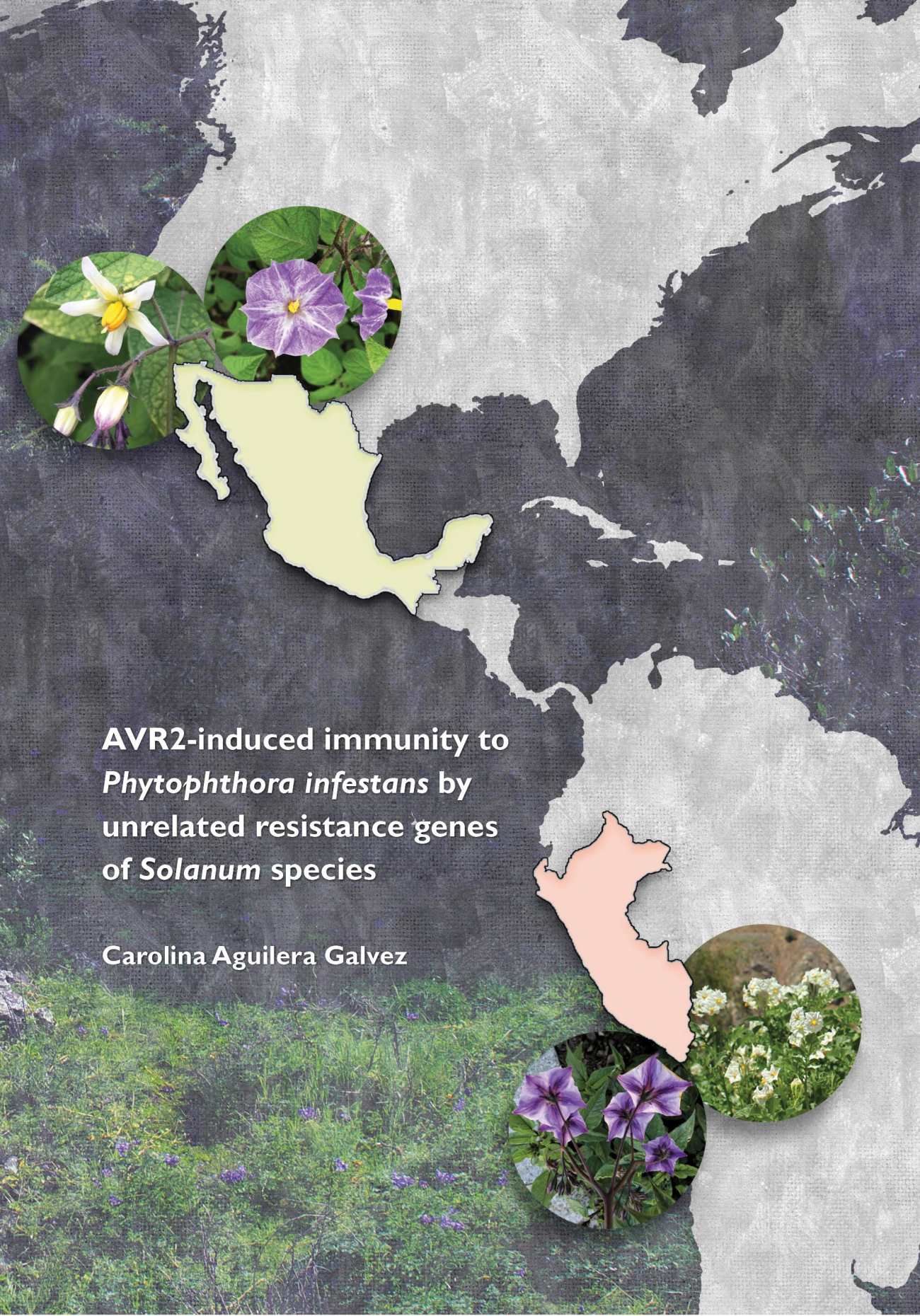

AVR2-induced immunity to *Phytophthora infestans* by unrelated resistance genes of *Solanum* species

Carolina Aguilera Galvez

Propositions

1. The recognition of the same effector mediated by unrelated *Solanum R* genes is not always related with identical resistance specificity to *P. infestans*.
   (this thesis)

2. Targeting effectors which are required for *P. infestans* virulence is crucial to get broad-spectrum disease resistance in potato.
   (this thesis)

3. Overpopulation is a major threat for biodiversity.

4. Antibacterial soaps should be forbidden for their link with antibiotic resistance and environmental damage.

5. The challenge of the new genome sequencing platforms is to know what to do with the information rather than generate it.

6. Limited access to research only stimulates the monopoly of knowledge.

7. The democratic state of a country represents the moral status of that countries society.

Propositions belonging to the thesis, entitled AVR2-induced immunity to *Phytophthora infestans* by unrelated resistance genes of *Solanum* species

Carolina Aguilera Galvez
Wageningen, 4 September 2019

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AVR2-induced immunity to *Phytophthora infestans* by unrelated resistance genes of *Solanum* species

Carolina Aguilera Galvez

Thesis

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A mis padres Alberto Aguilera Garcia
y Beatriz Galvez de Aguilera con amor y gratitud

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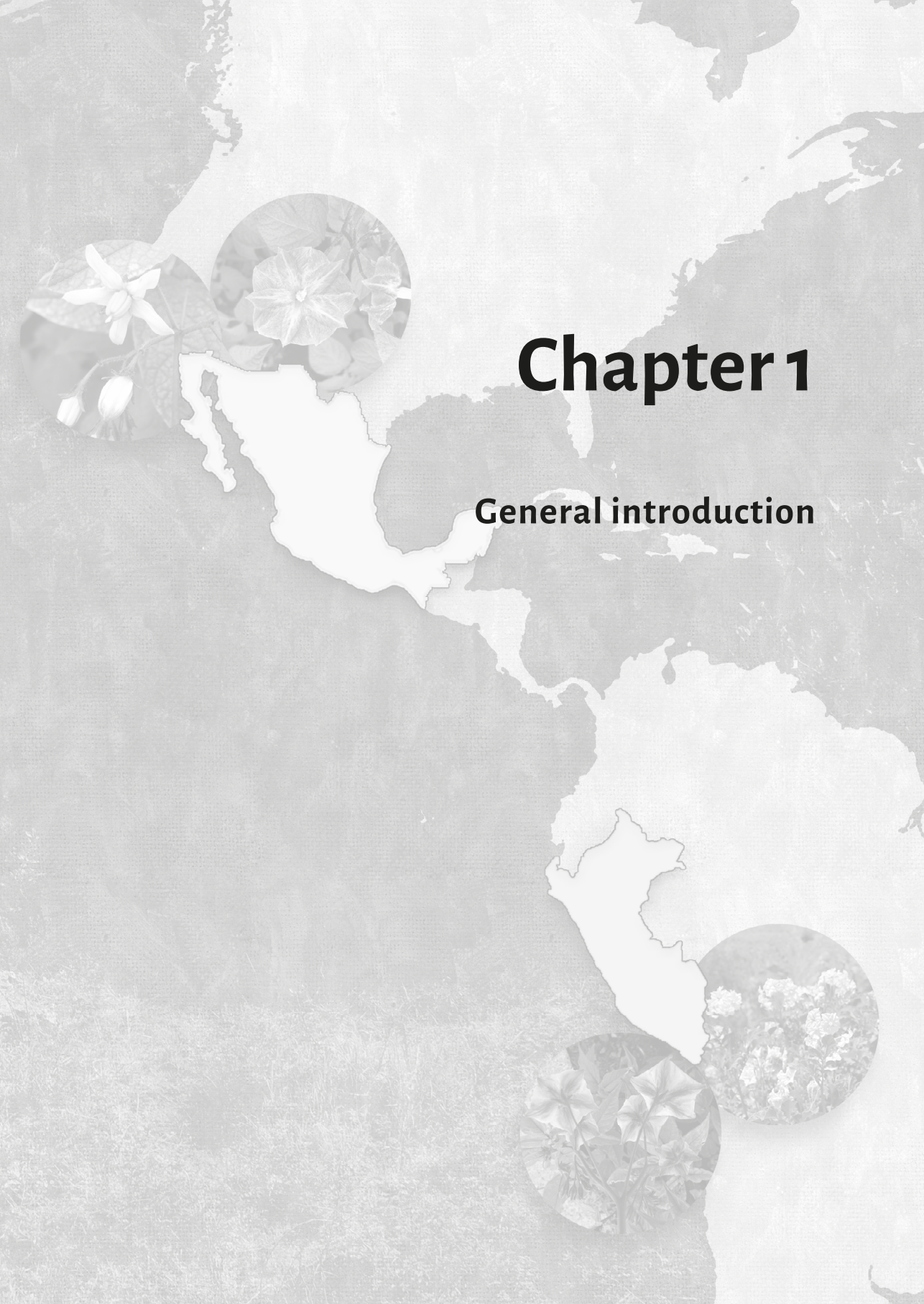

Chapter 1

General introduction

The potato crop

Potato (*Solanum tuberosum* L) is the most important non-cereal crop worldwide. It has been recommended as a food security crop because of its high yield and excellent nutritional value (Jansky & M Spooner, 2018).

Wild and cultivated potatoes are grouped in the genus *Solanum* section *Petota* (Hawkes, 1990). It has been proposed that the ancestral wild species were diploids and originated in Central America. Subsequently, these species migrated to South America where they were introduced to the Andes Mountains. Later, some of these species re-migrated to North and Central America. The hybridization and allopolyploidization with species that are native to Mexico or Central America led to species with different ploidy levels (Hawkes, 1990).

The taxonomy of the section *Petota* has been difficult due to its hybridization and morphological plasticity. The first comprehensive taxonomic treatment was provided by Hawkes (1990). It recognized seven cultivated species and 228 wild species divided in 21 taxonomic series. Currently, the number of species has been reduced to 107 wild and four cultivated species, and it continues to be refined (Spooner *et al.*, 2014). Overall, wild *Solanum* species provide a rich source of genetic variation. Mexico and the Andes in South America have been recognized as the two centers of diversity. Wild species from these locations, especially from Mexico have been widely used for over 100 years in potato breeding. However, only a small sample of the available diversity has been exploited. The incorporation of more diversity in breeding programs would help to expand the repertoire of sources for different agronomic traits, such as disease resistance. (Spooner & Hetterscheid, 2007; Machida-Hirano, 2015).

Phytophthora infestans: the causal agent of potato late blight

Potato is vulnerable to several diseases. Late blight caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary is the most destructive. In Ireland, it 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 remains 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).

P. infestans is a near-obligate hemi-biotrophic pathogen that needs living plant tissue for the initial colonization phase and it switches to a necrotrophic phase during later infection. The life cycle of this pathogen begins with the production of sporangia, which germinate either directly at high temperatures (20-25°C) or indirectly by producing zoospores at lower temperatures (10-15°C). The zoospores are motile for a short time and encyst on the host surface. The cysts and sporangia can germinate on the leaf surface and form an appressorium to penetrate the leaf. Upon host cell penetration, an infection vesicle is formed inside the

plant cell, from which hyphae emerge, enter the apoplast and grow in between the mesophyll cells. From these ramifying hyphae, haustoria, are formed and penetrate the mesophyll cells. This intimate interface with the host cell is also the site of secretion of various effector proteins (Avrova *et al.*, 2008; Whisson *et al.*, 2016). In the final stage, sporangiophores and sporangia are formed and the pathogen switches to a necrotrophic stage where the plant cells can be killed. This asexual reproductive cycle of *P. infestans* cycle is completed rapidly and is repeated multiple times during one growing season leading to devastating epidemics (Fry, 2008). For the sexual reproduction of *P. infestans* two mating types, A1 and A2, co-occur. The hyphal structures antheridia and oogonia are formed and fused together to create oospores. Oospores are thick-walled spores with the ability to survive during winter and cause infection (Judelson & Blanco, 2005). The occurrence of the sexual reproduction in *P. infestans* populations has contributed a lot to the genetic variation of the pathogen (Drenth *et al.*, 1994).

The multilayered plant immune system

Plants have evolved a sophisticated immune system to defend themselves against numerous pathogens such as bacteria, fungi, oomycetes, viruses and nematodes. Pathogens secrete apoplastic effectors and enzymes to promote virulence. Some of these enzymes damage the cell membranes, or bind to molecules that are required for pathogen growth (e.g. necrosis and ethylene inducing peptide1-like proteins (NLPs), endo-polygalaracturonases (ePGs) and elicitins) (Bishop *et al.*, 2005; Oome *et al.*, 2014; Derevnina *et al.*, 2016). These molecules are known as pathogen-, microbe- or damage-associated molecular patterns (PAMPs/MAMPs/DAMPs) and induce immune responses in the first layer of defense. Examples of well conserved apoplastic effectors of *Phytophthora* species are elicitins (Derevnina *et al.*, 2016). Some apoplastic effectors of *P. infestans* are highly diverse such as the small-cysteine rich (SCR) proteins and the protease inhibitors EPI1, EPI10, EPIC1 and EPIC2 (Liu *et al.*, 2005; Tian *et al.*, 2007). Typically, apoplastic effectors are recognized by apoplastic immune receptors, such as pattern recognition receptors (PRRs), which are either receptor kinases (RLKs) or receptor-like proteins (RLPs). An example is the elicitin receptor (ELR) from *Solanum microdontum*) (Du *et al.*, 2015). The activation of this defense response leads to PAMP/MAMP/DAMP-triggered immunity (PTI, MTI, DTI) which confers basal immunity to adapted pathogens and non-host resistance to non-adapted pathogens (Fig. 1) (Couto & Zipfel, 2016; Boutrot & Zipfel, 2017).

To circumvent PTI, adapted pathogens secrete effector proteins in the cytoplasmic space. Inside the host cells, these effectors can be recognized by a highly polymorphic superfamily of nucleotide-binding (NB) and leucine-rich repeat (LRR) receptors (NLR) that are encoded by disease resistance (*R*) genes. This recognition activates a second layer of defense response known as effector-triggered immunity (ETI). The response often produces a form of programmed cell death known as a hypersensitive response (HR) (Fig.1) (Dodds & Rathjen, 2010).

Cytoplasmic effector proteins

Cytoplasmic effectors of oomycetes are mainly represented by the crinkling- and necrosis-inducing (CRN) effectors and the RXLR effectors. The latter class consist of modular proteins with an N-terminal signal peptide, a conserved Arg-X-Leu-Arg (RXLR) motif, which is required for translocation inside host cells, and a highly polymorphic C-terminal domain (Rehmany *et al.*, 2005; Whisson *et al.*, 2007; Win *et al.*, 2007). Some of the RXLR effectors are known to act as avirulence (Avr) factors when they are recognized by the corresponding resistance (R) protein inside the cell. Typically, *Avr* genes reside in gene-sparse regions and are up-regulated during the early biotrophic phase of infection. Examples of *Avr* genes of *P. infestans* are *Avr1*, *Avr2*, *Avr3a*, *Avr4*, *Avrblb1*, *Avrblb2* and *Avrvnt1* (Vleeshouwers *et al.*, 2011).

The AVR2 protein is a member of a highly diverse RXLR effector family of *P. infestans*. AVR2 accumulates at the site of haustoria formation and is up-regulated at an early stage of potato infection. The recognition of AVR2 is mediated by the *R2* family from Mexican wild *Solanum* species (Gilroy *et al.*, 2011). *R2*-mediated recognition of AVR2 leads to resistance to *P. infestans*. However, this resistance is overcome by *P. infestans* isolates that contain the virulent allele of AVR2. This allelic form is AVR2-like and differs in 13 amino acids with AVR2 (Gilroy *et al.*, 2011).

The AVR3a protein is another well-studied RXLR effector of *P. infestans*. AVR3a has two major allelic variants that encode for AVR3a^{KI} and AVR3a^{EM}. These allelic variants differ in two amino acids in the mature protein and are differentially recognized by R3a from *Solanum demissum* (Armstrong *et al.*, 2005). AVR3a^{KI} induces R3a-mediated resistance and confers avirulence to *P. infestans* isolates. In addition, AVR3^{KI} suppresses the cell death induced by the elicitin INF1 (Bos *et al.*, 2009; Bos *et al.*, 2010).

Similar to AVR2 and AVR3a, other RXLR effectors of *P. infestans* are found to trigger *R* gene-mediated cell death responses and they also suppress immune pathways in the plant (Wang *et al.*, 2019). The suppression of plant immune responses can be achieved in two ways: either by reducing the activity of a positive immune regulator, or by enhancing the function of a negative immune regulator. *Phytophthora infestans* has been shown to use both strategies (Whisson *et al.*, 2016). Whilst the RXLR effectors AVR3a, PexRD2, and Pi03192 reduce immune responses by means of interaction with positive regulators of immunity (Bos *et al.*, 2010; McLellan *et al.*, 2013; King *et al.*, 2014), Pi04089, Pi04314, Pi02860 and Pi17316 interact with negative regulators of immunity (Wang *et al.*, 2015; Boevink *et al.*, 2016; Yang *et al.*, 2016; Murphy *et al.*, 2018). The understanding of the function of RXLR effectors is important to improve the identification, functional characterization and deployment of *R* genes that recognize effectors which are required for the benefit of the pathogen.

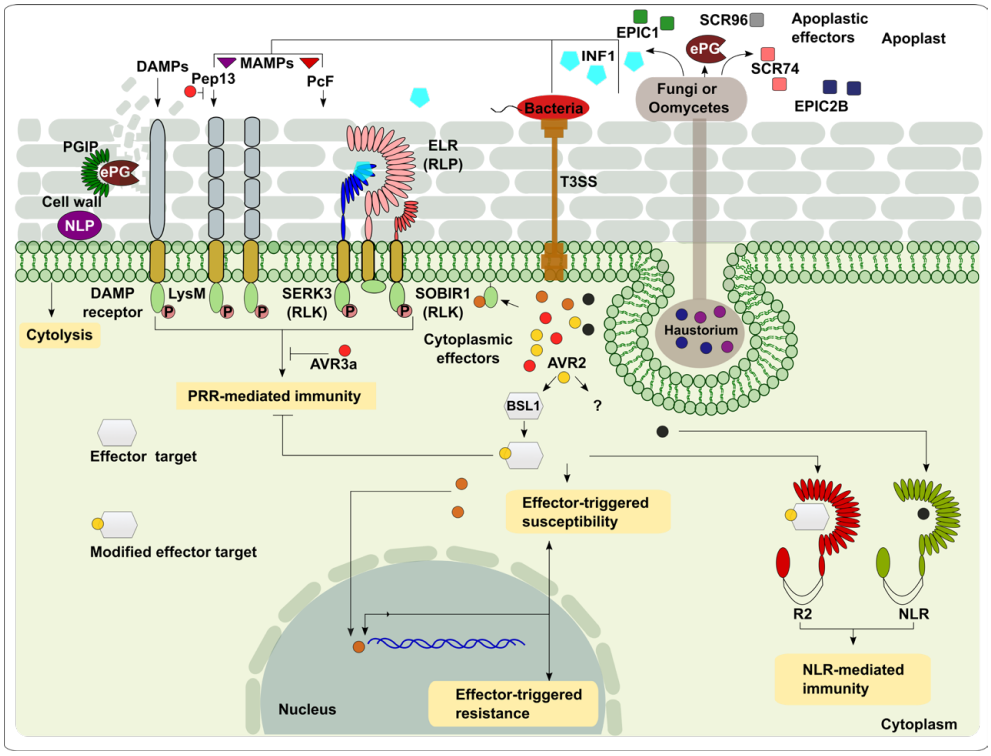

FIGURE 1 | Overview of the molecular players involved in plant-pathogen interactions (adapted from Wirthmueller *et al.* (2013)). Plant pathogens secrete apoplastic effectors (e.g. SCR74, SCR96, EPIC1 and EPIC2B) and cytoplasmic effectors (e.g. AVR2 and AVR3a) to manipulate different plant process. They also secrete enzymes, toxins and nutrient uptake proteins such as endo-polygalacturonasas (ePGs), the ethylene-induced Nep1-like proteins (NLP) and elicitins, respectively. Plants have evolved protease inhibitors (e.g. PG-inhibiting proteins (PGIPs)), cell surface recognition receptors (PRRs) (e.g. ELR), and cytoplasmic receptors (*R* genes) to trigger immune responses by recognition of microbe-associated molecular patterns (MAMPs) or apoplastic/cytoplasmic effectors. Effectors can also associate with specific host targets (e.g. brassinosteroid-insensitive1 suppressor-like1 (BSL1)) for the benefit of the pathogen. Some *R* genes, such as *R2*, indirectly recognize effectors by perception of host target modifications leading to *R*-mediate immunity. Plants may also sense self-derived components called damage-associated molecular patterns (DAMPs). Other abbreviations: phosphate (P), leucine-rich repeat (LRR), Lysin motif (LysM), Type III secretion system (T3SS), somatic-embryogenesis receptor kinase (SERK), suppressor or BIR1-1 (SOBIR1), BRI1-associated kinase (BAK1).

NLR proteins

NLRs are multi-domain proteins with a conserved architecture. Many of these proteins contain a variable N-terminal, a central nucleotide-binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain. The N-terminal domain exhibits either a Toll/interleukin-1 receptor-like domain (TIR) or a coil-coil (CC) domain (Meyers *et al.*, 1999; Pan *et al.*, 2000). The NB domain includes a conserved region ARC, which was found in Apaf-1 (apoptotic protease-activating factor-1) in humans, R proteins in plants and Ced4

(*Caenorhabditis elegans* death-4 protein) in nematodes (Van der Biezen & Jones, 1998). The LRR domain is typically composed of a variable number of repeats fitting the amino acid consensus sequence LxxLxxLxxLxLxxC/Nxx, in which the x represents amino acids available for interaction with ligands, and L represents any hydrophobic amino acid, such us (F, I, L, M or V) (McHale *et al.*, 2006). In addition, this domain has been considered to be the major determinant of specificity in NLR genes, that is caused by interallelic recombination and gene conversion that alter the organization of solvent-exposure residues in the LRR region (Michelmore & Meyers, 1998).

NLRs-gene complexes are highly dynamic, unstable and are organized in gene clusters which facilitates a rapid gene evolution. *NLRs* clusters are reservoirs of variation for resistance specificities and are required for the adaptation to new pathogen populations (Hulbert *et al.*, 2001). The *R2* cluster is located in a major late blight (MLB) locus on chromosome IV of potato and contains at least 10 members which display identical recognition specificity to AVR2 (Park *et al.*, 2005; Lokossou *et al.*, 2009; Champouret, 2010).

Virulence targets

Effectors of *P. infestans* can target host proteins to achieve virulence. Some of these proteins have been designated as susceptibility (S) factors because they are involved in activities that support pathogen infection, suppress or antagonize immunity and provide nutrition to pathogen growth (van Schie & Takken, 2014).

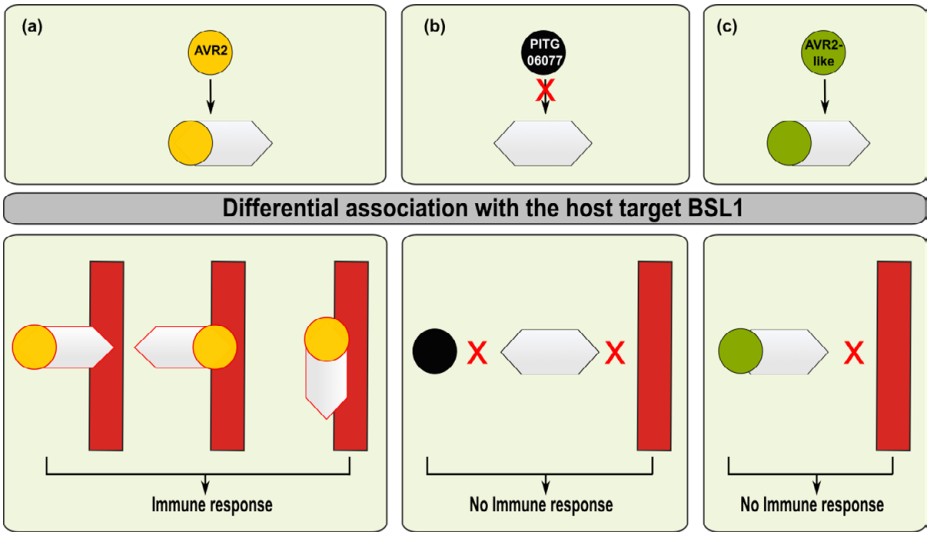

FIGURE 2 | Model illustrating the potential interaction of AVR2 family with BSL1 to promote R2-mediated recognition and resistance to *P. infestans* (Adapted from Saunders *et al.* (2012)). (a) AVR2 associates with BSL1 and R2 is activated by either an AVR2-modified BSL1, BSL1-modified AVR2, or AVR2-BSL1 complex. (b) PITG_06077 does not associates with BSL1 and R2 is not activated. (c) AVR2-like associates with BSL1 and R2 is not activated. BSL1 (white hexagons) and R2 (red rectangles) and potential modifications (highlighted in red) are indicated.

AVR2 of *P. infestans* targets BSL1 which is a homolog of the *Arabidopsis* brassinosteroid-insensitive1 suppressor (BSU1) and belongs to the phosphoprotein phosphatase (PPP) family of Serine/Threonine phosphatases (Mora-García *et al.*, 2004). The BSL family represent protein phosphatases with a kelch-like domain (PPKL) and they are implicated in growth-promoting brassinosteroid (BR) signaling in plants (Saunders *et al.*, 2012; Maselli *et al.*, 2014). In potato, the activation of BR-signaling by the BSLs proteins results in up-regulation of the transcription factor StCHL1 which leads to enhanced leaf colonization by *P. infestans* as well as suppression of Pattern-Triggered Immunity (PTI) responses. This points to the BSLs proteins as susceptibility (S) factors in late blight virulence and AVR2 to be a crucial player in the cross talk between BR-signaling and innate immunity in *Solanum* species (Turnbull *et al.*, 2017; Turnbull *et al.*, 2019). AVR2 and AVR2-like of *P. infestans* both interact with the potato BSL1 protein, however, only the interaction with AVR2 leads to R2-mediated recognition and resistance to *P. infestans* (Fig.2) (Saunders *et al.*, 2012).

Mechanisms of NLR activation

NLRs proteins can be activated directly by physical interaction with the pathogen effector following the 'gene-for-gene' model described by Flor (1971). In this model for a specific plant-pathogen interaction, the plant *R* gene matches with the corresponding *Avr* gene of the pathogen strain. Some direct interactions have been detected for the interaction between the flax rust fungus (*Melampsora lini*) and flax (*Linum usitatissimum*) (Dodds *et al.*, 2006; Catanzariti *et al.*, 2009), the rice blast fungus (*Magnaporthe grisea)* and rice (*Oryza sativa*) (Jia *et al.*, 2000), the bacterial wilt (*Ralstonia solanacerum*) and *Arabidopsis thaliana* (Deslandes *et al.*, 2003). For some of them, the physical interaction has been demonstrated to be required for resistance (Fig. 3).

For many of R-AVR interactions, physical interactions have not been observed and they are believed to be indirect interactions. The 'guard model' describes the indirect perception mechanism which requires the interaction with additional proteins in the host, namely the effector targets (Tameling & Takken, 2008). Such proteins can be genuine virulence targets or decoy proteins that have evolved to mimic the effector target (Fig. 3) (van der Hoorn & Kamoun, 2008). In filamentous pathogens, the perception of AVR2 of *P. infestans* by the *Solanum* R2 protein is the first evidence for an indirect recognition. AVR2 associates with the plant phosphatase BSL1 and mediates the interaction of BSL1 with R2 in planta (Saunders *et al.*, 2012). A third model has been proposed as an integrated decoy model. In this model, some NLRs protein present integrated domains (IDs) located at different positions in the classical NLR architecture. For instance, rice R proteins integrated a heavy metal-associated (HMA) domain that directly binds to unrelated *M. oryzae* effectors (Fig. 3) (Cesari *et al.*, 2013; Maqbool *et al.*, 2015).

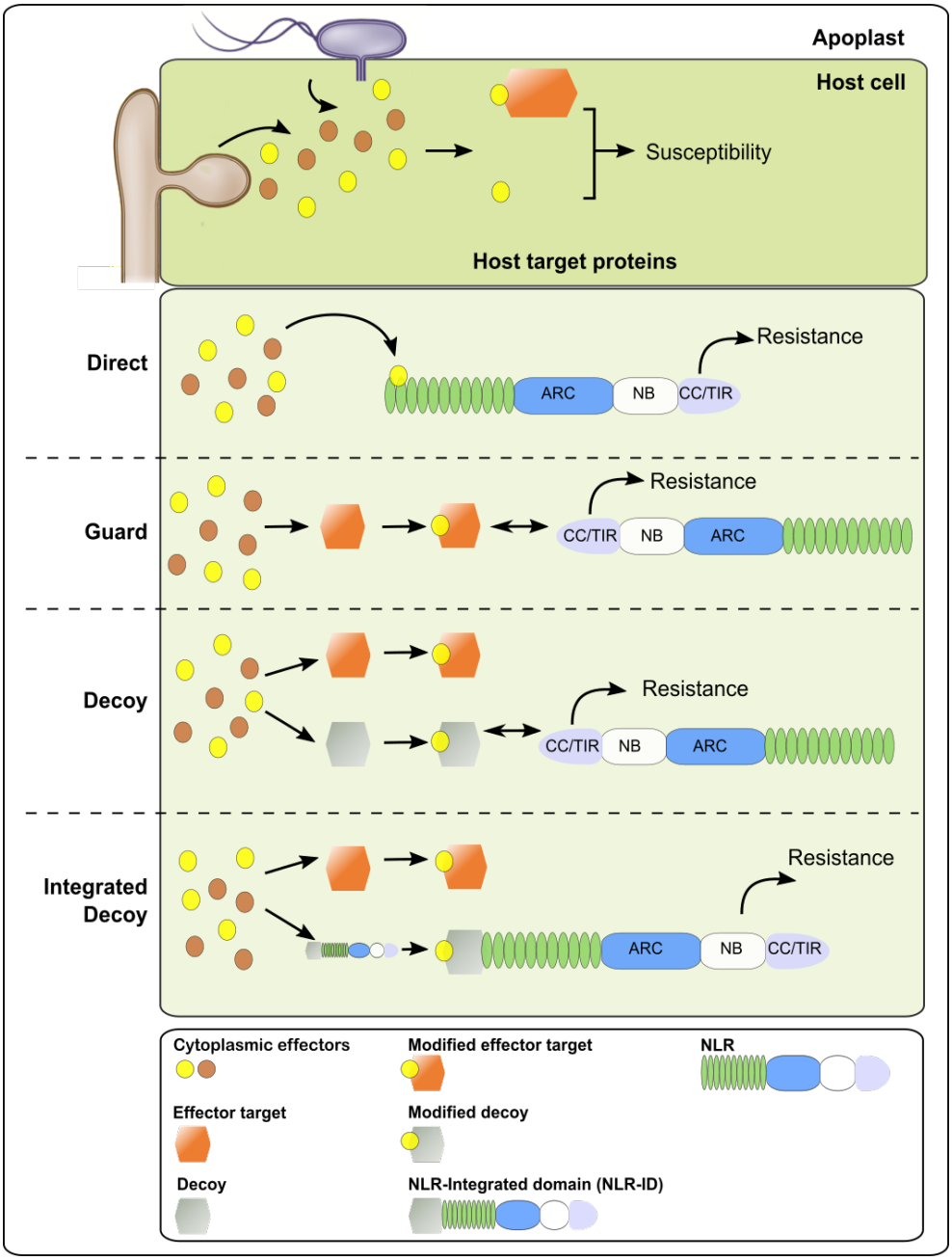

FIGURE 3 | A simplified view of the effector recognition inside the cell upon pathogen infection (adapted from Cesari (2018)). Pathogen effectors target and modify host proteins to promote susceptibility. NLR-mediated recognition of effectors induces resistance by either direct or indirect recognition which involves the interaction with effector targets or decoy proteins. Decoys are in some cases integrated in the NLR structure allowing the direct binding with the effector.

Breeding for disease resistance to *P. infestans*

Breeding for disease resistance has been focused on the introgression of *R* genes from wild *Solanum* species into potato cultivars. *R1* to *R11* from the Mexican *Solanum demissum* were the first introgressed *R* genes with showed successful resistance to *P. infestans* (Malcolmson & Black, 1966). However, these resistances have been defeated by new adapted *P. infestans* isolates (Hein *et al.*, 2009). Effectoromics has accelerated the identification of *Avr* genes in *P. infestans* and the cloning of *R* genes from wild *Solanum* species. For instance, this tool has been applied to identify *R* gene homologs (*RGH*) with differential recognition specificities to AVR proteins of *P. infestans*. This identification is crucial to avoid extensive efforts in the cloning of *R* genes with similar specificity of recognition. In addition, functional assays of *R* gene candidates can be assessed efficiently when the corresponding *Avr* gene is available. Finally, the knowledge of effectors can be used to detect effector diversity in pathogen populations and therefore, assist breeders in the evaluation of the *R* gene potential (Vleeshouwers *et al.*, 2011). Nowadays, effectoromics is complemented with new sequencing technologies to efficiently map and clone *R* genes from wild *Solanum* species (e.g. the resistance gene enrichment sequencing (RenSeq)) (Jupe *et al.*, 2012; Witek *et al.*, 2016).

Scope of this thesis

The knowledge about the molecular R- AVR interactions is crucial for a more educated exploitation of *R* genes in the *P. infestans- Solanum* pathosystem. In this thesis, I focussed my research on AVR2 and its interaction with wild *Solanum* species. This project started with a functional screening for cell death responses to AVR2 in a highly diverse set of wild *Solanum* genotypes. We found two different geographic locations, Mexico and Peru in which native *Solanum* genotypes recognize AVR2. The recognition of AVR2 in Mexican *Solanum* species is mediated by the *R2* family, which is located on chromosome IV of potato. **In chapter 2**, we investigate the recognition of AVR2 in *Solanum* species from Peru. We found a new *R* gene family which resides on chromosome IX and we cloned three members of this family that confer resistance to *P. infestans*. **In chapter 3**, we study the relationship between the AVR2-recognizing genotypes from Mexico and Peru and also the relationship among the *R* genes that were cloned from those genotypes. We investigate the race-specificity to *P. infestans* of the Mexican and Peruvian *R* genes. **In chapter 4**, we search for proteins related to AVR2. We found that AVR2 belongs to a highly polymorphic gene family in *P. infestans* and we studied the genetic diversity of AVR2 family in a set of *P. infestans* isolates. In **Chapter 5**, we further study the differential signaling pathways that are activated upon recognition of AVR2 by *Solanum* R proteins. **Chapter 6** summarizes and discusses the main findings of this thesis. In addition, the future prospects in breeding for resistance to *P. infestans* are discussed.

