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Two different *R* gene loci co-evolved with *Avr2* of *Phytophthora infestans* and confer distinct resistance specificities in potato

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Abstract: Late blight, caused by the oomycete pathogen *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 *P. infestans* have evolved in wild tuber-bearing *Solanum* species from North, Central and South America, upon co-evolution with cognate avirulence (*Avr*) genes. Here, we report how effectoromics screens with *Avr2* of *P. infestans* revealed defense responses in diverse *Solanum* species that are native to Mexico and Peru. We found that the response to AVR2 in the Mexican *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 *Solanum* species is mediated by *Rpi-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 *Solanum* species, respectively. Detached leaf tests on potato cultivar 'Désirée' transformed with *R* genes from either the *R2* or the *Rpi-mcq1* locus revealed an overlapping, but distinct resistance profile to a panel of 18 diverse *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, *Phytophthora infestans*, Potato, *R* gene, Resistance, *Solanum*.

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INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most important non-cereal crop consumed worldwide and is affected by the destructive late blight disease. The oomycete pathogen *Phytophthora infestans* is the causal agent of the disease, which destroys leaves, stems and tubers from growing potato plants (Fry 2008). 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 (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 (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 (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 *Phytophthora infestans* (*Rpi*) have been introgressed into potato cultivars from *Solanum* species native to Mexico (Malcolmson & Black 1966). The Toluca Valley in Mexico is a center of diversity for *P. infestans* and suggested to be its center of origin (Goodwin *et al.* 1992, Fry *et al.* 1993, Grunwald & Flier 2005). The Mexican resistance (*R*) genes include *R1-R11* from *Solanum demissum*, *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* from *Solanum bulbocastanum, Rpi-sto1* and *Rpi-pta* from *Solanum stoloniferum*, *Rpi-amr3* from *Solanum americanum, Rpi-mch1* from *Solanum michoacanum* and *Rpi1* from *Solanum pinnatisectum* (Kuhl *et al.* 2001, Hein *et al.* 2009, de Vetten *et al.* 2011, Vleeshouwers *et al.* 2011b, Jo *et al.* 2015, Witek *et al.* 2016, 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 (Park *et al.* 2005a, Lokossou *et al.* 2009). In the Andean region in South America, the other center of genetic diversity of tuber-bearing *Solanum* (Hijmans & Spooner 2001, Spooner *et al.* 2004) as well as *P. infestans* (Abad & Abad 1997, Alpizar-Gomez *et al.* 2007), additional *R* genes have been identified. These include *Rpi-mcq1*, *Rpi-vnt1*, *Rpi-ber, Rpi-chc1, Rpi-tar1*, *Rpi-rzc1* from *Solanum mochiquense, Solanum venturii*, *Solanum berthaultii,*

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Solanum chacoense, *Solanum tarijense* and *Solanum sparsipilum*, respectively (Smilde *et al.* 2005, Jones *et al.* 2007, Foster *et al.* 2009, Park *et al.* 2009, Pel *et al.* 2009, Vossen *et al.* 2009, Jones *et al.* 2014a, Sliwka *et al.* 2012a).

R gene-mediated resistance is generally based on a strong hypersensitive response (HR), but in potato, single *R* genes have failed to provide durable resistance against late blight. Therefore, the modern breeding approach is to isolate a variation of *R* genes and deploy them in pyramids. This is expected to lead to broad-spectrum recognition of *P. infestans* isolates and might provide a more durable resistance (Jo *et al.* 2016). The originally laborious job of cloning new *R* genes has accelerated in recent years. Map-based cloning approaches have been greatly facilitated by the availability of the potato genome sequence, and modern approaches such as *R* gene enrichment sequencing (RenSeq) promise to speed up the *R* gene identification to unprecedented rate (Jupe *et al.* 2013, Witek *et al.* 2016). In addition, functional genomics approaches such as effectoromics can be exploited to probe resistant germplasm for specific recognition to *P. infestans* effectors to identify new *R* genes and speed up complementation studies (Vleeshouwers *et al.* 2008, Haas *et al.* 2009). Functional studies on effectors of *P. infestans* are key to understanding the specificity and potential durability of *R* genes (Vleeshouwers *et al.* 2011b).

