Functional Genomics of *Phytophthora infestans* Effectors and *Solanum* Resistance Genes

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CONTENTS

ABSTRACT

Potato (*Solanum tuberosum* L.) is nowadays the most important non-cereal food crop in the world. It is prone to huge annual losses due to late blight, the disease caused by the oomycete pathogen *Phytophthora infestans*. Modern management of late blight necessitates the use of multiple resistance (*R*) genes, which requires efficient pipelines for identification, isolation and characterization of *R* genes. This thesis employs effectoromics, i.e. the use of effectors (pathogenic secreted protein) to probe corresponding *R* gene(s) in a host plant and sort out their functional redundancy and specificity. Using cytoplasmic RXLR effectors of *P. infestans* to probe resistant *Solanum* germplasm for late blight *R* genes, we were able to: (i) assess the biodiversity of *Avr-blb1*, characterize the genomic structure of virulent *P. infestans* isolates on *Rpi-blb1* plants and thus provide a technical solution for long-term disease management; (ii) identify the centre of origin of *R3a*, characterize *R3a* gene homologues and a functional *R* gene (*Rpi-sto2*), and (iii) uncover the potential co-evolution at both *R* and *Avr* side for the *R2/PiAvr2-PexRD11* interactions, providing more diversity and specificity of *R2* homologues, which may be valuable for potato breeding.

CHAPTER 1

General Introduction

Potato

Potato (*Solanum tuberosum* L.) is currently, the most consumed crop worldwide by men after wheat and rice. In the early ages, potato was already an important food crop for people inhabiting the South American Andes region, and excavated food plant remains from archaeological sites date back as far as 2500 BC and 5000 BC (Hawkes 1990). The exact origin of European potato is still unclear, since Chile and Peru are both competing for this honour. However, potato was brought from South America to Europe in the late 16th century, in Spain (1570) and England (between 1588 and 1593) (Hawkes 1990). Since then European potato breeding came up and nowadays, the European Cultivated Potato Database (ECPD) records 4,100 cultivated varieties. In 2005, the estimated potato cultivation area was almost 20 million hectares with a total world production of over 300 million tons (Haverkort et al. 2009). Potato is used for human consumption (boiled, steamed, baked, "sauté" or fried, and processed into French fries, chips or noodles) or for industrial purposes mainly for starch production which can be processed in textile, papermaking, glue, coating, sizing, flocculating agents and building materials. More uses can be anticipated for the future such as biopharmaceuticals for encapsulation and controlled release of functional ingredients (Bradshaw et al. 2006a; Li et al. 2009).

Potato is part of the tuber-bearing *Solanum* species that belong to the section *Petota*. *Solanum* section *Petota* species occur in the Andes of South America and a secondary centre of diversity exists in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004). Hawkes postulated that the ancestral potato species originated from Central America, subsequently migrated to South America, and a return migration back to Mexico, followed by hybridizations and allopolyploidisations within Central American taxa, then led to species with various ploidy levels (Hawkes 1990). *Solanum* species can easily hybridize with each other, making classification challenging (Hawkes 1990; Jacobs et al. 2008; Spooner and Hijmans 2001). Due to increased insights in potato taxonomy, the number of species is being reduced, in recent reviews. Hawkes (1990) recognized 227 tuber bearing species (7 cultivated species included) and 9 non-tuber-bearing species within section *Petota*. Spooner and Hijmans (Spooner and Hijmans 2001) recognized 203 tuber-bearing species including 7 cultivated species. Finally, Spooner and Salas (2006), reduced the number further to 189 species (including 1 cultivated species) in section *Petota*. However, one thing for sure, wild *Solanum* species are well spread from North to South America and highly diverse. Diploid, triploid, tetraploid, pentaploid and hexaploid species exist, and a polyploid species can contain genes with high homology to genes in one of its diploid progenitors, which increases the potential of diversification of genes in polyploid species to adapt to there environment. Consequently more diversity can occur for each gene homologue in the tuber-bearing *Solanum* species gene-pool, which can be used to improve various quality traits in breeding programs.

Phytophthora infestans

Phytophthora infestans (Mont.) de Bary, the responsible pathogen for the Irish famine in the middle of the 1840's, was the first 'plant destroyer' described (Fry 2008; Large 1940; Schumann 1991). *P. infestans* is the causal agent of late blight, which is nowadays the most devastating disease on potato and tomato. It infects foliage, stems and can also infect fruits and tubers (Fry 2008). This notorious disease entails global yield losses of 16% of the potato crop, representing an annual financial loss of € 5.2 billion worldwide (Haverkort et al. 2009).
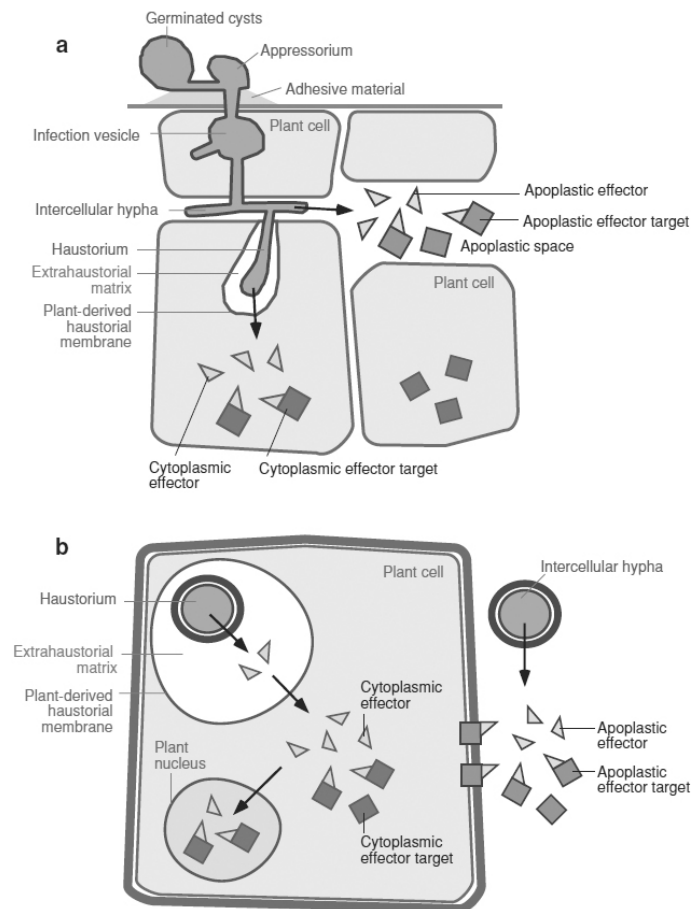
Figure 1. Plant pathogenic oomycetes secrete apoplastic effectors into the plant extracellular space and cytoplasmic effectors inside the plant cell.
A) Schematic view of the early stages of infection by *Phytophthora infestans* of a host plant illustrating the sites of action of the apoplastic and cytoplasmic effectors. B) Cross-section of the view in panel A illustrating the delivery of apoplastic and cytoplasmic effectors to their cellular target. One class of cytoplasmic effectors is known to target the plant nucleus. Apoplastic effectors interact with extracellular targets or surface receptors. Pathogen structures are shown in *dark grey* and plant structures in *clear grey*. Apoplastic effector and effector targets are outside of the plant cell, whereas cytoplasmic effectors and their targets are in the plant cell. Adapted from Kamoun (2006).

