désirée-Rpi-blb3 and Désirée-Rpi-mcq1 (Fig. 5). Group I contains seven isolates that are avirulent on both Désirée-Rpi-blb3 and Désirée-Rpi-mcq1, whereas Group III
contains eight isolates that are virulent on these plants. Interestingly, group II consists of three isolates that display a distinct virulence profile on D/C19cesir/C19ee-Rpi-blb3 compared with D/C19cesir/C19ee-Rpi-mcq1. All of the three isolates are avirulent on D/C19cesir/C19ee-Rpi-blb3 but virulent on D/C19cesir/C19ee-Rpi-mcq1. Considering the virulence pattern observed, D/C19cesir/C19ee-Rpi-blb3 displays a slightly broader and partly overlapping disease resistance spectrum as compared to D/C19cesir/C19ee-Rpi-mcq1.

**DISCUSSION**

This manuscript presents a study of AVR2 effector recognition in a wide diversity of wild *Solanum* species. We detected AVR2 responses in *Solanum* genotypes from two different geographical locations, Mexico and Peru, which are both recognized as centers of diversity of *P. infestans* (Goodwin et al. 1992, Fry et al. 1993, Abad & Abad 1997, Grunwald & Flier 2005, Alpizar-Gomez et al. 2007). The recognition in Mexican *Solanum* species is conferred by genes from the R2 family that resides at an MLB locus on the short arm of chromosome IV (Lokossou et al. 2009, Champouret 2010, Lokossou et al. 2010). In contrast, the AVR2 response in Peruvian *Solanum* species is conferred by Rpi-mcq1 or allelic variants, which exhibits distinct resistance specificities to a range of *P. infestans* isolates. Rpi-mcq1 belongs to the CNL4 family (Fig. 3) and is located on chromosome IX (Smilde et al. 2005).

The AVR2-responding *Solanum* species identified in this study occur in separate groups based on geographic origin (Fig. 1), taxonomic classification (Table 1) and phylogenetic analysis using AFLP data (Fig. 2). Several studies point the origin of *P. infestans* to Mexico and to the Andes, and as a consequence, Mexican and South American *Solanum* may have independently evolved distinct R genes to adapt to local pathogen populations (Westerdijk 1917, Grunwald & Flier 2005, Alpizar-Gomez et al. 2007, Goss et al. 2014). The fact that Rpi genes from Mexican and Peruvian *Solanum* species are present in different loci and belong to different classes (Fig. 3), supports the hypothesis that recognition of AVR2 has evolved independently in those geographic regions and has led to the evolution of two different R genes that mediate AVR2-based resistance to *P. infestans*. Comparably, in *Phytophthora sojae*, two distinct genes conferring resistance to *Phytophthora sojae* (Rps genes), Rps3a and Rps5, were found to mediate recognition of the product of the AVR3a/5 alleles from *P. sojae*. These Rps genes are located on different chromosomes (Li et al. 2016) and specific residues of AVR3a/5 were identified that are required for recognition by Rps5, but not Rps3a (Dong et al.
suggesting that Rps3a and Rps5 evolved independently. Research using other systems show that the recognition of an AVR protein by multiple, unrelated, R proteins is sometimes also observed in other plant-pathogen systems (Feyter et al. 1993, Ashfield et al. 2004, Anh et al. 2015). Recently, it was found that distinct immune receptors can be involved in the recognition of conserved molecules like bacterial flagellin as well (Hind et al. 2016).

\[ \text{R gene specificity is known to be determined by specific recognition of AVR proteins of pathogens. The largely overlapping resistance spectra mediated by Rpi-mcq1 and R2/Rpi-bib3 can be explained by Avr2, which was found to be the} \]

\[ \text{Clade 1} \]

\[ \text{Mexican and Central American species} \]

\[ \text{Clade 3} \]

\[ \text{Ecuador and Northern Peru} \]

\[ \text{Clade 4} \]

\[ \text{S. verrucosum in Mexico} \]

\[ \text{South American diploids exclusive of clade 3} \]

\[ \text{Complex clade} \]

\[ \text{Clade 1 + 4} \]

\[ \text{Complex clade} \]

\[ \text{Clade etuberosum} \]
cognate Avr for both R genes (Gilroy et al. 2011). AVR2 is a member of a highly diverse gene family (Champouret 2010, Vleeshouwers et al. 2011b) and the difference in resistance specificity between Rpi-bib3 and Rpi-mcq1 might be explained by differential recognition of other AVR2 family members, or additional alleles of AVR2. It has been demonstrated in P. sojae that recognition of the same effector is not always linked with the same race specificity and the differential specificities in effector recognition may be attributed to the presence of additional alleles or paralogs of the effector (Kaitany et al. 2001, Dong et al. 2011). Therefore, the study of recognition of AVR2 family members and their allelic variants in diverse P. infestans isolates by Rpi-bib3 and Rpi-mcq1 could contribute to better understanding of race-specific resistances and subsequently contribute to more educated deployment of respective R genes.