AVR2, a cytoplasmic RxLR-EER effector from *P. infestans,* is the cognate avirulence protein matching R2 (Gilroy *et al.* 2011). Overexpression of AVR2 in potato plants results in enhancement of susceptibility to *P. infestans* isolates (Turnbull *et al.* 2017), and therefore, AVR2 is considered an important effector for *P. infestans*. We hypothesize that host species evolve immune receptors that target important effectors such as AVR2 during the tight co-evolution with the pathogen in centers of diversity.

In this study, a diverse collection of wild *Solanum* genotypes was screened for responses to AVR2 in order to identify AVR2-responding genotypes. Cell death responses were found in Mexican, as well as in South American *Solanum* spp. We studied the genetic basis of the response to AVR2 in both centres of diversity, and investigated the spectrum of resistance caused by respective *R* genes. The data show that *R* genes mediating the recognition of AVR2 have evolved independently, resulting in different genes at unrelated genetic loci in two different centers of diversity of *Solanum* spp. and cause different resistance specificities.

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* (Hjt) 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* (Mcq) 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 *Demissa*, *Longipedicellata* and *Bulbocastana*, 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 interference. 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 **A**paf-1 in humans, **R** proteins in plants and **C**ed4 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 (Rairdan & 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 on chromosome IV is present in various Mexican *Solanum* spp. including *S. demissum, S. bulbocastanum, S. edinense, S. schenckii* and *S. hjertjingii,* which are, respectively, the donors of *R2, Rpi-blb3, 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-blb2*, *Rpi-mch1* and *Rpi1*, *Rpi-blb1, 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. chacoense*, *Rpi-ber* from *S. berthaultii* and *Rpi-tar1* from *S. tarijense* from Bolivia, (Vossen *et al.* 2009), *Rpi-rzc1* 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	*Solanum* species	GenBank accession	Genotype	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	CGN 18310	520-21	+	−	+	Mexico	8
	S. bulbocastanum	GLKS 31741	522-1	−	−	+	Mexico	
	S. bulbocastanum	CGN 22367	946-1	−	−	+	Mexico	
	S. bulbocastanum	PI 275199	947-1	−	−	+	Mexico	
	S. bulbocastanum	CGN 23010	948-1	−	−	+	Mexico	
	S. bulbocastanum	CGN 23010	948-2	−	−	+	Mexico	
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 22387	541-2	−	−	+	Mexico	
	S. cardiophyllum	CGN 18326	337-2	−	−	+	Mexico	
	S. cardiophyllum	GLKS 30099	124-1	−	−	+	Mexico	
	S. cardiophyllum	CGN 18326	337-1	−	−	+	Mexico	
	S. cardiophyllum	BGRC 55227	539-2	−	−	+	Mexico	
	S. pinnatisectum	CGN 17742	775-1	−	−	+	Mexico	
	S. pinnatisectum	GLKS 31586	204-1	−	−	+	Mexico	
	S. trifidum	CGN 22371	882-4	−	−	+	Mexico	
	S. tarnii	PI 545742	226-3	−	−	+	Mexico	
	S. tarnii	PI 545808	229-2	−	−	+	Mexico	
	S. jamesii	CGN 18349	355-10	−	−	+	USA	
	S. jamesii	CGN 18349	355-1	−	−	+	USA	
	S. jamesii	CGN 18346	674-1	−	−	+	USA	
IV. *Polyadenia*	*S. lesteri*	CGN 18337	358-2	−	−	+	Mexico	
	S. lesteri	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 quimense	CGN 18158	567-1	−	−	+	Bolivia	
IX. *Yungasensa*	*S. chacoense*	CGN 18365	544-5	−	−	+	Bolivia	
	S. arnesii	CGN 23986	4-11	−	−	+	Bolivia	
	S. huancabambense	CGN 18306	353-8	+	−	+	Peru	9
	S. huancabambense	CGN 17719	354-1	+	−	+	Peru	10
	S. huancabambense	CGN 18306	354-2	−	−	+	Peru	
	S. huancabambense	CGN 17719	354-10	−	−	+	Peru	
X. *Megistacroloba*	*S. astleyi*	GLKS 32836	114-4	−	−	+	Bolivia	
XVI. *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	12
	S. mochiquense	GLKS 32319	186-2	+	−	+	Peru	11
	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	R3a/AVR3a	Country	Collection site
	S. gourlayi vidaurrei	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 18279	741-1	−	−	+	Argentina	
XVIII. *Longipedicellata*	*S. fendleri*	CGN 18116	596-2	−	−	+	USA	
	S. papita	CGN 17830	369-7	−	−	+	Mexico	
	S. papita	CGN 18303	765-1	−	−	+	Mexico	
	S. papita	CGN 17832	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	838-5	−	−	+	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*	*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-coinfiltration with R3a/Avr3a were included as negative and positive controls, respectively. Collection sites 1–12 correspond to Figs 1 and 2.