P. infestans is a near-obligate hemi-biotrophic pathogen. The infection is based on a first biotrophic stage which is followed by a necrotrophic phase. Infection starts with the formation of an appressorium which breaks through the first physical layer of defence of the host (Figure 1). During the biotrophic stage, an intracellular infection vesicle is formed. From there, hyphae emerge, and hyphal growth expands in the extracellular space of the host. Then specialized infection structures called haustoria invaginate host cells and make near-direct contact with the host plasma membrane (Birch et al. 2006). From that point on, the infection switches to a necrotrophic phase and mycelium spreads rapidly through the host tissue and forms spores. During its life cycle *P. infestans* adopts two types of reproduction. The asexual cycle, as described above, enables dramatically rapid population growth in susceptible host tissue (Fry 2008). The sexual cycle, which requires both mating types (A1 and A2), leads to the formation of oospores that serve both as a survival structure and as a source of variation via sexual recombination (Fry 2008).

P. infestans belongs taxonomically to the oomycetes phylum. Traditionally *Phytophthora* was classified as a fungus due to its filamentous growth habit and common infection strategies (Latijnhouwers et al. 2003). However, genetic structure (Tyler et al. 2006), phylogeny (Baldauf 2003) and life cycle (Judelson and Blanco 2005) studies have demonstrated that oomycetes are closer to heterokont algae than to fungi (Figure 2). In the last decade various *Phytophthora* species such as *P. sojae, P. ramorum* and *P. infestans* have entered the genomics era (Haas et al. 2009; Lamour et al. 2007; Tyler et al. 2006). The major discovery has unquestionably been the variability and diversity of effectors these species contain.
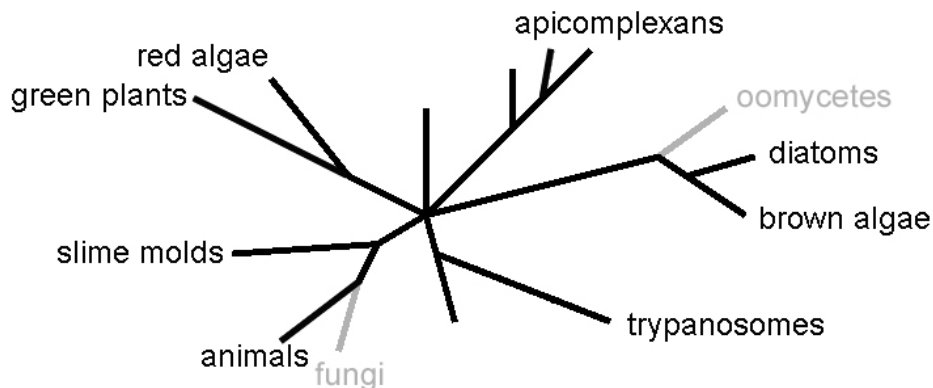
Figure 2. Schematic phylogenetic tree of the eukaryotes.
The tree is adapted from that of Baldauf et al. (Baldauf 2003) that is based on a concatenation of six highly conserved proteins. Oomycetes are more related to brown algae and diatoms (heterokonts) in the Stramenopiles, than fungi.

Effectors

Effectors are pathogen molecules that manipulate host cell structure and function thereby facilitating infection and/or triggering defence responses. Unlike the terms "avirulence", "elicitor", "toxin", and "virulence", the term effector is neutral and does not imply a negative or positive impact on the outcome of the disease interaction. Two classes of effectors target distinct sites in the host plant: apoplastic effectors are secreted into the plant extracellular space, where they interact with extracellular targets and surface receptors; and cytoplasmic effectors are translocated inside the plant cell presumably through specialized structures like infection vesicles and haustoria that invaginate living host cells (Figure 1) (Kamoun 2006).

Apoplastic effectors contain N-terminal signal peptides for secretion, followed by C-terminal effector module(s) (Figure 3), but are not known to carry additional host targeting signals (Damasceno et al. 2008; Tian et al. 2005; Tian et al. 2007; Tian et al. 2004). Some apoplastic effectors such as INF elicitins are down-regulated in early infection stages perhaps to avoid recognition. For example, INF elicitins are known to induce cell death when infiltrated on some *Solanum* species or *Nicotiana benthamiana* (Kamoun et al. 1998; Kamoun et al. 1997; van West et al. 1999; Vleeshouwers et al. 2006). Also others, like the NEP1-Like Family (necrosis- and ethylene-inducing peptides) are mainly expressed in late stages of infection (Kanneganti et al. 2006) when they might function in triggering tissue necrosis and enable necrotrophic colonization at later infection stages. Two other well-studied apoplastic effector classes of *P. infestans*, are the Serine and Cysteine protease
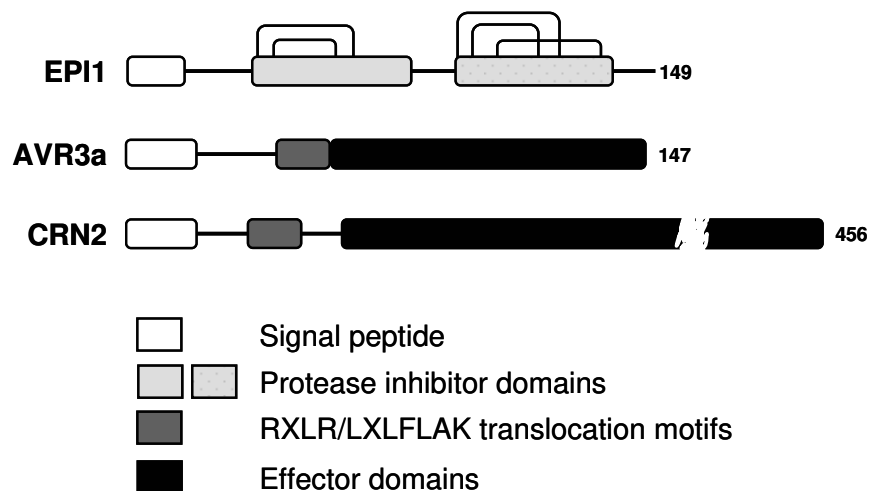
Figure 3. Oomycete effectors are modular.
All known effectors carry N-terminal signal peptides for secretion (white box). Cytoplasmic effectors, in addition, have conserved motifs in their N-termini (RXLR or LXLFLAK) that are required for host translocation. The C-terminal domain carries the module with biochemical effector activity. Adapted from Schronack et al. (2009b).