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Chapter 2

Rpi-mcq1 represents a new family of resistance genes against *Phytophthora infestans* from South American wild *Solanum* species

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Abstract

- Potato late blight, which is caused by the destructive oomycete pathogen *Phytophthora infestans*, is a major threat to global food security. Several nucleotide binding site-leucine-rich repeat (NLR) genes against *P. infestans* (*Rpi*) have been introgressed into potato cultivars from wild *Solanum* species that are native to Mexico, but these were quickly defeated.
- A map-based cloning approach in *Solanum mochiquense*, combined with allele mining in *Solanum huancabambense* were used to identify a new family of *Rpi* genes from Peruvian *Solanum* species. The *Rpi* genes were characterized by disease tests with *P. infestans* isolates and effector assays.
- *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* confer race-specific resistance to *P. infestans* isolates. The Rpi-mcq1 family mounts a hypersensitive response upon recognition of the RXLR effector AVR2, which had previously been found exclusively to be recognized by R2 resistance proteins. *Rpi-mcq1* and *R2* are sequence-unrelated and reside at different *R* loci on chromosome IX and IV, respectively.
- This is the first report of two unrelated R protein families that recognize the same AVR protein. We anticipate that this likely is a consequence of a geographically separated dynamic co-evolution of *R* gene families of *Solanum* with an important effector gene of *P. infestans*.

Key words: *Avr2*, co-evolution, chromosome IX, *Phytophthora*, potato, resistance gene, *Rpi-hcb1*, *Rpi-mcq1*.

Introduction

Potato (*Solanum tuberosum* L.) is the most important non-cereal crop directly consumed worldwide, and plays a pivotal role in food security. To date, the major threat for potato production is the devastating late blight disease, which is caused by the Irish famine pathogen *Phytophthora infestans* (Fry, 2008). Breeding for resistance to late blight began in the 1850s, when high levels of resistance were found in wild *Solanum demissum* that is native to the highlands of Toluca Valley in Mexico (Muller & Black, 1952). As a result of a tight co-evolution between wild *Solanum* and *P. infestans* in this center of genetic diversity, a wealth of resistance (*R*) genes have evolved in Mexican *Solanum* species (Hawkes, 1990; Fry *et al.*, 1993; Hijmans & Spooner, 2001; Grunwald & Flier, 2005). Identified *R* genes include *R1-R11* from *S. demissum*, and one or multiple members from the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* family from *S. bulbocastanum* and *S. stoloniferum*, *Rpi-amr3* from *S. americanum*, and few more (Vleeshouwers *et al.*, 2011b; Witek *et al.*, 2016). Unfortunately, all *R* genes identified so far have failed to provide durable resistance to the '*R* gene destroyer' *P. infestans* (Fry, 2008). A second center of genetic diversity of *P. infestans* and tuber-bearing *Solanum* species occurs in South America (Hijmans & Spooner, 2001). More recently, new *R* genes from those regions have been isolated as well, such as the *Rpi-vnt1* family from *Solanum venturii* in Argentina and *Rpi-ber* family from Bolivian *S. chacoense*, *S. berthaultii* and *S. tarijense* (Sliwka *et al.*, 2012; Jo *et al.*, 2016). In addition to these, various other South American wild *Solanum* species are resistant to *P. infestans* and could provide useful *R* genes against late blight (Vleeshouwers *et al.*, 2011a).

The oomycete pathogen *P. infestans* secretes an arsenal of effectors in order to manipulate host defense responses. Cytoplasmic effectors are typically members of the RXLR class, consisting of modular proteins with an N-terminal signal peptide, a conserved Arg-X-Leu-Arg (RXLR) motif required for translocation inside of the host cells and a highly polymorphic C-terminal domain (Whisson *et al.*, 2007). *Avr2* of *P. infestans* is a well characterized RXLR effector and member of a highly diverse gene family. AVR2 targets the plant phosphatase BSL1 that is implicated in positive regulation of the brassinosteroid (BR) signaling. BR-signaling elevates expression of the transcription factor CHL1, which acts as a negative regulator of immunity. AVR2 was found to play an important role promoting *P. infestans* virulence and suppressing the effect of Pattern-triggered immunity (PTI), and therefore, *Avr2* is considered an important effector gene and thus drives selection for *R* genes that recognize it (Gilroy *et al.*, 2011; Turnbull *et al.*, 2017).

R2 resistance genes which mediate recognition of AVR2 have been cloned from a major late blight resistance locus (MLB) on the short arm of chromosome IV (Park *et al.*, 2005; Lokossou *et al.*, 2009). Ten *R2* homologs were identified from the Mexican *S. bulbocastanum, S. demissum*, *S. edinense*, *S. hjertingii*, and *S. schenckii* (Champouret, 2010; Aguilera-Galvez *et al.*, 2018). In addition to these, AVR2 was recently found to be also recognized by *Solanum*

mochiquense (mcq) and *Solanum huancabambense* (hcb) that originate from Peru (Aguilera-Galvez *et al.*, 2018). The activation of hypersensitive cell death responses upon delivery of AVR2 suggests that additional *R* genes are present in these *Solanum* species.

In this study, we focused on a new family of *Rpi* genes from the Peruvian *S. mochiquense* and *S. huancabambense*. *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* were cloned by map-based cloning in combination with a gene allele mining approach. The newly isolated *Rpi* genes encode NLR proteins and are sequence-unrelated with the *R2* family and confer resistance to *P. infestans* isolates. Positive selection analysis shows that diversifying selection is acting in those genes, which may be a consequence of an independent molecular arms race between wild populations of *P. infestans* and the three *Solanum* species from South America.

Materials and Methods

Plant materials

The interspecific *Solanum mochiquense* BC1 mapping population was developed by crossing the *P. infestans*-resistant parent A988 (CGN18263) and susceptible parent A966 (CGN17731) (Smilde *et al.*, 2005). The seeds were routinely treated with 1000 ppm gibberellic acid (GA3) for 24 hours to break dormancy and were sown on MS20 medium. Recombinants seedling plants were transferred to greenhouse and treated regularly with fungicides and pesticides to control other pests as described previously by Foster *et al.* (2009).

DNA isolation and sequencing

DNA isolation was performed using a Retch protocol (Park *et al.*, 2005). BAC and cosmid clone DNA were isolated using the Qiagen Midi Prep Kit ® (Qiagen). BACs and cosmids ends, PCR products and shortgun clones were sequenced using the ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystens) using manufacturer instructions.

PCR-based marker development

The PCR-based markers used in this study are listed in supporting information, Table **S1**. Cleaved amplified polymorphic sequence (CAPS) markers T0156 and TG328 were developed according with Smilde *et al.* (2005). TG591N and S1D11 were developed from restriction fragment length polymorphism (RFLP) markers annotated on Tomato-EXPEN map or Potato Maps (Riaño-Pachón *et al.*, 2009). Blast searches with the tomato gene sequences were performed on the *Solanum tuberosum* unigenes database at Sol genomics network (Fernandez-Pozo *et al.*, 2015) and the obtained Expressed Sequenced Taq (EST) homologs were used for CAPS markers development. U286446, U296361 and U272857 were developed from a released tomato BAC sequence (HBa_165P17) which overlaps with the *Rpi-mcq1* region.

The CAPS marker 9C23R was developed from the BAC end sequence of the candidate 9C23 BAC clone. PCR products from resistance bulks and susceptible bulks were sequenced for each pair of primers. The identified SNPs were used to develop additional CAPS markers which were mapped on chromosome IX by using the 163 recombinants identified with T0156 and S1D11.

BAC library construction and screening

A BAC library was constructed with pIndigoBAC-5 (*Hind III*-Cloning Ready) for a heterozygote resistance plant K182 carrying the *Rpi-mcq1* followed the instrument (Epicentre, WI, USA). The library is approximately 9× coverage with an average insert size of 85 kb. The BAC library was pooled for each 384-well plate and screened by PCR using the flanking and co-segregating makers. Positive clones were validated by singleton PCR.

Allele mining

The *Rpi-mcq1_RGH* primers including the CACC site for Gateway® cloning purposes, were designed on *Rpi-mcq1* sequence (Supporting information, Table S1). Genomic DNA of the resistant genotype hcb 353-8 was used as template in a PCR reaction (98C: 30"', 25X [98C: 10"', 64C: 30"', 72C: 1'30"] 72C; 10"') using Phusion® high-fidelity DNA polymerase (Thermo Fisher Scientific). Amplicons were separated on agarose gel and purified using the QIAquick Gel Extration Kit® (Qiagen). Purified products were cloned in pENTR/D-TOPO® (Invitrogen) using DH10B *E. coli* competent cells. The inserts of the entry clones were checked by PCR and Sanger sequencing. Unique sequences were transferred to the pK7WG2 destination vector (Karimi *et al.*, 2002) by an LR reaction using LR-clonase II® (Invitrogen) and plasmids were used for *A. tumefaciens* transformation strain AGL1.

Binary vectors for *Agrobacterium tumefaciens*-mediated transient transformation

Avr2, *Avr3a*, *Rpi-hcb1.1* and *Rpi-hcb1.2* were cloned in the binary vector pK7WG2 (Karimi *et al.*, 2002). *R2*, *Rpi-mcq1* and *R3a* were previously cloned in the binary vector pBINPLUS (van Engelen *et al.*, 1995). The NLR candidate genes cosA1, cosA2 and cosD5 were cloned in the binary cosmid vector pCLD04541 (Tao & Zhang, 1998).

Agroinfiltration

Agroinfiltration was performed as described by Domazakis *et al.* (2017). Briefly, young and fully expanded leaves of 4-5-week-old potato and *N. benthamiana* plants were used. A suspension of *A. tumefaciens* strain AGL1 containing the appropriate expression vectors at an OD_{600} of 0.2 for potato and at an OD_{600} of 0.5-1 for *N. benthamiana* were infiltrated in leaf panels. Three plants per genotype and three leaves per plant were used in two biological replicates. Local symptoms were assessed at 3-4 dpi. The percentage of cell death was quantified using scores of 0%, 25%, 50%, 75% and 100%, based on observation of the infiltrated area.

Transient complementation

Agroinfiltration of *A. tumefaciens* strains carrying *Rpi-mcq1*, *Rpi-hcb1.1* or *Rpi-hcb1.2* were performed in four-week-old *N. benthamiana* plants according with method described by Domazakis *et al.* (2017). Two days after inoculation, infiltrated leaves were detached and inoculated with *P. infestans* isolates IPO-0 and IPO-C (Supporting information, Table S2). Infection symptoms were scored between 4-8 dpi.

Transformation of potato cv. 'Désirée' and tomato cv. 'Moneymaker'

A. tumefaciens-mediated stable transformation was performed with potato cv. 'Désirée' and tomato cv. 'Moneymaker'. The cosmid vector pCLD04541 harboring the cosmids A2, A1 and D5 under the control of their native promoters, were used in potato and tomato transformation to test the function of the NLR candidate genes. pK7WG2:*Rpi-hcb1.1* and pK7WG2:*Rpi-hcb1.2* under the control of 35S promoter were used in potato transformation to test the function of *Rpi-hcb* genes. The transformation was performed using routine transformation protocols (Fillatti *et al.*, 1987). Transformants were selected after growth in greenhouse conditions (18-22°C, 16 h of light and 8 h of dark).

Phytophthora infestans isolates, culture conditions and inoculum preparation

The *P. infestans* isolates used in this study are listed in supporting information, Table S2. Isolates were retrieved from our in-house stock collection. Isolates were 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, sporulation mycelium was flooded with cold water and incubated at 4°C for 1-3 hours.

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 10µl droplets containing $5^{*}10^{4}$ zoospores per ml (in tap water). 12 inoculations in each leaf were used for potato (Désirée) and tomato (Moneymaker) transformants and 3 inoculations for *N. benthamiana* experiments. In all the cases, from three to five leaves per isolate and three independent experiments were performed. After inoculation, the trays were incubated in a climate chamber at 15°C with a 16h photoperiod. Development of lesions and presence of sporulation was determined at 5-6 dpi. Disease index was estimated using a scale from 1 to 9, ranging from expanding lesions with massive sporulation (1 to 3, susceptible), sporulation no clearly visible (4), sporadic sporulation only visible under the microscope (5), lesion with a diameter size ≥ 10 mm (6), occurrence of hypersensitive response (HR) between 3 to 10 mm (7), less abundant sporulation and smaller lesions, occurrence of HR (8, resistant) and to no symptoms (9, fully resistant).

Phylogenetic and positive selection analysis

A UPGMA-based tree was generated with the full amino acid sequence of 16 proteins, including Rpi-mcq1 and R2 family members. Bootstrap value was set equal to 100. The obtained UPGMA-based tree was displayed as circular unrooted cladogram using Geneious®9.1.2.

To test for amino acid under purifying or diversifying selection, a codon-based analysis was conducted using PAMLX1.3.1 package (Xu & Yang, 2013). Maximum-likelihood codon substitution models M0, M1, M2, M7 and M8 were used for the analysis. Positively selected sites detected by, Models M2 and M8 were identified using Empirical Bayes Statistics (Yang *et al.*, 2005).

Results

High resolution mapping and cloning of *Rpi-mcq1*

Rpi-mcq1 (formerly named *Rpi-moc1*) was previously mapped to a region of 15.8 cM on chromosome IX, flanked by the CAPS marker T0156 and an AFLP marker (Smilde *et al.*, 2005). In order to fine map *Rpi-mcq1*, a BC1 mapping population from *S. mochiquense* accessions A988 (CGN 18263) and A966 (CGN 17731) was developed. A recombinant screen on 2502 individuals from the mapping population using the flanking markers S1D11 and T0156 was performed. 163 recombinant individuals were identified and characterized for resistance to *P. infestans* isolates 90128 and EC1, resulting in resistant and susceptible bulks. Subsequently, five CAPS markers including TG328, U286446, U296361, TG591N and U272857 were screened on the recombinants (Supporting information, Table S1). *Rpi-mcq1* was mapped to a narrow region flanked by U286446 and S1D11, and co-segregates with TG591N and U282757 (Supporting information, Fig. S1). U286446, TG591N and U282757 were tested on a BAC library, and two overlapping BAC clones 9C23 and 43B09 derived from the resistant haplotype were identified (Fig. 1a). A new CAPS marker, 9C23R, was obtained from the end sequence of BAC clone 9C23 and was screened, revealing segregation of *Rpi-mcq1* with six recombinants in the bulks. The marker 9C23R is located on the opposite orientation of *Rpi-mcq1* at a distance of 0.24 cM (Fig. 1a, supporting information, Fig. S1). *Rpi-mcq1* was fine-mapped on the two overlapping BACs, 9C23 and 43B09. Subsequently, the two BACs were sequenced by TIGR and as a result two contigs that shared 62,395 and 114,083 bp, respectively, were obtained. In total eight ORFs were predicted in the two contigs, including four *Tm2²* - like NLR candidate genes and four non-NLRs, including a putative NAD dependent epimerase, a RNA-directed DNA polymerase, a retransposon, and a protein with unknown function. Two NLR candidate genes, cosA2 and cosA1, have full length ORFs with ~80-85% identity to *Tm2²* at the amino acid level. A third NLR candidate

gene, cosD5, represents a partial NLR with a truncated Coil-Coil (CC) domain. The fourth NLR candidate gene, cosE7, contains an early stop codon and was omitted from further study.

Rpi-mcq1 confers late blight resistance in potato and tomato

To determine whether the genes cosA2, cosA1 and cosD5 confer resistance to *P. infestans* in potato and tomato, stable potato and tomato transformants cv. 'Désirée' and cv. 'Moneymaker', respectively, were generated. In total, 22, 24, and 20 independent primary transformants were obtained in potato that express the cosA2, cosA1 and cosD5 genes, respectively, and 8, 7, and 10 independent tomato transgenic lines were obtained. Following transfer to the greenhouse, a detached leaf assay (DLA) was performed on putative transformants and in the wild type 'Desiree'. Detached leaves of the selected lines were inoculated with the avirulent *P. infestans* isolates EC1 and 90128 (Supporting information, Table S2). Macroscopic observations were carried out at 6 days post inoculation (dpi). On potato, 13 out of 22 putative transformants derived from cosA2 showed hypersensitive response (HR) and were resistant to the tested *P. infestans* isolates, whereas abundant sporulation was found in the 44 putative transformants derived from cosA1 and cosD5 and in the wild type 'Désirée' (Fig. 1b). Upon inoculation with the virulent *P. infestans* isolate IPO-C (Supporting information, Table S2), all plants were infected (Fig. 1b). Consistent with these results, only the putative transformants derived from cosA2 showed race-specific resistance to EC1 and 90128 in tomato cv. 'Moneymaker' (Supporting information, Fig. S2). Altogether, the results indicate that cosA2 can complement the late blight susceptible phenotype in potato and tomato, and we designated it *Rpi-mcq1*.

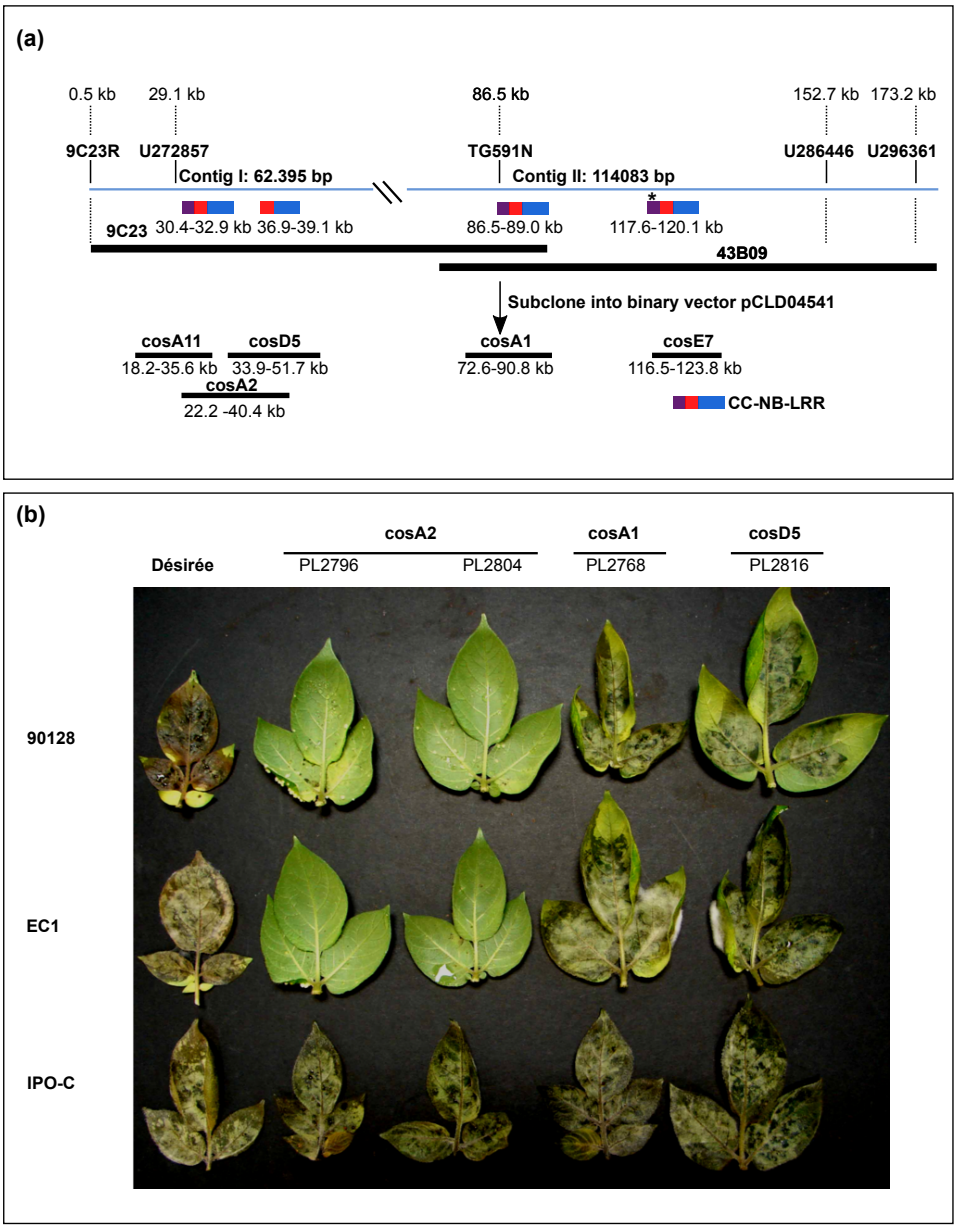

FIGURE 1 | Physical map of the *S. mochiquense* genomic region containing *Rpi-mcq1* and genetic complementation. (a) Black rectangles represent bacterial artificial chromosome (BAC) clones (9C23 and 43B09) covering the region of *Rpi-mcq1* and cosmid clones carrying the NLR candidate genes. Blue lines indicate two contigs sequences from two BAC clones. Colored segments represent four NLR candidate genes, two of them encoded a complete CC-NB-LRR genes, represented as cosA2 and cosA1, respectively. Asterisk indicates an early stop codon in one NLR candidate gene. (b) Representative pictures of the disease symptoms of potato transgenic plants inoculated with *P. infestans* isolates 90128, EC1 and IPO-C, respectively. Pictures were taken at 6 dpi. PL2796 and PL2804 represent two transgenic lines obtained from cosA2 construct, PL2768 and PL2816 represent transgenic lines obtained with cosA1 and cosD5 constructs, respectively.

Solanum huancabambense recognizes AVR2 and is resistant to *P. infestans*

The *Rpi-mcq1* bearing *S. mochiquense* plant was previously shown to mount AVR2-specific cell death upon agroinfiltration (Aguilera-Galvez *et al.*, 2018). In these high throughput effector screens in wild *Solanum* germplasm, also *S. huancabambense* (hcb) was found to show AVR2-triggered cell death. To confirm the specificity of this response in independent experiments, *Avr2* was transiently expressed by agroinfiltration in the hcb 353-8 and in the wild type 'Désirée'. Single infiltrations of empty vector pK7WG2 and co-agroinfiltrations of *R3a*/*Avr3a* were included as negative and positive controls, respectively. Specific cell death responses to AVR2 were evident in hcb 353-8 and no cell death responses were identified on 'Désirée', suggesting the presence of an AVR2-recognizing R protein in hcb 353-8 (Supporting information, Fig. S3a).

To determine whether hcb 353-8 is resistant to *P. infestans*, a detached leaf assay was performed with a panel of 12 *P. infestans* isolates, from which 4 and 8 are virulent or avirulent on *R2* plants, respectively (Supporting information, Table S2). Macroscopic observations were carried out at 6 dpi. Hcb 353-8 showed a typical HR and was resistant to 8 isolates, while large sporulating lesions were found in the cv. 'Désirée' (Supporting information, Fig. S3b). Among the 12 isolates, resistance phenotypes on hcb353-8 were fully correlating with *R2*-specific resistance (Supporting information, Table S2). These results indicate that a race-specific *Rpi* gene is present in hcb 353-8, which exhibits similarities to AVR2-based resistance.

PCR-base cloning in hcb 353-8 reveals new variants of the *Rpi-mcq1* family

To investigate whether the *Rpi* gene in hcb 353-8 is homologous to *Rpi-mcq1*, a genetic analysis was performed. The resistant hcb 353-8 was crossed with the susceptible hcb 354-2 and together with 18 offspring genotypes were tested for resistance to *P. infestans* using the aggressive and the moderately aggressive isolates 90128 and IPO-0, respectively (Table S2). Detached leaves of the parents and the progeny were inoculated with *P. infestans* isolates. Macroscopic observations were carried out at 6 dpi. The susceptible hcb 354-2 was infected by both isolates, whereas hcb 353-8 showed HR and was resistant. No disease symptoms were found in ten genotypes of the progeny, whereas eight genotypes showed abundant sporulation and disease symptoms. This result confirms an expected 1:1 segregation ratio (x^2= 0.11, P=0.74), suggesting the presence of a single dominant *Rpi* gene in hcb 353-8 (Supporting information, Table S3a).

Subsequently, to determine the genetic position of the *Rpi* in hcb 353-8, a set of PCR markers that reside on LG IX of potato were tested on the F1 progeny (Supporting information, Table S1). The marker GP41 revealed polymorphism between the parents and progeny of the population and a complete co-segregation of marker patterns with *P. infestans* resistance was observed (Supporting information, Table S3b). Additionally, we tested the progeny

plants for AVR2 response by agroinfiltration and the response segregates with resistance to *P. infestans* (Supporting information, Table S3c). In summary, the *Rpi* in hcb 353-8 is located on LG IX and together with the AVR2-based race-specific resistance, we hypothesize that the *Rpi* gene in hcb 353-8 is homologous to *Rpi-mcq1*.

We followed a homology-based cloning approach to identify the *Rpi* gene. PCR with the conserved *Rpi-mcq1* primers (*Rpimcq1_RGH*) (Supporting information, Table S1) on genomic DNA of hcb 353-8 plants yielded amplicons of 2577 bp, which is the range of the expected size of an *Rpi-mcq1* homologue. Sequence analysis of the amplicons revealed five additional *Rpi-mcq1* gene homologues (*RGH*) from hcb 353-8 with amino acid identities from 79.7 to 91.6% between *RGH* and *Rpi-mcq1* (Fig. 2a, supporting information, Table S5). The phylogenetic relationship between *Rpi-mcq1*, *RGH* and the *R2* homolog *Rpi-blb3* was examined. A neighbor joining (NJ) tree grouped *Rpi-mcq1* and the *RGH* in a single clade, separate from *Rpi-blb3* (Supporting information, Fig. S5). This result confirms that the *Rpi-blb3*/*R2* and *Rpi-mcq1* families are sequence unrelated.

Two *RGH* respond to AVR2 and confer resistance to *P. infestans*

To test the function of the *RGH*, the five identified homologues were cloned in binary vector pK7WG2. *Rpi-mcq1* was previously cloned in the binary vector pBINPLUS (Aguilera-Galvez *et al.*, 2018). The constructs were transferred to *A. tumefaciens* strain AGL1. Co-agroinfiltrations were performed in potato cv. 'Bintje' leaves with *A. tumefaciens* expressing each *RGH* or *Rpi-mcq1* with *Avr2*. Single infiltrations of *Avr2*, *RGH* or *Rpi-mcq1* and empty vector were included as negative controls. Co-agroinfiltration of *R3a*/*Avr3a* was included as positive control. Two *RGH, i.e. RGH4* and *RGH5* induced specific cell death with AVR2 and we designated them *Rpi-hcb1.1* and *Rpi.hcb1.2*, respectively (Fig. 2b).

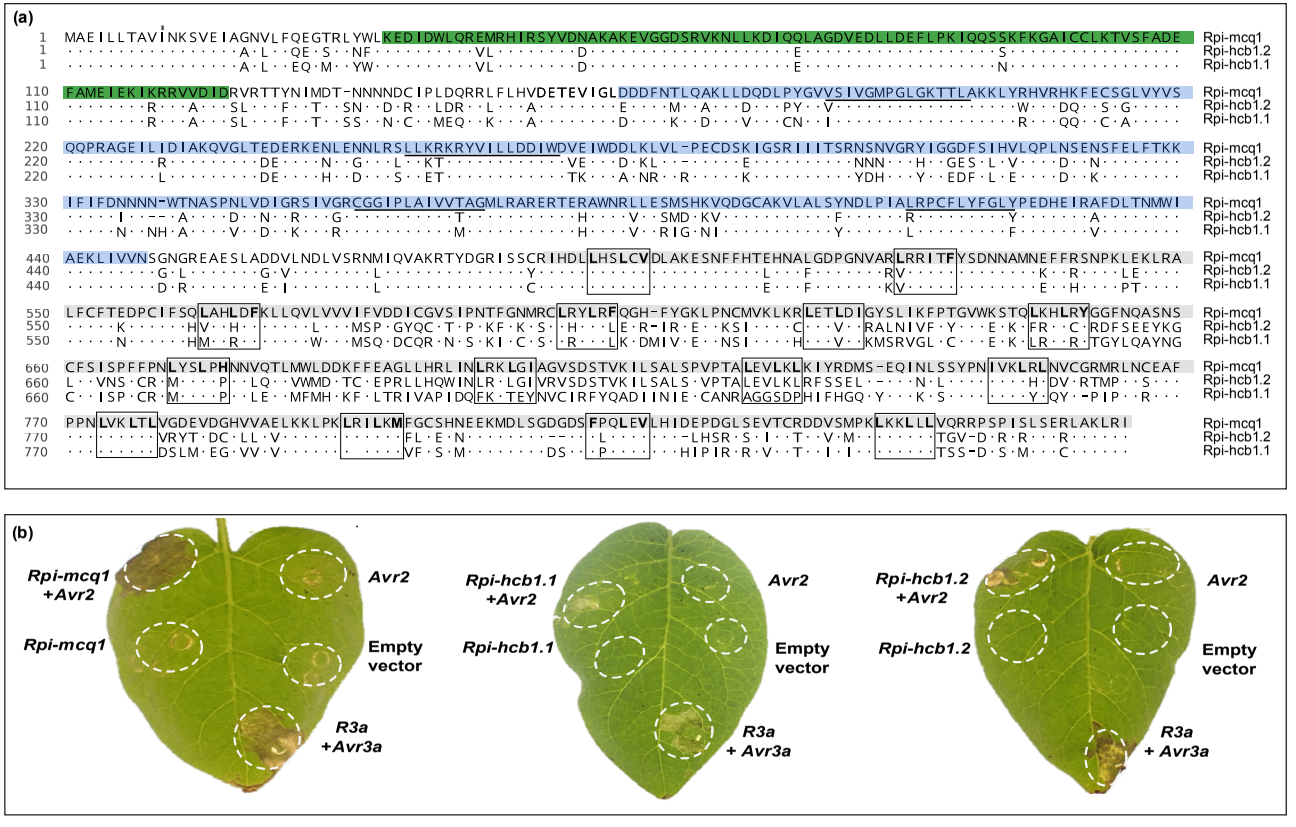

FIGURE 2 | Protein alignment of the Rpi-mcq1 family and their specificity of recognition to *Avr2*. (a) Multiple sequence alignment of Rpi-mcq1, Rpi-hcb1.1 and Rpi-hcb1.2, using the Rpi-mcq1 amino acid sequence as reference. The CC, NB-ARC and LRR domains are shaded in green, blue and grey, respectively. The characteristics conserved domains including the P-loop (Kinase 1a), Kinase 2, kinase 3 and the RNBS-D (resistance domain) in the NB-ARC are underlined. The 14 LRR repeats and the hydrophobic amino acids are represented with black rectangles and bold letters, respectively. (b) *Agrobacterium*-mediated expression of *Avr2* with *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* in potato cv. 'Bintje'. Single infiltrations of each *R* gene alone, *Avr2* and empty vector were performed as negative controls. Co-agroinfiltrations of *R3a*/*Avr3a* were included as positive control.

For assessing whether the *Rpi-hcb* genes confer resistance to *P. infestans*, a transient complementation assay was conducted. *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* were agroinfiltrated in *N. benthamiana* leaves. Additionally, agroinfiltrations with *Rpi-blb3*, empty vector, and infiltration medium (MMA) were used as a positive and negative controls, respectively. Two days later, zoospore suspensions of the avirulent IPO-0 and virulent IPO-C *P. infestans* isolates (Supporting information, Table S2) were spot-inoculated on the agroinfiltrated leaf panels (Aguilera-Galvez *et al.*, 2018). After 8 days, *Rpi*-mcq1, *Rpi-hcb1.1*, *Rpi-hcb1.2*, and *Rpi-blb3*-treated leaf panels display a HR to IPO-0 (race 0), whereas large expanding necrotic lesions surrounded by sporulation zone were observed on leaves with the complex race IPO-C. Leaf panels agroinfiltrated with the empty vector and MMA medium show large sporulating lesions for both isolates (Supporting information, Fig. S4). These data show that *Rpi-hcb1.1* and *Rpi-hcb1.2* confer a race-specific resistance to *P. infestans*, similar to *Rpi-mcq1*- and *Rpi-blb3*-mediated resistance.

Rpi-hcb1.1 and *Rpi-hcb1.2* confer resistance to *P. infestans* in potato stable transformants

To confirm the transient complementation assay by *Rpi-hcb1.1* and *Rpi-hcb1.2* in potato, stable potato transformants cv. 'Désirée' were generated that express the *Rpi-hcb* genes under the control of the 35S constitutive promoter. 18 *Rpi-hcb1.1* and 24 *Rpi-hcb1.2* independent primary transformants were selected and cultured in greenhouse. Subsequently, they were tested for AVR2 response by agroinfiltration. Four independent transgenic lines expressing *Rpi-hcb1.1* and five expressing *Rpi-hcb1.2* were selected. Detached leaves of the selected lines were inoculated with two avirulent *P. infestans* isolates PIC99183 and IPO-0 (Supporting information, Table S2). Stable transformed plants containing the *R* genes were resistant to both tested isolates. This result confirms that *Rpi-hcb1.1* as well as *Rpi-hcb1.2* confer resistance to *P. infestans* in potato (Fig. 3).

Rpi-mcq1 is under diversifying selection

The obtained *RGH* sequences harbor all characteristics of NLR genes, similar to *Rpi-mcq1*. Overall, the six *Rpi-mcq1* family members display considerable sequence conservation in the CC and NB-ARC domain. In contrast, the LRR domain, that is typically involved in effector recognition, contained multiple single nucleotide polymorphisms (SNPs). To get further insights on the region of *Rpi-mcq1* that is under diversifying selection, the PAML method was conducted (Yang *et al.*, 2005). Several positive selected amino acid residues were identified on the CC, the NB-ARC and the LRR domain (Supporting information, Table S5). Model M2 identified 11 amino acid residues under positive selection, 10 amino acids were present in the LRR domain and 1 in the NB-ARC domain. The selection model M8 predicted the same 11 amino acids identified in M2 model and 6 additional amino acids. 1 out of 6 was present in CC domain, and the other amino acids were present in the LRR domain,

indicating a strong diversifying selection on the LRR domain (Supporting information, Table S5). This result suggests that the polymorphisms in this region could be driven by the evolution of differential specificities of AVR recognition that is typically located in the LRR domain (Moffett, 2009).