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, we performed an agroinfiltration experiment in potato cv. 'Bintje' (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 infiltrations 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

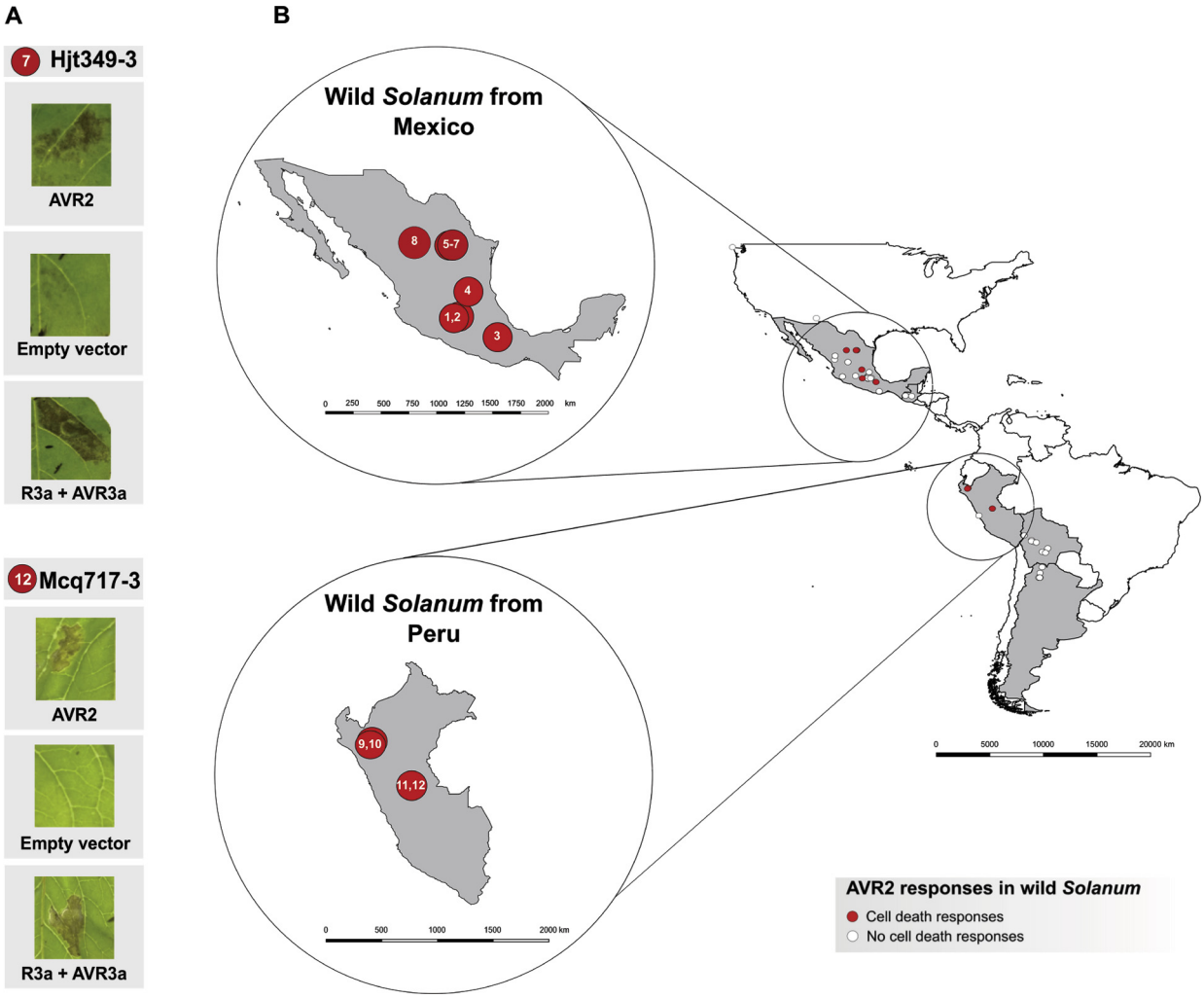

Fig. 1. *Solanum* species that respond to AVR2 occur in Mexico and Peru. (A) Representative leaf panels of AVR2-recognizing *Solanum* species from Mexico (Hjt349-3) and Peru (Mcq717-3). Leaves were agro-infiltrated with pK7WG2:AVR2, with pK7WG2: empty and co-infiltrated *R3a*/AVR3a as negative and positive controls, respectively. Pictures were taken at 4 dpi. **(B)** Geographic map representing the origins of all tested *Solanum* genotypes (white circles) including those that respond to AVR2 (red circle), listed in Table 1.

contains eight isolates that are virulent on these plants. Interestingly, group II consists of three isolates that display a distinct virulence profile on Désirée-*Rpi-blb3* compared with Désirée-*Rpi-mcq1.* All of the three isolates are avirulent on Désirée-*Rpi-blb3* but virulent on Désirée-*Rpi-mcq1.* Considering the virulence pattern observed, Désirée-*Rpi-blb3* displays a slightly broader and partly overlapping disease resistance spectrum as compared to Désirée-*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.*