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inhibitors EPI1, EPI10 and EPIC1, EPIC2B respectively, which inhibit protease defence layers of the pathogen. Kazal-like extracellular serine protease inhibitors are e.g. EPI1 and EPI10, which bind and inhibit the pathogenesis-related P69B subtilisin-like serine protease from tomato (Song et al. 2009; Tian et al. 2005). Cystatin-like protease inhibitors, EPIC1 and EPIC2B, are subject to degradation by P69B. However, EPI1 protects the EPICs from degradation. EPIC2B binds and inhibits a salicylic acid–induced papain-like protease PIP1, and the related RCR3 in tomato, which are both under diversifying selection, but no virulent function for EPIC1 was found yet (Tian et al. 2007). These findings demonstrate a cascade of protein interactions between the pathogen and the host, to be able to establish infection (Song et al. 2009).

Oomycete cytoplasmic effectors (Figure 3) are characterized by an N-terminal signal peptide for type II secretion, a translocation motif (mainly RXLR and LXLFLAK) for trafficking inside host cells and a C-terminal domain for biochemical effector activity (Birch et al. 2008; Birch et al. 2006; Kamoun 2006; Morgan and Kamoun 2007; Tyler et al. 2006; Whisson et al. 2007). The RXLR effectors are defined by the amino acid (a.a.) sequence Arginine-X-Leucine-Arginine (where X is any residue) that designates a domain involved in translocation inside host cells (Dou et al. 2008; Whisson et al. 2007), this motif in an effector class is unique to oomycetes, revealing another clear distinction between oomycetes and true fungi. Elevated levels of polymorphism and signatures of positive (adaptive) selection have
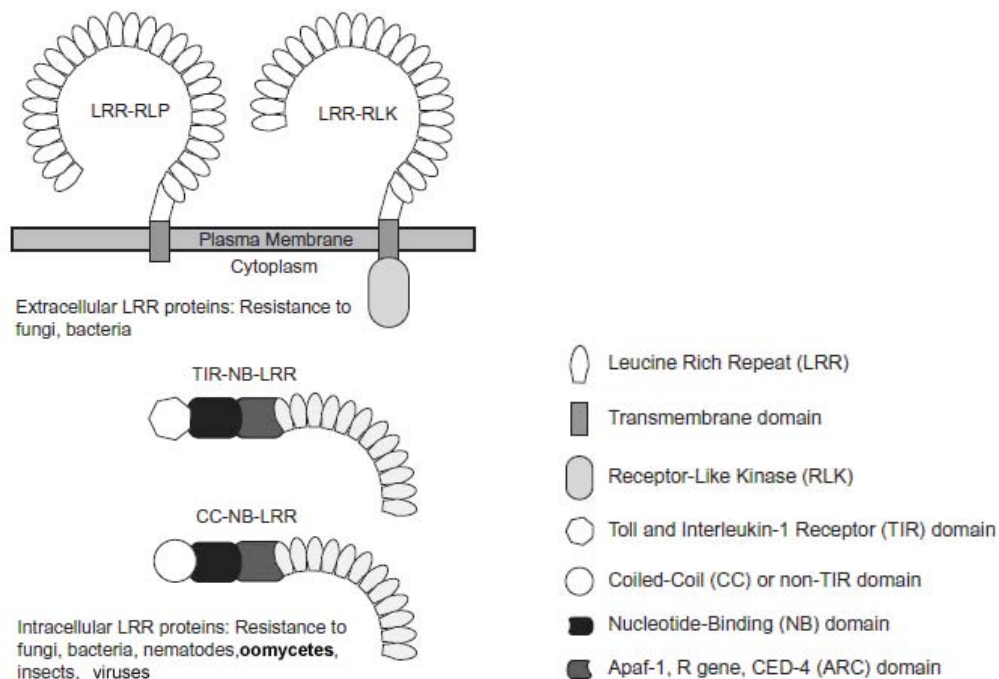
Figure 4. Schematic diagram of the proteins encoded by disease resistance genes along with a list of the types of pathogens recognized by these proteins.
These include proteins with extracellular moieties (top) or intra-cellular proteins (middle). The identity of individual protein domains are indicated at the bottom. Adapted from Moffett (2009).

been observed in the C-terminal half of RXLR effectors, consistent with the view that this region executes the effector activity inside plant cells and coevolves with host factors (Allen et al. 2008; Oh et al. 2009; Rehmany et al. 2005; Win et al. 2007). The CRN effectors also contain N-terminal sequence domains, including the conserved LXLFLAK motif, which are implicated in host translocation (Haas et al. 2009; Win et al. 2007). Some of these cytoplasmic effectors are even known to target the nucleus of the host, like the NUK effectors of *P. infestans* that contain nuclear localization signals (NLSs) (Kanneganti et al. 2007). Interestingly, cytoplasmic RXLR effectors of *P. infestans* are the only effectors until now that were found to have an avirulence activity in potato, like AVR3a, PiAVR2, AVR-blb1, AVR4, AVR1 and more recently AVR-blb2 and AVR-vnt1 (Table 1) (Armstrong et al. 2005; Lokossou et al. 2009; Oh et al. 2009; Pel 2010; Tyler et al. 2006; Vleeshouwers et al. 2008). With the recent completion of the *P. infestans* genome (Haas et al. 2009), we can use effectors of this pathogen to functionally profile resistant *Solanum* plants for response to these effectors and mine their active *R* genes. This approach is known as effector genomics or effectoromics (Oh et al. 2009; Vleeshouwers et al. 2008). The characterisation of resistance genes by effectoromics is more accurate and provides more molecular insight in the potato/*P. infestans* pathosystem compared to determination of resistance spectra with *P. infestans* isolates.