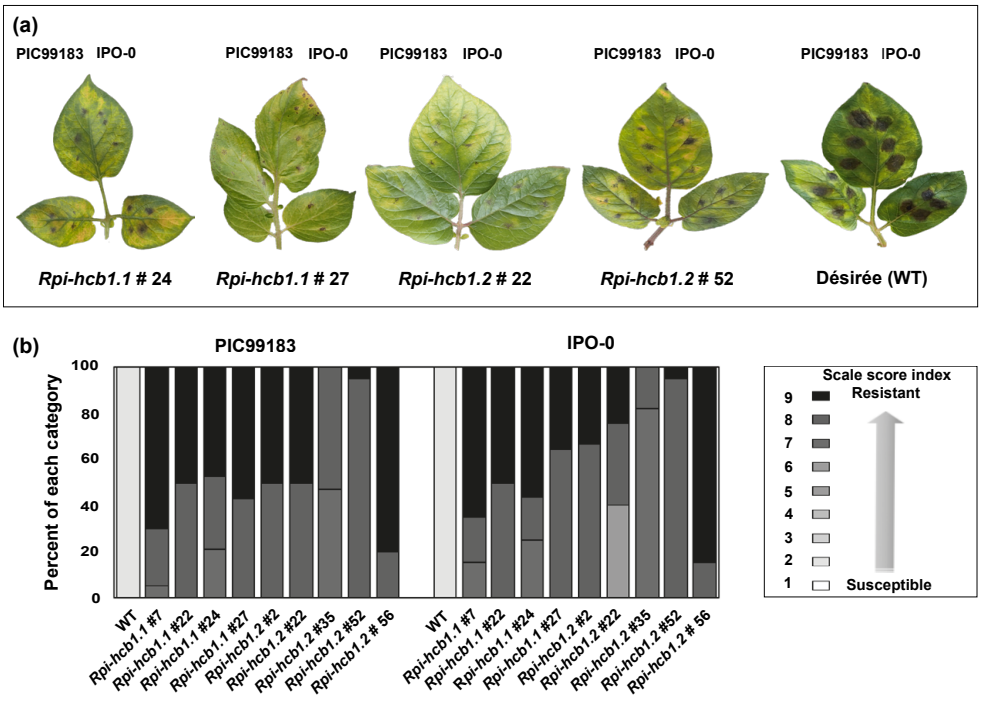

FIGURE 3 | Genetic complementation for late blight resistance. Four and five transgenic Désirée lines expressing *Rpi-hcb1.1* or *Rpi-hcb1.2*, respectively, are resistant upon inoculation of *P. infestans* isolates PIC99183 and IPO-O. (a) Representative photographs are shown for Désirée expressing *Rpi-hcb1.1* lines 24 and 27 and *Rpi-hcb1.2* lines 22 and 52, respectively, at six dpi. Wild type (wt) 'Désirée' represents a susceptible control. (b) Disease symptoms on 'Désirée' (WT), Désirée-*Rpi-hcb1.1* lines 7, 22, 24 and 27 and Désirée-*Rpi-hcb1.2* lines 2, 22, 35, 52 and 56, are scored on a scale from 1 (susceptible) to 9 (resistant).

Discussion

Despite cloning of a number of *Rpi* genes in the past years (Jo *et al.*, 2016), the fast-evolving *P. infestans* remains the most threatening pathogen of potato worldwide. To avoid losing the arms-race with this fast-evolving oomycete pathogen, it is necessary to intensify the search for new sources of resistance and extend the repertoire of available *Rpi* genes for breeding. The majority of introgressed resistance genes into potato cultivars, have been cloned from *Solanum* species native to Mexico, however, South America that is considered

the second center of genetic diversity has been much less explored. In this manuscript, we report the cloning and functional characterization of a new family of late blight resistance genes from the Peruvian *S. mochiquense* and *S. huancabambense*. *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* confer race-specific resistance to *P. infestans* and belong to the *Rpi-mcq1* locus on linkage group (LG) IX (Smilde *et al.*, 2005). The three *Rpi-mcq1* homologs share high levels of amino acid sequence identity, and we found that all three Rpi proteins mediate response to AVR2 of *P. infestans*.

Known *Avr* genes of *P. infestans* typically seem to have co-evolved with a single *R* locus in potato, such as *R3a* and *Avr3a* (Armstrong *et al.*, 2005; Huang *et al.*, 2005). However, AVR2 is the first example of an AVR protein that is recognized by two different, sequence-unrelated R protein families in tuber-bearing *Solanum* species (Aguilera-Galvez *et al.*, 2018). In Mexican *Solanum* species, AVR2 recognition is conferred by *R2* family on chromosome IV, whilst in Peruvian *Solanum* genotypes AVR2 response is mediated by *Rpi-mcq/hcb* family located on chromosome IX. Since *Avr2* has a strong virulence function and is broadly spread among the pathogen strains, multiple *R* gene families seem to have evolved to recognize this effector (Gilroy *et al.*, 2011; Turnbull *et al.*, 2017). Since the *R* gene families display dissimilar sequences, the *Solanum* species occur in different phylogenetic clades and the plants occur in different geographic regions, indicating that they have evolved independently (Aguilera-Galvez *et al.*, 2018). Our evolutionary analyses with Rpi-mcq/hcb sequence indicate that the LRR motif, which plays a pivotal role in effector recognition specificity, is shaped by a strong diversifying selection that could result in new specificities of effector recognition (McDowell & Simon, 2006; Champouret, 2010). Similar properties were found for the *R2* family (Champouret, 2010). These findings are in line with the concept of fast nucleotide evolution and sequence interchange between *R* gene homologs, as major mechanism shaping *R* gene diversity in plants, and especially targeted at the LRR (Meyers *et al.*, 1999; Scot H. Hulbert *et al.*, 2001).

The recognition of an AVR protein by different R proteins has been reported for other plant-pathosystems, however, their relationship has so far been unknown. For instance, *Avr3a/5* of *Phytophthora sojae* is recognized by *Rps3* as well as *Rps5* resistance genes from soybean from which the sequence is still unreported (Dong *et al.*, 2011). *Avr-Rmg7/8* of *Magnaporthe grisea* is recognized by the wheat resistance genes *Rmg7* and *Rmg8*, which are located in homeologous chromosomes 2B and 2A, respectively (Anh *et al.*, 2015). Furthermore, some rice *R* genes were recently found to interact with divergent *Magnaporthe oryzae* effectors via different binding surfaces (Guo *et al.*, 2018; Varden *et al.*, 2019). The effectors have evolved independently to unconventional *R* gene domains and target them with different binding-specificities (Białas *et al.*, 2017). These studies are providing more insights in the antagonistic interplay between pathogen and host that is driven by co-evolutionary forces targeted at *R* and *Avr* genes.

Pathogen populations in European countries, the USA and Canada have developed virulence to potato plants carrying the *S. demissum*-derived *R1*, *R3*, *R4*, *R7*, *R10* and *R11*. However, lower frequencies were noted for virulence on *R2*, and *R2* significantly delays the onset of epidemics in field trails (Andrivon, 1994; Pilet *et al.*, 2005). Since these characteristics heavily rely on the matching *Avr* gene, also *Rpi-mcq/hcb* are expected to display extended latency periods in practice and contribute to late blight resistance.

Our improved understanding of the recognition of *Avr2* by *R2* and *Rpi-mcq/hcb* confirms that effectors are a powerful tool to accelerate the identification of *R* genes. This study is sharpening the concept that AVR2-based breeding should be complemented with additional molecular techniques, e.g molecular markers and cloning to securely determine the identity of the matching *R* genes. Combination of *Rpi* genes from distinct loci but with similar race-specific resistance specificities, such as *R2* and *Rpi-mcq1*, could be more effective than using single *R* genes, however, a broader resistance spectrum is not expected based on AVR2 recognition alone. *Avr2* is member of a highly diverse gene family, and molecular studies on *Avr2* variants will reveal deeper insights underlying recognition specificities and pathogenicity in *P. infestans* populations. Such knowledge should contribute to wiser strategies for efficient deployment of *R* genes in potato.

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Supporting information

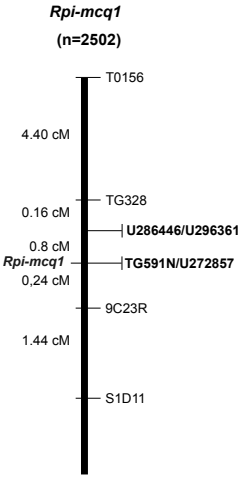

FIGURE S1 | High-resolution genetic linkage map of the *Rpi-mcq1* locus on chromosome IX. Genetic distances and marker names are indicated on the left and right of the map, respectively. *Rpi-mcq1* co-segregates with polymorphism makers TG591N and U272857, flanked by 9C23R and U286446 (or U296361), respectively.

FIGURE S2 | Genetic complementation for late blight resistance on tomato with NLR candidate genes. Representative pictures of the disease symptoms of PL3320, PL2741 and PL2759 tomato transgenic lines expressing the NLR candidate genes cosA2, cosA1 and cosD5, respectively. Detached leaves were inoculated with *P. infestans* isolates 90128 and EC1 and macroscopic observations for disease symptoms were carried out at 6 dpi.

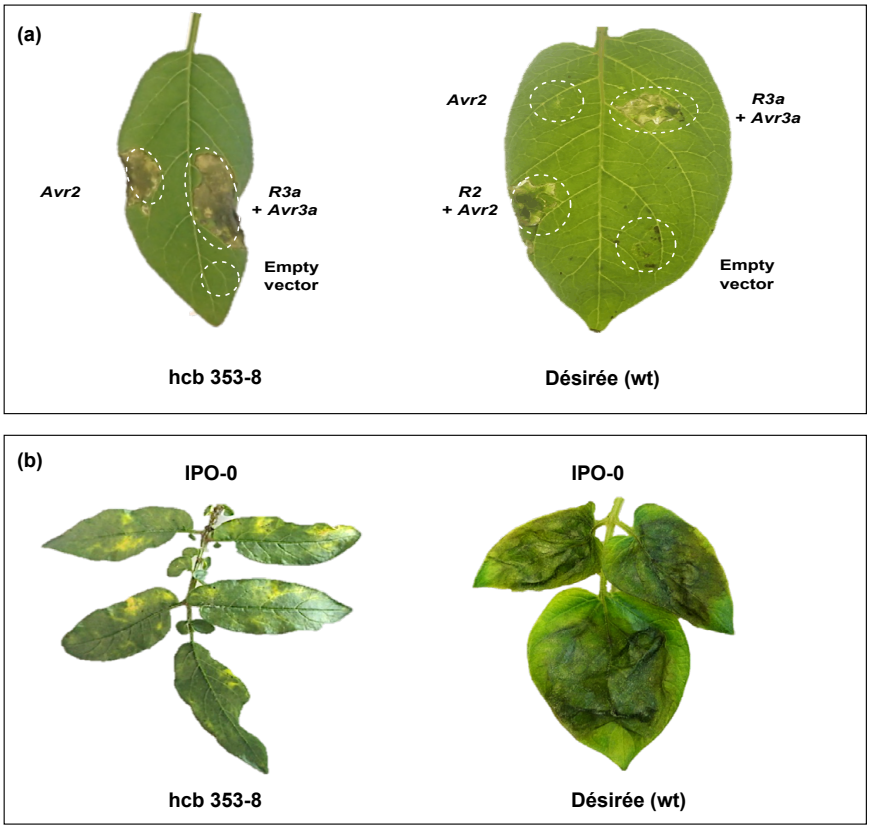

FIGURE S3 | *Solanum huancabambense* recognizes AVR2 and is resistant to *P. infestans*. (a) Agroinfiltration of *A. tumefaciens* carrying pK7WG2:*Avr2* and co-infiltrations of *R3a*/*Avr3a* on hcb 353-8. Single infiltrations of the pK7WG2 empty vector was included as a negative control and co-infiltrations of *R3a*/*Avr3a* and *R2*/*Avr2* were included as positive controls on the wild type 'Désirée'. (b) hcb 353-8 shows no lesion development upon inoculation with *P. infestans* isolate IPO-O, whereas 'Désirée' (wt) shows large sporulating lesions at 6 dpi. Representative pictures are presented.

FIGURE S4 | Genetic complementation of *Rpi-mcq1, Rpi-hcb1.1* and *Rpi-hcb1.2* in *N. benthamiana*. *Rpi-mcq1, Rpi-hcb1.1* and *Rpi-hcb1.2* were transiently expressed in *N. benthamiana* leaves, followed by inoculation with the *P. infestans* isolates IPO-0 and IPO-C. *Rpi-blb3* was included as a resistant control and infiltrations with medium (MMA) and with empty vector were used as negative controls. Race-specific resistance to IPO-O was observed with *Rpi-mcq1*, *Rpi-hcb1.1*, *Rpi-hcb1.2* and in the resistant control *Rpi-blb3*, whereas IPO-C caused expanding lesions.

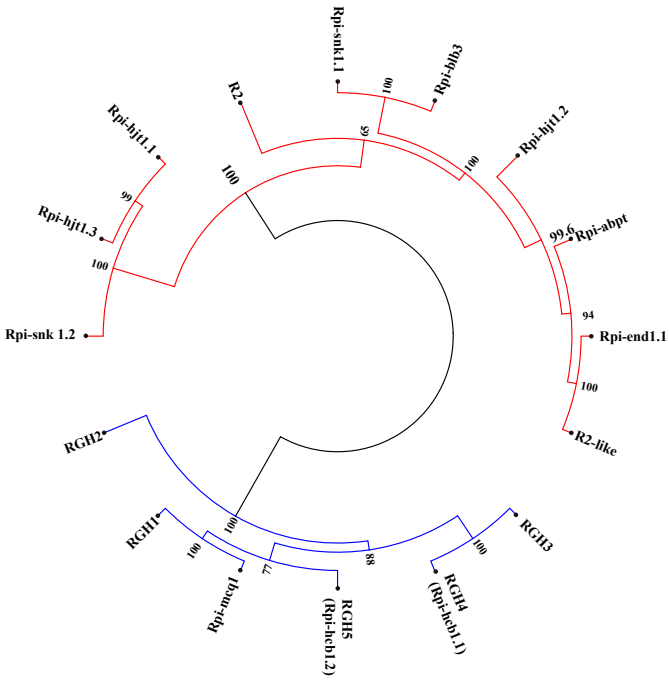

FIGURE S5 | Phylogenetic relationship and clustering of Rpimcq1 family. The UPGMA-based tree illustrates the phylogenetic relationship at the amino acid level of Rpi-mcq1 and RGH cloned from hcb 353-8 (blue) and the R2 family (red). Numbers on each node represent bootstrap values based on 100 replicates.

TABLE S1 | Overview of CAPS markers and primers used in this study. Primer sequence, annealing temperature and restriction enzyme are indicated. [a] Orientation of the primer: F, Forward; R, reverse. [b] Annealing temperature. [C] Restriction enzyme that reveals polymorphism between alleles linked to resistance or susceptibility.

Name	F/R[a]	Primer sequence (5'-3')	Tm (°C) [b]	Restriction enzyme [c]
T0156	F	AAGGCAGGAACAAGATCAGG	55	*RsaI*
	R	TTGACAGCAGCTGGAATTG		
S1D11	F	CTGGTCCTATAGGGTTACCATT	55	*ApoI*
	R	AGAACCGCACCATCATTTCTTG		
TG328	F	AATTAAATGGAGGGGGTATC	50	*AluI*
	R	CCTTTGAATGTCTAGTACCAG		
U286446	F	GCACAAGCACAGTCTGGAAA	55	*HaeIII*
	R	GCTGCATTAATAGGGCTTGC		
U296361	F	CAGAAGCAGCTGACTCCAAA	55	*HincII*
	R	TTCAACAGTGAGAGAGCCACA		
TG591N	F	TACTCGTGCAAGAAGGAACG	55	*HaeIII, HpaII*
	R	CCAACTTGTTTGGCTATGTCA		
U272857	F	GTGGTCTTTTGAGGCAGAGC	55	*XhoI*
	R	AGATTCGCCGTCTGTGAAGT		
9C23R	F	TCTTGCCAAGCAGGTCTTTT	55	*HinfI*
	R	CAGCCATTAGGCATTTGACA		
GP41	F	CAGGAGATCCATCTCTCAAG	55	*Hpy1881*
	R	CTGCAGTAAAGTGCATTCGG		
Rpi-mcq1_RGH	F	CACCATGGCTGAAATTCTTCTTACAGCA	64	
	R	TCATATTCTGAGCTTTGCAAGACG		

TABLE S2 | List of *P. infestans* isolates used in this study. The country, year, the genotype source of collection, the race, as well as the virulence on hjt 349-3 and hcb 353-8 plants are indicated. Hjt349-3 contains an *R2* homolog (Aguilera-Galvez *et al.*, 2018).

Isolate name	Origin			Race	Virulence	
	Country	Year	Source		hjt349-3	hcb353-8
90128	The Netherlands	1990	Potato	1.3.4.7.8.10.11	R	R
EC1	Ecuador	n.d.	Potato	1.3.4.7.10.11	R	R
IPO-O	The Netherlands	n.d.	Potato	0	R	R
88069	The Netherlands	1988	Tomato	1.3.4.7	R	R
91011	The Netherlands	1991	Potato	3.4.5.10	R	R
H30P04	The Netherlands	1995	F1 progeny from a cross between isolates 88029 and 88133	3a.7.10.11	R	R
EC3364	Ecuador	2001	*S. betaceum*	non-host	R	R
PIC99183	Mexico	1999	*S. stoloniferum*	1.3.4.7.8.10.11	R	R
UK7824	United Kingdom	1978	Potato	1.2.3.6.7	S	S
IPO-C	Belgium	1982	Potato	1.2.3.4.5.6.7.10.11	S	S
PIC99189	Mexico	1999	*S. stoloniferum*	1.2.5.7.10.11	S	S
UK3928A	United Kingdom	2006	Potato	1.2.3.4.5.6.7.10.11	S	S

TABLE S3 | Co-segregation for resistance, AVR2 response and genetic marker in hcb 7393 population. (a) Resistance (R), susceptibility (S) or quantitative resistance (Q) against *P. infestans* isolates IPO-0 and 90128. (b) Presence (1) or absence (0) of the polymorphic band of the genetic marker on chromosome IX GP41. (c) Presence (+) or absence (-) of cell death response after agroinfiltration with *Avr2*, empty vector (negative control) and co-infiltration with *R3a/Avr3a* (positive control) at 4 dpi. n.d. not determined.

	a		**b**		**c**		
Genotype	**Disease test**		**Marker Chr IX**		**AVR2 response**		
	***P. infestans* inoculation**				**Agroinfiltration ATTA**		
	IPO-0	**90128**	**GP41**	**Hpy1881**	***Avr2***	***R3a+ Avr3a***	**Empty vector**
Hcb 353-8	R	R	1		56	67	0
Hcb 354-2	S	S	0		0	100	0
Offspring							
1	R	R	1		83	92	10
2	R	Q	1		n.d	n.d	n.d
12	R	R	1		100	50	10
16	n.d	R	1		100	67	0
29	R	R	1		67	100	0
52	R	R	1		80	80	16
57	R	R	1		75	80	0
72	R	R	1		100	100	0
79	Q	R	1		100	90	40
89	R	R	1		56	100	0
4	S	S	0		0	100	0
9	S	S	0		0	100	0
13	S	S	0		40	100	0
21	S	S	0		10	100	0
56	S	S	0		0	100	0
65	S	S	0		0	100	0
75	S	S	0		0	100	0
80	S	S	0		10	90	10

TABLE S4 | Positive selection analysis of *Rpi-mcq1* loci.

Model of selection	Estimated parameters	l*	Model comparison	2Dl (df.p)**	Positively diversified codons ***
M0: one ratio	ω= 0.636	-8845.73			Not allowed
M1: neutral	r0 = 0,505; r1 = 0,495; ω0,038 = 0; ω1= 1	-8693.64			Not allowed
M2: positive selection	r0 = 0,448; r1= 0,475; r2= 0,076; ω1= 0,0227; ω2= 1; ω3=4,314;	-8657.60	M1 vs M2	72,08 (2<0,001)	26T, 573V, 577C, **697A**, **723K**, 728M, 774G, **793G**, 820E, 847R, 850P
M7: beta	r= 0,040; q=0,043	-8697.36			Not allowed
M8= beta and ω	r0= 0,9004; r= 0,041; q= 0,042; r1= 00995; ω=3,711	-8658.42	M7 vs M8	77,88 (2<0,001)	26T, 341N, **573V**, 576I, **577C,** 601Y, 621Y, 628T, 645N, **697A**, **723K**, 728M, **774G**, **793G**, 820E, **847R**, 850P

* Log likelihood value. ** Likelihood radio test: $2\Delta l = 2(lnl_{alternative\ hypothesis} - lnl_{null\ hypothesis})$, with significance evaluated from distribution: df is degree of freedom and p is the probability. *** Bayes Empirical Bayer (BEB) analysis; amino acid sites, based on Rpi-hcb1.1 sequence, inferred to be under diversifying selection with probability >95%, and 99% in bold.

TABLE S5 | Percentage of similarity between Rpi-mcq1 and RGH. Amino acid sequence similarity is indicated.

	Rpi-mcq1	RGH2	RGH3	RGH1	RGH5	RGH4	Rpi-blb3
Rpi-mcq1	***	89.7	91.6	90.1	90.2	85.8	57.9
RGH2	89.7	***	87.6	84.2	88.6	84.4	57.8
RGH3	91.6	87.6	***	84.8	88.2	88.9	57.3
RGH1	90.1	84.2	84.8	***	84.2	79.7	56.3
RGH5	90.2	88.6	88.2	84.2	***	85.4	57.5
RGH4	85.8	84.4	88.9	79.7	85.3	***	56.2
Rpi-blb3	57.9	57.8	57.3	56.3	57.5	56.2	***

Chapter 3

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*.

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; Sliwka *et al.*, 2012b; Jo *et al.*, 2015; Witek *et al.*, 2016). 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, T. H. *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*, *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; Sliwka *et al.*, 2012a; Jones, JDG *et al.*, 2014).

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).

TABLE 1 | List of *Solanum* genotypes used in this study. 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 Fig. 1 and 2.

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	

Series	*Solanum* species	GenBank accession	Genotype	Agro infiltration			Accession origin	
				pK7WG2::AVR2	pK7WG2:: empty	R3a/AVR3a	Country	Collection site
	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	
	S. gourlayi vidaurrei	CGN 23045	626-2	-	-	+	Argentina	
	S. microdontum gigantophyllum	CGN 18200	712-6	-	-	+	Bolivia	
	S. microdontum gigantophyllum	CGN 23050	714-1	-	-	+	Argentina	

Series	***Solanum* species**	**GenBank accession**	**Genotype**	**Agro infiltration**			**Accession origin**	
				pK7WG2::AVR2	pK7WG2:: empty	R3a/AVR3a	Country	Collection site
	S. microdontum gigantophyllum	CGN 18295	956-1	-	-	+	Argentina	
	S. microdontum gigantophyllum	CGN 18049	963-3	-	-	+	Argentina	
	S. okadae	PI 458368	283-1	-	-	+	Argentina	
	S. okadae	CGN 18109	366-1	-	-	+	Argentina	
	S. okadae	CGN 18108	367-1	-	-	+	Argentina	
	S. okadae	CGN 17998	368-6	-	-	+	Argentina	
	S. okadae	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	CGN17717	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	

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 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.

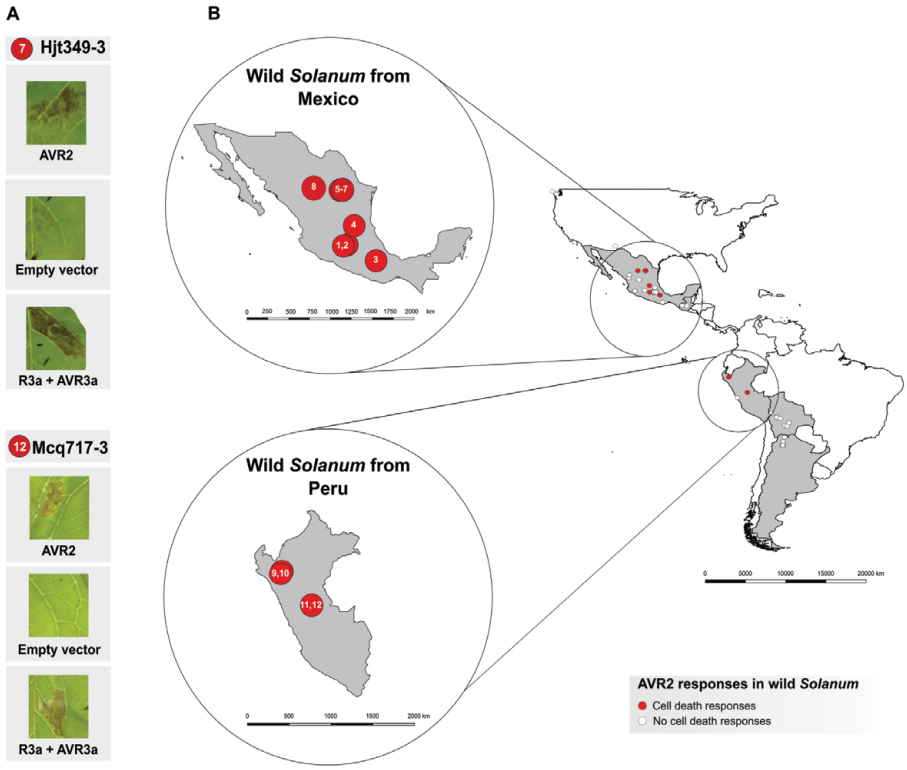

FIGURE 1 | *Solanum* species that respond to AVR2 occur in Mexico and Peru. (a) Representative leaf panels of AVR2-recognizing *Solanum* species from Mexico (Hjt349-3) and Peru (Mcq717-3). Leaves were agro-infiltrated with pK7WG2:AVR2, with pK7WG2: empty and co-infiltrated *R3a*/AVR3a as negative and positive controls, respectively. Pictures were taken at 4dpi. (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.

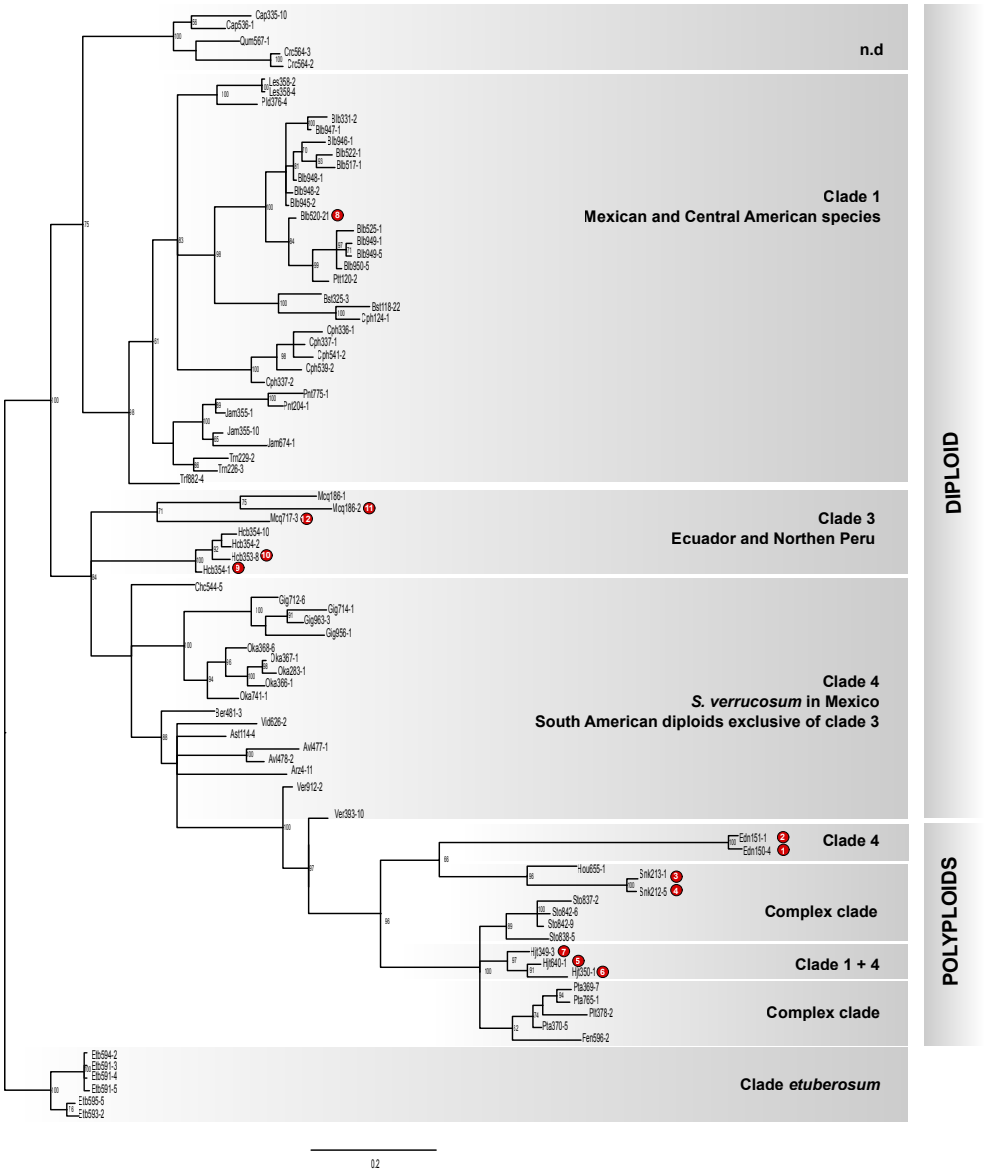

FIGURE 2 | Classification of tested wild *Solanum* genotypes. Bayesian rooted tree base on AFLP data of 80 screened *Solanum* genotypes and 6 *Solanum etuberosum* genotypes. The branch length represents expected changes per site and posterior probability values are shown near the respective nodes. AVR2-responding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

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).

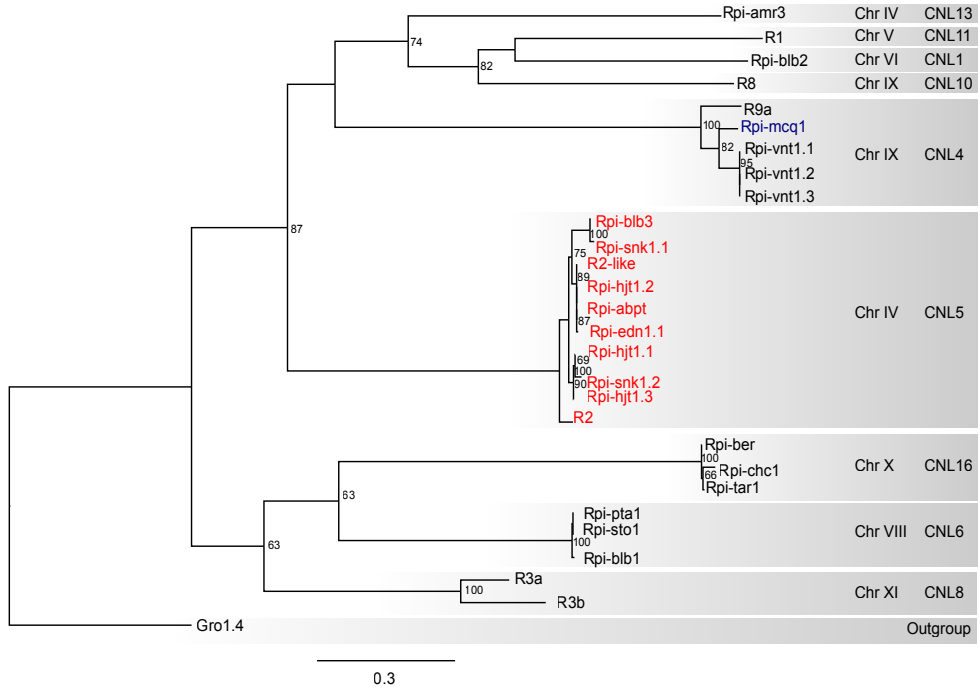

FIGURE 3 | Classification of Rpi proteins. Phylogenetic tree derived from the full NB-ARC domains (range of amino acid sequences in Supplemental Table 1) obtained from 27 Rpi proteins. *Rpi* cloned from Mexican (red) and South American (blue) *Solanum* are highlighted. CNL clades are indicated. The nematode 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.

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. hjertingii*, 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* from Peru (Smilde *et al.*, 2005; Jones, JDG *et al.*, 2014), 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 agro-coinfiltration 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.

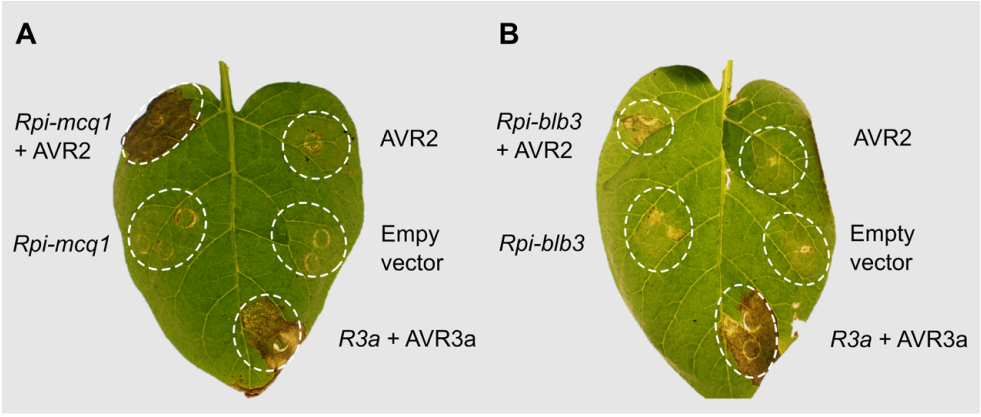

FIGURE 4 | Rpi-mcq1 and Rpi-blb3 confer response to AVR2. Leaves of potato cv. 'Bintje' were co-infiltrated with AVR2 and *Rpi-mcq1* (A) and *Rpi-blb3* (B) as a cell death control trigger by AVR2. Single infiltrations of AVR2, *Rpi-mcq1.1, Rpi-blb3* and empty vector were included as negatives controls 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.