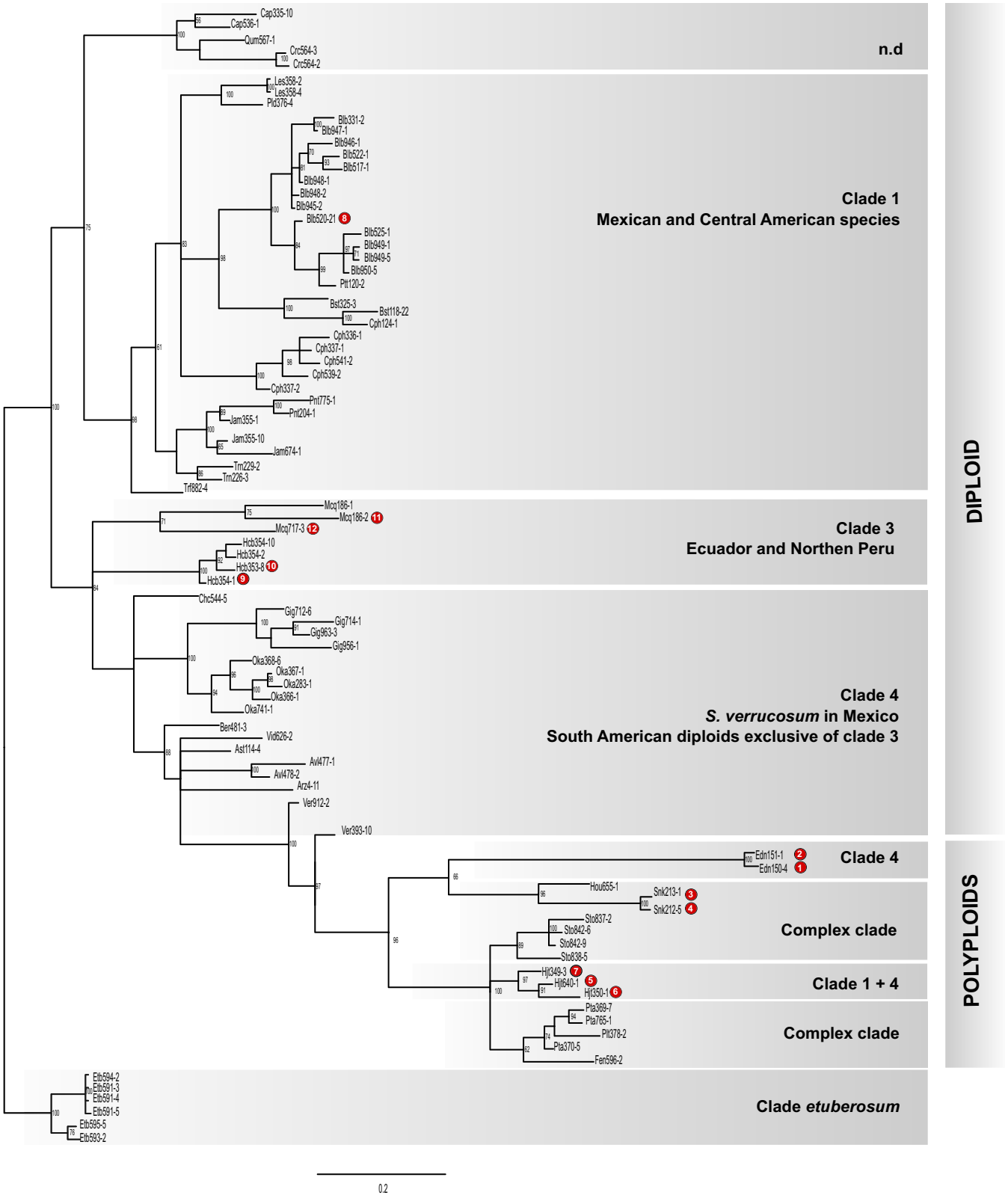

Fig. 2. Classification of tested wild *Solanum* genotypes. Bayesian rooted tree of 80 screened *Solanum* genotypes and 6 *Solanum etuberosum* genotypes. The branch length represents expected changes per site and posterior probability values are shown near the respective nodes. Indicated clades are based on Spooner *et al.* (2014). The AVR2-responding *Solanum* genotypes are marked with red dots, and numbers correspond to their geographic location (Fig. 1). n.d. not determined.

2011), 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).

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-blb3* can be explained by *Avr2,* which was found to be the