Resistance genes

R proteins, often known as NB-LRR proteins, consist of three domains, the N-terminal, the NB-ARC (Nucleotide-Binding domain found in proteins such as human Apaf-1, plant R proteins and *Caenorhabditis elegans* Ced-4) and the LRR (Leucine Rich Repeat) domain. Two types of N-terminal domains are commonly observed (Figure 4). The Toll and Interleukin-1 Receptor (TIR), which is a protein-protein interaction domain, known to be associated with innate immune systems in multiple phyla (Staal and Dixelius 2007), and the coiled-coil (CC) N-terminal domain, which is in fact highly variable and not fully conserved between CC-NBS-LRR proteins (Cannon et al. 2002) forming a significant source of variation for fast evolution in this class of proteins. The NB-ARC domain, also known as NBS (nucleotide-binding site), is made of multiple conserved motifs (Albrecht and Takken 2006; McHale et al. 2006; Ooijen et al. 2007), which create a functional nucleotide-binding pocket capable of binding and hydrolyzing ATP (Tameling et al. 2002). This ATP/ADP binding site in the NB-ARC domain is thought to be the activation site for signal transduction of the R protein (Takken and Tameling 2009). The LRR domain is made up of individual repeats with the consensus sequence LxxLxxLxxLxLxx(N/C/T)x(x)LxxIPxx (Jones et al. 1997). The number of repeats and primary structure is highly variable between family members, and domain swapping studies have shown that recognition specificity maps to the LRR domain (Dodds et al. 2006; Ellis et al. 2007b; Kamoun 2006). Nevertheless recent

studies let believe that not only the LRR but also the N termini of NB-LRR protein are involved in recognition of AVR protein and signal transduction, via a "bait" protein (Caplan et al. 2008; Sacco et al. 2009; Ueda et al. 2006). Collier and Moffett (2009) proposed a two-step recognition process to describe this mechanism, called "bait and switch" model, involving interactions of the AVR protein with a "bait" protein which could be the guardee or the decoy (see below) presented to the N terminal domain of the R protein. These inter-domain interactions bring the "bait" protein into close proximity with other domains of the NB-LRR protein which leads to a direct or indirect recognition of the AVR by the LRR domain and cause a flipping of the NB-LRR protein to a switch "on" mode starting the signal transduction for resistance. All these models try to explain the different mechanism observed in what is basically called R/AVR interaction.

During evolution, *R* genes under selective pressure can evolve at different rates, even within an individual cluster of similar sequences. Kuang et al. (2004) describe the fast and slow evolving extremes as type I and type II *R* genes, respectively. Type I *R* genes are characterized by frequent sequence exchanges between paralogs that obscure orthologous relationships, whereas type II resistance genes rarely undergo sequence exchanges with paralogs and maintain orthologous relationships (Friedman and Baker 2007). Within an *R* gene locus, the number of paralogs can vary considerably. For example, two *RPP8* homologous (type II) in *A. thaliana* (Kuang et al. 2008) and a few computationally recognized NBS-LRR singletons in sunflower (Radwan et al. 2008) represent rather small *R* gene families. In contrast, multi-copies of *R* genes can form highly extended *R* gene clusters, e.g. the maize *Rp1* (against *Puccinia sorghi*) cluster (Smith et al. 2004), the lettuce *Dm3* cluster (effective towards *Bremia lactucae*) and *RGC2* cluster (Kuang et al. 2004) and two potato Major *Late Blight* resistance (MLB) cluster at Linkage Group (LG) XI (Friedman and Baker 2007) harbouring up to 45 type I *R3a* homologous per haplotype, and at LG IV with the *R2* cluster of which up to 16 *R2* variants, or Resistance Gene Homologue (*RGH*), were observed in the Mastenbroek *R2* differential (Lokossou et al. 2009).

Defence mechanisms & Co-evolution potato – *P. infestans*

Pathogens exert significant constraints on host fitness. These pressures have consequently applied strong selection on the evolution of host genomes such that most hosts devote significant portions of their genomes to encoding defences against pathogens (Moffett 2009). In the last decade multiple theories have been put forward to describe this close co-evolution between the pathogen and its natural host. The most explicative mechanism is described by Zig-Zag model of Jones and Dangl (2006). In this model the first layer of defence is comprised of membrane-receptors that detect the widely conserved pathogen-associated molecular patterns (PAMPs), and activate responses that protect against colonization. This mechanism is called PAMP-Triggered Immunity (PTI). In some cases

pathogens are able to escape PTI in their hosts by secreting effectors that interfere with PTI, and due to this effector-triggered suppression (ETS), infection can proceed. As a second defence layer, plants employ resistance (*R*) genes to activate ETI (Effector Triggered Immunity) which is often characterized by the hypersensitive response (HR), resulting in localized programmed cell dead. The effectors in that case act as avirulent factors (*Avr*) and their recognition by *R* genes is based in the gene-for-gene model (Flor 1971).

Direct interactions between an R protein and its matching AVR have relatively rarely been demonstrated (Deslandes et al. 2003; Dodds et al. 2006; Ellis et al. 2007a; Jia et al. 2000). Therefore new hypotheses of interaction were presented, for example the guard model, where R proteins monitor or guard host proteins (van der Biezen and Jones 1998). In that case, R proteins do not directly detect the presence of the pathogen, but perceive alterations of the protein they guard when pathogen effectors interact with it. This model is well illustrated with effectors proteins from *Pseudomonas syringae* AvrB, AvrRpm1, AvrRpt2, interacting with RIN4, which cause the activation of RPM1 and RPS2 resistance gene; and AvrPphB, interacting with PBS1 resulting in the activation of RPS5 of *Arabidopsis thaliana* (Axtell and Staskawicz 2003; Mackey et al. 2003; Mackey et al. 2002; Shao et al. 2003). Yet recognition specificity, or avirulence function, does not always correlate with virulence activity. In the case of AvrPto and AvrPtoB of *P. syringae*, the host target was thought to be Pto protein in tomato which is guarded by the R protein Prf (Pedley and Martin 2003). Recent studies have demonstrated that the virulence targets of these two effectors are in fact the receptor-like kinases CERK1, BAK1, EFR1 and FLS2, which mediate PTI responses (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Shan et al. 2008; Xiang et al. 2008). Therefore Pto is used as a decoy protein by the host to trap AvrPto and AvrPtoB and then activate Prf responses. These recent discoveries are supporting the decoy model established by van der Hoorn and Kamoun (2008). In this model of evolution, guardees will be subject to alternating selective pressures depending on the presence or absence of their guarding R protein. In the presence of the R protein, the guardee would be optimized for Avr interaction, and hence detection. In the absence of the R protein, the guardee would be under pressure to evade interaction with pathogen effectors in order to reduce virulence of the pathogen.