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 the Rpi-mcq1 and Rpi-blb3 are functional in these plants and lead to the recognition of AVR2.

The resistance spectrum of Desirée-*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 Desirée-*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 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*.

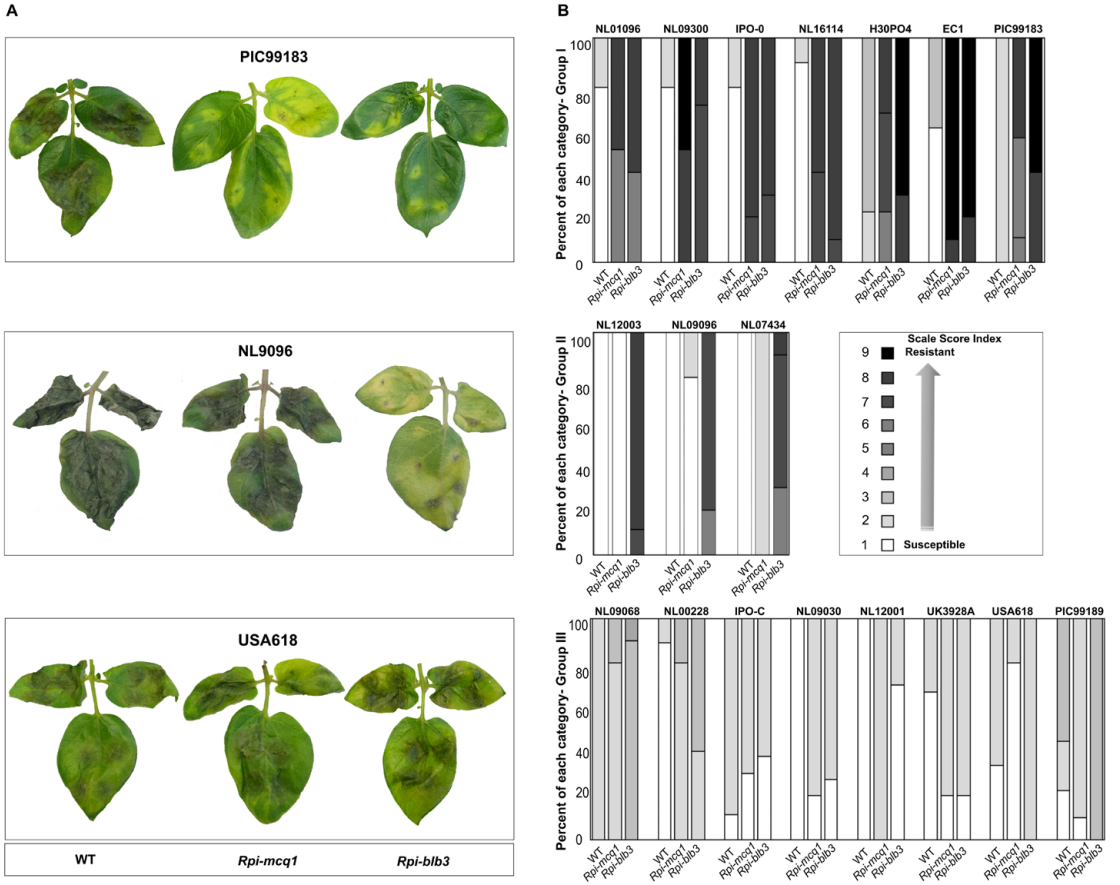

FIGURE 5 | Disease index on 'Désirée', Désirée-*Rpi-mcq1* and Désirée-*Rpi-blb3* with isolates from group I-III. Representative pictures of isolates from group I to III tested in 'Désirée' (WT), Désiree-*Rpi-mcq1* (*Rpi-mcq1*) and Désirée-*Rpi-blb3* (*Rpi-blb3*) are displayed. Pictures were taken after 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 ≥ 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.

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.*, 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 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, T. H. *et al.*, 2005a; Park, T. H. *et al.*, 2005b; Park, T.H. *et al.*, 2005; 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 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-coinfiltrations (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-coinfiltration 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, P *et al.*, 2014) (Supplemental 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 hours.

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 16h 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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Supporting information

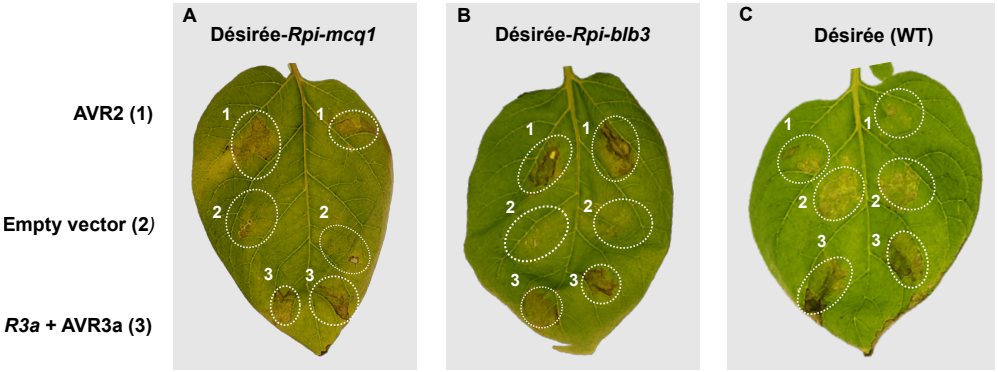

FIGURE S1 | Cell death responses upon expressing of AVR2 on Désirée-*Rpi-mcq1* and Désirée-*Rpi-blb3*. Agroinfiltration using *A. tumefaciens* strain carrying pK7WG2: AVR2, pK7WG2: empty and co-infiltrations of R3a/AVR3a on Désirée-*Rpi-mcq1* (A), Désirée-*Rpi-blb3* (B), and untransformed 'Désirée' (Wild type) (C). Each effector is tested twice on three leaves, over two plants and two biological replicates. Representative photographs of symptoms were taken at 4 dpi.

TABLE S1 | List of known *Rpi* genes cloned from wild *Solanum* genotypes. For each *Rpi* gene, the donor species, geographic origin, chromosome, and position of NB-ARC domain (used for generating Fig. 3) are presented.

Rpi gene	Donor species	Geographic origin	Chromosome location	NB-ARC domain	CNL Clades
R1	*S. demissum*	Mexico	V	548-825	CNL11
R2	*S. demissum*			165-445	
R2-like	Breeding lines AM78-3778 and ABPT			165-445	
Rpi-abpt				167-445	
Rpi-blb3	*S. bulbocastanum*			166-445	
Rpi-edn1.1	*S.edinense*	Mexico	IV	167-445	CNL5
Rpi-snk1.1	*S. schenkii*			166-445	
Rpi-snk1.2				166-442	
Rpi-hjt1.1				165-445	
Rpi-hjt1.2	*S. hjertingii*			167-445	
Rpi-hjt1.3				165-445	
R3a	*S. demissum*	Mexico	XI	181-462	CNL8
R3b				187-457	
R8	*S. demissum*	Mexico	IX	476-754	CNL10
Rpi-blb2	*S. bulbocastanum*	Mexico	VI	22-295	CNL1

***Rpi* gene**	**Donor species**	**Geographic origin**	**Chromosome location**	**NB-ARC domain**	**CNL Clades**
Rpi-blb1	*S. bulbocastanum*			156-435	
Rpi-sto1	*S. stoloniferum*	Mexico	VIII	156-435	CNL6
Rpi-pta1	*S. stoloniferum*			156-435	
Rpi-amr3	*S. americanum*	Mexico	IV	144-434	CNL13
Rpi-chc1	*S. chacoense*			213-495	
Rpi-ber	*S. berthaultii*	Bolivia	X	212-495	CNL16
Rpi-tar1	*S. tarijense*			213-496	
Rpi-mcq1	*S. mochiquense*	Peru		164- 441	
Rpi-vnt1.1				161-438	
Rpi-vnt1.2	*S. venturi*	Argentina	IX	208-485	CNL4
Rpi-vnt1.3				208-485	
R9a	*S. demissum*	Mexico		165-443	

TABLE S2 | List of *P. infestans* isolates used in this study. The country and year of collection, the genotype source, as well as the group classification (I-III) of the strain are indicated.

Isolate name	**Origin**			**Group**
	Country	**Year**	**Source**	
NL01096	The Netherlands	2001	Potato	
NL09300	The Netherlands	2009	cv.'Toluca'	
IPO-O	The Netherlands	n.d.	Potato	
NL16114	The Netherlands	2016	cv. 'Carolus'	I
H30P04	The Netherlands	1995	F1 progeny from a cross between isolates 88029 and 88133	
EC1	Ecuador	n.d.	Potato	
PIC99183	Mexico	1999	*S. stoloniferum*	
NL12003	The Netherlands	2012	cv. 'Frieslander'	
NL09096	The Netherlands	2009	Potato from C.Meijer B.V	II
NL07434	The Netherlands	2007	Potato from Averis seeds B.V	
NL09068	The Netherlands	2009	Désirée-*Rpi-blb2*	
NL00228	The Netherlands	2000	Potato	
IPO-C	Belgium	1982	Potato	
NL09030	The Netherlands	2009	cv. 'Bionica'	III
NL12001	The Netherlands	2012	cv. 'Eba'	
UK3928A	United Kingdom	2006	Potato	
USA618	USA	n.d.	Potato	
PIC99189	Mexico	1999	*S. stoloniferum*	

Chapter 4

Genetic diversity in *Avr2* family of *Phytophthora infestans* underpins differential recognition specificity by *Solanum* resistance genes

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Abstract

Pathogens secrete effector proteins to promote colonization and manipulate host cellular processes. AVR2 is an RXLR effector which promotes the virulence of *P. infestans* by suppressing pattern-triggered immunity (PTI). AVR2 also plays a role in avirulence and can be recognized by two sequence-unrelated proteins, R2 and Rpi-mcq1, which display an overlapping but distinct resistance pattern to various *P. infestans* isolates. Here, we study the genetic diversity of *Avr2* to understand the differences in race-specificity to *P. infestans* mediated by *R2* and *Rpi-mcq1*. Our results show that *Avr2* belongs to a highly diverse and polymorphic gene family which is clustered in three classes (I to III). Using RNA-seq data, we found variants of the *Avr2* family that are differentially expressed in *P. infestans* isolates that show a distinct virulence pattern on *R2* and *Rpi-mcq1* potato plants. We found that 14 *Avr2* family members are differentially recognized by *R2* versus *Rpi-mcq1* in transient expression assays. This is likely resulting from the tight co-evolution between *Avr2* family and two distinct *Solanum R* genes. Overall, our findings show that the diversity in *Avr2* family is associated with different recognition specificities and race-specificity to *P. infestans* mediated by *R2* and *Rpi-mcq1*.

Key words: *Avr2*, *Avr2* variants, co-evolution, diversity, late blight, *Phytophthora infestans*, potato, *R* genes, *R2*, resistance, *Rpi-mcq1*, *Solanum*.

Introduction

Late blight disease caused by the oomycete pathogen *Phytophthora infestans* is the most devastating disease of potato and a major threat to food security (Fisher *et al.*, 2012). In Ireland, late blight led to the Irish potato famine between 1845 and 1849, causing the death of one million people and migration of one million more (Zadoks, 2008). Currently, the disease is still hampering potato production. It has been estimated to cause global yield losses of around 16% and a financial cost of approximately € 6 billion (Haverkort *et al.*, 2016).

In order to promote colonization and manipulate host cellular processes, *Phytophthora infestans* secretes an arsenal of cytoplasmic and apoplastic effectors. Effectors are small molecules that can have dual roles and function as virulence as well as avirulence (AVR) factors (Kamoun, 2006). All the known AVR proteins belong to the cytoplasmic RXLR effectors, which are modular proteins characterized by a host translocation motif (RXLR) and a highly polymorphic C-terminal domain (Bhattacharjee *et al.*, 2006; Win *et al.*, 2007). Typically, RXLR effectors of *P. infestans* are diverse and reside in highly dynamic regions of the genome that probably enables the fast-evolutionary adaptation of this pathogen. More than 550 RXLR effectors have been predicted in the *P. infestans* genome, however the function of all these proteins remains to be elucidated (Haas *et al.*, 2009).

Plants have evolved immune receptors to detect invading pathogens and activate diverse defense responses (Dodds & Rathjen, 2010). Among these receptors are the resistance (R) proteins, which are typically members of the large family of nucleotide-binding leucine-rich-repeat (NLR) proteins. NLR proteins typically reside in the cytoplasm and can detect specific RXLR effectors inside the host cells, which is then leading to the activation of effector-triggered immunity (ETI). This ETI response is often associated with a form of programmed cell death known as hypersensitive response (HR) that restricts the spread of the pathogen from the infection site.

Avr2 (*PITG_22870*) is a well characterized RXLR effector of *P. infestans* (Gilroy *et al.*, 2011). AVR2 is recognized by two sequence-unrelated R protein families, R2 and Rpi-mcq1, from Mexican and South American *Solanum* species, respectively (Park *et al.*, 2005; Lokossou *et al.*, 2009; Champouret, 2010; Aguilera-Galvez *et al.*, 2018). *R2* and *Rpi-mcq1* potato plants display an overlapping but distinct resistance pattern to various *P. infestans* isolates. It has been suggested that the differential specificity in effector recognition mediated by *R2* and *Rpi-mcq1* may be attributed to additional alleles or paralogs of *Avr2*. However, the genetic diversity of *Avr2* remains to be understood (Aguilera-Galvez *et al.*, 2018).

Two RXLR effectors have been shown to be related with *Avr2*. *PexRD11* (*PITG_13930*) was predicted in the *P. infestans* reference genome and it was found to be induced at an early stage of potato infection (Haas *et al.*, 2009). *PexRD11* is recognized by *R2* as well (Champouret, 2010), although it shares only 63% of amino acid similarity at the C-terminal domain with

AVR2. *Avr2-like* was identified in *P. infestans* isolates which are virulent on *R2* potato plants and is known as the virulence allele of *Avr2*. *Avr2-like* differs from *Avr2* by 25 nucleotides (13 amino acids) and evades recognition of *R2* (Gilroy *et al.*, 2011). Understanding the recognition specificity of AVR2-related proteins mediated by R2 and Rpi-mcq1 will help to understand how *Avr2* effectors interact and co-evolve with *Solanum R* genes.

In this study, we characterize additional proteins that are related to AVR2 and PexRD11 in the proteome of *P. infestans* isolate T30-4. We found a highly polymorphic family in which the members are differentially expressed upon potato infection with various *P. infestans* isolates. We performed transient expression assays and we found a distinct specificity of recognition of *Avr2* family members mediated by *R2* and *Rpi-mcq1*. This result suggests that the genetic diversity of the *Avr2* family may be associated with the differences in race-specificity to *P. infestans* mediated by *R2* versus *Rpi-mcq1* in *Solanum* species.

Results

Avr2 and *PexRD11* belong to a diverse gene family in *P. infestans*

To mine for proteins with sequence similarity to AVR2 (PITG_22870) and PexRD11 (PITG_13930), a BLAST search was performed in the proteome of *P. infestans* isolate T30-4 (Haas *et al.*, 2009). The C-terminal domain amino acid sequence of AVR2 and PexRD11 was used as a query for the analysis. In total 21 proteins were retrieved. Among these, 13 proteins displayed percentages of amino acid identity ranging from 16 to 100% with AVR2. The remaining 8 proteins showed a percentage of identity from 39 to 100% with PexRD11 (Supporting information, Table S1). To identify proteins with potential avirulence activity, the sequences were filtered for the presence of the RXLR motif. Redundant sequences were removed. A total of 11 RXLR proteins were identified and included as members of the AVR2 family. These proteins are sharing a percentage of amino acid identity from 16 to 63% either with AVR2 or PexRD11 (Supporting information, Table S2).

To study the genetic variation of the AVR2 family, a sequence analysis was conducted. A multiple alignment of the 13 amino acid sequences revealed that the family is highly diverse (Fig.1). Each member of the family was predicted to contain a signal peptide (position 1 to 21), and the conserved RXLR-EER motif (position 50 to 66). High levels of polymorphism were noted at the C-terminal domain (position 67 to the end) (Fig.1). The genetic relationship between the AVR2 family was examined by constructing a Neighbor-Joining (NB) tree. Three major classes were identified and designated as I, II and III (Fig.1). PexRD11was grouped with four additional members in class I. AVR2 and PITG_05121 were grouped in class II and the remaining six members in class III. Altogether, these data illustrate the high diversity of the AVR2 family in *P. infestans* T30-4.

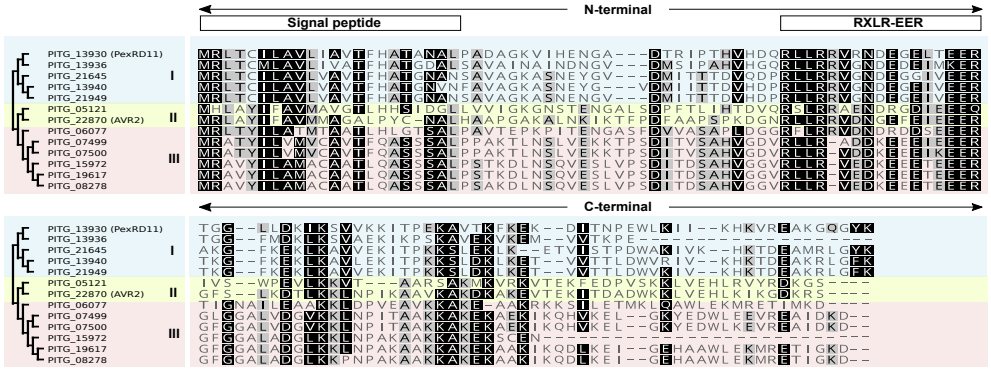

FIGURE 1 | Diversity of AVR2 family in *P. infestans* T30-4. Neighbor-Joining (NJ) tree and multiple sequence alignment of 13 AVR2 family members. Classes I, II and III are indicated. Identical amino acids residues are shaded in black, conserved and semi-conserved residues are shaded in grey. The N-terminal and C-terminal domains, the signal peptide and the RXLR-EER motif are indicated above the alignment

The AVR2 family displays expression polymorphism

Diverse *P. infestans* isolates T30-4, NL07434 and UK3928A display a distinct avirulence pattern on *R2* and *Rpi-mcq1* potato plants (Supporting information, Table S3) (Aguilera-Galvez *et al.*, 2018). To investigate the expression profile of the AVR2 family members in those *P. infestans* isolates, the NimbleGen microarray data from Haas *et al.* (2009) was analyzed. Briefly, the microarray data were obtained using samples from mycelium grown on Rye and V8 medium and from infected potato leaves at 0, 48, 72 and 96 hours post inoculation (hpi). The members of the AVR2 family were found to be differentially expressed within the family and between the three *P. infestans* isolates.

Most remarkably, AVR2 from class II was highly induced in all the *P. infestans* isolates at 48 hpi (Fig. 2). Other peaks of expression were found for class III in T30-4, where three members were induced at 48 hpi. In NL07434, some class III members were slightly induced at early stage of infection as well, whereas the remaining three members of class III were down-regulated. None of the class III members were induced in UK3929A, and most were down-regulated at 48-96 hpi (Fig. 2). For class I, PexRD11 and to a lesser extend PITG_13940 were induced at 48 hpi in UK3928A. In T30-4, PITG_13936 was slightly induced at 48 hpi whereas the other members were down-regulated and in NL07434, family members from class I were mostly down-regulated during early infection stages (Fig. 2). Overall, these data indicate that members of the AVR2 family are differentially expressed upon *P. infestans* infection on potato.

The expression of AVR2 in T30-4 and NL07434 can explain the avirulence of these isolates on *R2* plants, as reported earlier by Gilroy *et al.* (2011). The figure also shows a peak for AVR2

in UK3928A, however, this isolate is virulent on *R2* plants and expresses AVR2-like instead of AVR2 (Gilroy *et al.*, 2011). Therefore, we conclude that some allelic variants of the AVR2 family are not discriminated in the microarray and a complementary approach will be required.

To get more precise insight on the expression of individual AVR2 family members, an RNA sequencing (RNAseq) approach was implemented. Zoospore suspensions of the isolates PIC99183, NL07434, NL12003, NL09096 and PIC99189 that show different avirulence profiles on *R2* and *Rpi-mcq1* potatoes (Aguilera-Galvez *et al.*, 2018) were spot-inoculated on detached potato leaves (Supporting information, Table S3). At 24 hpi leaf discs were sampled, pooled and stored. To monitor the disease development process in this experiment, macroscopic observations were carried out every day until 6 days post inoculation (dpi). Abundant sporulation and infection symptoms were detected on Désirée leaves that were inoculated with all the *P. infestans* isolates. This result indicates the success of the infection. Subsequently, total RNA was isolated from mycelium and the leaf discs. The RNA was prepared for sequencing by Illumina HiSeq platform.

The raw RNA-seq reads were mapped to the RXLR effectors of *P. infestans* T30-4 isolate (Haas *et al.*, 2009), resulting in eleven transcripts that were associated with the AVR2 family. Among these, five transcripts correspond to the known effectors, AVR2, AVR2-like, PexRD11, PITG_13940, and PITG_08278. Two transcripts represent new variants of AVR2 effector and we designated them AVR2-1 and AVR2-2, respectively. The remaining four transcripts are variants of PITG_21949, PITG_05121, PITG_19617 and PITG_07499 and were designated PITG_21949-1, PITG_05121-1, PITG_19617-1 and PITG_07499-1, respectively (Fig.3a).

We generated a NJ tree for the 20 members of AVR2 family, and obtained the three phylogenetic classes as before (Fig. 1). The new variants were clustered along their most close family member (Fig.3a). This is in line with our previous findings that AVR2 family is highly diverse and is clustered in three classes (I to III).

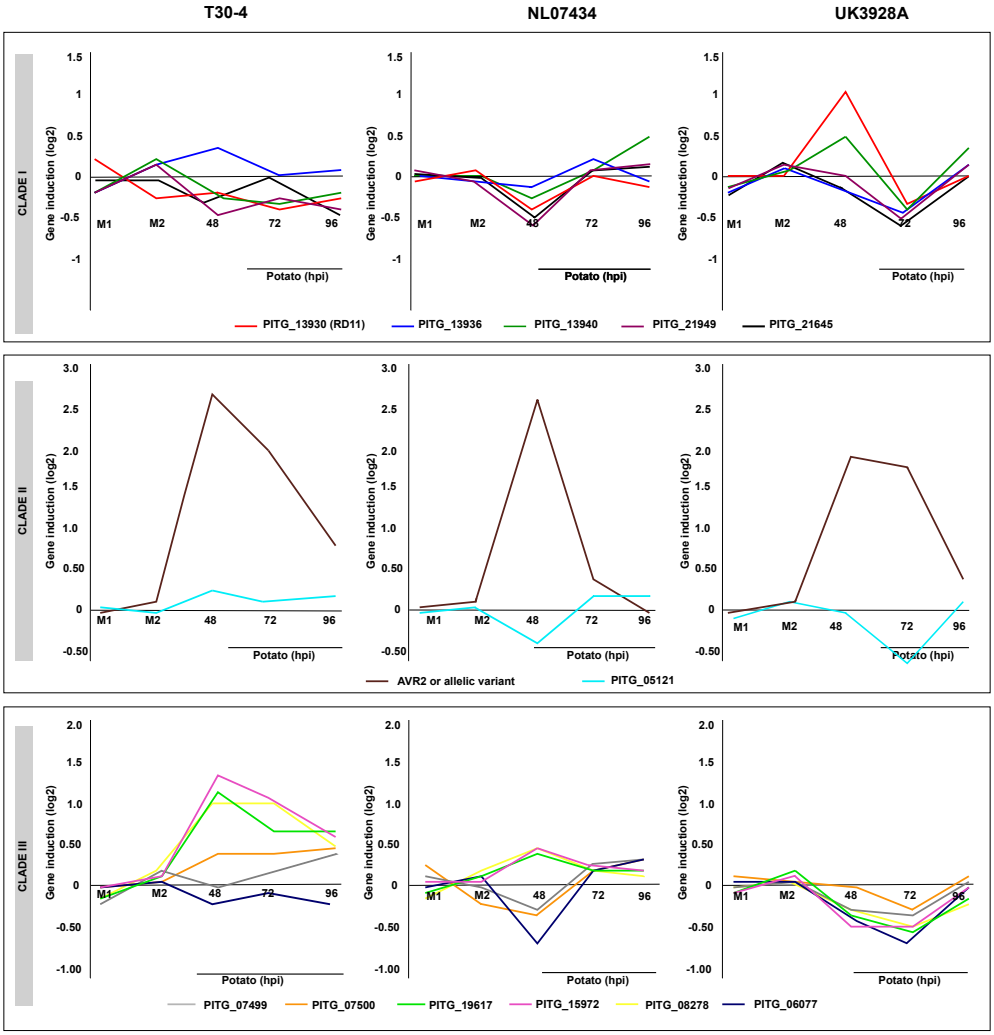

FIGURE 2 | Expression profile of AVR2 family upon infection on potato. Expression profile of the *Avr2* family based on microarray data after Log2 normalization(Haas *et al.*, 2009). The results were obtained from total RNA isolated from mycelium grown on Rye medium (M1) or V8 medium (M2) and from Désirée infected leaves at 48, 72 and 96 hours post inoculation (hpi) of the *P. infestans* isolates T30-4, NL07434 and UK3928A.

For class II, most new variants were obtained in the new *P. infestans* isolates. A multiple sequence alignment of AVR2 and its allelic variants, AVR2-like, AVR2-1 and AVR2-2, showed a total of 14 polymorphic amino acid sites. Eight of these are localized at the C-terminal domain, which is generally known for determining recognition specificity (Supporting information, Fig. S1). This result indicates that especially the *Avr2* gene is genetically different among *P. infestans* isolates.

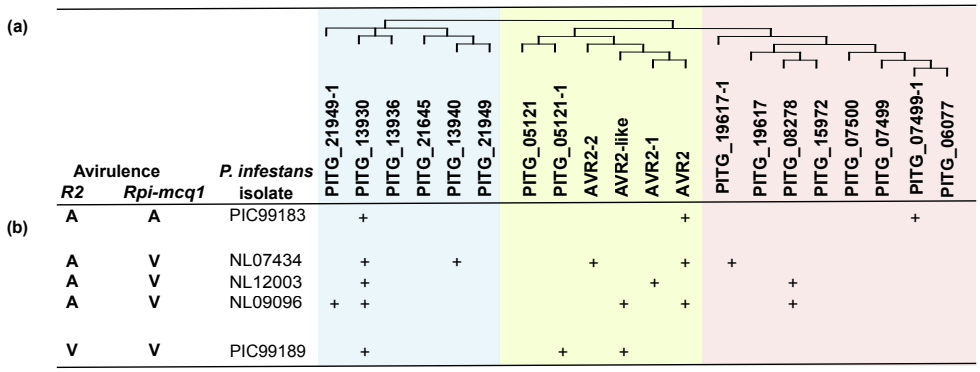

FIGURE 3 | AVR2 family is differentially expressed in *P. infestans* isolates. (a) The presence or absence of 20 AVR2 family members in *P. infestans* isolates PIC99183, NL07434, NL12003, NL09096 and PIC99189 and the virulence profile of each isolate on *R2* and *Rpi-mcq1* plants is indicated. (A) indicates avirulence and (V) indicates virulence. (b) NJ tree of 20 AVR2 family members.

Differential expression of AVR2 family leads to a distinct virulence patterns on *R2* and *Rpi-mcq1*

To determine the extent to which the 20 AVR2 family members are involved in the differential race-specificity to *P. infestans* mediated by *R2* and *Rpi-mcq1*, we analyzed the presence or absence of transcripts of the family members in *P. infestans* isolates using the RNAseq data previously obtained. The sequencing reads from the *P. infestans* isolates PIC99183, NL07434, NL12003, NL09096 and PIC99189 (Supporting information, Table S3) were aligned to the sequences of the 20 AVR2 family members. We again found that the AVR2 family members are differentially expressed in the tested isolates (Fig.3b). Transcripts of class I and II were represented in all isolates, and one or two members per class were found expressed for each isolate. Transcript of PexRD11 (PITG_13930) from class I was detected in all the *P. infestans* isolates. In addition, PITG_21949-1 and PITG_13940 class I sequences were detected in the NL09096 and NL07434 isolates, respectively. Class II, which contains various forms of AVR2, showed remarkable patterns. The avirulent isolate PIC99183 expressed AVR2, whereas the virulent isolate PIC99189 did not, along with expectations. Intriguingly, the isolates NL07434, NL09096 and NL12003 that are avirulent on *R2* but virulent on *Rpi-mcq1* plants, expressed other variants of AVR2, sometimes in combination with AVR2. The presence of AVR2 variants in these discriminating isolates may indicate that those effectors play a role in the distinct resistance specificity mediated by *R2* versus *Rpi-mcq1*. Class III was the least represented in the RNAseq analysis. No class III member was detected in RNA of PIC99189 which is virulent on *R2* and *Rpi-mcq1*. One variant from this class was detected for the other isolates, which might suggest that class III is required for *R2* and *Rpi-mcq1*-mediated resistance. Overall, the diversity of *Avr2* family and underlying expression polymorphism during early infection phases suggest the family was shaped by a tight co-evolution with *Solanum R* genes.

AVR2 family is under diversifying selection in *P. infestans*

To detect amino acid sites under diversifying selection in the AVR2 family, the maximum likelihood (ML) method implemented in PALMX 1.3 software package was applied. The discrete model M3 suggested that ~18% of the amino acid sites were under positive selection with ω_2=1.42 (Supporting information, Table S5). The likelihood ratio test (LRT) for comparing M3 with M0 is 2ΔL=2*[-3418,25-(-3446,63)] = 56,8 which is higher than the X^2 critical value (13,28 at the 1% significance level with 4 degrees of freedom). This indicates that the discrete model M3 fits better the data than the neutral model M0, which does not allow diversifying selection sites with ω >1 (Supporting information, Table S5). Subsequently, we used the empirical Bayes theorem to identify 12 amino acids under strong positive selection with >95% of confidence (28A, 31L, 41A, 78L, 84I, 88V 95K, 96E, 97V, 100K 113L, 119I). Nine of the amino acids are located in the C-terminal region, which is in line with previous findings indicating that positive selection has typically more affected the C-terminal region of the RXLR effectors (Win *et al.*, 2007). We also performed the LRT for comparing the alternative model M8 with the null model M7. However, the difference between M8 and M7 was not statistically significant (Supporting information, Table S5). Overall, these data show that 12 amino acids were found under diversifying selection supported by the discrete model M3.

To characterize the selection pressures underlying sequence diversification on the *Avr2* family, the rates of non-synonymous substitution (d_N) and synonymous substitution (d_S) were calculated and compared for every allele pair. We found that the d_N value was greater than d_S ($\omega = d_N/d_S$ >1) in 44 of 190 pairwise sequence comparisons of 20 complete nucleotide sequences of *Avr2* family (Fig. 4 and supporting information, Table S4). These results indicate that diversifying selection is acting on *Avr2* family.

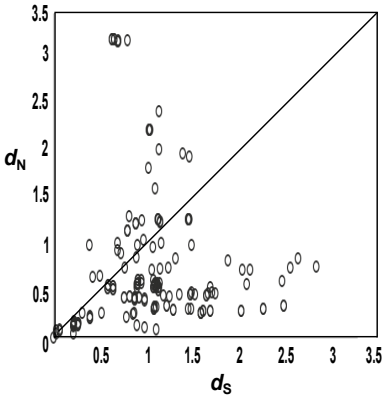

FIGURE 4 | Pairwise comparison of nucleotide substitution rates in 20 AVR2 sequences of *P. infestans*. The diagonal line indicates d_N=d_S, meaning neutral selection. Points above the line represent positive selection with $\omega = d_N/d_S$>1. Point below the lines indicate purifying selection with $\omega = d_N/d_S$<1

AVR2 family members are differentially recognized by *Solanum* R proteins

To gain insights on the recognition specificity of the AVR2 family by *Solanum* R proteins, agro-coinfiltrations were conducted in *N. benthamiana*. *Rpi-blb3* and *Rpi-mcq1* were previously cloned in the binary vector pBINPLUS (Aguilera-Galvez *et al.*, 2018) and 14 *Avr2* members were cloned in the vector pK7WG2 (Supporting information, Table S6). *N. benthamiana* leaves were agro-coinfiltrated with *A. tumefaciens* expressing either *Rpi-blb3* or *Rpi-mcq1* with each member of the *Avr2* family. Agro-coinfiltrations of *R3a/Avr3a* were used as a positive control for R/AVR-mediated cell death (Vleeshouwers *et al.*, 2011). Single infiltrations of each *Avr2* member were used as negative controls. Experiments were carried out in triplicate on 3 plants, using 3 leaves per plant. Local symptoms of cell death were monitored at 4 dpi. Confluent cell death responses were observed for members from class I in agro-coinfiltrations with *Rpi-blb3*, whereas no cell death responses were observed in agro-coinfiltrations with *Rpi-mcq1*. This result indicates that class I members are exclusively recognized by the *R2* family. For class II, *Avr2* induced cell death responses in agro-coinfiltrations with either *Rpi-blb3* or *Rpi-mcq1*. Interestingly, *Avr2-like* from the same class is recognized exclusively for *Rpi-mcq1*, indicating a new specificity of recognition mediated by *Rpi-mcq1*. Members from class III are not recognized by either *R2* or *Rpi-mcq1*, except for *PITG_08728* that is recognized by *Rpi-mcq1*. *PITG_07499* induces auto-necrosis in single agro-infiltrations with this effector (Fig. 5). For *R3a/Avr3a* agro-co-infiltrations, cell death responses were noted in all the inoculated spots. The finding that the *Avr2* family members are differentially recognized by *R2/Rpi-blb3* and *Rpi-mcq1* is in line with our earlier finding that the *R2* and *Rpi-mcq1* gene families have evolved independently.