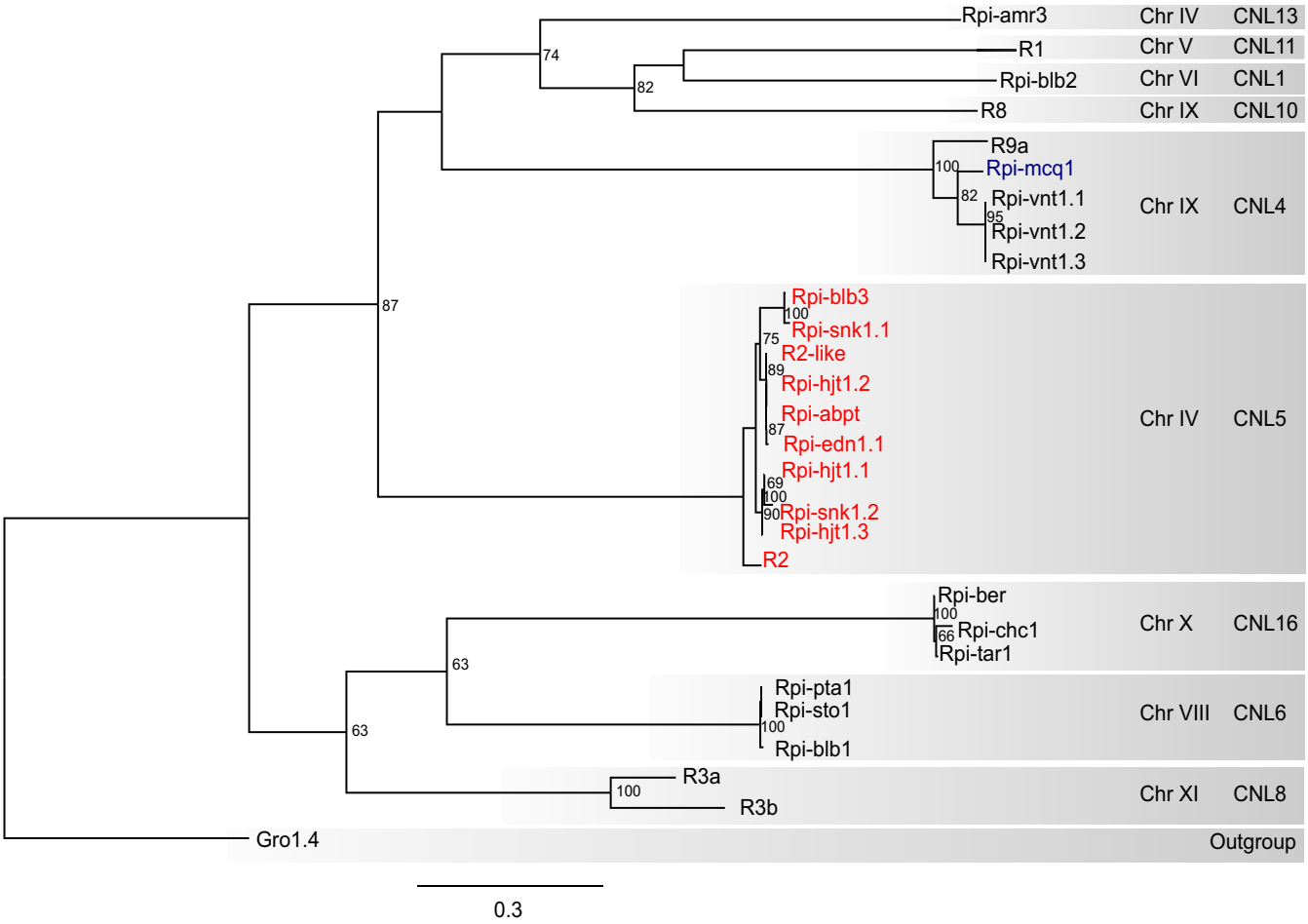

Fig. 3. Classification of Rpi proteins. Phylogenetic tree derived from the full NB-ARC domains (range of amino acid sequences in Supplemental Table 1) obtained from 27 Rpi proteins. *Rpi* cloned from Mexican (red) and South American (blue) *Solanum* are highlighted. CNL clades are indicated. The nematode resistance protein Gro1.4 was used as outgroup in a Maximum-Likelihood analysis. The Bootstrap values of 60 % and higher are indicated in the nodes. Horizontal branches lengths and scale bar correspond to the evolutionary distances that are measured as the proportion of amino acid substitutions between sequences.