For *Solanum* section *Petota* species, two centres of diversity exist, one in the Andes of South America and another in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004), as described above. Both areas have been suggested to be also the geographic origin of *P. infestans*, although this is still a subject of debate. The origin as Toluca Valley in Mexico is supported by the presence of sexual populations with both the A1 and A2 mating types in similar frequencies and the high degree of molecular genetic diversity in that region (Flier et al. 2003; Fry et al. 1992; Goodwin and Fry 1994; Grünwald and Flier 2005). The hypothetical South America origin is based on endemic coexistence of distinct lineages as well as nucleotide and haplotype diversity in the Andes (Gomez-Alpizar et al. 2007). These studies suggest that the *P. infestans* isolates may have adapted to different host range species

in both centres. As a consequence, *R* loci that co-evolved with *P. infestans* in South American *Solanum* species are not necessarily expected to display functional similarity to those from the Central American species, and vice versa. Therefore studying *R* gene diversity in these two centres of origin will provide insight into the co-evolution of this pathosystem and hopefully new tools to manage the disease.

Late blight potato breeding

Early potato breeding efforts focussed on the characterisation of the resistance spectrum and the introgression of 11 *R* genes from *Solanum demissum*, a wild relative of potato. The exploitation of new cultivars containing these *R* genes proved to be successful (Black et al. 1953; Malcolmson and Black 1966). However, rapidly changing populations of *P. infestans* overcame the *R1-R11* genes, even in pyramiding systems (Hein et al. 2009). In the second half of the twentieth century, potato farmers resorted largely to fungicides to control the disease. Nevertheless *P. infestans* continued to damage potato crops, and at the beginning of the 1980s even gained in resistance to metalaxyl, a phenylamide fungicide, in Europe (Cooke 1981; Davidse et al. 1981) and in Middle East (Cohen and Reuveni 1983), in the 1990s in North America (Deahl et al. 1993; Miller et al. 1997), and beginning of the 21st century in Asia (Deahl et al. 2002). At the end of the 20th century, a new interest for the protection of the environment and for a more economical management system by farmers, made such pesticide inputs undesirable. Contemporary potato breeding for *P. infestans* resistance is exploring the wealth of *R* gene diversity in a broad range of tuber-bearing *Solanum* section *Petota* species to build up a collection of diverse *Rpi* (*Resistance* to *P. infestans*) genes. A number of *R* genes and new *Rpi* genes have recently been cloned from Mexican *Solanum* species, i.e. *R1, R2* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009), *Rpi-blb1/RB* (Song et al. 2003; van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al. 2009), *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008), and *Rpi-abpt / R2-like* from unknown species used in a pre-breeding program (Lokossou et al. 2009). Also, the first *Rpi*-genes of South American origin are being cloned, such as *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009) (Table 1). Many more *Rpi* genes are expected to be cloned in the coming years so that the set of cloned *R* genes against late blight is rapidly growing (Hein et al. 2009; Hein et al. 2007; Jacobs et al. 2010; Kuhl et al. 2001; Rauscher et al. 2006; Sandbrink et al. 2000; Smilde et al. 2005; Tan et al. 2008; Villamon et al. 2005) (Table 1). Interestingly all cloned *R* genes from wild *Solanum* species against late blight are intracellular LRR proteins from the CC-NBS-LRR category (Figure 4).

Table 1. Late blight *R* genes, their origin, cloning status and their cognate *Avr*

Gene	Species	Comments	Matching *Avr*	References
R genes from Central America				
R1	*S. demissum*	Cloned	*Avr1*	Ballvora et al. 2002; Tyler 2009
R2	*S. demissum*	Cloned	*PiAvr2,*	Lokossou et al. 2009; This thesis chapter 3
R2-like	*Unknown*	Cloned	*PexRD11,*	Lokossou et al. 2009; This thesis chapter 3
Rpi-abpt	*Unknown*	Cloned	*PITG_21949,*	Lokossou et al. 2009; This thesis chapter 3
Rpi-blb3	*S. bulbocastanum*	Cloned	*PITG_21645*	Lokossou et al. 2009; This thesis chapter 3
Rpi-edn1.1	*S. edinense*	Cloned		This thesis chapter 3 and 4
Rpi-snk1.1	*S. schenckii*	Cloned	*PiAvr2,*	This thesis chapter 3 and 4
Rpi-snk1.2	*S. schenckii*	Cloned	*PexRD11,*	This thesis chapter 3 and 4
Rpi-hjt1.1	*S. hjertingii*	Cloned	*PITG_21949,*	This thesis chapter 3 and 4
Rpi-hjt1.2	*S. hjertingii*	Cloned	*PITG_21645,*	This thesis chapter 3 and 4
Rpi-hjt1.3	*S. hjertingii*	Cloned	*PITG_13940*	This thesis chapter 3 and 4
Rpi-dmsf1	*S. demissum*	Mapped		Hein et al. 2007
R3a	*S. demissum*	Cloned	*Avr3a*	Armstrong et al. 2005; Huang et al. 2005
Rpi-sto2	*S. stoloniferum*	Cloned	*Avr3a*	This thesis chapter 5
R3b, R5-R11	*S. demissum*	Mapped		Bradshaw et al. 2006b; El-Kharbotly et al. 1996; Huang et al. 2005; Huang et al. 2004
Ma-R4	*S. demissum*		*Avr4*	van Poppel et al. 2008
Rpi-blb1/RB	*S. bulbocastanum*	Cloned	*Avr-blb1 (ipiO)*	Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008
Rpi-sto1	*S. stoloniferum*	Cloned	*Avr-blb1 (ipiO)*	Vleeshouwers et al. 2008; Wang et al. 2008
Rpi-pta1	*S. papita*	Cloned	*Avr-blb1 (ipiO)*	Vleeshouwers et al. 2008; Wang et al. 2008
Rpi-plt1	*S. polytrichon*	Mapped		Wang et al. 2008
Rpi-blb2	*S. bulbocastanum*	Cloned	*Avr-blb2*	van der Vossen et al. 2005
Rpi-bst1	*S. brachistotricum*	Mapped		Hein et al. 2009
Rpi1	*S. pinnatisectum*	Mapped		Kuhl et al. 2001
R genes from South America				
Rpi-mcd1	*S. microdontum*	Mapped		Sandbrink et al. 2000; Tan et al. 2008
Rpi-mcq1	*S. mochiquense*	Mapped		Smilde et al. 2005
Rpi-vnt1.1	*S. venturii*	Cloned	*Avr-vnt1*	Foster et al. 2009; Pel 2010; Pel et al. 2009
Rpi-vnt1.2	*S. venturii*	Cloned	*Avr-vnt1*	Foster et al. 2009; Pel 2010; Pel et al. 2009
Rpi-vnt1.3	*S. venturii*	Cloned	*Avr-vnt1*	Foster et al. 2009; Pel 2010; Pel et al. 2009
Rpi-cap1	*S. capsicibaccatum*	Mapped		Jacobs et al. 2010
R_{ber}	*S. berthaultii*	Mapped		Rauscher et al. 2006
Rpi-pcs	*S. paucissectum*	Mapped		Villamon et al. 2005

   P. infestans populations exhibit a high risk of evolution owing to high mutation rates, large effective populations, a high gene/genotype flow, a mixed reproduction system and an efficient directional selection (McDonald and Linde 2002). Moreover, the revelation of *P. infestans* genome plasticity and its abundance of putative effectors, 563 genes predicted for the RXLR family (Haas et al. 2009), show that this oomycete remains a formidable foe. This explains why single-dominant host *R* genes, that were deployed separately, have been rapidly overcome (Wastie 1991). Also pyramiding approaches with the first R1 to R11 *R* genes

rendered only little success (Hein et al. 2009). Nevertheless, in natural populations, *Solanum* host species are not extinct in areas where *P. infestans* is dominantly present. This co-evolution situation is most-likely the one we have to be inspired by for future breeding, and we postulate that understanding the molecular insight of R/AVR interactions in this pathosystem can provide rational solutions for application of a durable resistance strategy to manage late blight in agriculture.