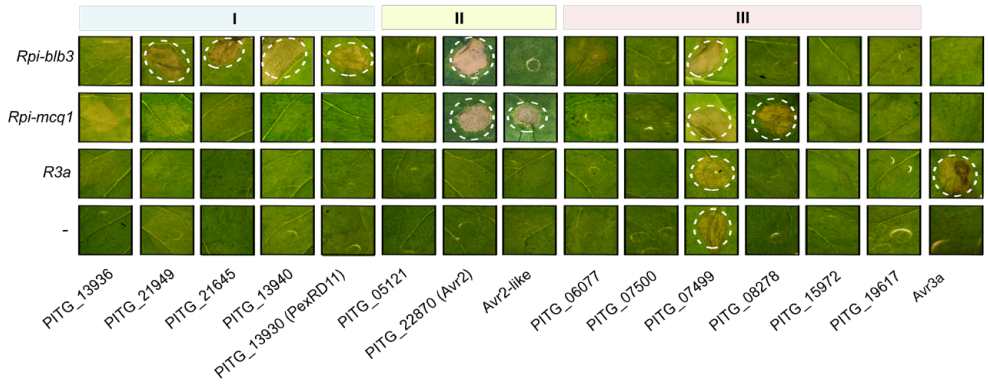

FIGURE 5 | *Avr2* family members are differentially recognized by *R2* and *Rpi-mcq1*. *Agrobacterium*-mediated co-expression of *R2*, *Rpi-mcq1* and *R3a* with 14 *Avr2* family members in *N. benthamiana*. Co-agroinfiltrations of *R3a-Avr3a* and single infiltrations of all the effector clones were performed as positive and negative controls, respectively. Representative photographs of cell death symptoms were taken at 4 dpi

Discussion

AVR2 is important for virulence of *P. infestans* and it promotes late blight disease development by suppressing pattern-triggered immunity (PTI) (Turnbull *et al.*, 2017). In addition, AVR2 is recognized by two sequence-unrelated R proteins, R2 and Rpi-mcq1, which display an overlapping but distinct virulence profile to diverse *P. infestans* isolates (Aguilera-Galvez *et al.*, 2018). Here, we study the genetic diversity of the *Avr2* family of *P. infestans* to unravel the differences in race-specificity mediated by *R2* versus *Rpi-mcq1*. Overall, we showed that *Avr2* family is highly diverse in *P. infestans* and has likely co-evolved separately with two distinct *Solanum R* gene families.

We found that *Avr2* family members display high levels of polymorphisms and show evidence for diversifying selection. These features may be involved in the differential race-specificity to *P. infestans* mediated by *Solanum R* genes. Intriguingly, *P. infestans* isolates virulent on *Rpi-mcq1* plants contain more than one class II member. One potential explanation is that *Avr2* members of this class are important components to avoid recognition mediated by *Rpi-mcq1*. For example, members of class II may be suppressing the recognition of *Avr2* effector that is leading to a loss of resistance mediated by *Rpi-mcq1*. The class I members, such as PexRD11, that are recognized by R2 could then explain the *R2*-specific resistance to *P. infestans*. Also for other *R-Avr* interactions, effectors families are reported to act as suppressors, even when the resistance protein has been activated. For instance, the highly diverse *Avrblb1* family of *P. infestans* is grouped in three classes (I to III) (Champouret *et al.*, 2009) and the presence of members from class III allows the pathogen to overcome resistance triggered by *Rpi-blb1*, even when members from class I are present (Halterman *et al.*, 2010). It has been proposed that the suppression mechanism may be associated with competition for host recognition sites which results in blocking of recognition. Some effectors from diverse pathogens such as *Melampsora lini*, *Hyaloperonospora parasitica* and *Pseudomonas syringae*, are able to act as suppressors as well, indicating that some effectors can modify the typical result of an *R-Avr* interaction from resistance to susceptibility (Pedley & Martin, 2003; Kim *et al.*, 2005; Rehmany *et al.*, 2005; Ellis *et al.*, 2007). Further studies should address whether the differences in virulence profile to *P. infestans* mediated by *R2* versus *Rpi-mcq1* are attributed to suppression of *Avr2* by its allelic variants and to recognition of *PexRD11* by *R2*. To test the negative interaction between class II members, the new variants from this class should be cloned in an expression vector and agro-coinfiltrated with either *R2* or *Rpi-mcq1* and *Avr2*. The absence of *Avr2*-mediated cell death in agro-coinfiltrations with *Rpi-mcq1* may be an indication of *Avr2* suppression. In addition, complementation with the new allelic variants of *P. infestans* isolates which are avirulent on *Rpi-mcq1* potatoes, such as PIC99183 could compromise the avirulence phenotype, validating the suppression of *Avr2*.

Our results also show that in *P. infestans* isolates which are virulent on *R2* and *Rpi-mcq1*, class III members are completely absent, which might suggest that the presence of class III is

required for *R2* and *Rpi-mcq1*-mediated resistance. However, class III is hardly expressed in *P. infestans* isolates, so perhaps it is not the most likely explanation.

The diverse members of the *Avr2* family showed different recognition specificities mediated by *Solanum R* genes. Using co-agroinfiltrations of *R2* or *Rpi-mcq1* with 14 *Avr2* family members, different patterns of recognition were displayed. Class I members were purely recognized by *R2*. In contrast, *Avr2-like* and *PITG_08278* from class II and III respectively, were exclusively recognized by *Rpi-mcq1*. Based on these features, we hypothesize that *Avr2* family members have separately co-evolved with the *Solanum R2* and *Rpi-mcq1*, e.g. to avoid host recognition (Gilroy *et al.*, 2011). In turn, similar evolution features have been observed in *R2* and *Rpi-mcq1* families that display sequence polymorphism, positive selection and sequence exchange between paralogs to evolve new capabilities of effector recognition (Lokossou *et al.*, 2009) (Chapter 1, this thesis). For other *R-Avr* interactions in the *Solanum* – *P. infestans* pathosystem, similar characteristics have been reported. For example, the highly diverse *Avrblb1* and *Avrblb2* families of *P. infestans* have co-evolved with the *Solanum Rpi-blb1* and *Rpi-blb2* to avoid recognition by resistant *Solanum* plants (Oh *et al.*, 2009). This is consistent with an arm-race model in which the host-pathogen co-evolution is driven by selection for resistance in the host and virulence in the pathogen (Bergelson *et al.*, 2001). To test the recognition specificity of the entire *Avr2* family mediated by *R2* and *Rpi-mcq1*, the remaining six members of the family can be cloned in an expression vector. Subsequently, they can be agro-coinfitrated with either *R2* or R*pi-mcq1* and each additional member of the *Avr2* family.

Our findings add to the biology of RXLR effectors. The knowledge of the effector diversity is critical to understand the dynamics of natural pathogen populations and will help to monitor the potential occurrence of *P. infestans* isolates that can overcome *R2* and *Rpi-mcq1*. We propose that allelic variants of *Avr2* may be responsible for the differential virulence profile to *P. infestans* mediated by *R2* versus *Rpi-mcq1*.

Materials and methods

Plant Materials

Potato plants of the cultivar Désirée 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 greenhouse compartments. *Nicotiana benthamiana* plants were grown from seeds. In the greenhouse compartments, the temperature range from 18 to 22°C and day/night regime of 16h/8h at 70% relative humidity.

Phytophthora infestans isolates, culture conditions, and inoculum preparation

The *P. infestans* isolates used in this study are listed in Supporting information, Table S3 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 hours as previously described by Aguilera-Galvez *et al.* (2018).

Plasmid construction

Construction of pBinplus:*Rpi-blb3*, pBinplus:*Rpi-mcq1*, pBinplus:*R3a*, pK7wg2:*Avr2*, pK7wg2:*PexRD11*, pk7wg2:*Avr3a* was previously described (Vleeshouwers *et al.*, 2008; Gilroy *et al.*, 2011; Aguilera-Galvez *et al.*, 2018) (Supporting information, Table S6). For cloning *PITG_13936*, *PITG_21949*, *PITG_21645*, *PITG_13940*, *PITG_05121*, *AVR2-like*, *PITG_06077*, *PITG_07500*, *PITG_07499*, *PITG_08278*, *PITG_15972* and *PITG_19617*, the sequences of the C-terminal domain including the *att*L recombination sites were synthesized at Genewiz (UK) and cloned in the entry vector pUC57-amp. Subsequently, the entry clones were transferred to the destination vector pK7WG2 (Karimi *et al.*, 2002) by an LR reaction using LR-Clonase II enzyme® (Invitrogen), resulting in the constructs listed in Supporting information, Table S6.

Sequence analysis

Similarity searches to AVR2 and PexRD11 were performed using a basic local alignment search tool (BLAST). The E-value cutoff was 10 using a BLOSUM62 matrix, no filter, and an ungapped alignment (Altschul *et al.*, 1990). Multiple amino acid alignments were carried out using Muscle program (Edgar, 2004). Phylogenetic analyses were conducted using the Neighbor-joining method (Nei & Li, 1979) in Geneiuos® 9.1.2 (www.geneious.com). 1000 replicates of bootstrap were used for resampling. The evolutionary distances were measured as the proportion of nucleotide substitutions between sequences. The prediction of the signal peptide of the AVR2 family was performed by SignalP 4.1 server (Nielsen, 2017).

The raw RNA-seq reads were mapped to the RXLR effectors of *P. infestans* using the BWA-MEM algorithm implemented in BWA software v 0.7.12 with the default settings (Li & Durbin, 2010)England.

RNAseq of *P. infestans* isolates

Potato leaves from the cultivar Désirée from 6-8-week-old plants grown in greenhouse conditions were detached and placed in water-saturated oasis trays. The leaves were spot-inoculated at the abaxial leaf side with 10 µl droplets containing 5 *10^4 zoospores per ml for each isolate. After inoculation, the leaves were placed in water-saturated oasis trays and were incubated in a climate chamber at 15°C with a 16h photoperiod. RNA was isolated from the leaf discs sampled at 24 hpi and from mycelium grown on Rye medium and V8 medium. Samples were grinded by TissueLyser II (Qiagen) and RNA extraction was performed using

the RNAeasy Plant mini kit (Qiagen) following manufacturer's instructions. The gDNA eliminator spin column from the kit was used to remove the gDNA. The quality of the RNA samples was tested by agarose electrophoresis and quantified by Nanodrop (ThermoFisher). The RNA samples were sent to Novogene (Beijing, China) with dry ice. The RNA sequencing was performed by Ilumina Hiseq platform with a 150-paired end read metric.

Positive selection analysis

The rate of nonsynonymous nucleotide substitutions per synonymous site (d_N) and the rate of synonymous nucleotide substitutions per synonymous site (d_S) across all the amino acid sites in pairwise comparisons between nucleotide sequences were calculated using the approximate methods of Yang and Nielsen (2000) implemented in the YN00 program in PAMLX 1.3.1 software package (Xu & Yang, 2013). The amino acid sites that have been affected by diversifying selection, were identified by a maximum likelihood model of codon substitution (CODEML) implemented in PAMLX 1.3.1 software package by using the substitution models M0, M3, M7 and M8. Models M3 and M8 allow for heterogeneous selection pressures across codon sites, while their respective null models M0 and M7 only allows ratio classes with ω<1. Statistical significance was tested by comparing the null models M0 and M7 with their respective alternative models M3 and M8 using an LRT. Twice the difference in log likelihood ratio was compared with a chi-squared (X^2) distribution with degrees of freedom equaling the difference in the number of parameters estimated from the pair of models. The likelihood rations of the two models test whether an alternative model fits the data better than the null model (Anisimova *et al.*, 2001). Maximum-likelihood codon substitution models M0, M1, M2, M7, and M8 were used for the analysis. Positively selected sites were detected using Empirical Bayes statistic. Amino acids with high posterior probability for an advantageous class of sites (ω>1) were deemed more likely to be under diversifying selection ((Yang *et al.*, 2005).

Agrobacterium transient cell death assays

Agro-coinfiltrations were performed as previously described by (Domazakis *et al.*, 2017). Briefly, agro-coinfiltrations were performed on 3-4-week-old *N. benthamiana* plants using a suspension of *Agrobacterium tumefaciens* AGL1 strain containing the appropriate expression vectors (Supporting information, Table S6). Local symptoms of cell death responses were assessed at 3-4 days post-inoculation.

Acknowledgements

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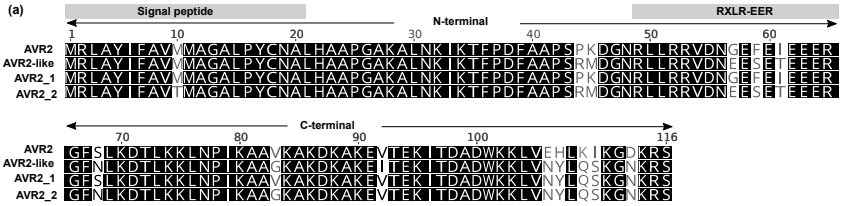
Supporting information

(a)



(b)

AVR2 Variant	Sequence number of Amino acid													
	N-terminal						C-terminal							
	10	44	45	57	59	61	68	83	92	106	107	109	110	113
AVR2	M	P	K	G	F	I	S	V	V	E	H	K	I	D
AVR2-like	M	R	M	E	S	T	N	G	I	N	Y	Q	S	N
AVR2_1	M	P	K	G	F	I	S	V	V	N	Y	Q	S	N
AVR2_2	T	R	M	E	S	T	L	G	V	N	Y	Q	S	N

FIGURE S1 | AVR2 family members from class II are polymorphic. (a) Multiple sequence alignment of AVR2, AVR2-like, AVR2-1 and AVR2-2. Identical amino acids are shaded in black. The N-terminal and C-terminal domains are indicated above the alignment. (b) Positions of polymorphic amino acid sites.

TABLE S1 | List of proteins with sequence similarity to AVR2 and PexRD11 from *Phytophthora infestans* T30-4. Results are based on a Blast search. The protein name, the protein description, the score, E-value, alignment length, identities and similarities are indicated.

Blastp	Protein name	Protein description	Score (bits)	E-value	Alignment length (%)	Identity (%)	Similarity (%)
AVR2	PITG_22870	Secreted RxLR effector peptide, putative	117.6	4.99e-28	100	100	100
	PITG_08943	Secreted RxLR effector peptide, putative	117.6	4.99e-28	100	100	100
	PITG_05121	Secreted RxLR effector peptide, putative	43.1	1.3e-5	80.4	41.2	51
	PITG_19617	Secreted RxLR effector peptide, putative	40.0	1.13e-4	64.7	33.3	47.1
	PITG_07500	Secreted RxLR effector peptide, putative	38.1	2.1e-4	64.7	31.4	49
	PITG_07499	Secreted RxLR effector peptide, putative	38.1	2.1e-4	64.7	31.4	49
	PITG_08278	Secreted RxLR effector peptide, putative	37.3	7.23e-4	64.7	31.4	45.1
	PITG_20025	Secreted RxLR effector peptide, putative	37.3	7.23e-4	64.7	31.4	45.1
	PITG_15972	Secreted RxLR effector peptide, putative	36.9	9.48e-4	51	33.3	39.2
	PITG_16221	Crinkler (CRN) family	29.7	0.138	64.7	27.5	29.4
	PITG_00323	Conserved hypothetical protein	29.3	0.188	78.4	25.5	47.1
	PITG_21949*	Secreted RxLR effector peptide, putative	21.3	0.263	23.5	15.7	17.6
	PITG_06077	Secreted RxLR effector peptide, putative	28.4	0.349	49	21.6	35.3
PexRD11	PITG_13930	Secreted RxLR effector peptide, putative	121.0	4.46e-29	100	100	100
	PITG_21949	Secreted RxLR effector peptide, putative	66.2	1.4e-12	100	51	74.5
	PITG_21645	Secreted RxLR effector peptide, putative	66.2	1.4e-12	96.1	51	70.6
	PITG_23009	Secreted RxLR effector peptide, putative	66.2	1.4e-12	96.1	51	70.6
	PITG_23008	Secreted RxLR effector peptide, putative	66.2	1.4e-12	96.1	51	70.6
	PITG_13956	Secreted RxLR effector peptide, putative	66.2	1.4e-12	96.1	51	70.6
	PITG_13940	Secreted RxLR effector peptide, putative	63.6	9.06e-12	100	47.1	74.5
	PITG_13936	Secreted RxLR effector peptide, putative	49.2	1.92e-12	64.7	39.2	47.1

TABLE S2 | List of proteins belonging to AVR2 family. Protein name and percentages of amino acids identity with AVR2 or PexRD11 are indicated.

Protein name	Percentage of identity at the Amino Acid Level	
	AVR2	PexRD11
PITG_13936	31.0	60.6
PITG_21645	21.1	51.0
PITG_13940	23.4	47.1
PITG_21949	21.3	51.0
PITG_13930 (PexRD11)	25.5	100
PITG_22870 (Avr2)	100	25.5
PITG_05121	43.8	15.9
PITG_06077	26.0	22.4
PITG_08278	35.3	26.5
PITG_19617	37.3	26.5
PITG_15972	63.0	37.9
PITG_07499	37.3	24.5
PITG_07500	35.3	24.5

TABLE S3 | List of isolates used in this study. The country, year of collection as well as the genotype source are indicated. Virulence data on transgenic potatoes expressing *R2* or *Rpi-mcq1* were obtained from Aguilera-Galvez *et al.* (2018).

Isolate name	Origin			Virulence	
	Country	Year	Source	*R2*	*Rpi-mcq1*
T30-4	The Netherlands	1995	F1 progeny from a cross between isolates 88029 and 88133	R	R
PIC99183	Mexico	1999	*S. stoloniferum*	R	R
NL07434	The Netherlands	2007	Potato from Averis seeds B.V	R	S
NL12003	The Netherlands	2012	cv. Frieslander	R	S
NL09096	The Netherlands	2009	Potato from C.Meijer B.V	R	S
PIC99189	Mexico	1999	*S. stoloniferum*	S	S
UK3928A	United Kingdom	2006	Potato	S	S

TABLE S4 | Pairwise comparison of the ratios (ω= d_N/d_S) of nonsynonymous (d_N) to synonymous nucleotide (d_S) substitution rates and d_N and d_S values among 20 sequences *Avr2* sequences. d_N and d_S substitutions are indicated below the diagonal. The d_N/d_S ratio is indicated above the diagonal.

						I				
		PITG 13930	**PITG 13936**	**PITG 13940**	**PITG 15972**	**PITG 19617**	**PITG 19617_1**	**PITG 21645**	**PITG 21949**	**PITG 21949_1**
	PITG_13930		0.857	0.275	0.535	0.535	0.553	0.425	0.255	**1.357**
	PITG_13936	0.264		0.551	0.301	0.301	0.298	0.512	0.505	**1.496**
		0.308								
	PITG_13940	0.259	0.226		0.431	0.431	0.446	N/A	N/A	**1.344**
		0.941	0.410							
	PITG_15972	0.548	0.456	0.505		N/A	N/A	0.482	0.572	**1.809**
		1.024	1.512	1.172						
I	**PITG_19617**	0.548	0.456	0.505	0.000		N/A	0.482	0.572	**1.809**
		1.024	1.512	1.172	0.000					
	PITG_19617_1	0.575	0.478	0.530	0.142	0.142		0.498	0.591	**1.827**
		1.038	1.600	1.187	0.000	0.000				
	PITG_21645	0.250	0.208	0.041	0.541	0.541	0.566		N/A	**2.903**
		0.589	0.406	0.000	1.123	1.123	1.136			
	PITG_21949	0.239	0.207	0.013	0.537	0.537	0.561	0.027		**2.383**
		0.937	0.409	0.000	0.938	0.938	0.949	0.000		
	PITG_21949_1	2.118	1.863	2.079	2.042	2.042	2.052	2.623	2.233	
		1.562	1.246	1.547	1.129	1.129	1.123	0.903	0.937	
	Avr2	0.528	0.430	0.405	0.308	0.308	0.327	0.447	0.433	3.223
		0.587	0.795	0.868	1.167	1.167	1.194	0.811	0.870	0.684
	Avr2_1	0.528	0.430	0.405	0.308	0.308	0.327	0.447	0.433	3.223
		0.587	0.795	0.868	1.167	1.167	1.194	0.811	0.870	0.684
	Avr2_2	0.522	0.416	0.395	0.262	0.262	0.281	0.435	0.422	3.221
		0.652	0.943	0.977	1.738	1.738	1.817	0.920	0.980	0.656
II	**Avr2_like**	0.522	0.416	0.395	0.262	0.262	0.281	0.435	0.422	3.221
		0.652	0.943	0.977	1.739	1.739	1.818	0.920	0.980	0.656
	PITG_05121	0.864	0.834	0.756	1.283	1.283	1.255	0.751	0.747	1.739
		0.911	1.456	2.738	1.475	1.475	1.488	1.316	2.653	1.090
	PITG_05121_1	0.889	0.851	0.731	1.330	1.330	1.291	0.738	0.730	1.547
		0.791	1.552	2.160	1.094	1.094	1.097	1.215	2.046	1.157
	PITG_06077	0.747	0.668	1.005	0.468	0.468	0.466	0.898	0.934	3.228
		0.774	0.510	0.735	1.380	1.380	1.396	0.764	0.728	0.870
	PITG_07499	0.593	0.554	0.638	0.133	0.133	0.149	0.608	0.601	1.835
		1.246	1.178	1.020	0.281	0.281	0.283	1.177	1.020	2.471
III	**PITG_07499_1**	0.713	0.537	0.603	0.151	0.151	0.167	0.572	0.569	1.789
		1.129	2.220	1.063	0.228	0.228	0.230	1.256	1.059	1.718
	PITG_07500	0.581	0.480	0.612	0.119	0.119	0.134	0.582	0.577	1.830
		1.170	2.052	1.005	0.225	0.225	0.227	1.171	1.000	1.591
	PITG_08278	0.579	0.485	0.535	0.014	0.014	0.029	0.573	0.567	2.046
		1.021	1.511	1.173	0.000	0.000	0.000	1.126	0.939	1.125

		II					III			
Avr2	**Avr2_1**	**Avr2_2**	**Avr2 like**	**PITG 05121**	**PITG 05121_1**	**PITG 06077**	**PITG 07499**	**PITG 07499_1**	**PITG 07500**	**PITG 08278**
0.898	0.898	0.800	0.800	0.949	**1.124**	0.966	0.476	0.631	0.497	0.566
0.540	0.540	0.441	0.441	0.573	0.548	**1.311**	0.471	0.242	0.234	0.321
0.467	0.467	0.404	0.404	0.276	0.338	**1.366**	0.626	0.567	0.609	0.456
0.264	0.264	0.151	0.151	0.870	**1.216**	0.339	0.473	0.659	0.527	N/A
0.264	0.264	0.151	0.151	0.870	**1.216**	0.339	0.473	0.659	0.527	N/A
0.274	0.274	0.155	0.155	0.843	**1.177**	0.334	0.527	0.726	0.592	N/A
0.551	0.551	0.473	0.473	0.570	0.607	**1.175**	0.516	0.455	0.497	0.509
0.497	0.497	0.431	0.431	0.282	0.357	**1.282**	0.589	0.537	0.577	0.604
4.714	**4.714**	**4.908**	**4.908**	**1.596**	**1.337**	**3.712**	**0.742**	**1.041**	**1.150**	**1.819**
	N/A	**1.909**	**1.909**	**1.073**	**1.182**	0.848	0.265	0.198	0.157	0.281
0.000		**1.909**	**1.909**	**1.073**	**1.182**	0.848	0.265	0.198	0.157	0.281
0.000										
0.069	0.069		N/A	**1.295**	**1.435**	0.740	0.206	0.261	0.218	0.165
0.036	0.036									
0.069	0.069	0.000		**1.295**	**1.435**	0.740	0.206	0.261	0.218	0.165
0.036	0.036	0.000								
1.122	1.122	1.103	1.103		0.126	0.560	0.669	0.762	**1.111**	0.884
1.046	1.046	0.852	0.852							
1.077	1.077	1.067	1.067	0.086		0.510	0.908	0.993	**1.417**	**1.237**
0.911	0.911	0.743	0.743	0.680						
0.544	0.544	0.529	0.529	0.959	0.882		0.281	0.339	0.310	0.364
0.641	0.641	0.715	0.715	1.713	1.729					
0.297	0.297	0.290	0.290	0.955	1.000	0.453		**1.759**	**1.070**	0.537
1.120	1.120	1.404	1.404	1.428	1.101	1.609				
0.331	0.331	0.323	0.323	0.931	0.973	0.443	0.067		N/A	0.740
1.671	1.671	1.240	1.240	1.222	0.979	1.307	0.038			
0.284	0.284	0.275	0.275	1.189	1.252	0.436	0.040	0.054		0.602
1.802	1.802	1.262	1.262	1.069	0.884	1.403	0.037	0.000		
0.330	0.330	0.284	0.284	1.288	1.339	0.497	0.150	0.168	0.135	
1.174	1.174	1.721	1.721	1.457	1.082	1.365	0.280	0.228	0.225	

TABLE S5 | Likelihood Ratio Test Results for *Avr2* family. a) lnL, Log likelihood value, (b) Amino acid sites, based on *Avr2* sequence, inferred to be under diversifying selection with a probability >95%, and 99%, (c) Likelihood radio test: $2L=2(lnl_{alternative\ hypothesis}-lnl_{null\ hypothesis})$.

Model	Estimated parameters	lnL [a]	Positively selected sites [b]	Model comparison	2ΔL [c]	X^2 Critical value	Degrees of freedom
M0: one ratio	ω=0,54	-3446.63	Not allowed				
M3: discrete	P_0=0,253, P_1=0,563, P_2= 0,184, ω_0=0,165, ω1= 0,628 ω2=1,417	-3418.25	28A, 31L, 41A, 78L, 84I,88V 95K, 96E, 97V, 100K 113L, 119I	M3 Vs M0	56.76	13.28	4
M7: beta	P= 1,135, q=0,775	-3419.73	Not allowed				
M8: beta + ω	P_0= 0,823, P=1,492, q=1,482, P1=0,177, ω=1,357	-3418.68	28A, 31L, 41A, 78L, 84I,88V 95K, 96E, 97V, 100K 113L, 119I	M8 Vs M7	2.1	9.21	2

TABLE S6 | List of constructs used for *Agrobacterium*-mediated transient expression. The purpose, the insert, the binary vector and the reference are indicated.

Purpose	Insert	Binary vector	Reference
Cell death assays	*Rpi-blb3*	pBinplus	(Aguilera-Galvez et al., 2018)
	Rpi-mcq1	pBinplus	(Aguilera-Galvez et al., 2018)
	R3a	pBinplus	(Vleeshouwers, 2008)
	Avr3a	pK7WG2	(Vleeshouwers, 2008)
	Avr2	pK7WG2	(Gilroy, 2011)
	PITG_13936	pK7WG2	This study
	PITG_21949	pK7WG2	This study
	PITG_21645	pK7WG2	This study
	PITG_13940	pK7WG2	This study
	PITG_13930	pK7WG2	(Champouret 2010)
	PITG_05121	pK7WG2	This study
	Avr2_like	pK7WG2	This study
	PITG_06077	pK7WG2	This study
	PITG_07500	pK7WG2	This study
	PITG_07499	pK7WG2	This study
	PITG_08278	pK7WG2	This study
	PITG_15972	pK7WG2	This study
	PITG_19617	pK7WG2	This study

Chapter 5

Different signaling pathways are activated upon recognition of *AVR2* of *Phytophthora infestans* by *Solanum* resistance proteins

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Abstract

Plant pathogens secrete and translocate an arsenal of RXLR effector proteins to promote infection of the host. The RXLR effector AVR2 of *Phytophthora infestans* targets the potato BSU-like 1 (BSL1), a phosphatase which is involved in brassinosteroid (BR) hormone signaling, and this leads to enhanced infection. Besides, AVR2 can be recognized by two evolutionary unrelated immune receptors, R2 and Rpi-mcq1, from different wild *Solanum* species. The mechanism of AVR2 recognition mediated by R2 is known to require the interaction with the BSL1 protein, however AVR2 signaling mediated by Rpi-mcq1 remains to be understood. Here we investigate whether BSL1 is required for AVR2 recognition mediated by Rpi-mcq1. Virus-induced gene silencing (VIGS) and genetic complementation indicated that Rpi-mcq1-mediated recognition of AVR2 is independent of BSL1. In line with these findings, we found that BSL1 is not required for Rpi-mcq1-mediated resistance to *P. infestans*. Chemical inhibition of plant phosphatases with okadaic acid demonstrated that phosphatase activity is required for Rpi-mcq1-mediated recognition of AVR2, however, the phosphatase involved remains unknown. Overall, our study shows that differential signaling pathways are activated upon recognition of AVR2 from *P. infestans* by R2 versus Rpi-mcq1.

Keywords: AVR2, BSL1, effector target, guard model, late blight, *Phytophthora infestans*, potato, resistance, R2, Rpi-mcq1, Serine/Threonine phosphatases, signaling pathways, *Solanum*.

Introduction

The oomycete pathogen *Phytophthora infestans* is the causal agent of late blight, the most devastating disease of potato. It remains as a major threat for potato production, which results in yield losses of 16% and a total annual cost of more than €6 billion (Haverkort *et al.*, 2016). Breeding for disease resistance has started since the mid-19th century, when the Mexican wild *Solanum demissum* was identified as a strong source of resistance to late blight. Since then, resistance breeding strategies have been focused on the introgression of resistance (*R*) genes into potato cultivars (Malcolmson & Black, 1966), such as *R1* to *R11* from *Solanum demissum*, and many more (Aguilera-Galvez *et al.*, 2018). However, these *R* genes are typically quickly overcome by new adapted *P. infestans* strains (Fry, 2008).

Plants have evolved a multilayered immune system to prevent pathogen colonization. The pathogens in turn, have developed effector proteins to manipulate host cellular processes, including suppression of plant defense responses (Win *et al.*, 2012). Effectors are small secreted molecules which interact with their host either inside the host cell or in the apoplast (Kamoun, 2006). Many of these effectors are characterized by the presence of an Arg-X-Leu-Arg (RXLR) motif, that serves as a signal for translocation (Bhattacharjee *et al.*, 2006). Inside the host cells, effectors are recognized by R proteins, leading to the activation of effector-triggered immunity (ETI), which often leads to a form of programmed cell death that is known as a hypersensitive response (HR) (Dodds & Rathjen, 2010). Effectors can be recognized either directly through a physical binding with the R protein, or indirectly by the interaction with additional proteins that are called effector targets in the host (Tameling & Takken, 2008). Significant progress has been made in understanding the mechanism of indirect effector recognition mediated by R proteins, which monitor effector-induced modifications in host proteins. Such proteins can be genuine virulence targets or decoy proteins that have evolved to mimic the effector target (van der Hoorn & Kamoun, 2008). Some effector targets are required for host resistance or susceptibility, and therefore the effectors act as positive or negative regulators of immunity (Rovenich *et al.*, 2014; van Schie & Takken, 2014).

AVR2 of *P. infestans* is a well-studied RXLR effector of *P. infestans* that plays an important role in promoting pathogen virulence (Gilroy *et al.*, 2011). *Avr2* is up-regulated during the early biotrophic infection phase on potato host plants and enhances pathogen colonization (Turnbull *et al.*, 2017). AVR2 interacts with a potato homolog of the *Arabidopsis* brassinosteroid-insensitive1 suppressor (BSU1), StBSL1 (BSU1-like) (Turnbull *et al.*, 2019). The BSL family represent protein phosphatases with a kelch-like domain (PPKL) and belong to the phosphoprotein phosphatase (PPP) family of Serine/Threonine phosphatases. The BSLs are implicated in growth-promoting brassinosteroid (BR) signaling in plants (Saunders *et al.*, 2012; Maselli *et al.*, 2014), and the members BSL1, BSL2 and BSL3 are assumed to display overlapping roles in the BR pathway (Mora-García *et al.*, 2004). The activation of BR-

signaling in potato up-regulates the transcription factor StCHL1 which leads to enhanced leaf colonization by *P. infestans* as well as suppression of Pattern-Triggered Immunity (PTI) responses. This points to AVR2 being implicated in the cross talk between BR-signaling and plant innate immunity (Turnbull *et al.*, 2017).

AVR2 is recognized by two unrelated R protein families, R2 and Rpi-mcq1, that originate from diverse wild *Solanum* species (Aguilera-Galvez *et al.*, 2018). *R2* is representing an extensive gene family of at least 10 members, which resides in the major late blight locus on chromosome IV of a number of Mexican *Solanum* species (Park *et al.*, 2005; Lokossou *et al.*, 2009; Champouret, 2010; Aguilera-Galvez *et al.*, 2018). In contrast, *Rpi-mcq1* belongs to an evolutionary unrelated gene family on chromosome IX and occurs in Peruvian *Solanum* species, *S. mochiquense* and *S. huancabambense* (Chapter 2, this thesis). The mechanism of AVR2 recognition mediated by R2 requires the interaction with StBSL1 (Saunders *et al.*, 2012). However, the signaling pathway activated upon AVR2 recognition by Rpi-mcq1 remains to be understood. In this study, we investigate whether recognition of AVR2 by Rpi-mcq1 is also dependent on BSL1. We performed virus-induced gene silencing (VIGS) experiments of BSL1, genetic complementation approaches and chemical inhibition studies of plant phosphatases to test whether BSL1 is required for Rpi-mcq1-mediated recognition of AVR2 and resistance to *P. infestans*.

Results

Rpi-mcq1-mediated recognition of AVR2 is independent of BSL1 expression

To study the role of BSL1 in AVR2 recognition mediated by Rpi-mcq1, a tobacco rattle virus (TRV)-mediated VIGS approach was applied. The construct pTRV:5'*NbBSL1* was agro-infiltrated in *N. benthamiana* seedlings and pTRV:*GUS* and pTRV:*PDS* were included as a negative and positive control of the silencing, respectively (Kumagai *et al.*, 1995). Experiments were carried out in triplicate on 15 plants, using 3 leaves per plant. Two weeks post-inoculation (wpi) with VIGS constructs, phenotypic observations were carried out. No differences in morphology were observed between *BSL1*-silenced plants and those silenced with the negative control pTRV:*GUS*. All plants that had been treated with pTRV:*PDS* showed a photobleaching phenotype, which indicates the efficiency of the VIGS treatment. Subsequently, *Avr2* was agro-co-infiltrated either with the *R2* homolog *Rpi-blb3* or with *Rpi-mcq1* in the VIGS-treated plants. Single infiltrations of *Rpi-blb3*, *Rpi-mcq1* and *Avr2* were included as negative controls. Co-infiltrations of *Rpi-sto1*/*Avr-blb1*, were included as a positive control for R/AVR-mediated cell death (Vleeshouwers *et al.*, 2011). The infiltrated leaves were monitored for local symptoms at 4 days post-inoculation (dpi). Confluent cell death responses were observed for *Rpi-mcq1*/*Avr2* agro-coinfiltrations in *BSL1* and *GUS*-silenced plants (Fig.1), which indicates that Rpi-mcq1-mediated cell death is not compromised by silencing of *BSL1*. In contrast, no cell

death responses were observed in agro-co-infiltrations performed with *Rpi-blb3*/*Avr2* in *BSL1*-silenced plants, whereas cell death responses were observed in *GUS*-silenced plants as previously described by Saunders *et al.* (2012). For *Rpi-sto1*/*Avrblb1* agro-co-infiltrations, cell death responses were noted in leaves of *BSL1*-silenced plants as well as *GUS*-silenced plants, which confirm that BSL1 silencing is not required for this R/AVR pathway.