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-blb3* 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-blb3 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-blb1, 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

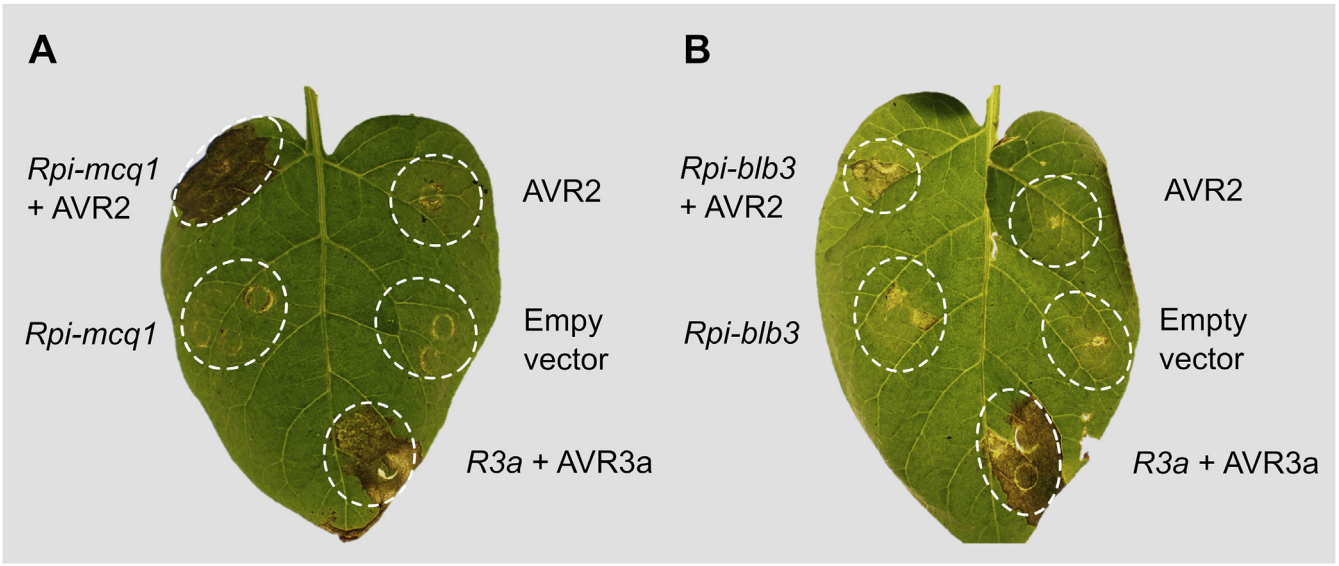

Fig. 4. Rpi-mcq1 and Rpi-blb3 confer response to AVR2. Leaves of potato cv. 'Bintje' were co-infiltrated with AVR2 and *Rpi-mcq1* (**A**) and *Rpi-blb3* (**B**) as a cell death control trigger by AVR2. Single infiltrations of AVR2, *Rpi-mcq1*, *Rpi-blb3* and empty vector were included as negatives controls 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.