First of all, increasing information about the molecular basis for *R* and *Avr* genes and their interactions is being uncovered (Oh et al. 2009; Vleeshouwers et al. 2008). New *R* genes with broad or less broad spectrum are being cloned, and *RGH* identification and cloning is also providing new tools to breeders. For example, *Rpi-sto1* and *Rpi-pta1* from the crossable *S. stoloniferum,* which are homologues of *Rpi-blb1* from *S. bulbocastanum*, can be used for breeding programs because *S. bulbocastanum* is less easy crossable with *S. tuberosum* (Vleeshouwers et al. 2008). To contour classical breeding problems, like time and non desired genome linkage dragging, a new Cisgenesis technology is being developed (Jacobsen and Schouten 2007; Vetten et al. 2003). In this technique plants are genetically modified by cisgene only. A cisgene is a natural gene, coding for an (agricultural) trait, from the crop plant itself or from a sexually compatible donor plant that can be used in traditional plant breeding. In potato breeding against late blight, *R* genes from crossable or crossable by bridging *Solanum* species is inserted in susceptible or new cultivars by genetic modification without any bacterial markers. This technique also provides new opportunities for *R* gene stacking (Jacobsen and Schouten 2007). Another example is the quest for complementary *R* genes, which are targeted at the full spectrum of allelic variants of an Avr gene, for stacking approaches to provide broad-spectrum resistance. In the case of R3a/AVR3a, with only *Avr3a^{KI}* and *Avr3a^{EM}* present in *P. infestans* population (Armstrong et al. 2005), combining an R gene responding to AVR3a^{EM} with R3a, is likely to cover a full spectrum of resistance against *P. infestans*. For the deployment of *R* genes in agriculture, the availability of effectors that match particular *R* genes are useful for example via effectoromics studies (Oh et al. 2009; Vleeshouwers et al. 2008). The diversity of effector variants across pathogen populations, coupled with functional assays, can be used to monitor pathogen populations for the potential occurrence or emergence of races that overcome the *R* genes (Schornack et al. 2009b; Vleeshouwers et al. 2008). Also the identification of an *R* gene responding to an effector with a virulent function crucial for *P. infestans* infection process could be perceived as a "Holy Grail" for potato breeders. All these strategies and ideologies to manage late blight require the identification and characterization of more *R* genes and *RGH*, as well as the diversity of the corresponding *Avr* genes in the *P. infestans* natural population to be able to setup a durable strategy against late blight.

Scope of this thesis

The aim of this thesis was to provide new insights in diversity and co-evolution of *Solanum* section *Petota* resistance genes and corresponding *Phytophthora infestans* effectors by functional genomics to provide new genetic tools for management of late blight and potato resistance breeding. We focussed our research on three diverse R-AVR interactions, namely *Rpi-blb1 – Avr-blb1, R2 – Avr2/PexRD11,* and *R3a-Avr3a* and dedicated effectoromics in combination with disease tests and population studies to uncover new diversities on *R* and *Avr* gene families by targeted allele mining. Our finding with genotypes originating from natural environments brought new genetic material, information regarding gene stacking, copy numbers of resistance gene homologues and tools to potato breeders for predicting and deploying a durable, broad-spectrum resistance in contemporary agricultural practice.

In Chapter 2, we challenge the classification of *Rpi-blb1* from *S. bulbocastanum* as a broad spectrum *R* gene using information on the matching effector *Avr-blb1* (*ipiO*). We examined the diversity of *ipiO* in a wide set of *P. infestans* isolates and tested them for virulence on potatoes expressing *Rpi-blb1*. We found the first *P. infestans* isolates that defeated *Rpi-blb1* plants, and showed that profiling the *ipiO* variation is instrumental for predicting the effectiveness of *Rpi-blb1* in potato.

Chapter 3 presents an effectoromics approach to identify R-Avr interactions and rapidly characterize functional RGH from other wild *Solanum* species. By functional assays we detect that two effectors of *P. infestans*, PiAVR2 and PexRD11, interact with R2 homologues. We amplified a number of *R2GH* from various wild *Solanum* species, and uncovered a Mexican origin of this *R2* gene family. Among the members, we identified six new functional *R2* homologues, i.e. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3* that confer resistance to *P. infestans* Positive selection modelling on the R2GH revealed amino acids under diversifying selection in the N-terminus, the NB and the LRR domain, and we postulated a guard model function for *R2* with a bait and switch mechanism for recognition and signal transduction. In addition a discussion on copy number of functional *R* homologues in wild *Solanum* species is presented.

In Chapter 4 we further investigate the R2-PiAvr2/PexRD11 interaction, and use the *P. infestans* genome sequence to retrieve the *PiAvr2/PexRD11* gene family. We analyzed the diversity and functionality of *PiAvr2/PexRD11* and *R2* gene families for avirulence activity, and discussed functional evidence for an arm-race or co-evolution between *P. infestans* and Mexican *Solanum* species.

Chapter 5 presents effectoromics using different transient assays in combination with disease tests to characterize *Solanum* species containing functional homologues of *R3a*. One functional R3a homologue, Rpi-sto2 that interacts with Avr3a, was cloned from *S. stoloniferum*. The origin of *R3a*, its diversity and the implications of for breeding purposes are discussed.