To quantify the silencing levels of BSL1 in *N. benthamiana* (NbBSL1), quantitative real time PCR (qRT-PCR) was performed. Leaf discs of three leaves per plant, from 15 plants were sampled and pooled at two weeks post-inoculation with the VIGS constructs. The experiment was conducted in three biological replicates. The accumulation of NbBSL1 transcripts was measured and the results were normalized against the accumulation of the NbEF1α transcripts (reference gene). The qRT-PCR data confirmed a reduction in the accumulation of NbBSL1 transcripts in *BSL1*-silenced plants compared to *GUS*-silenced plants (Supporting information, Fig. S1).

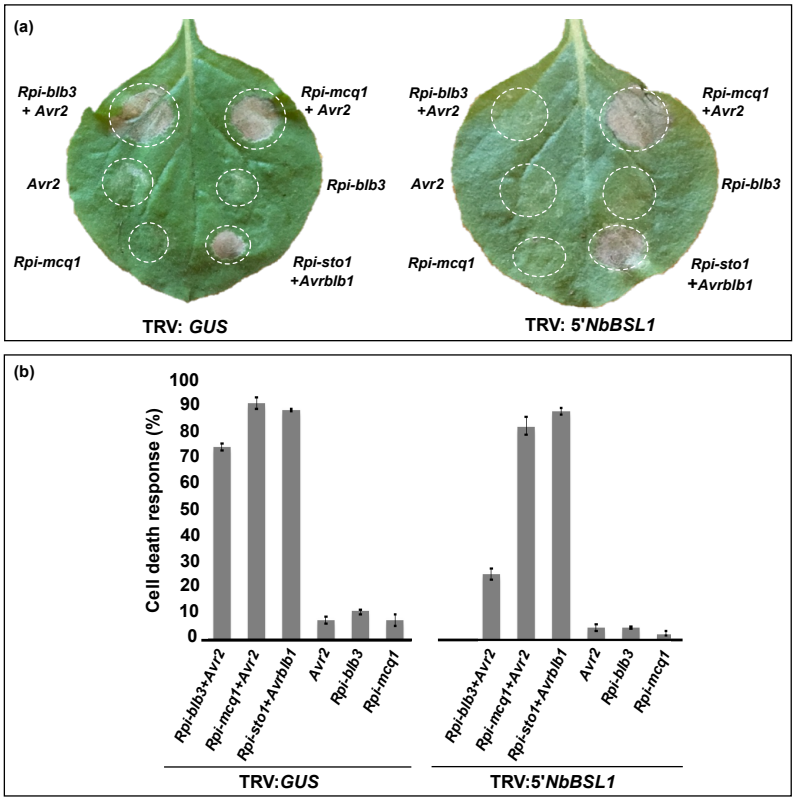

FIGURE 1 | BSL1 is not required for Rpi-mcq1- mediated recognition of AVR2. *GUS* and *BSL1*-silenced plants were agro-co-infiltrated with *Rpi-blb3*/*Avr2* and *Rpi-mcq1*/*Avr2*. Agro-infiltrations of *Rpi-sto1*-*Avrblb1* and *Avr2*, *Rpi-blb3 and Rpi-mcq1* were included as positive and negative controls, respectively (a) Representative pictures of VIGS-treated leaves at 4 dpi. (b) Percentage of infiltrations resulting in a cell death response. Error bars represent the standard error.

Rpi-mcq1-mediated recognition of AVR2 is independent from StBSL1 and StBSL1^{H648V}

We investigated whether the phosphatase activity of BSL1 is required for Rpi-mcq1-mediated cell death in *N. benthamiana*. To this aim, site-directed mutagenesis was performed on BSL1 to generate a variant with modifications in the active site within the phosphatase domain. Active site predictions were done in the Pfam protein family database (Finn *et al.*, 2010). The histidine residue at position 648 was selected and mutated to valine to generate the phosphatase-dead mutant StBSL1^{H648V} (Turnbull, 2016). To assess the effect of StBSL1 and StBSL1^{H648V} in Rpi-mcq1-mediated cell death responses, independent agro-co-infiltrations of *StBSL1*, *StBSL1*^{H648V} and empty vector with either *Rpi-blb3*/*Avr2* or *Rpi-mcq1*/*Avr2* were conducted in *N. benthamiana*. Agro-co-infiltrations with *Rpi-sto1*/*Avrblb1* were included as a negative control. Since high levels of cell death incidence from 80-100% usually occur upon *Rpi-mcq1*/*Avr2* and *Rpi-blb3*/*Avr2* agro-co-infiltrations (Figure 1), which can be too high to detect increased cell death, we adjusted the concentration of *Agrobacterium* cultures for each construct to a final OD_{600} of 0.5, compared to the usual OD_{600} of 1. Three independent experiments were performed, with each four plants and three leaves per plant. At two dpi, *Rpi-blb3*/*Avr2*/empty vector agro-co-infiltrated leaf panels showed a cell death response index of 47% (Fig. 2). A significant increase in cell death responses index to 81% was observed in *Rpi-blb3*/*Avr2*/*StBSL1* agro-co-infiltrations, compared with empty vector agro-co-infiltrations ($p<0,05$). In contrast, a reduction in cell death responses to 16% was noted in agro-co-infiltrations of *Rpi-blb3*/*Avr2*/*StBSL1*^{H648V} compared with empty vector and *StBSL1* agro-co-infiltrations ($p<0,05$) (Fig. 2). The boost in cell death responses observed in agro-co-infiltrations of *Rpi-blb3*/*Avr2*/*StBSL1* and suppression of cell death responses by *StBSL1*^{H648V}, confirmed that the BSL1 kinase activity is required for Rpi-blb3-mediated cell death upon recognition of AVR2. In contrast, for the Rpi-mcq1/AVR2 and Rpi-sto1/AVRblb1 interactions, similar levels of death responses were noted when *StBSL1*, *StBSL1*^{H648V} and the empty vector were overexpressed and no statistically significant differences were noted between the agro-co-infiltrations ($p<0,05$) (Fig. 2). These results further confirm that BSL1 is not required for Rpi-mcq1-mediated cell death, but it is required for Rpi-blb3, in which overexpression of StBSL1 and StBSL1^{H648V} leads to enhanced and suppressed cell death responses, respectively.

StBSL1^{H648V} or empty vector were agro-co-infiltrated with *Rpi-blb3*/*Avr2*, *Rpi-mcq1*/*Avr2* or *Rpi-sto1*/*Avrblb1* in *N. benthamiana* leaves. Average cell death index is indicated in the graph and error bars indicate the standard error of three biological repeats. Statistical differences were assessed using analysis of variance at $p<0.05$ (One-way ANOVA with LSD test). Representative pictures of treated leaves were taken at 2 dpi.

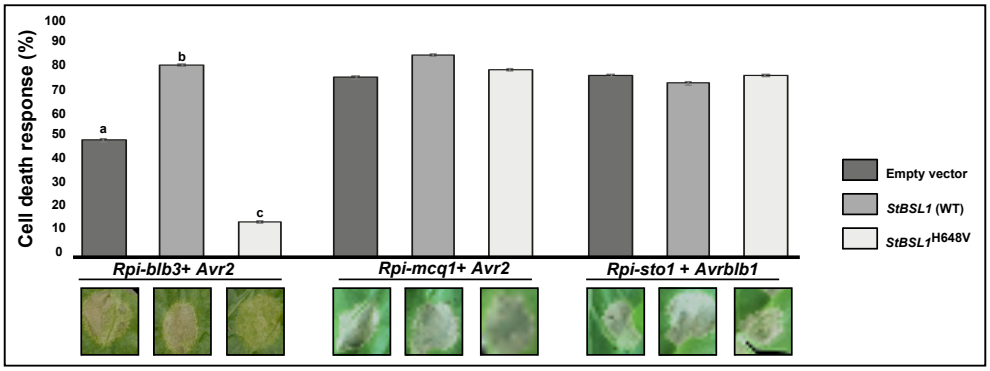

FIGURE 2 | Overexpression of *StBSL1*^{H648V} leads to suppression of AVR2-induced cell death with Rpi-blb3 but not with Rpi-mcq1. *Agrobacterium* cultures expressing *StBSL1*,

BSL1 silencing did not perturb Rpi-mcq1-based resistance to *P. infestans*

To investigate whether BSL1 is involved in *Rpi-mcq1*-mediated resistance of *P. infestans*, we conducted a VIGS experiment, followed by a transient complementation assay and a disease test. First, *N. benthamiana* seedlings were agro-infiltrated with pTRV-*5'NbBSL1*, and agro-infiltrations with pTRV:*GUS* and pTRV:*PDS* were performed as negative and positive controls for the silencing, respectively (Kumagai *et al.*, 1995). At two weeks post-inoculation with the VIGS constructs, all *PDS*-silenced plants showed a photobleaching phenotype. Subsequently, leaf panels of TRV-treated plants were agro-infiltrated with *Rpi-blb3* and *Rpi-mcq1*. *Rpi-sto1* and empty vector agro-infiltrations were included as positive control of resistance and negative control, respectively. The plants were incubated in the greenhouse and after 2 days, agro-infiltrated leaf panels were spot-inoculated with spore suspensions of *P. infestans* isolate NL90128. Macroscopic observations were carried out at 8 dpi. After visual scoring, the leaves were stained with trypan blue to further assess the extent of the *P. infestans* colonization. Three independent experiments were carried out with 5 plants and 3 replicates per plant. In all experiments, we observed contrasting phenotypes between *BSL1*- silenced plants and *GUS* controls for *Rpi-blb3*-treated leaf tissue. No symptoms of infection were found in leaf panels from *GUS*-silenced plants (Fig. 3a), whereas lesions with abundant sporulation of *P. infestans* were noted in leaf panels from *BSL1*-silenced plants (Fig. 3c). These results indicate that *R2*-mediated resistance to *P. infestans* is compromised by silencing of *BSL1*, in line with the previous findings of Saunders *et al.* (2012). In contrast, for *Rpi-mcq1* agro-infiltrated leaf panels, no lesions were observed for either *GUS* or *BSL1*-silenced plants (Figs. 3a and 3c). The same was found for the resistant positive control treatment with *Rpi-sto1*. For the negative control leaves that were agro-infiltrated with the empty vector, abundant sporulation and infection symptoms were observed (Figs. 3b and 3d) for both *GUS* and *BSL1*-silenced plants. From these findings, we conclude that silencing of BSL1 does not compromise resistance to *P. infestans* mediated by *Rpi-mcq1*.

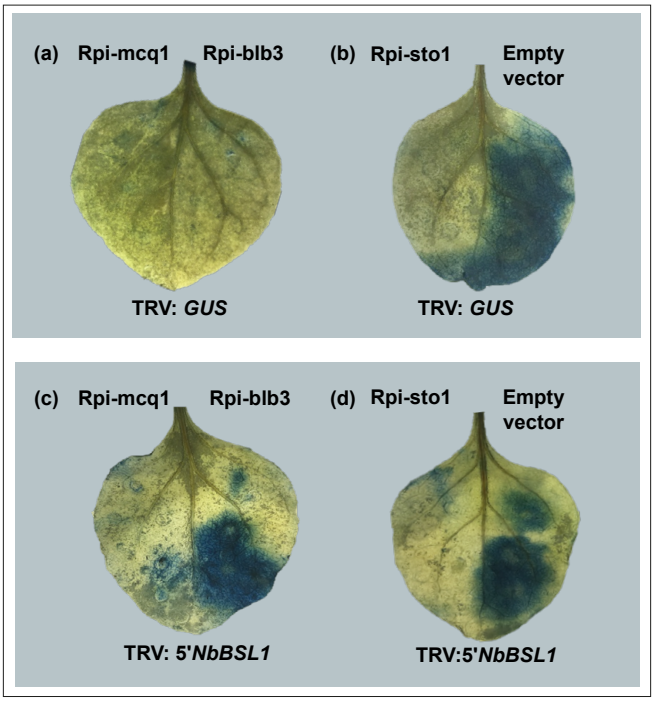

FIGURE 3 | Silencing of BSL1 does not compromise Rpi-mcq1-mediated resistance to *P. infestans* in *N. benthamiana*. *GUS* and *BSL1*-silenced plants were agro-infiltrated with (a,c) *Rpi-blb3* and *Rpi-mcq1* and (b,d) *Rpi-sto1* and empty vector. Two days later, *P. infestans* isolate NL90128 was spot-inoculated on agro-infiltrated leaf panels. No lesions of *P. infestans* were observed on *BSL1*-silenced plants transiently expressing Rpi-mcq1 visualized by trypan blue staining.

Rpi-mcq1-mediated cell death does not occur in absence of Serine/Threonine phosphatases

Okadaic acid (OA) is a potent inhibitor of the protein phosphatases 1 (PP1) and 2A (PP2A) (Swingle *et al.*, 2007). To get further evidence for requirement of phosphatase activity for Rpi-blb3 or Rpi-mcq1-mediated recognition of AVR2, a chemical phosphatase inhibition assay was performed. OA was mixed with agro-suspensions of either *Rpi-blb3/Avr2*, *Rpi-mcq1/Avr2* or *Rpi-sto1/Avr2*. Ethanol, the solvent of OA, was included as negative control and mixed with the same *Agrobacterium* suspensions. The resulting mixtures were incubated for 2 hours at room temperature. Subsequently, the mixtures were infiltrated in 3-week-old *N. benthamiana* leaves. The treatment with OA or ethanol was repeated in the infiltrated leaves at 24 hours post-infiltration (hpi). The plants were monitored for occurrence of cell death responses at 3 dpi. In presence of OA, a significant decrease in cell death responses was observed in the leaf panels co-infiltrated with *Rpi-blb3/Avr2* and *Rpi-mcq1/Avr2*. (Fig. 4). In contrast, in the negative control treatments with just the ethanol solvent, *Rpi-blb3/Avr2* and *Rpi-mcq1/Avr2* co-infiltrations led to strong cell death responses (Figs. 4b and 4c). The suppression of R2-mediated cell death

responses by phosphatase inhibition with OA was previously described (Turnbull, 2016), however, it had not yet been shown that phosphatase inhibition can also compromise *Rpi-mcq1*-mediate cell death. The leaf panels that were co-infiltrated with *Rpi-sto1/Avrblb1* showed confluent cell death everywhere, irrespective of the presence of OA or ethanol (Figs. 4a-b). Overall, the reduction of cell death responses for *Rpi-blb3/Avr2* as well as *Rpi-mcq1/Avr2* in OA-treated tissue indicates that phosphatase activity is required for AVR2 recognition mediated by both Rpi-blb3 and Rpi-mcq1. Further studies should be conducted in order to identify the exact identity of the phosphatase that is required for Rpi-mcq1-mediated cell death.

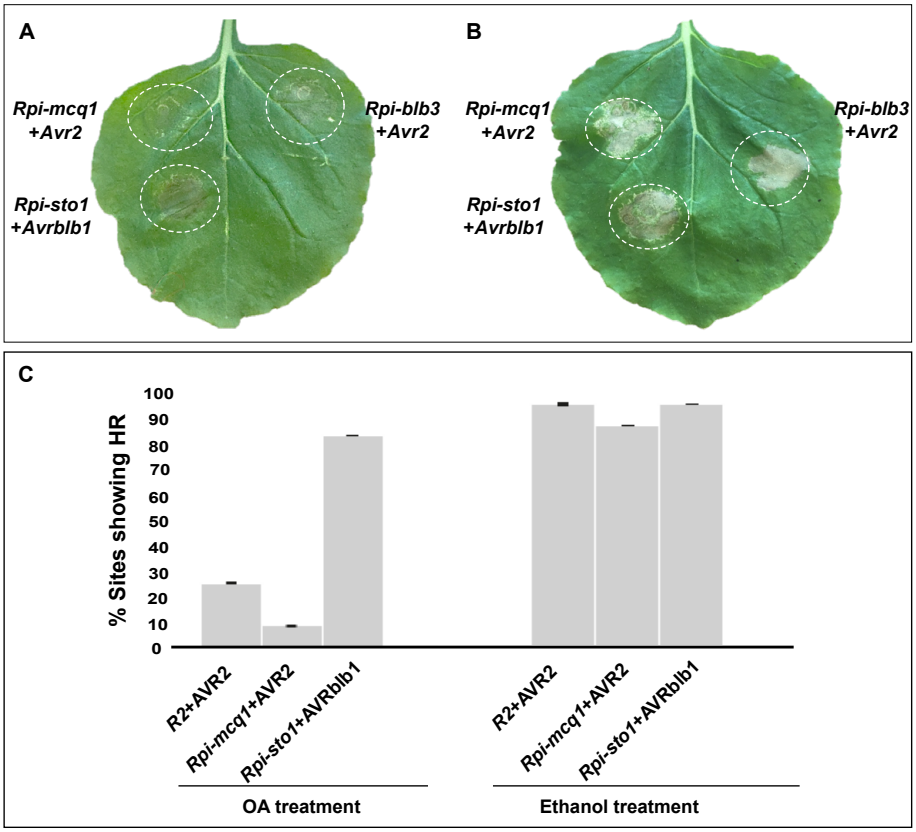

FIGURE 4 | Phosphatase inhibition compromise Rpi-blb3 and Rpi-mcq1-medicate cell death. Representative pictures of *N. benthamiana* leaves in presence of (a) OA, or (b) ethanol. (c) Percentage of cell death responses. Error bars indicate the standard error of three biological repeats.

Discussion

The plant phosphatase BSL1 is known to be required for Rpi-blb3-mediated cell death to AVR2 and resistance to *P. infestans* (Saunders *et al.*, 2012). In this study, we found that BSL1 is not required for Rpi-mcq1-mediated defense. In *BSL1*- silenced plants, we detected a decrease in cell death after agro-co-infiltrating Rpi-blb3/AVR2, but not for Rpi-mcq1/AVR2. Overexpression of StBSL1 and StBSL1^{H648V} led to an increase and a reduction of cell death for Rpi-blb3/AVR2 treatments, respectively, but not for Rpi-mcq1/AVR2, which confirms that Rpi-mcq1/AVR2 responses are not dependent on BSL1. We conclude that the recognition of AVR2 mediated by the R2 homolog Rpi-blb3 versus Rpi-mcq1, involves the activation of two different pathways. This is not unexpected, since AVR2 recognition has evolved independently from *Rpi-blb3* and *Rpi-mcq1* and thus, the signaling pathways are not unlikely to be different (Aguilera-Galvez *et al.*, 2018). The results of our experiments with altered *BSL1* expression are in line with the evidence obtained using a chemical inhibitor of phosphatases. We found that the inhibition of the plant phosphatases PP1 and PP2 with okadaic acid results in a reduction of Rpi-blb3/AVR2 cell death as well as Rpi-mcq1/AVR2, suggesting that another plant phosphatase may be involved in Rpi-mcq1-mediated recognition of AVR2. It remains to be determined whether this unknown phosphatase is another member of the BSL family, or whether it represents a different phosphatase.

Elucidating the mechanism of effector recognition is crucial to understand the effector-triggered immunity. In recent years, some studies have revealed a few cases for direct pathogen recognition mediated by *R* genes (Jia *et al.*, 2000; Deslandes *et al.*, 2003; Dodds *et al.*, 2006), however, indirect recognition has been shown to occur more often (van der Hoorn & Kamoun, 2008; Saunders *et al.*, 2012; Cesari, 2018). Interestingly, pathogen effectors are often highly specific and can discriminate between related proteins and e.g. precisely associate with different members of a host protein family. For example, the recognition of AVR2 of *Cladosporium fulvum* by Cf-2 from tomato is mediated by the inhibition of two papain-like cysteine proteases RCR3 and PIP1 (Shabab *et al.*, 2008). Our studies confirm previous findings that the recognition of AVR2 of *P. infestans* by R2 depends on the BSL1 member of the BSL family. We provide evidence that Rpi-mcq1-mediated recognition of AVR2 is also dependent of phosphatase activity, but not BSL1. We argue that other members of the BSL family, such as BSL2, or BSL3 might be good candidates for interacting with Rpi-mcq1/AVR2, however, we cannot exclude that other plant phosphatases may be involved in the mechanism of AVR2 recognition. Several members of the Serine/Threonine protein kinase family have been associated with negative regulation of immune responses at diverse pathways (He *et al.*, 2004; Segonzac *et al.*, 2014). This could be consistent with AVR2 targeting by related members of the BSL family to ensure the infection process and the suppression of host defense responses.

Some effectors target host proteins with that facilitate the pathogen infection (*S*-genes), such as negative regulators of immunity (van Schie & Takken, 2014). For example, the bHLH transcription factor has a central role in the crosstalk between growth and immunity in *Arabidopsis* (Fan *et al.*, 2014) and the same was found for the potato homolog *StCHL1* (Turnbull *et al.*, 2017). Several breeding strategies for durable disease resistance are focused on disabling susceptibility factors. To overcome *S* gene-based resistance for the pathogen would imply the acquisition of a new virulence function, which is potentially more difficult to accomplish. Ultimately, for educated resistance breeding, multiple approaches can be combined and *R* genes as well as *S* genes can be exploited. Detailed mechanistic knowledge about the signaling pathways that are activated during pathogen infection will be key to achieve a more durable resistance to late blight in potato.

Materials and methods

Plant material

Nicotiana benthamiana plants were grown from seeds and maintained in climate-regulated greenhouse compartments within the temperature range of 18-22 °C and under 16h/8h day/night regime at 70% relative humidity.

Plasmid construction

Construction of pBinplus:*Rpi-blb3*, pBinplus:*Rpi-mcq1*, pBinplus:*Rpi-sto1*, pK7WG2:*Avr2*, pK7WG2:*Avrblb1*, pTRV2:*GUS* and pTRV2:*PDS* was previously described (Vleeshouwers *et al.*, 2008; Gilroy *et al.*, 2011; Aguilera-Galvez *et al.*, 2018; Domazakis *et al.*, 2018) (Supporting information, Table S1). For cloning of *5'NbBSL1*, the sequence was amplified from *N. benthamiana* cDNA with Phusion® proofreading polymerase (Thermo Fisher Scientific), using the primers listed in Table S2. The 281pb fragment was cloned in pENTR/D-TOPO vector (Invitrogen). The insert of the entry clone was checked by sequencing after which it was transferred to the destination vector pTRV2 by an LR reaction using LR-Clonase II enzyme® (Invitrogen) resulting in the construct pTRV2:5'*NbBSL1* (Table S1). For cloning of *BSL1* from *Solanum tuberosum* (*StBSL1*), the entire sequence was amplified from *S. tuberosum* cDNA using gene specific primers listed in Table S2. The 2616 pb fragment was cloned in the pENTR/D-TOPO vector (Invitrogen) and transferred to the destination vector pB7WGF2 by an LR reaction resulting in the construct: pB7WGF2:*StBSL1*. PCR-based site-directed mutagenesis was used to introduce the mutation in the sequence of *StBSL1*. The mutant site was incorporated using the primers listed in Table S2 and the Quik-Change XL kit (Agilent technologies) following the manufacturers' instructions. The resulting vector was used as a template for cloning $StBSL1^{H648V}$ in the expression vector pB7WGF2. All the details about the vectors used in this study are listed in Table S1.

VIGS assay

VIGS experiments were performed using 2-week-old *N. benthamiana* seedlings. The *Agrobacterium tumefaciens* TRV constructs were mixed with pTRV1 at a final OD_{600} of 1 (Liu *et al.*, 2002), except when indicated otherwise. The details of the constructs used in this study are listed in supporting information, Table S1.

Agrobacterium transient cell death assays

Agro-infiltrations were performed as previously described by Domazakis *et al.* (2017) Briefly, agro-infiltrations were performed on 3-4-week-old *N. benthamiana* plants using a suspension of *Agrobacterium tumefaciens* strain containing the appropriate expression vector (Supporting information, Table S1). Local symptoms of cell death responses were assessed at 3-4 dpi.

For genetic complementation assays, *Agrobacterium* strains carrying *StBSL1*, *StBSL1*^{H648V} and empty vector controls were independently co-infiltrated with the respective agro-suspension at a final OD_{600} of 0.5. Macroscopic observations of the infiltrated area were carried out at 2 dpi and averaged over the number of replicates. In all cases, a cell death index was estimated using a scale from 0% to 100%, where 0% corresponds to no visible symptoms, 50% to intermediate responses displaying chlorosis and increasing levels of cell death and 100% to confluent cell death (Domazakis *et al.*, 2017). A one-way ANOVA test with a Fisher's Least Significant Difference (LSD) post hoc was performed to identify statistically significant differences using GraphPad Prism 7 for Mac (www.graphpad.com).

RNA extraction and quantitative PCR analyses

RNA was isolated from TRV-inoculated *N. benthamiana* plants at 2 weeks post inoculation. RNA extraction was performed using the RNAeasy Plant mini kit (Qiagen) and used to synthesize cDNA with Superscript II reverse transcriptase (Invitrogen), according to manufacturer's instructions. Quantitative RT-PCRs were performed on a CFX96 Real-Time PCR machine (BioRad). Each 10µl of reaction contained 300nM of each primer, 2µl (20ng) of cDNA template and 1x iQ SYBR Green Supermix (BioRad). Reactions were incubated at 95°C for 3 minutes, followed by 40 cycles of 10 seconds of denaturation at 95°C, and 1 minute of annealing and extension at 60°C, finished by a melt cycle of 0.5°C increment per 10 seconds from 65°C to 95°C. Data were analyzed using the ΔΔCt method (Schmittgen & Livak, 2008), with expression normalized to the reference gene elongation factor 1α of *N. benthamiana* (*NbEF1α*) (Nicot *et al.*, 2005). Primers used in this experiment are listed in supplementary information, Table S2.

Transient complementation assay and inoculations with *P. infestans*

The appropriate constructs were transiently expressed in VIGS plants at a final OD_{600} of 0.5. At 2 dpi, each infiltration site was inoculated with zoospores from *P. infestans* isolate NL90128 as described by Aguilera-Galvez *et al.* (2018). Development of lesions and presence

of sporulation were determined at 8 dpi. Next, leaves were stained with trypan blue solution (50 mL dH2O, 50 mL phenol, 50 mL lactic acid, 50 mL glycerol, and 100 mg Trypan Blue) for 3 days and were destained with chloral hydrate at 25% for 4 days. Representative pictures were taken after 6 days.

Chemical inhibition of phosphatases

3-week-old fully expanded *N. benthaminana* leaves were used in the experiment. OA (Cayman chemical ®) was dissolved in ethanol by the manufacturer to $1.2x10^3$µM and later, it was diluted with water to 250µM. Ethanol at 0,05% was used as a negative control of the co-infiltrations. OA was mixed with the appropriate agro-suspensions to a final concentration of 250nM. The percentage of cell death was quantified according the *Agrobacterium* transient cell death assays.

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Supporting information

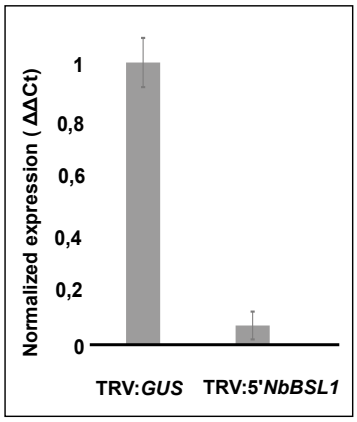


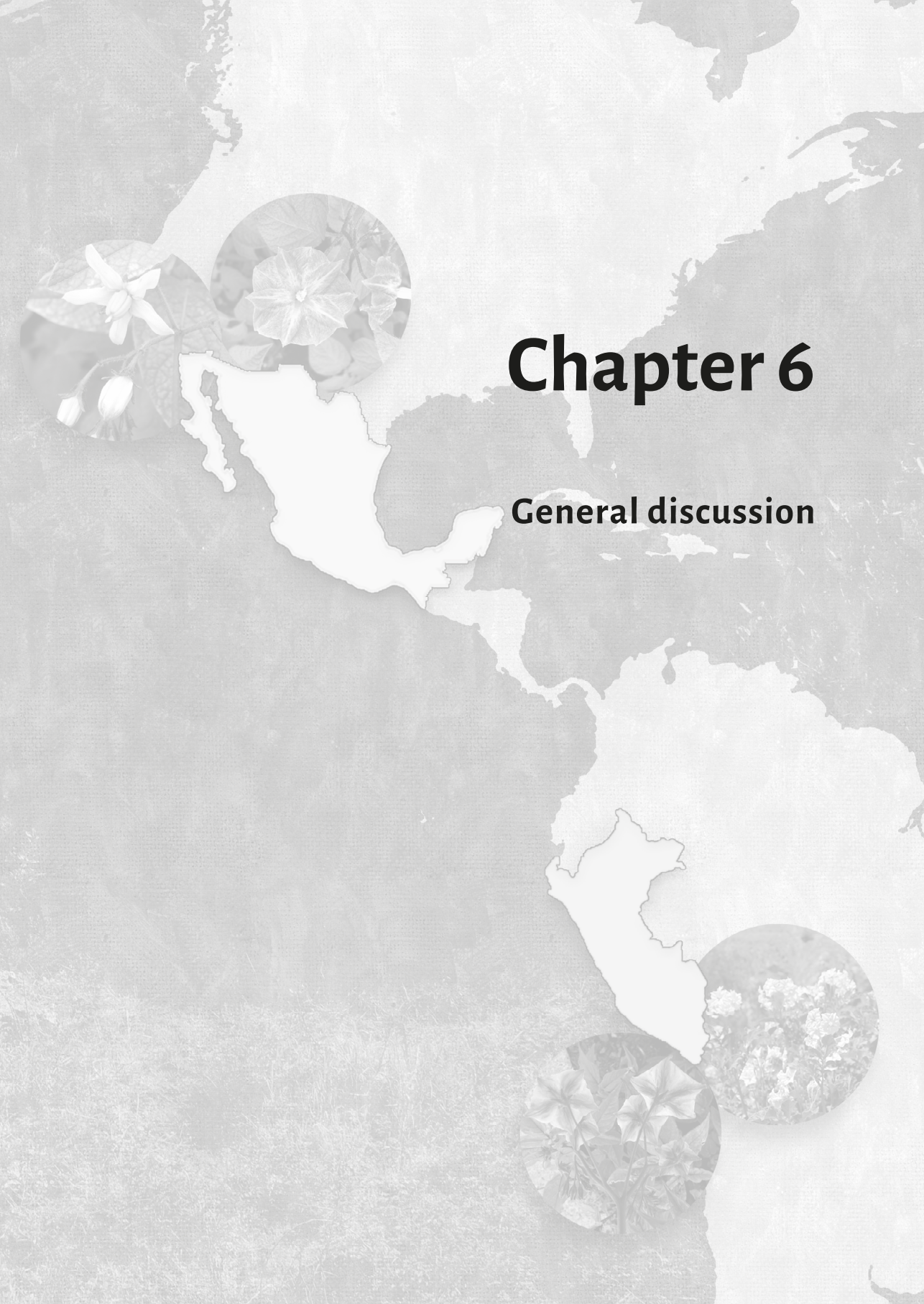
FIGURE S1 | NbBSL1 is successfully silenced in TRV:5'*NbBSL1*-inoculated *N. benthamiana* plants. The relative expression of NbBSL1 in *GUS* and *BSL1*-inoculated plants was determined. Data represents the average of biological replicates +/- the standard error.

TABLE S1 | List of constructs used for *Agrobacterium*-mediated transient expression. The purpose, the insert, the binary vector, the *A. tumefaciens* strain and the reference are indicated.

Purpose	Insert	Binary vector	*A. tumefaciens* strain	Reference
		pTRV1	GV3101	(Liu et al., 2002)
Gene silencing (VIGS)	*GUS*	pTRV2	AGL1	(Domazakis et al., 2018)
	PDS	pTRV2	AGL1	(Domazakis et al., 2018)
	5'NbBSL1	pTRV2	LBA4404	This study
Cell death assays and transient complementation	*Rpi-blb3*	pBinplus	AGL1	(Aguilera-Galvez et al., 2018)
	Rpi-mcq1	pBinplus	AGL1	(Aguilera-Galvez et al., 2018)
	Rpi-sto1	pBinplus	AGL1	(Vleeshouwers et al., 2008)
	Avr2	pK7WG2	AGL1	(Gilroy et al., 2018)
	Avrblb1	pK7WG2	AGL1	(Vleeshouwers et al., 2008)
	StBSL1 (WT)	pB7WGF2	LBA4404	(Turnbull, 2016)
	StBSL1^{H648V}	pB7WGF2	LBA4404	(Turnbull, 2016)
	Empty vector	pBinplus	AGL1	This study

TABLE S2 | List of primers used in the study. The primer name, the purpose and the sequence are indicated.