According to the Achilles’ heel theory (Homer 1999), proteins that fulfill essential functions for a pathogen are less likely to become mutated or lost from the invaders genome. Therefore, targeting such proteins is expected to lead to more broad-spectrum, and even more sustainable disease resistance (Laugé et al. 1998). AVR2 interacts with the host target StBSL1, a putative phosphatase that acts as a positive regulator of the brassinosteroid (BR) pathway. Enhanced BR-signaling results in up-regulation of the basic-Helix-Loop-Helix transcription factor StCHL1, which acts as a negative regulator of immunity (Saunders et al. 2012, Turnbull et al. 2017). AVR2 was found to contribute to virulence of P. infestans (Gilroy et al. 2011). The fact that two independent R gene families have evolved in Solanum to detect AVR2, supports the idea that AVR2 is an important effector of P. infestans. Avr2 thus seems an important target for obtaining resistance.

Besides targeting important or conserved effectors, it has been proposed that the stacking of R genes can contribute to obtaining a broader and more durable type of resistance (Pink & Puddephat 1999). In the past, some breeders have used the geographic origin of the resistant genotypes as a criterion to decide which resistance sources to include in their breeding program. However, since allelic variants of R genes are found across Solanum spp., e.g. like Rpi-bib1, Rpi-sto1 and Rpi-pta1 from S. bulbocastanum and S. stoloniferum (Vleeshouwers et al. 2008, Champouret et al. 2009) and the members of R2 from S. demissum, from at least 5 Mexican Solanum species (Park et al. 2005a, Park et al. 2005b, Park et al. 2005c, Vleeshouwers et al. 2008, Lokossou et al. 2009, Champouret 2010), this appears not a very robust criterion. In more modern breeding approaches, breeders select R genes by locus, as it has been proposed that R genes that originate from different R gene clusters recognize different effectors and are thus preferred (Zhu et al. 2012). Marker-assisted breeding is then considered efficient for breeding, although R gene activity by functional effector assays seems the best method to distinguish between mechanistically different R genes (Vleeshouwers et al. 2011b, Jo et al. 2016). In this study however, we show that R genes that recognize the same effector (AVR2) can still confer different
Fig. 4. *Rpi-mcq1* and *Rpi-blb3* confer response to AVR2. Leaves of potato cv. ‘Bintje’ were co-infiltrated with AVR2 and *Rpi-mcq1* (A) and *Rpi-blb3* (B) as a cell death control trigger by AVR2. Single infiltrations of AVR2, *Rpi-mcq1*, *Rpi-blb3* and empty vector were included as negatives controls and co-infiltration of R3a/AVR3a was included as positive control. Each effector is tested twice on three leaves, over two plants and two biological replicates. Representative photographs of cell death symptoms were taken at 4 dpi.

Fig. 5. Disease index on ‘Desirée’, ‘Desirée-*Rpi-mcq1*’ and ‘Desirée-*Rpi-blb3*’ with isolates from group I–III. (A) Representative pictures of isolates from group I to III tested in ‘Desirée’ (WT), ‘Desirée-*Rpi-mcq1*’ (*Rpi-mcq1*) and ‘Desirée-*Rpi-blb3*’ (*Rpi-blb3*) are displayed. Pictures were taken after 6 dpi. (B) Disease symptoms were scored on a scale from 1 to 9: 1 represents intensive sporulation; 2–3, macroscopically visible sporulation, but to a less extend as 1. 4–5, represent sporulation only visible under the binocular; 6–7 represent necrotic lesion ≥ 10 mm of diameter and between 4–10 mm, respectively; 8, small necrotic lesion not exceeding 4 mm and 9 represents no symptoms. The percent of each category is shown with isolates of group I–III.
resistance patterns, which further nuances the strategy to discriminate race-specificity of R genes.

To conclude, the effectoromics approach can aid identification of R genes with new resistance specificities and facilitates the detailed characterization of R genes. A better understanding of how R genes contribute to resistance is essential to select the best genes for resistance breeding. This information can be the basis for an educated breeding effort, which will contribute to the goal of obtaining broad-spectrum and durable resistance against *P. infestans*.