CHAPTER 2

Phytophthora infestans isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato

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Phytophthora infestans isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato

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Abstract

A strategy to control the devastating late blight disease is providing potato cultivars with *R gene*s that are effective to a broad spectrum of *Phytophthora infestans* isolates. Thus far, most late blight *R* genes that were introgressed in potato were quickly defeated. In contrast, the *Rpi-blb1* gene originating from *Solanum bulbocastanum* has performed as an exclusive broad-spectrum *R* gene for many years. Recently, the RXLR effector family *ipiO* was identified to contain *Avr-blb1*. Monitoring the genetic diversity of the *ipiO* family in a large set of isolates of *P. infestans* and related species resulted in 16 *ipiO* variants in three distinct classes. The class I and class II, but not class III, *ipiO* variants induce cell death when co-infiltrated with *Rpi-blb1* in *Nicotiana benthamiana*. Class I is highly diverse and represented in all analyzed *P. infestans* isolates except in two Mexican *P. infestans* isolates, and these were found virulent on *Rpi-blb1* plants. In its C-terminal domain, IPI-O contains a W-motif that is essential for triggering Rpi-blb1-mediated cell death and is under positive selection. This study shows that profiling the variation of *Avr-blb1* within a *P. infestans* population is instrumental for predicting the effectiveness of *Rpi-blb1*-mediated resistance in potato.

Introduction

Late blight caused by the oomycete *Phytophthora infestans* is one of the most severe threats to potato production worldwide (Fry 2008). Despite many efforts, effective methods to control late blight epidemics are still not available. In recent years, however, intensified research on both the pathogen and the host plant has deepened our insight into the molecular basis of virulence and avirulence determinants in *P. infestans,* and of host defense responses. This knowledge is instrumental for obtaining genetic resistance in potato (Bryan and Hein 2008; Govers and Gijzen 2006; Park et al. 2009).

In order to invade plant cells without hindrance, pathogens secrete effector proteins that can manipulate host defense responses thus resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Kamoun 2006; Kamoun 2007). When an effector is recognized by a resistance (R) protein, effector triggered immunity (ETI) is activated often resulting in a hypersensitive response (HR). Effectors then act as avirulence (Avr) factors and the encoding *Avr* genes interact with *R* genes according to the gene-for-gene model (Flor 1971). The molecular arms race between the pathogen and its host drives co-evolution of *R-Avr* gene pairs, as has been clearly demonstrated with the *Arabidopsis R* gene *RPP13* and the corresponding *Avr* factor *ATR13* of the oomycete *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*) (Allen et al. 2008). Both *RPP13* and *ATR13* are highly variable and by examining natural variants of ATR13 key amino acids were identified that are functionally essential for interaction with RPP13 (Allen et al. 2008). In *P. infestans* only a few *Avr* genes have been studied at the molecular level, so far. *Avr3a*, the counterpart of the *Solanum demissum* gene *R3a*, has two alleles – a virulent and avirulent one – that differ only two amino acids (Armstrong et al. 2005). The latter, *Avr3a^{KI}*, not only triggers a *R3a*-dependent HR; it is also capable to suppress a cell death response induced by the elicitin INF1. These two activities, however, are conditioned by distinct amino acids (Bos et al. 2009). On the plant side, *R3a* unleashed its evolutionary potential with numerous *R3a*-like genes, which resulted in a major late blight locus on chromosome 11 of *Solanum demissum* (Friedman and Baker 2007; Huang 2005; Huang et al. 2005). *Avr4*, which interacts with *S. demissum R4*, has one predominant avirulent allele in nature but unlike *Avr3a*, the virulent allele has frameshift mutations and can no longer produce an effector protein (van Poppel et al. 2008). Recently, we described the identification of another potential *P. infestans Avr* gene, i.e. *ipiO*, the *in planta induced* gene that was postulated as being involved in pathogenicity based on its expression profile (van West et al. 1998). The identification of *ipiO* as *Avr-blb1* resulted from an effector genomics approach that is based on high throughput functional profiling of effector genes in plants carrying *R* genes (Vleeshouwers et al. 2008). In the effector screening, two variants of *ipiO*, i.e. *ipiO1* and *ipiO2,* triggered a cell death response in *S. bulbocastanum* plants carrying the late blight *R* gene *Rpi-blb1* (alternatively named RB) (Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008). Cell death

responses to *ipiO1* and *ipiO2* were also noted in *S. stoloniferum,* which is the source of the *Rpi-blb1* homologues *Rpi-sto1* and *Rpi-pta1*. Accordingly, agro-coinfiltration of *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* with *ipiO1* and *ipiO2* in *Nicotiana benthamiana* resulted in specific cell death, and provided functional evidence for the *R-Avr* interaction. However, *ipiO4* – a genetically more distant variant – did not elicit cell death when agro-coinfiltrated with *Rpi-blb1*, nor with its homologues. This suggested that alleles or variants of the *ipiO* gene family vary with respect to recognition by *Rpi-blb1* and hence, in avirulence to *Rpi-blb1*.

Like Avr3a and Avr4, IPI-O contains at its N-terminus a signal peptide for type II secretion and a RXLR-dEER motif for host cell internalization whereas the C-terminal domain is required for effector functions (Govers and Bouwmeester 2008; Rehmany et al. 2005; Whisson et al. 2007). The RXLR domain of IPI-O partly overlaps with a RGD cell adhesion motif, which has been shown to bind to a lectin receptor kinase in *Arabidopsis* that may function as an effector target (Gouget et al. 2006). This lectin receptor kinase participates in protein-protein interactions to mediate cell wall-plasmamembrane adhesions and it has been observed that IPI-O can disrupt these adhesions (Senchou et al. 2004). In many of the RXLR-dEER effectors the C-terminal domain consists of a variable number of motifs that occur in a repeated fashion (Jiang et al. 2008). This allows rapid evolution and diversification within this effector family, which is consistent with a role for *Avr* genes in gene-for-gene interactions with their hosts.

A wealth of resistance (*R*) genes is present in botanical *Solanum* species, but in spite of that, resistance breeding has thus far been unsuccessful. Introgressed *R* genes from e.g. *S. demissum* and *S. berthaultii* were quickly defeated and virulent *P. infestans* isolates were detected in the field sometimes even before introduction into cultivated potato (Flier et al. 2003; Grünwald et al. 2001; Rauscher et al. 2006; Wastie 1991). Recently, renewed hope for resistance breeding emerged with the identification of the so-called broad-spectrum *R* genes *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). Since the introduction in potato, *Rpi-blb1* appears to have remained effective in various geographical areas and over several growing seasons (Colton et al. 2006; Helgeson et al. 1998; Naess et al. 2000). The identification of *ipiO* as the candidate for *Avr-blb1* enables us to address the question how widespread avirulent alleles or variants occur in *P. infestans* populations, and related to this, how likely it is that *Rpi-blb1*-mediated resistance will last in the field. In this study, we monitored the genetic variation of *ipiO* in a highly diverse set of *P. infestans* isolates and related *Phytophthora* species, and identified 16 naturally occurring *ipiO* variants that could be grouped in three different classes. The *P. infestans* isolates lacking one specific class of *ipiO* variants appeared to be virulent on plants carrying *Rpi-blb1*, thus confirming the gene-for-gene interaction between *ipiO* and *Rpi-blb1*. We also show that IPI-O contains a W-motif in the C-terminal domain that is subject to positive selection and that this domain is sufficient to trigger *Rpi-blb1*-dependent cell death.