Primer name	Purpose	Sequence 5'→3'
5'NbBSL1_fwd	Cloning	CACCATGGGTTCAAAGCCATGGCT
5'NbBSL1_rv	Cloning	TCACCGGCAGGTCTAAGTCT
StBSL1_fwd	Cloning	AAAGCAGGCTTCATGGTGAGGCAATTGTCA
StBSL1_rv	Cloning	GAAAGCTGGGTATTAAATATAGGCAAGTGAGCT
StBSL1_H648V_fwd	Mutagenesis	CATTTGATAAGAGGGAATGTTGAAGCTGCTGATATTAATGC
StBSL1_H648V_rv	Mutagenesis	GCATTAATATCAGCAGCTTCAACATTCCCTCTTATCAAATG
qRT-PCR BSL1 Fwd	qPCR	GTGAGTCAGCTGTCCGCC
qRT-PCR BSL1 Rv	qPCR	CAATCCTTGAGGTGATTTCTGCC
qRT-PCR EF1a Fwd	qPCR	ATTGGAAACGGATATGCTCCA
qRT-PCE EF1a Rv	qPCR	TCCTTACCTGAACGCCTGTCA



Chapter 6

General discussion

Late blight caused by the oomycete pathogen *Phytophthora infestans* remains the most devastating disease for potato (Haverkort *et al.*, 2009). Breeding for disease resistance against late blight started early to mid-20th century with the introgression of resistance (*R*) genes from wild *Solanum* species into potato cultivars (Malcolmson & Black, 1966; Aguilera-Galvez *et al.*, 2018). However, these *R* genes are typically quickly overcome by new adapted *P. infestans* strains (Fry, 2008). For wise *R* gene stewardship and deployment, molecular knowledge of the corresponding avirulence (*Avr*) gene is essential. AVR2 is an RXLR effector of *P. infestans* with an important role in late blight virulence. With the work presented here we have identified wild *Solanum* species that recognize AVR2. In addition to known Mexican *Solanum* species that contain the *R2* gene, we identified recognition in *Solanum* species from Peru and we cloned a number of *R* genes from these plants. We found that the new *R* genes, represented by *Rpi-mcq1*, are sequence-unrelated to *R2* and we unravelled the race-specificity of both *R* genes to *P. infestans* isolates. Subsequently, we explored the genetic diversity of *Avr2* effector family and we found that *R2* and *Rpi-mcq1* display differences in the recognition pattern to various members of the *Avr2* family. In addition to genetically exploring recognition specificities by *R* and *Avr* families, we studied the virulence targets of AVR2 and we found different signaling pathways that are activated upon recognition of AVR2 by *Solanum* R proteins. Overall, this thesis contributes to our ever expanding knowledge of R-AVR interactions in the *P. infestans – Solanum* pathosystem, which is crucial to improve *R* gene-based breeding and deployment of resistance genes against late blight in potato.

Solanum R genes have evolved independently to recognize AVR2 of *P. infestans*

Resistance to *P. infestans* has been identified in various wild *Solanum* species that belong to the section *Petota*. Early potato breeding started with the introgression into potato cultivars of eleven resistance (*R*) genes (*R1* to *R11*) from the Mexican *Solanum demissum* (Malcolmson & Black, 1966). To date, additional *R* genes have been identified from *Solanum* species that are native to Mexico, such as *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum*, *Rpi-sto1* and *Rpi-pta* from *Solanum stoloniferon* and *Rpi-amr3* from *Solanum americanum*. In addition, some *R* genes have been cloned from South American *Solanum* species (e.g *Rpi-vnt1* and its homologues from *Solanum venturii*, *Rpi-ber1* from *Solanum berthaultii*, *Rpi-chc1* from *Solanum chacoense* and *Rpi-tar1* from *Solanum tarijense*) (Hein *et al.*, 2009; Jo *et al.*, 2016; Aguilera-Galvez *et al.*, 2018). It has been proposed that the diversity and distribution of these *R* genes in the section *Petota* might be shaped by the geographic distribution of the originating *Solanum* species (Fig. 1). For instance, *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* occur in few Mexican diploid and polyploid species closely related to *S. bulbocastanum*. These *R* genes are described as ancient genes which have evolved in *Solanum* species from Central America, except of *Rpi-blb2* which has been suggested to have emerged more recently. These data indicate that the distribution and diversity of *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* have been restricted to Central America (Lokossou *et al.*, 2010). This geographic restriction seems to be true for other *R*

genes as well. The *R2* family emerged and remained restricted to Mexico (Champouret, 2010), one of the centres of *P. infestans* genetic diversity (Grunwald & Flier, 2005). Comparable, *Rpi-mcq1* and its homologues from Peruvian *Solanum* species and *Rpi-vnt* and its homologues from *Solanum* species which are native to Argentina, emerged and have been restricted to Peru and Argentina in South America which is the other centre of genetic diversity of *P. infestans* (Fig.1) (Abad & Abad, 1997; Hijmans & Spooner, 2001; Alpizar-Gomez *et al.*, 2007).

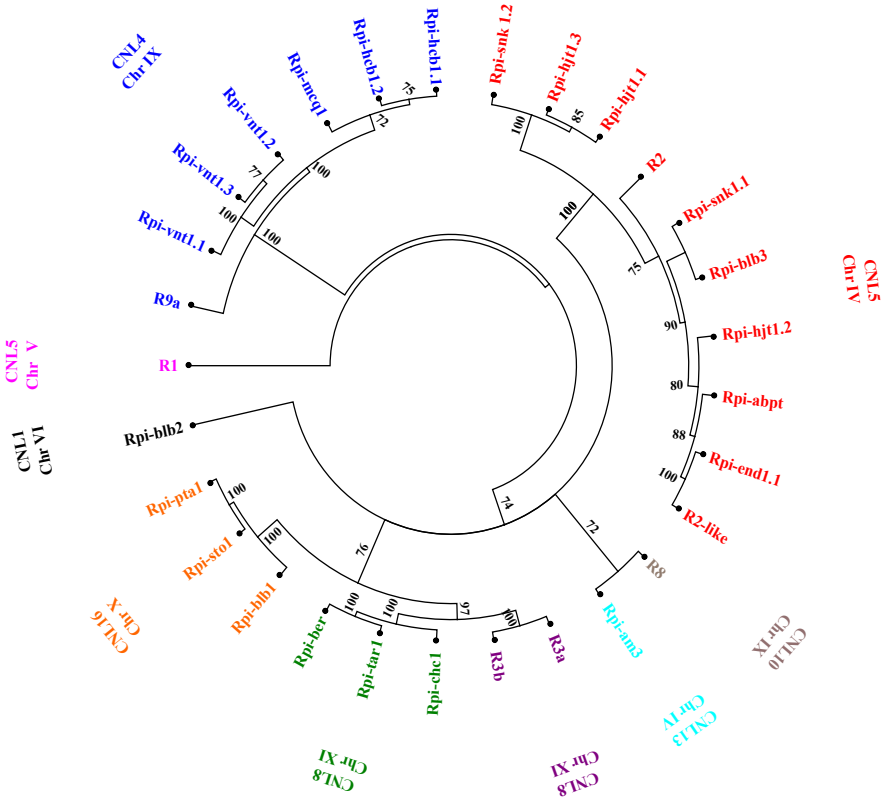


FIGURE 1 | Classification of the *Solanum* R proteins. Phylogenetic tree derived from the full amino acid sequences of the *Solanum* R proteins. The *R* genes cloned from Mexican (red) and South American (blue) *Solanum* species, the CNL clades and the chromosome location are indicated. The bootstrap values of 60% or higher are indicated in the nodes and the R1 protein was used as outgroup.

The fast adaptability and evolution of *P. infestans* suggest that the identification of more *R* genes with broad-spectrum resistance is essential (Fry, 2008). Effector genomics (Effectoromics) has been widely used to explore the wealth of *R* gene diversity in wild *Solanum* species and to accelerate the cloning of *R* genes by specific recognition to effectors of *P. infestans* (Vleeshouwers *et al.*, 2011). At the start of this project it was known that the RXLR effector AVR2 of *P. infestans* is recognized by the R2 protein from *Solanum demissum* (Gilroy *et al.*, 2011).

R2 resides at a major late blight locus (MLB) on chromosome IV of potato and represents an extensive gene family of at least 10 members of a number of Mexican *Solanum* species which display identical recognition specificity to AVR2 (e.g. *Rpi-blb3* from *Solanum bulbocastamum*) (Fig.1) (Park *et al.*, 2005; Lokossou *et al.*, 2009; Champouret, 2010; Aguilera-Galvez *et al.*, 2018).

In this thesis, we found that AVR2 is also recognized by *Solanum* species from South America. We cloned *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* from the Peruvian *S. mochiquense* and *S. huancabambense* and found that these represent a new family of *R* genes (**Chapter 2**). *Rpi-mcq1* is located on chromosome IX (Smilde *et al.*, 2005). From our studies, we concluded that AVR2 is recognized from wild *Solanum* species from two geographic locations, Mexico and Peru. These *Solanum* species have been classified in separated taxonomic series (Hawkes, 1990) and cluster in different phylogenetic groups based on the most recent classification of the section *Petota* (Spooner *et al.*, 2014). Figure 1 indicates the genetic relationship between all the cloned *R* genes from potato. The *R* genes from wild *Solanum* species that are native to Mexico and Peru cluster in separate groups, belong to distinct CNL clades and the resistances reside at different chromosomes. Altogether, our studies revealed that the AVR2 recognition has co-evolved independently with two sequence-unrelated *R* genes, *R2* and *Rpi-mcq1* in two distinct geographic locations, Mexico and Peru (Aguilera-Galvez *et al.*, 2018).

Does the recognition of AVR2 family by *Rpi-mcq1* lead to resistance to *P. infestans*?

R2 and *Rpi-mcq1* potato plants revealed an overlapping and dissimilar race-specificity to various *P. infestans* isolates (Aguilera-Galvez *et al.*, 2018) (**Fig. 5, Chapter 3**). Some *P. infestans* isolates are avirulent on both plants, *R2* and *Rpi-mcq1*. These isolates express AVR2, which triggers cell death responses in agro-coinfiltrations with *R2* and *Rpi-mcq1* (**Chapter 3**). Thus, a likely explanation would be to attribute the avirulence phenotype to the presence of the AVR2 protein, and this would be in line with the resistance profiles that are found for 15 out of 18 tested *P. infestans* isolates (Fig. 2a). Yet, the contribution of AVR2 in disease resistance to *P. infestans* has only been validated for *R2*-mediated resistance (Gilroy *et al.*, 2011), and still needs to be confirmed for *Rpi-mcq1*. Although, a bit unlikely, it cannot be excluded that *Rpi-mcq1*-mediated resistance is triggered by a yet unidentified different effector than AVR2. Transformations with AVR2 of *P. infestans* isolates virulent on *Rpi-mcq1* potatoes can help to determine whether the recognition of AVR2 by Rpi-mcq1 leads to resistance *to P. infestans*.

Other *P. infestans* isolates are virulent on *R2* and *Rpi-mcq1* potato plants (Fig. 2c). The virulence on *R2* plants has previously been attributed to the absence of AVR2 and the presence of AVR2-like, an allelic form of AVR2. In line with this, AVR2-like was shown to not be recognized by R2 (Gilroy *et al.*, 2011). In this thesis, we showed that AVR2-like is recognized by *Rpi-mcq1* in agro-coinfiltrations assays (**Fig. 5, Chapter 4**), however, it seems that presence of AVR2-like does not lead to resistance to *P. infestans* in *Rpi-mcq1* plants (**Fig. 5, Chapter 3).** It might be possible that the strength of Rpi-mcq1/AVR2-like cell death is

too weak to elicit resistance responses. A second option might be that Rpi-mcq1/AVR2-like cell death is uncoupled of resistance and therefore, the cell death responses may take place in an inadequate spatio-temporal frame that compromises resistance. Comparable with our observations, the recognition of AVR3a^{EM} of *P. infestans* by the R3a+ mutant triggers cell death responses, however this recognition does not lead to resistance (Century *et al.*, 1995; Segretin *et al.*, 2014). A second possibility is that class III members are instrumental in determining avirulence. AVR2 family members of class III are not expressed in the virulent *P. infestans* isolate PIC99189, whereas all avirulent isolates do express a member of class III. This may suggest that class III could be required for *R2* and *Rpi-mcq1*-mediated resistance to *P. infestans* (Fig. 2). A similar finding was reported for avirulence on Rpi-blb1, which is determined by one class of Avrblb1 (Champouret *et al.*, 2009). A third possibility is that additional members of the AVR2 family are suppressing AVR2-like and thus, the activation of *Rpi-mcq1*-mediated immunity.

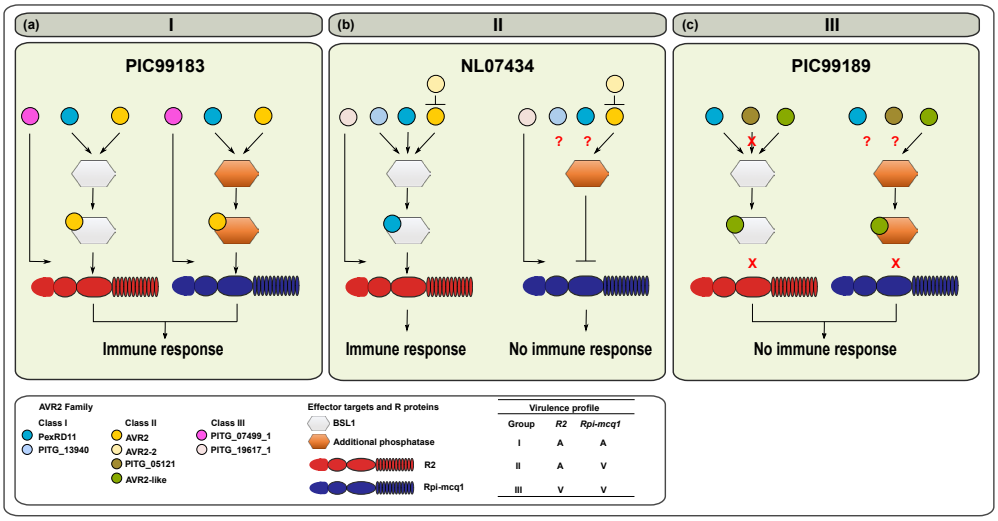


FIGURE 2 | Schematic summary of the interactions between AVR2 family of *P. infestans* and *Solanum* R proteins. The representative isolates PIC99183, NL07434 and PIC99189 of *P. infestans* display a differential virulence pattern on *R2* and *Rpi-mcq1* potato plants (Aguilera-Galvez *et al.*, 2018). (a) R2 and Rpi-mcq1-mediated immunity is activated by the association of AVR2 with the plant phosphatase BSL1 and another plant phosphatase in presence of AVR2 and/or a member from class I and III (b) AVR2 causes resistance in *R2* but not in *Rpi-mcq1* potatoes. Hypothetically this can have several reasons. For example, AVR2-2 from class II might suppress AVR2 and its association with the phosphatases. PITG_13930 binds BSL1 and R2-mediated immunity is activated in presence of an AVR2 member from class I. PITG_13930 is not recognized by Rpi-mcq1and Rpi-mcq1-mediated immunity is not activated. (c) AVR2-like from class II associates with BSL1 and another plant phosphatase in presence of an AVR2 from class I and II, however R2 and Rpi-mcq1-mediated immunity is not activated. An AVR2 member from class III is present in the isolates in which R2 immunity is activated (a, b) but absent in the virulent isolate PIC99189 (c). Solid arrows indicate activation and lines ending with a cross bar indicate suppression. Cell death responses are based on agro-coinfiltrations of *R2* or *Rpi-mcq1* with members of the *Avr2* family (**Chapter 4**) and the virulence profile of the *P. infestans* isolates is based on the disease test performed on R2 and Rpi-mcq1 potato plants (**Chapter 3**).

The dissimilar virulence pattern of *P. infestans* isolates on *R2* and *Rpi-mcq1* potato plants may be attributed to additional members of AVR2 family

A set of three *P. infestans* isolates were found avirulent on *R2* and virulent on *Rpi-mcq1* potatoes (**Chapter 3**). This dissimilar pattern could be potentially attributed to the expression of additional effectors that are related to AVR2. In **Chapter 4** we found that AVR2 family members are differentially expressed in *P. infestans* isolates and we found novel AVR2 variants that are expressed in the discriminating *P. infestans* isolates, NL07434, NL12003 and NL09096. We can hypothesize that in such *P. infestans* isolates, AVR2 variants might have evolved to remain undetected by *Solanum* R proteins but have maintained their contribution to virulence. Further studies should address whether those variants are recognized by R2 or Rpi-mcq1. Alternatively, it would be possible that AVR2 variants are suppressing the recognition of AVR2 mediated by R2 and Rpi-mcq1. AVR2 members from class I are recognized by R2 and thus, the activation of R2 when AVR2 is suppressed might be attributed to the presence of class I members (e.g. PITG_13930) (Fig. 2b). It could be that AVR2 family members are competing for the effector target and without the suppressor effector AVR2 and its variants are always associated with the effector target, but when AVR2 is suppressed other members such as members for class I can associate with the target.

The suppression of *R*-mediated immunity by effectors has been demonstrated for some effectors of *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*). The activity of the tomato I-2 and I-3 proteins is suppressed by AVR1 which is secreted by some fungal strains even in presence of the corresponding AVR (Rep *et al.*, 2005; Houterman *et al.*, 2008). Similarly, resistance to *P. infestans* mediated by Rpi-blb1 from potato is inactivated by the RXLR effector IPI-O4 of *P. infestans* that blocks the recognition of IPI-O1 (Chen *et al.*, 2012). Some flax R proteins are not recognizing the corresponding AVR proteins of flax rust (*Melampsora lini*) in the presence of an inhibitor of avirulence genes (*I* gene) (Jones, 1988; Ellis *et al.*, 2007). It seems that the suppression of *R*-mediated cell death by effectors even in the presence of the matching *R* gene is a mechanism of the pathogen to avoid recognition by the plant.

It has been proposed that the combination ('stack'or 'pyramid') of different *R* genes in a single cultivar could lead to broader resistance to *P. infestans* (Zhu *et al.*, 2012). Considering that some effectors are able to suppress host defense responses, durable pyramids should include *R* genes that are not suppressed by the same effectors (Stam & McDonald, 2018). In addition, based on our studies (**Chapter 4**), *R* genes with differential recognition specificity to effectors of *P. infestans* should be combined to expand broader resistance. *R2* and *Rpi-mcq1* recognize differential members of the AVR2 family, however this differential specificity does not lead to increased resistance to *P. infestans* and thus, pyramiding *R2* with *Rpi-mcq1* is not expected to expand the resistance. In contrast, *R2* could be combined with one or more *R* genes that recognize the AVR2 variants, however these *R* genes have been not identified yet. Further effectoromic screens with the AVR2 allelic variants are required to identify additional *R* genes that recognize AVR2 variants.

Plant phosphatases are required for AVR2 recognition mediated by *Solanum* R proteins

Plants have evolved immune receptors to recognize pathogen effectors. Typically, the recognition of an AVR protein from the pathogen by the corresponding R protein in the plant can be direct by a physical interaction or indirect through detection of modification in specific host targets (van der Hoorn & Kamoun, 2008). Direct perception of filamentous AVR proteins has been demonstrated for AvrL567 of the flax rust fungus *Melampsora lini* which physically interacts with the resistance proteins L5 and L6 (Dodds & Thrall, 2009). Comparable, AvrPi-ta of *Magnaporthe grisea* is directly recognized by the rice Pi-ta protein (*Oryza sativa*) and ATR1 of *Hyaloperonospora parasitica* by RPP1 from *Arabidopsis thaliana* (Jia *et al.*, 2000; Krasileva *et al.*, 2010). In addition, research on bacterial systems has also provided evidence for direct protein-protein interactions. For instance, AvrPto from *Pseudomonas syringae* pv. *tomato* is directly recognized by the tomato bacterial speck resistance protein Pto. Comparable, PopP2 of *Ralstonia solanaceraum* directly binds to RRS1-R from *Arabidopsis thaliana* (Scofield *et al.*, 1996; Deslandes *et al.*, 2003).

Significant progress has been made in understanding the mechanism of indirect effector recognition mediated by R proteins that monitor effector-induced modifications in host proteins (van der Hoorn & Kamoun, 2008). AVR2 of *P. infestans* interacts with the plant phosphatase BSU1-like 1 (BSL1) in potato which activates the brassinosteroid (BR) signaling pathway. This activation leads to upregulation of the transcription factor CHL1, which acts as a negative regulator of immunity. Thus, AVR2 exploits an antagonistic crosstalk between growth and innate immunity in *Solanum* species (Fig.3) (Mora-García *et al.*, 2004; Saunders *et al.*, 2012; Turnbull *et al.*, 2017). Many other effectors of *P. infestans* interact with negative regulators of immunity, such as Pi04089 which targets the RNA-binding protein K-homology RNA-binding protein1 (Wang *et al.*, 2015), Pi04314 that targets three host protein phosphatase catalytic isoforms (Boevink *et al.*, 2016), and Pi02860 which interacts with the predicted CULLIN3-associated ubiquitin E3 ligase NRL1(Yang *et al.*, 2016). These effector targets are required for *P. infestans* virulence and therefore, they are considered susceptibility (S) factors in late blight infection (van Schie & Takken, 2014).

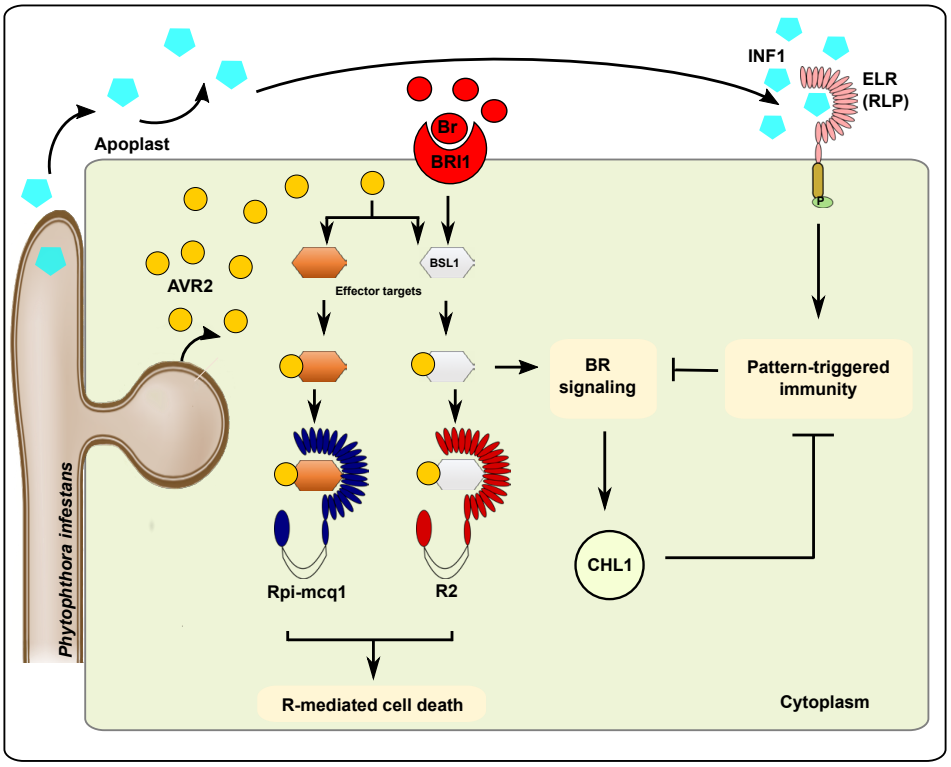


FIGURE 3 | Proposed model of the dual function of AVR2. AVR2 of *P. infestans* is translocated into the plant cell where it interacts with the host BSL1 and another plant phosphatase. The association of AVR2 with BSL1 is required for R2-mediated cell death and the association with another phosphatase may be required for Rpi-mcq1-mediated dell death. The plant phosphatases activate the brassinosteroid signaling pathway, leading to up-regulation of the transcription factor CHL1, which suppresses pattern-triggered immunity.

In this thesis, we confirmed that BSL1 is required for recognition of AVR2 mediated by the *Solanum* R2, but is not involved in Rpi-mcq1-mediated recognition of AVR2 (**Chapter 5**). The host target required for Rpi-mcq1-mediated cell death remains unknown, however we found that a plant phosphatase is required for this interaction. Recently, it has been demonstrated that AVR2 not only interacts with the BSL1 protein, but also with other members of the BSL family, such as BSU1-like 2 and 3 (BSL2 and BSL3) in potato (Turnbull *et al.*, 2019). Potentially, another member of the BSL family could be involved in Rpi-mcq1-mediated recognition of AVR2, yet this remains to be determined. Further studies should address whether BSL2 or BSL3 are required for AVR2 recognition mediated by Rpi-mcq1. One approach is the silencing BSL2 and BSL3 in *N. benthamiana* and the subsequent agro-coinfiltrations of *Rpi-mcq1* and *Avr2*. The possible suppression of the Rpi-mcq1/AVR2 cell death would then be an indication of the role of BSL2 and BSL3 in the recognition of AVR2 mediated by Rpi-mcq1. In addition to the silencing, the possible association of BSL2 or BSL3 with AVR2 and Rpi-mcq1 should be tested by co-immunoprecipitation experiments.

In Figure 3, we schematically describe a general model for the interaction of AVR2 family members with *Solanum* R proteins by the association with the BSL1 protein and another phosphatase. From our experiments, we concluded that two different plant phosphatases are required for AVR2 recognition mediated by *Solanum R* genes, which is in line with our previous findings of independent co-evolution between *Avr2* and *R2* and *Rpi-mcq1*.

Future perspectives on resistance breeding to late in potato

Over the last 100 years, breeding for resistance to late blight has been focused on the introgression of *R* genes from wild *Solanum* species into potato cultivars. However, this resistance has proven to be defeated by new pathogen populations (Fry, 2008). Therefore, we advise to select *R* genes based on pathogen population studies rather than based on geographical origin of the resistance genotypes or by *R* gene locus cluster, as it has been doing before with the past breeding strategies. It has also been shown that in some cases the disease resistance is already broken in wild pathogen populations before the *R* genes are introgressed into the cultivars, thus the study of effector function and diversity is required for more educated deployment of *R* genes (Stam & McDonald, 2018).

Effectoromics has contributed to identifying and characterizing *R* genes in potato in the last decade (Vleeshouwers *et al.*, 2008; Oh *et al.*, 2009; Vleeshouwers *et al.*, 2011). We also exploited effectoromics to monitor for cell death responses to AVR2 in *Solanum* species. We found recognition to AVR2 in *Solanum* from Mexico and Peru which is mediated by two unrelated R proteins (**Chapter 3**). From our experiments, we concluded that effectors are a powerful tool to identify *R* genes in *Solanum* species, however AVR2 is the first example in which an AVR is not able to discriminate between two different *R* genes in a single effector assay. Therefore, we advise to complement AVR2-based breeding with additional molecular techniques, e.g molecular markers to securely determine the identity of *R* genes.

Recently, studies on effector biology have revealed the function of some effectors which are required for pathogen virulence (Wang *et al.*, 2019). Targeting effectors which are required for the benefit of the pathogen might help to predict the *R* gene durability in field (Bai *et al.*, 2000). AVR2 targets the plant phosphatase BSL1which is a susceptibility (S) factor required for late blight virulence (**Chapter 5**). This criterion has been used to select *R* genes in pepper. *AvrBs1*, *AvrBs2* and *AvrBs3* of *X. campestris* pv. *vesicatoria* are recognized by *Bs1*, *Bs2* and *Bs3*, respectively. However, the function of *AvrBs3* and *AvrBs1* are not related with virulence and thus, it has been proposed that *Bs1* and *Bs3* genes would not provide long term resistance in the field. In contrast, *AvrBs2* contributes to pathogen virulence and *Bs2* has been predicted to be more durable (Leach *et al.*, 2001).

An interesting new field of research is focused on engineering of *R* genes to improve their capabilities of effector recognition (Rodriguez-Moreno *et al.*, 2017). For instance, it has been demonstrated that single-residue mutations in the *Solanum* R3a expand the response

to AVR3a^{EM} while retaining the recognition to AVR3^{KI} of *P. infestans* (Segretin *et al.*, 2014). Comparably, the same mutations in the tomato I2 protein which is an ortholog of R3a have revealed an expanded response spectrum to effectors of the fungus *Fusarium oxysporum* f.sp l*ycopersici*, indicating that *R* genes can be engineered to confer resistance to different pathogens (Giannakopoulou *et al.*, 2015). In a more recent work, the recognition specificity of the rice Pikp protein was expanded to different AVR-pik variants of the rice blast pathogen *Magnaporthe oryzae* by engineering the binding interface between these proteins (De la Concepcion *et al.*, 2019). We have shown that AVR2 and its allelic variants play an important role in avoiding recognition mediated by *Solanum* R proteins, however the molecular mechanism of AVR2 recognition mediated by *R2* and *Rpi-mcq1* remains unknown. Further studies should address this mechanism. Later, protein engineering could be used as a new strategy in AVR2-based breeding to late blight by expanding the recognition capabilities of *R2* and *Rpi-mcq1*.

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Summary

Potato (*Solanum tuberosum* L.) is the most important non-cereal crop worldwide. It has been recommended as a food security crop considering its high yield and excellent nutritional value. Potato is susceptible to several diseases among which late blight is the most devastating. This disease is caused by the oomycete pathogen *Phytophthora infestans*, which severely damages leaves, stems and tubers from potato plants and ultimately causes the complete loss of the crop. In Ireland, late blight destroyed a large portion of the crop and led to the Irish potato famine between 1845 and 1849. Nowadays, late blight still remains a major threat to potato production. Several *R* genes from wild *Solanum* species have been introgressed into different potato cultivars over the last 100 years. However, most of the single introduced *R* genes have been defeated by adapted *P. infestans* isolates. The identification of *R* genes with broader resistance spectra is required for sustainable management strategies to control late blight in potato.

P. infestans secretes an arsenal of apoplastic and cytoplasmic effectors to manipulate the plant cells and promote infection. In turn, wild *Solanum* species have evolved immune receptors (e.g. resistance (*R*) genes) to recognize these effectors. In the presence of the matching *R* gene, the effectors can induce avirulence and therefore, they are commonly known as avirulence (*Avr*) genes. All the known AVR proteins belong to the cytoplasmic RXLR effectors, which are modular proteins characterized by a host translocation motif (RXLR) and a highly polymorphic C-terminal domain. These effectors can be recognized directly by physical interaction with the plant R protein or indirectly by the interaction with additional proteins in the host, that are called effector targets. The effector recognition by R proteins leads to effector-triggered immunity (ETI) which is a fast and strong immune response in plants. ETI is frequently associated with hypersensitive response (HR), a form of programmed cell death that prevents the spread of the pathogen infection (**Chapter 1**).

Effector genomics (effectoromics) has proven to be a powerful tool to identify immune receptors in wild *Solanum* species. We employed this strategy to perform a high-throughput screening for effector responses in a large set of *Solanum* genotypes representing the broad section *Petota* gene pool. Our research was focused on responses to AVR2 of *P. infestans*. AVR2 is an RXLR effector that promotes late blight virulence by suppressing plant immune responses in potato. We found cell death responses after agroinfiltration with AVR2 in*Solanum* genotypes from Mexico and Peru. This finding indicates that *Solanum* genotypes from two different geographic locations recognize this important effector. The recognition of AVR2 in Mexican *Solanum* genotypes is mediated by the *R2* family which resides on chromosome IV of potato. The *R2* family contains at least 10 members restricted to Mexican *Solanum* species. In **Chapter 2**, we explored the recognition of AVR2 in *Solanum* species which are native to Peru. We cloned *Rpi-mcq1*, *Rpi-hcb1.1* and *Rpi-hcb1.2* using a map-based cloning approach in *Solanum mochiquense*, combined with allele mining in *Solanum huancabambense*.

These *R* genes represent a new family located on chromosome IX. The R proteins mount a hypersensitive response upon recognition of AVR2 and confer race-specific resistance to *P. infestans*. In addition, we performed a sequence analysis of *R2* and *Rpi-mcq1*. We found that *Rpi-mcq1* is sequence-unrelated to *R2*. We provide the first report of two unrelated R protein families that recognize the same AVR as a consequence of a separated co-evolution of *Solanum R* gene families with an important *Avr* gene of *P. infestans*.

In **Chapter 3,** we further studied the recognition of AVR2 in Mexican and Peruvian *Solanum* species. We revealed that AVR2-recognizing genotypes belong to different taxonomic series, the Mexican genotypes belong to *Demissa*, *Longipedicellata* and *Bulbocastana*, whereas the Peruvian genotypes belong to *Yungasensa* and *Tuberosa*. In addition, these genotypes cluster in separate groups based on phylogenetic analysis using AFLP data. We studied the relationship between *R* genes from Mexican and Peruvian *Solanum* genotypes and we identified that *R2* and *Rpi-mcq1* families cluster in different groups based on the phylogenetic tree derived from the full nucleotide binding and ARC (NB-ARC) domain of 27 R proteins. We generated stable potato transformants expressing either *R2* or *Rpi-mcq1* to identify the resistance specificity to *P. infestans* of these *R* genes. A detached leaf test on *R2* and *Rpi-mcq1* potatoes revealed an overlapping but still distinct resistance profile to a set of 18 diverse *P. infestans* isolates, indicating that the recognition of AVR2 by *Solanum* R proteins is not fully linked with same race-specificity to *P. infestans*. Altogether, these findings show that *R2* and *Rpi-mcq1* from Mexican and Peruvian wild *Solanum* species have evolved independently to recognize AVR2.

We studied the genetic diversity of *Avr2* to unravel the differences in race-specificity to *P. infestans* mediated by *R2* and *Rpi-mcq1*. In **Chapter 4**, we showed that *Avr2* belongs to a highly diverse and polymorphic gene family that is clustered in three classes (I to III). Using RNA-seq data, we found variants of the *Avr2* family that are differentially expressed in *P. infestans* isolates that show a distinct virulence pattern on *R2* and *Rpi-mcq1* potato plants. We investigated the recognition specificity of *R2* and *Rpi-mcq1* to *Avr2* family members by transient expression assays in *N. benthamiana*. Individual members of the *Avr2* family were transiently expressed with either *R2* or *Rpi-mcq1*. We found that *Avr2* family members are differentially recognized by *R2* versus *Rpi-mcq1*. For instance, members of class I are exclusively recognized by the *R2* family. *Avr2* from class II, is recognized by *R2* and *Rpi-mcq1*, but *Avr2-like* from the same class is recognized exclusively by *Rpi-mcq1*. Members from class III are not recognized by either *R2* or *Rpi-mcq1* and only one member is recognized by *Rpi-mcq1*. These results indicate that *R2* and *Rpi-mcq1* have evolved different recognition capabilities to AVR2 family members which are associated with the differential race-specificity to *P. infestans* mediated by *Solanum* R proteins.