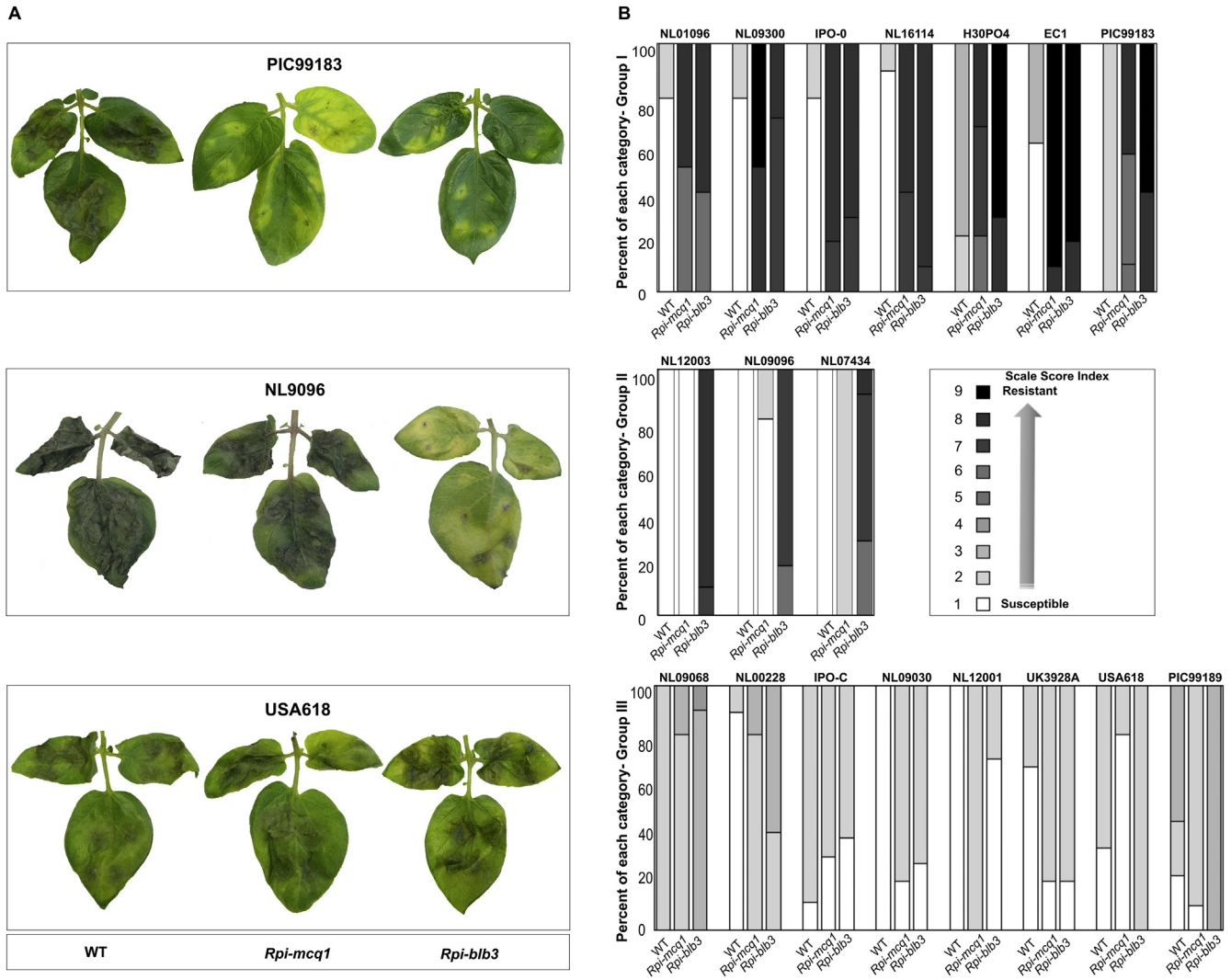

Fig. 5. Disease index on 'Désirée', Désirée-*Rpi-mcq1* and Désirée-*Rpi-blb3* with isolates from group I–III. (**A**) Representative pictures of isolates from group I to III tested in 'Désirée' (WT), Désiree-*Rpi-mcq1* (*Rpi-mcq1*) and Désirée-*Rpi-blb3* (*Rpi-blb3*) are displayed. Pictures were taken 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 $\geq$ 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 4–5-week-old potato plants using a suspension of *A. tumefaciens* strain AGL1 containing the appropriate expression vectors at an OD_{600} of 0.2. Each individual effector was tested twice on three leaves of two plants in two separated experiments. Local symptoms of cell death responses were assessed at 3–4 dpi.

Phylogenetic data analysis

A phylogenetic tree of 80 screened *Solanum* genotypes and *Solanum etuberosum* (Etb) 594-2, 591-3, 591-4, 591-5, 595-5 and 593-2 was constructed by MrBayes v3.2.6 (Huelsenberck & 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 MrBayes was used to estimate the posterior distribution by Markov Chian Monte Carlo (MCMC) methods (Larget & Simon 1999). Trees were sampled every 1000 generations from four chains run for 10 000 000 generations with a temperature setting for the heated chains of 0.25. *Solanum etuberosum* 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 three 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 (Fillatti *et al.* 1987, Hoekema *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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