**MATERIALS AND METHODS**

**Plant material**

The wild *Solanum* plant material used in functional effector screening for cell death responses to AVR2 is listed in Table 1 (Vleeshouwers et al. 2011a). Plant genotypes were maintained *in vitro* in sterile jars containing MS20 medium (Murashige & Skoog 1962) at 24 °C under 16/8h day/night regime. Top shoots were transferred to fresh medium for rooting, and 2 weeks later transferred to pots containing sterilized soil in climate regulated greenhouse compartments within the temperature range of 18–22 °C and under 16 h/8 h day/night regime.

**Agroinfiltration**

AVR2 from *P. infestans* (NCBI Genbank code XM_002902940.1) was previously cloned in the pK7WG2 vector (Karimi et al. 2002) and was transiently expressed in *Solanum* plants using Agro-infiltrations (Vleeshouwers & Rietman 2008). Single infiltrations of pK7WG2: empty were included as a negative control and R3a/AVR3a were co-expressed as a positive control. Agro-infiltration was performed on 4-week-old potato plants using a suspension of *A. tumefaciens* strain AGL1 containing MS20 medium (Murashige & Skoog 1962) at 24 °C under 16/8h day/night regime. Top shoots were transferred to fresh medium for rooting, and 2 weeks later transferred to pots containing sterilized soil in climate regulated greenhouse compartments within the temperature range of 18–22 °C and under 16 h/8 h day/night regime.

**Phylogenetic data analysis**

A phylogenetic tree of 80 screened *Solanum* genotypes and *Solanum tuberosum* (Etb) 594-2, 591-3, 591-4, 591-5, 595-5 and 593-2 was constructed by MrBayes v3.2.6 (Huelsenbeck & Ronquist 2001) using 224 AFLP markers scored as presence/absence of polymorphisms (Jacobs et al. 2008). Mesquite v3.3 (Maddison & Maddison 2017) was used for formatting data and 1000 generations from four chains run for 10 000 000 generations with a temperature setting for the heated chains of 0.25. *Solanum tuberosum* genotypes represented the outgroup.

A Maximum-Likelihood (ML) tree was generated with the NB-ARC domains of 27 Rpi proteins obtained by InterProScan (Jones et al. 2014b) (Supplementary Table 1). The domain sequences were aligned using Muscle (Edgar 2004) and the resulting alignment was used for phylogenetic analysis. The ML tree was built in PhyML v3.0 (Guindon et al. 2010) using the nearest Neighbor Interchange (NNI) as the heuristic method for finding the best tree topology. The tree was rooted using Gro1.4 (NCBI Genbank code AAP44390.1) and was visualized by Figtree v1.4.3 (Rambaut 2009).

**Generation of transgenic Rpi-blb3 and Rpi-mcq1 potato cv. ‘Désirée’**

Stable transformation of potato cv. ‘Désirée’ (event A03-142) was previously performed using *A. tumefaciens* strain AGL1 harboring pBINPLUS: Rpi-blb3 under the control of native expression elements (Zhu et al. 2012). For Rpi-mcq1 transformation to Désirée, Rpi-mcq1 was subcloned from the library clone pSLJ2115 (Jones et al. 2007) into the binary vector pBINPLUS under the control of native regulatory elements and was transferred to *A. tumefaciens* strain AGL1. The transformation of potato cv. ‘Désirée’ was performed using routine transformation protocols (Filatti et al. 1987, Hoeckema et al. 1991). Among 35 independent primary transformants, the resistant event A31-47 was selected after growth under greenhouse conditions (18–22 °C, 16 h of light and 8 h of dark) and field condition.

**Phytophthora infestans** isolates, culture conditions and inoculum preparation

The *P. infestans* isolates used in this study are listed in Supplemental Table 2 and were retrieved from our in-house collection. Isolates were routinely grown in the dark at 15 °C on solid rye sucrose medium prior to the disease test (Caten & Jinks 1968). To isolate zoospores for plant inoculations, sporulating mycelium was flooded with cold water and incubated at 4 °C for 1–3 h.

**Disease test**

Leaves from 6–8-week-old plants grown in greenhouse conditions (18–22 °C, 16 h of light and 8 h of dark) were detached and placed in water-saturated oasis in trays. The leaves were spot-inoculated at the abaxial leaf side with 10 μl droplets containing 5*10^4 zoospores per ml. 12 inoculations in each leave, three leaves per isolate and 3 independent experiments were performed. After inoculation, the trays were incubated in a climate chamber at 15 °C with a 16 h photoperiod. Development of lesions and presence of sporulation was determined at 5 dpi (Vleeshouwers et al. 1999, Champouret 2010). Disease index was estimated using a scale ranging from 1 to 9 scale, where 1 corresponds to expanding lesions with massive sporulation (susceptible), 7–8 to occurrence of the hypersensitive response (resistant) and 9 to no symptoms (fully resistant).

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Appendix A. Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.simyco.2018.01.002.

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