In **Chapter 5,** we studied the virulence targets required for recognition of AVR2 by *Solanum* R proteins. AVR2 of *Phytophthora infestans* targets potato BSU-like 1 (BSL1) protein, a

phosphatase which is involved in brassinosteroid (BR) hormone signaling, and this leads to enhanced infection by suppression of defense responses in potato. We confirmed that the mechanism of AVR2 recognition mediated by R2 requires the interaction with the BSL1 protein. We investigated whether BSL1 is required for AVR2 recognition mediated by Rpi-mcq1. Virus-induced gene silencing (VIGS) and genetic complementation indicated that Rpi-mcq1-mediated recognition of AVR2 is independent of BSL1. In line with this, we found that BSL1 is not required for Rpi-mcq1-mediated resistance to *P. infestans*. Chemical inhibition of plant phosphatases with okadaic acid demonstrated that phosphatase activity is required for Rpi-mcq1-mediated recognition of AVR2. However, the identity of the phosphatase involved remains unknown. Overall, our findings showed that differential signaling pathways are activated upon recognition of AVR2 from *P. infestans* by R2 versus Rpi-mcq1.

Finally, in **Chapter 6** the findings of this thesis are discussed and placed in a broader perspective. We provide the latest phylogenetic tree of all the potato *R* genes that have been cloned to date. The *R* gene families from Mexico and Peru that recognize the AVR2 cluster in separate groups as a consequence of the independent co-evolution of the AVR2 recognition in *Solanum* species. A model for the interaction of the AVR2 family of *P. infestans* with *R2* and *Rpi-mcq1* was proposed, including members of AVR2 family which are expressed in a set of *P. infestans* isolates that display a differential virulence profile on *R2* and *Rpi-mcq1*. We discuss that the interaction between the individual AVR2 family members and the R proteins might be different. In addition, we provide a schematic overview of the association of AVR2 with two different plant phosphatases, among which BSL1 is required for the activation of *R*-mediated immunity in potato.

In addition to the distinctive finding that unrelated *R* genes in wild *Solanum* species have co-evolved with the same *Avr* of *P. infestans*, our studies contribute to the characterization of the interaction of AVR2 of *P. infestans* with *Solanum* R proteins, which is crucial for AVR2-based breeding for disease resistance in potato.

Samenvatting

Aardappel (*Solanum tuberosum* L.) is wereldwijd het belangrijkste niet-graangewas. Gezien de hoge opbrengst en uitstekende voedingswaarde is de aardappel van essentieel belang voor de wereldvoedselvoorziening. Aardappel is vatbaar voor verschillende ziektes, waarvan de aardappelziekte het meest verwoestend is. Deze ziekte wordt veroorzaakt door de oömyceet *Phytophthora infestans*, die bladeren, stengels en knollen van aardappelplanten ernstig aantast en uiteindelijk het volledige verlies van het gewas kan veroorzaken. In Ierland heeft de aardappelziekte een groot deel van het gewas vernietigd en tussen 1845 en 1849 de Ierse hongersnood veroorzaakt. Tegenwoordig is aardappelziekte nog steeds een grote bedreiging voor de aardappelproductie. Verschillende resistentiegenen (*R*-genen) van wilde *Solanum*-soorten zijn de laatste 100 jaar ingebracht in verschillende aardappelrassen. De meeste van de enkelvoudig geïntroduceerde *R*-genen zijn echter doorbroken door aangepaste *P. infestans*-isolaten. De identificatie van *R*-genen met bredere resistentiespectra is vereist voor duurzame beheersstrategieën om de aardappelziekte te voorkomen.

P. infestans scheidt een arsenaal aan effectoren af om de plantencellen te manipuleren en infectie te bevorderen. Op hun beurt hebben wilde *Solanum*-soorten immuunreceptoren (bijvoorbeeld *R*-genen) ontwikkeld om deze effectoren te herkennen. In de aanwezigheid van het bijpassende *R*-gen kunnen de effectoren avirulentie induceren en daarom zijn ze algemeen bekend als avirulentie (*Avr*) -genen. Alle bekende AVR-eiwitten behoren tot de cytoplasmatische RXLR-effectoren. Dit zijn modulaire eiwitten die worden gekenmerkt door een gastheer-translocatie-motief (RXLR) en een variabel C-terminaal domein. De AVR eiwitten kunnen direct worden herkend door fysieke interactie met het R-eiwit van de plant, of indirect door de interactie met extra eiwitten in de gastheer die 'effector targets' worden genoemd. De effectorherkenning door R-eiwitten leidt tot effector-gestuurde immuniteit (ETI), met een snelle en sterke immuunrespons in de plant. ETI wordt vaak geassocieerd met overgevoeligheidsreacties (HR), een vorm van geprogrammeerde celdood die de verspreiding van het pathogeen verhindert (**hoofdstuk 1**).

Effector-genomics (effectoromics) is een krachtig hulpmiddel om immuunreceptoren in wilde *Solanum*-soorten te identificeren. We gebruikten deze strategie om een snelle screening uit te voeren voor detectie van effectorresponsen in een groot aantal knoldragende *Solanum* genotypen. Ons onderzoek was gericht op AVR2 van *P. infestans*. AVR2 is een RXLR-effector die de virulentie van de oömyceet bevordert door de immuunreacties van de aardappel te onderdrukken. We vonden celdoodresponsen na agro-infiltratie met AVR2 in *Solanum*-genotypen uit Mexico en Peru. Deze waarneming geeft aan dat *Solanum*-genotypen uit twee verschillende geografische locaties dezelfde belangrijke effector herkennen. De herkenning van AVR2 in Mexicaanse *Solanum*-genotypen wordt gemedieerd door de *R2*-genfamilie op chromosoom IV van aardappel. Deze *R2*-familie bevat ten minste 10 leden

die beperkt zijn tot Mexicaanse *Solanum*-soorten. In **hoofdstuk 2** hebben we de herkenning van AVR2 in *Solanum*-soorten onderzocht die inheems zijn in Peru. We hebben *Rpi-mcq1*, *Rpi-hcb1.1* en *Rpi-hcb1.2* gekloneerd met behulp van een op genetische kaarten gebaseerde kloneringsmethode in *Solanum mochiquense*, gecombineerd met "allele mining" in *Solanum huancabambense*. Deze *R*-genen vertegenwoordigen een nieuwe familie op chromosoom IX. De *R*-eiwitten geven een overgevoeligheidsreactie na herkenning van AVR2 en verlenen isolaatspecifieke resistentie tegen *P. infestans*. Uit een sequentieanalyse bleek dat *R2* en *Rpi-mcq1* niet aan elkaar verwant zijn. Wij rapporteren hier voor de eerste keer dat twee niet-verwante *R*-eiwitfamilies dezelfde AVR herkennen, waarschijnlijk als resultaat van een onafhankelijke co-evolutie van twee *Solanum R*-genfamilies.

In **Hoofdstuk 3** hebben we de herkenning van AVR2 in Mexicaanse en Peruviaanse *Solanum*-soorten verder bestudeerd. We hebben ontdekt dat AVR2-herkennende genotypen behoren tot verschillende taxonomische reeksen. De Mexicaanse genotypen behoren tot *Demissa*, *Longipedicellata* en *Bulbocastana*, terwijl de Peruaanse soorten behoren tot de taxonomische klassen *Yungasensa* en *Tuberosa*. Bovendien clusteren deze genotypen in afzonderlijke groepen op basis van fylogenetische analyse met behulp van AFLP-gegevens. We bestudeerden de relatie tussen *R*-genen van Mexicaanse en Peruviaanse *Solanum*-genotypen en we ontdekten dat de aan *R2*- en *Rpi-mcq1*-gerelateerde genfamilies zich in verschillende groepen clusterden op basis van de fylogenetische boom die was afgeleid van het volledige nucleotide-bindende (NB) en ARC (NB-ARC) domein van 27 R-eiwitten. We hebben stabiele aardappeltransformanten gegenereerd die ofwel *R2* of *Rpi-mcq1* tot expressie brengen om hun specificiteit van deze *R*-genen te vast te stellen. Een bladtest op *R2*- en *Rpi-mcq1*-aardappelen liet een overlappend, maar nog steeds verschillend resistentieprofiel zien voor een reeks van 18 verschillende *P. infestans*-isolaten. Dit geeft aan dat de herkenning van AVR2 door *Solanum R*-eiwitten niet volledig is gekoppeld aan dezelfde specificiteit voor *P. infestans*. Al met al laten deze resultaten zien dat *R2* en *Rpi-mcq1* van Mexicaanse en Peruaanse wilde *Solanum*-soorten onafhankelijk zijn geëvolueerd om AVR2 te herkennen.

We hebben de genetische diversiteit van *Avr2* onderzocht om verschillen in specificiteit van *P. infestans* isolaten met behulp van *R2* en *Rpi-mcq1* te ontrafelen. In **Hoofdstuk 4** hebben we aangetoond dat *Avr2* tot een zeer diverse en polymorfe genfamilie behoort die is geclusterd in drie klassen (I tot en met III). Met behulp van RNA-seq-gegevens vonden we varianten van de *Avr2*-familie die differentieel tot expressie komen in *P. infestans*-isolaten die verschillen in virulentie op aardappelplanten met *R2*- versus *Rpi-mcq1*. We hebben individuele leden van de *Avr2*-familie werden samen met *R2* of *Rpi-mcq1* tot transiënt tot expressie gebracht in *N. benthamiana* en we ontdekten dat leden van de *Avr2*-familie differentieel worden herkend door *R2* versus *Rpi-mcq1*. Leden van klasse I worden bijvoorbeeld exclusief herkend door de *R2*-familie. Avr2 uit klasse II wordt herkend door *R2* en *Rpi-mcq1*, maar een *Avr2*-achtig allel uit dezelfde klasse wordt exclusief herkent door *Rpi-mcq1*. Leden uit klasse III worden niet herkend door *R2* of *Rpi-mcq1*, met uitzondering van één lid dat herkend wordt

door *Rpi-mcq1*. Deze resultaten laten zien dat *R2* en *Rpi-mcql* een verschillende specificiteit hebben ontwikkeld voor AVR2-familieleden, en deze is geassocieerd zijn met differentiële resistentiepatronen.

In **Hoofdstuk 5** hebben we de virulentie bestudeerd die nodig is voor de herkenning van AVR2 door *Solanum R*-eiwitten. AVR2 van *Phytophthora infestans* richt zich op een BSU-achtig 1 (BSL1) –eiwit uit aardappel, een fosfatase die betrokken is bij brassinosteroïde (BR) hormoonsignalering. Dit leidt tot een verhoogde infectie door onderdrukking van normale afweerreacties in aardappel. We hebben bevestigd dat interactie met het BSL1-eiwit nodig is voor AVR2-herkenning door R2. We hebben vervolgens onderzocht of BSL1 nodig is voor AVR2-herkenning gemedieerd door Rpi-mcq1. Virus-geïnduceerde gen silencing (VIGS) en genetische complementatie gaven aan dat *Rpi-mcq1*-gemedieerde herkenning van AVR2 onafhankelijk is van BSL1. In overeenstemming hiermee vonden we dat BSL1 niet vereist is voor *Rpi-mcq1*-gemedieerde resistentie tegen *P. infestans*. Chemische remming van plantenfosfatasen met okadaïnezuur toonde aan dat fosfatase-activiteit vereist is bij *Rpi-mcq1*-gemedieerde herkenning van AVR2. De identiteit van de betrokken fosfatase blijft echter onbekend. Over het algemeen lieten onze waarnemingen zien dat differentiële signaleringsroutes worden geactiveerd na herkenning van AVR2 van *P. infestans* door *R2* versus *Rpi-mcq1*.

Ten slotte worden in **hoofdstuk 6** de bevindingen van dit proefschrift besproken en in een breder perspectief geplaatst. We bieden de nieuwste fylogenetische boom van alle aardappel-*R*-genen die tot op heden zijn gekloneerd. Dat de *R*-genfamilies uit Mexico en Peru die de AVR2-cluster herkennen afzonderlijke groepen vormen is een gevolg van onafhankelijke co-evolutie van de AVR2-herkenning in verschillende *Solanum*-soorten. Een model voor de interactie van de AVR2-familie van *P. infestans* met *R2* en *Rpi-mcq1* werd geponeerd, met inbegrip van leden van de AVR2-familie die tot expressie worden gebracht in een reeks *P. infestans*-isolaten die een differentieel virulentieprofiel vertonen met *R2* en *Rpi-mcq1*. We geven aan dat de interactie tussen de individuele AVR2-familieleden en de *R*-eiwitten anders kan zijn. Daarnaast bieden we een schematisch overzicht van de associatie van AVR2 met twee verschillende plantenfosfatasen, waarvan BSL1 vereist is voor de activering van *R*-gemedieerde immuniteit in aardappel.

Naast de belangrijke bevinding dat niet-verwante *R*-genen in wilde *Solanum*-soorten mede-geëvolueerd zijn met dezelfde A*vr* van *P. infestans*, dragen onze studies ook bij aan de karakterisering van de interactie van AVR2 van *P. infestans* met *Solanum R*-eiwitten, wat cruciaal is voor de op AVR2-gebaseerde veredeling voor ziekteresistentie in aardappel.

Resumen

La papa (*Solanum tuberosum* L.) es el cultivo no cereal más importante del mundo. Se ha recomendado como un cultivo de seguridad alimentaria debido a su alto rendimiento y excelente valor nutricional. La papa es susceptible a varias enfermedades, entre las cuales el tizón tardío es la más devastadora. Esta enfermedad es causada por el patógeno oomycete *Phytophthora infestans*, que afecta las hojas, tallos y tubérculos de las plantas de papa y finalmente causa la pérdida completa del cultivo. En Irlanda, el tizón tardío destruyó una gran parte de la cosecha y provocó la hambruna Irlandesa entre 1845 y 1849. Hoy en día, esta enfermedad continua siendo una gran amenaza para la producción de papa. Varios genes de resistencia (*R*) provenientes de las especies silvestres de *Solanum* han sido intogresados en diferentes cultivares en los últimos 100 años. Sin embargo, la mayoría de estos genes *R* han sido derrotados por nuevos aislamientos del patógeno *P. infestans*. La identificación de genes *R* con amplio espectro de resistencia es indispensable para el establecimiento de estrategias de control del tizón tardío en papa.

P. infestans secreta un arsenal de efectores apoplasticos y citoplásmicos para promover la infección. A su vez, las especies silvestres de *Solanum* han desarrollado receptores inmunes (por ejemplo, genes de resistencia) para reconocer estos efectores. En presencia del gen *R* correspondiente, los efectores pueden inducir avirulencia y por lo tanto, se conocen comúnmente como genes de avirulencia (*Avr*). Todas las proteínas AVR conocidas pertenecen a los efectores citoplásmicos del tipo RXLR, que son proteínas modulares caracterizadas por un motivo de translocación (RXLR) y un dominio en la region C-terminal altamente polimórfico. Estos efectores pueden ser reconocidos directamente por la interacción física con la proteína R de la planta o indirectamente por la interacción con proteínas adicionales en el huésped. El reconocimiento del efector por las proteínas R conduce a la inmunidad inducida por el efector (ETI), que es una respuesta inmune rápida y fuerte en las plantas. La ETI se asocia frecuentemente con la respuesta hipersensible (HR), una forma de muerte celular programada que evita la propagación de la infección por patógeno (**Capítulo 1**).

La genómica de efectores (efectorómica) ha demostrado ser una herramienta poderosa para identificar receptores inmunes en especies silvestres de *Solanum*. En este trabajo, empleamos esta estrategia para realizar una detección de alto rendimiento para las respuestas de efectores en un amplio conjunto de genotipos *Solanum* que representan la amplia gama de la section *Petota*. Nuestra investigación se centró en las respuestas a AVR2 de *P. infestans*. AVR2 es un efector del tipo RXLR, que promueve la virulencia del tizón tardío al suprimir las respuestas inmunes de las plantas en la papa. Encontramos respuestas de muerte celular después de la agroinfiltración con AVR2 en genotipos de *Solanum* de México y Perú. Este hallazgo indica que los genotipos *Solanum* de dos ubicaciones geográficas diferentes reconocen este importante efector. El reconocimiento de AVR2 en los genotipos mexicanos de *Solanum* está mediado por la familia *R2*, que reside en el cromosoma IV de

la papa. La familia *R2* contiene al menos 10 miembros restringidos a la especie mexicana *Solanum*. En el **Capítulo 2**, exploramos el reconocimiento de AVR2 en las especies de *Solanum* que son nativas del Perú. Clonamos *Rpi-mcq1*, *Rpi-hcb1.1* y *Rpi-hcb1.2* utilizando un enfoque de clonación basado en un mapa genetico en *Solanum mochiquense*, combinado con clonación de alelos en *Solanum huancabambense*. Estos genes *R* representan una nueva familia ubicada en el cromosoma IX. Las proteínas R desencadenan una respuesta hipersensible tras el reconocimiento de AVR2 y confieren resistencia específica de raza a *P. infestans*. Además, realizamos un análisis de secuencia de *R2* y *Rpi-mcq1*. Encontramos que *Rpi-mcq1* no está relacionado con la secuencia con *R2*. Proporcionamos el primer informe de dos familias de proteínas R no relacionadas que reconocen el mismo AVR como consecuencia de una co-evolución independiente de las familias de genes *R* en especies *Solanum* con un importante gen *Avr* de *P. infestans*.

En el **Capítulo 3**, estudiamos más a fondo el reconocimiento de AVR2 en las especies *Solanum* mexicanas y peruanas. Revelamos que los genotipos que reconocen AVR2 pertenecen a diferentes series taxonómicas. Los genotipos mexicanos pertenecen a las series *Demissa*, *Longipedicellata* y *Bulbocastana*, mientras que los genotipos peruanos pertenecen a *Yungasensa* y *Tuberosa*. Además, estos genotipos se agrupan en grupos separados basados en el análisis filogenético utilizando datos de AFLP. Estudiamos la relación entre los genes *R* de los genotipos *Solanum* mexicanos y peruanos e identificamos que las familias R2 y Rpi-mcq1 se agrupan en diferentes grupos según el árbol filogenético derivado de la unión de nucleótidos (NB) y el dominio ARC (NB-ARC) de 27proteínas R. Generamos transformantes de papa estables que expresan *R2* o *Rpi-mcq1* para identificar la especificidad de la resistencia a *P. infestans* de estos genes *R*. Una prueba de enfermedad en hojas de papas *R2* y *Rpi-mcq1* reveló un perfil de resistencia superpuesto pero diferente a un conjunto de 18 aislamientos de *P. infestans*, lo que indica que el reconocimiento de AVR2 por las proteínas R de especies *Solanum* no está completamente vinculado con la misma especificidad de raza de *P. infestans*. En conjunto, estos hallazgos muestran que *R2* y *Rpi-mcq1* de las especies silvestres de *Solanum* mexicanas y peruanas han evolucionado independientemente para reconocer AVR2.

Estudiamos tambien la diversidad genética de *Avr2* para identificar las diferencias en la especificidad de la raza a *P. infestans* por *R2* y *Rpi-mcq1*. En el **Capítulo 4**, mostramos que *Avr2* pertenece a una familia de genes altamente diversa y polimórfica que se agrupa en tres clases (I a III). Usando datos de RNA-seq, encontramos variantes de la familia *Avr2* que se expresan diferencialmente en aislamientos de *P. infestans* muestran un patrón de virulencia distinto en las plantas de papa *R2* y *Rpi-mcq1*. Investigamos la especificidad de reconocimiento de *R2* y *Rpi-mcq1* a los miembros de la familia *Avr2* mediante ensayos de expresión transitoria en *N. benthamiana*. Los miembros de la familia *Avr2* se expresaron de forma transitoria con *R2* o *Rpi-mcq1*. Encontramos que los miembros de la familia *Avr2* son

reconocidos diferencialmente por *R2* versus *Rpi-mcq1*. Por ejemplo, los miembros de la clase I son reconocidos exclusivamente por la familia *R2*. Algunos Miembros de la clase II, son reconocidos por *R2* y *Rpi-mcq1*. *Avr2-like* de la misma clase es reconocido exclusivamente por *Rpi-mcq1*. Los miembros de la clase III no son reconocidos ni por *R2* ni por *Rpi-mcq1* y *Rpi-mcq1* solo reconoce un miembro. Estos resultados indican que *R2* y *Rpi-mcq1* han evolucionado diferentes capacidades de reconocimiento a los miembros de la familia AVR2 que están asociadas con diferente especificidad de raza a *P. infestans* mediada por proteínas R de especies *Solanum*. En el **Capítulo 5**, estudiamos los blancos de virulencia necesarios para el reconocimiento de AVR2 por las proteínas R de especies *Solanum*. AVR2 de *Phytophthora infestans* se dirige a la proteína 'BSU-like 1' (BSL1), una fosfatasa que está involucrada en la señalización de la brassinosteroides (BR), y esto conduce a un aumento de la infección por la supresión de las respuestas de defensa en papa. Nosotros confirmamos que el mecanismo de reconocimiento AVR2 mediado por R2 requiere la interacción con la proteína BSL1e investigamos si BSL1 es necesaria para el reconocimiento de AVR2 mediado por Rpi-mcq1. El silenciamiento génico inducido por virus (VIGS) y la complementación genética indicaron que el reconocimiento de AVR2 mediado por Rpi-mcq1 es independiente de BSL1. De acuerdo con esto, encontramos que la proteina BSL1 no es necesaria para la resistencia a *P. infestans* mediada por Rpi-mcq1. La inhibición química de las fosfatasas con ácido okadaico demostró que la actividad fosfatasa es necesaria para el reconocimiento de AVR2 mediado por Rpi-mcq1. Sin embargo, la identidad de la fosfatasa involucrada sigue siendo desconocida. En general, nuestros hallazgos mostraron que differentes vías de señalización son activadas activan al reconocer AVR2 de *P. infestans* por R2 and Rpi-mcq1.

Finalmente, en el **Capítulo 6**, discutimos los resultados de esta tesis se discuten. Proporcionamos el último árbol filogenético de todos los genes *R* de papa clonados hasta la fecha. Las familias de genes *R* de México y Perú que reconocen AVR2 forman grupos independientes. Se propuso un modelo para la interacción de la familia AVR2 de *P. infestans* con R2 y Rpi-mcq1, incluidos los miembros de la familia AVR2 que se expresan en un conjunto de aislados de *P. infestans* que muestran un perfil de virulencia diferencial en *R2* y *Rpi-mcq1*.

Discutimos que la interacción entre los miembros de la familia AVR2 y las proteínas R podría ser diferente. Además, proporcionamos una descripción general de la asociación de AVR2 con dos fosfatasas diferentes e indicamos que la proteina BSL1 es necesaria para la activación de la inmunidad mediada por proteinas R en la papa.

Además del distintivo hallazgo de que los genes *R* no relacionados en las especies silvestres de *Solanum* han evolucionado conjuntamente con el mismo *Avr* de *P. infestans*, nuestros estudios contribuyen a la caracterización de la interacción de AVR2 de *P. infestans* con las proteínas R de especies *Solanum*, lo cual es crucial para el mejoramiento genetico resistencia a enfermedades en la papa basado en AVR2.

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And here I am, writing the end of one of the most important chapters in my life. I would like to acknowledge everyone who decided to believe in this dream and contributed to the completion of this thesis.

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Finalmente quiero agradecer a mi familia, quienes son el motor de mi vida y mi mayor fuente de inspiración. A mis padres **Alberto** y **Beatriz** por ser la columna vertebral de nuestra familia. Gracias por estar siempre conmigo a pesar de la distancia, por apoyarme en cada paso, por su confianza, su ejemplo y su amor incondicional. Para ustedes todo mi amor y gratitud. Gracias a mis hermanos: **Gabriel** por cuidarme y enseñarme que la disciplina es la clave del éxito y que no existen imposibles, a **Christian** por ser una persona incondicional en cualquier circunstancia y a **Andrés** por ser valiente y luchar siempre por sus sueños.

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Mil gracias!

Carolina

About the author

Carolina Aguilera Galvez was born in San Juan de Pasto (Colombia) on 25th November 1983. After finishing her primary and secondary education she moved to Medellin for a 5-year BSc program in Pharmaceutical Chemistry at the University of Antioquia. During her bachelor, she was a young researcher at the Bioactive Substances Research Group and together with the Program for the Study and Control of Tropical Diseases she worked on the characterization of enzymes from *Leishmania* and its evaluation as drug targets.

In 2008, she joined the National Coffee Research Center (Cenicafé) in Manizales (Colombia). She worked in the Breeding Program in several projects which aimed to develop new resistance coffee varieties against the most important pest of coffee crops worldwide, the coffee berry borer (*Hypothenemus hampei*). In 2009, while working in Cenicafé she started a MSc in Plant Biotechnology with a specialization in Plant Breeding at the National University of Colombia in Medellin.

In 2012, after finishing her MSc studies, she was appointed as lecturer in the Faculty of Sciences at the University of Santa Rosa in Pereira (Colombia). Working in the University inspired her to pursue an academic career and in 2014 she moved to Plant Breeding at Wageningen University & Research (The Netherlands) for doing a PhD. During her PhD studies, she worked with the major pathogen affecting potato production, *Phytophthora infestans* under the supervision of Dr Vivianne G.A.A Vleeshouwers and Prof. Dr Richard G.F. Visser. The outcome of this research is described in this thesis.

In May 2019, after finalizing the writing of her thesis, she started as a postdoc at the Laboratory of Phytopathology of Wageningen University & Research. Her new topic is on a different crop plant, banana, but on a related subject that also involves understanding the molecular interaction between pathogen effectors and host plants.

Publications

Aguilera-Galvez, C, Champouret N, Rietman H, Lin X, Wouters D, Chu Z, Jones J.D.G, Vossen J.H, Visser R.G.F, Wolters P.J, Vleeshouwers V.G.A.A. Two different *R* gene loci co-evolved with *Avr2* of *Phytophthora infestans* and confer distinct resistance specificities in potato. Studies in Mycology. 2018, 89:1-11.

Domazakis E, Lin X, Aguilera-Galvez C, Wouters D, Bijsterbosch G, Wolters P.J, Vleeshouwers V.G.A.A. Effectoromics-Based identification of cell surface receptors in potato. Methods in Molecular Biology. 2017. 1578: 337-353.

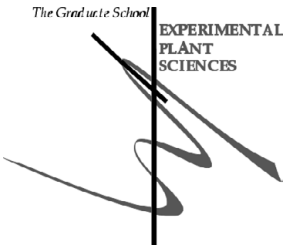
Derevnina L, Dagdas YF, De la Concepcion JC, Petre B, Domazakis E, Du J, Wu C-H, Lin X, Aguilera-Galvez C, Cruz-Mireles N, Vleeshouwers VGAA, Kamoun S. Nine things to know about elicitins. New Phytologist. 2016, 212: 888-895.

Aguilera-Galvez C, Vasquez-Ospina J, Gutierrez-Sanchez P, Acuña- Zornosa R. Cloning and biochemical characterization of an endo-1,4-β- Mannanase from the coffee berry borer *Hypothemus hampei*. BMC Research Notes. 2013; 6:333

Osorio E, Aguilera C, Naranjo N, Marin M, Muskus C. Biochemical characterization of the bifunctional enzyme dihydrofolate reductase tymidylate synthase from *Leishmania* (Viannia) and its evaluation as drug target. Biomédica. 2013; 33 (3).

Padilla-Hurtado B, Florez-Ramos C, Aguilera-Galvez C, Medina - Olaya J, Ramirez – Sanjuan A. Cloning and expression of an endo-1,4-beta-xylanase from the coffee Berry borer, *Hypothenemus hampei*. BMC Research Notes. 2012, 5: 1-23.

Education Statement of the Graduate School Experimental Plant Sciences



Issued to: Carolina Aguilera Gálvez
Date: 4 September 2019
Group: Plant Breeding
University: Wageningen University & Research

1) Start-Up Phase	*date*	*cp*
▶ **First presentation of your project**		
Understanding the mechanism of *Phytophthora infestans* RXLR effectors in potato	2 December, 2014	1.5
▶ **Writing or rewriting a project proposal**		
Specificity of *R* gene- mediated recognition of RXLR effectors of *P. infestans* in *Solanum* species	29 February, 2016	6.0
▶ **Writing a review or book chapter**		
▶ **MSc courses**		
Subtotal Start-Up Phase		7.5

2) Scientific Exposure	*date*	*cp*
▶ **EPS PhD student days**		
EPS PhD student day, Get2Gether, Soest, NL	29-30 January, 2015	0.6
EPS PhD student day, Get2Gether, Soest. NL	28-29 January, 2016	0.6
▶ **EPS theme symposia**		
EPS theme 2: 'Interactions between Plants and Biotic Agents' Utrecht, NL	20 February, 2015	0.3
EPS theme 2: 'Interactions between Plants and Biotic Agents' Leiden, NL	22 January, 2016	0.3
▶ **Lunteren Days and other national platforms**		
Annual meeting 'Experimental Plant Sciences' Lunteren, NL	14-15 April, 2014	0.6
Annual meeting 'Experimental Plant Sciences' Lunteren, NL	13-14 April, 2015	0.6
Annual meeting 'Experimental Plant Sciences' Lunteren, NL	11-12 April, 2016	0.6
Annual meeting 'Experimental Plant Sciences' Lunteren, NL	10-11 April, 2017	0.6
Annual meeting 'Experimental Plant Sciences' Lunteren, NL	9-10 April, 2018	0.6
Annual meeting 'Experimental Plant Sciences' Lunteren, NL	8-9 April, 2019	0.6
▶ **Seminars (series), workshops and symposia**		
EPS Flying seminar Dr Sophien Kamoun Genome and effector evolution in the irish potato famine pathogen lineage	28 May, 2014	0.1

3rd transPLANT training Workshop: Exploiting and understanding Solanaceous genomes	13-14 October, 2014	0.5
Plant Breeding Research day, 2014	30 September, 2014	0.3
EPS Flying seminar Prof.Dr. Jane Parker Plant intracellular immunity: evolutionary and molecular underpinnings	21 January, 2016	0.1
EPS Flying seminar Dr Wembo Ma Effectors as molecular probes to understand pathogenesis	20 June, 2016	0.1
EPS Flying seminar Dr Martin Cann The immune receptor Rx1 remodels chromatin and chromatin interactors in immunity	11 July, 2017	0.1
Westerdijk Symposium "Leading Women in Fungal Biology" Utrecht (NL)	30-31 August, 2017	0.6
Mini-symposium EPS polyploidy genetics and breeding	14 June, 2018	0.2
Seminar Professor Paul R.J Birch FRSE. Secretion and deliverty of effectors that suppress host immunity and shape host range.	2 July, 2018	0.1
Seminar Dr Yang Wang. A leucine-rich repeat receptor-like protein as PAMP receptor reconising XEG1, a Phytophthora glicoside hidrolase 12.	10 September, 2018	0.1
Seminar Dr. Ralph Panstruga. Phenotypic and molecular characterization of partially mlo-virulent isolates of the barley powdery mildew pathogen (*Blumeria graminis* f.sp. hordei)	23 May, 2019	0.1
▶ **Seminar plus**		
▶ **International symposia and congresses**		
Workshop of COST Action Sustain: 'Pathogen- informed crop improvement'. Wageningen, The Netherlands	8-10 April, 2015	0.9
IS-MPMI XVII Congress. Portland, Oregon.	17-21 July, 2016	1.5
3rd Annual Conference SUSTAIN COST Action (FA1208). Banyuls, France.	17-19 February, 2016	0.9
4rd Annual Coference SUSTAIN COST Action (FA1208). Bled, Slovenia.	1-3 March, 2017	0.9
IS-MPMI XVII Congress. Glasgow, Scotland.	14-18 July, 2019	1.5
▶ **Presentations**		
Poster entitled: Recognition of *Phytophthora infestans* RXLR effectors by R protein families in *Solanum*. Workshop Plant microbe interaction. Norwich, United Kingdom. 2015.	17-28 August, 2015	1.0
Poster entitled: Recognition specificity of *Phytophthora infestans* AVR2/PexRD11 effectors in wild *Solanum* species. 3rd Annual Conference SUSTAIN COST Action. Banyuls, France. 2016.	17-19 February, 2016	1.0
Poster entitled: Recognition specificity of AVR2/RD11 effectors from *Phytophthora infestans* in *Solanum* native to Mexico and Peru. IS-MPMI XVIII Congress. Portland, Oregon. 2016.	17-21 July, 2016	1.0
Poster entitled: Characterisation of AVR2 effectors from *Phytophthora infestans* for understanding differential R-gene mediated resistance. Cost Action Sustain (FA1208). Bled, Slovenia. 2017	1-3 March, 2017	1.0

	Presentation entitled: Two *R* gene loci co-evolved with Avr2 of *P. infestans* and confer distinct resistance specificities in potato. Experimental Plant Sciences meeting- Lunteren, The Netherlands. 2018	10 April, 2018	1.0
	Presentation entitled: AVR2-induced immunity to *P. infestans* by unrelated resistance genes of *Solanum* species. Experimental Plant Sciences meeting -Lunteren, The Netherlands. 2019	9 April, 2019	1.0
	Presentation entitled: AVR2-mediated resistance to *P. infestans* by unrelated *R* genes of *Solanum* species. Host-Microbe Co-evolution seccion. IS-MPMI XVIII Congress. Glasgow, Scotland. 2019	16 July, 2019	1.0
▶	**IAB interview**		
▶	**Excursions**		
	Subtotal Scientific Exposure		20.4

3) In-Depth Studies	*date*	*cp*
▶ **Advanced scientific courses & workshops**		
Phylogenetics: Principles and Methods	17-19 May, 2016	1.0
Summer School "Plant microbe interaction" (the Sainsbury lab). Norwich (UK)	17-28 August 2015	3.0
▶ **Journal club**		
Member of literature discussion of Plant Breeding	2015- 2017	2.3
▶ **Individual research training**		
Subtotal In-Depth Studies		6.3

4) Personal Development	date	cp
▶ **General skill training courses**		
EPS introduction course	22 September, 2015	0.2
The essentials of scientific writing and presenting	13-25 November, 2015	1.2
The Final Touch: Writing the General Introduction and Discussion	9 May, 2017	0.6
WGS course: Career Perspectives	9 Nov - 7 Dec, 2017	1.6
▶ **Organisation of meetings, PhD courses or outreach activities**		
▶ **Membership of EPS PhD Council**		
Subtotal Personal Development		3.6

TOTAL NUMBER OF CREDIT POINTS*	**37.8**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

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