Results

The *ipiO* gene is highly diverse

For assessing natural genetic variation of *ipiO*, we compiled a set of 29 *P. infestans* isolates that were collected from various geographic regions, including the European potato growing areas and the Central Highlands of Mexico that are both known to harbour genetically highly diverse *P. infestans* populations (Flier 2001; Flier et al. 2003; Rivera-Pena 1990a) (Table 1). Furthermore, the *P. infestans* isolates originated from diverse host plants, including potato, tomato and botanical *Solanum* species, and they vary for year of collection, mating type, and virulence pattern on the potato *R1-R11* differential set. To examine the genetic diversity we fingerprinted 21 *P. infestans* isolates with SSR markers and showed that nearly all isolates had a unique genotype. Only two isolates, i.e. IPO-0 and UK7824, had identical genotypes, and PRC505705 and PRC506303 have a highly similar genotype. No clear supported branching was observed upon Neighbor Joining in the cluster analysis, illustrating that the assembled set of *P. infestans* isolates is genetically highly diverse (Figure 1).

Figure 1. Genetic diversity of the Phytophthora infestans isolates in this study. Bootstrap values in percentage (>60) are shown at the nodes.

Table 1. *Phytophthora* isolates used in this study

Phytophthora species	Isolate	Origin Country	Year	Source	MT	Race	Reference
P. infestans	F95573	The Netherlands	1995	Potato cull pile	A1	1.3.4.7.10.11	Flier et al. (2003)
	89148-09	The Netherlands	1989	Potato crop	A1	0	
	PIC99177	Mexico	1999	*S. stoloniferum*	A2	1.2.3.4.7.9[a].11	Flier et al. (2002)
	88069	The Netherlands	1988	Tomato crop	A1	1.3.4.7	van West et al. (1998)
	PIC99189	Mexico	1999	*S. stoloniferum*	A2	1.2.5.7.10.11	Flier et al. (2002)
	90128	The Netherlands	1990	Potato crop	A2	1.3.4.7.8.10.11	Vleeshouwers et al. (1999)
	EC1	Ecuador	n.d. [b]	unknown	n.d.	1.3.4.7.10.11	Armstrong et al. (2005)
	H30P04	The Netherlands	1995	- [c]	A1	3a.7.10.11	Drenth et al. (1995)
	USA618	Mexico	n.d.	Potato crop	A2	1.2.3.6.7.10.11	Fabritius et al. (2002)
	IPO-0	unknown	n.d.	unknown	n.d.	3b.4.7.10.11	
	IPO-C	Belgium	1982	Potato crop	A2	1.2.3.4.5.6.7.10.11	
	PIC99183	Mexico	1999	*S. stoloniferum*	A2	1.2.3.4.5.7.8.10.11	Flier et al. (2002)
	NL01096	The Netherlands	2001	Potato crop	A2	1.3.4.7.8.10.11	
	VK98014	The Netherlands	1998	Potato crop	A1	1.2.4.11	
	IPO428-2	The Netherlands	1992	Potato crop	A2	1.3.4.7.8.10.11	Flier et al. (2003)
	NL00228	The Netherlands	2000	Potato crop	A2	1.2.4.7	
	DDR7704	Germany (fGDR [d])	1977	Potato crop	A1	1.2.4	
	UK7824	United Kingdom	1978	Potato crop	A1	1.2.3.6.7	
	89094	The Netherlands	1989	Potato cull pile	A2	1.2.3.6.7.10.11	Kamoun et al. (1998)
	91011	The Netherlands	1991	Experimental field	A2	3.4.5.10	
	PIC97757	Mexico	1997	*S. demissum*	A1	n.d.	Flier et al. (2001)
	IPO98014	The Netherlands	1998	Potato crop	A1	1.2.3.4.7.11	Flier et al. (2003)
	NL050194	The Netherlands	2005	Potato crop	A2	n.d.	
	NL05105	The Netherlands	2005	Potato crop	A2	n.d.	
	PRC505705	China	2005	Potato crop	A2	1.2.3.4.5.6.7.9.10.11	
	PRC506303	China	2005	Potato crop	A2	1.2.3.4.5.6.7.9.10.11	
P. infestans s.l. [e]	EC3260	Ecuador	2001	*S. betaceum*	A1	non-host	Adler et al. (2004)
	EC3394	Ecuador	2001	*S. betaceum*	A1	non-host	Adler et al. (2004)
	EC3364	Ecuador	2001	*S. betaceum*	A1	non-host	Adler et al. (2004)
P. andina	EC3414	Ecuador	2001	*Anarrhichomenum* complex	A1	non-host	Gomez-Alpizar et al. (2008)
P. ipomoeae	PIC99193	Mexico	1999	*Ipomoea longipedunculata*	s.f. [f]	non-host	Flier et al. (2002)
P. phaseoli	CBS556.88	unknown	1988	unknown	s.f.	non-host	Flier et al. (2002)
P. mirabilis	PIC99111	Mexico	1999	*Mirabilis jalapa*	A2	non-host	Flier et al. (2002)
	CBS150.88	Mexico	1988	*Mirabilis jalapa*	A2	non-host	Kamoun et al. (1998)

[a] not conclusive, [b] n.d., not determined, [c] F1 progeny from a cross between *P. infestans* isolates 80029 and 88133 (Drenth et al. 1995), [d] fGDR, the former German Democratic Republic, [e] s.l., sensu lato, [f] s.f., self fertile

To determine the genetic variation at the *ipiO* loci, we PCR amplified *ipiO* on genomic DNA derived from the 29 *P. infestans* isolates and from five isolates of other clade 1c species, i.e. *P. andina*, *P. ipomoeae*, *P. phaseoli*, and *P. mirabilis* (Blair et al. 2008). Sequence analyses revealed 16 variants of *ipiO* with a minimum of one and a maximum of four *ipiO* variants per isolate (Table 2, Figure 2 & 3). So far, no *ipiO* homologues have been detected in *Phytophthora* species outside clade 1c (data not shown). As described previously, IPI-O1 and IPI-O2 are highly similar with only four different amino acids (Pieterse et al. 1994). Most of the newly identified IPI-O variants have amino acid changes due to point mutations, i.e. IPI-O3 to IPI-O9, IPI-O11, IPI-Om1 and IPI-Om2. IPI-O10 is identical to IPI-O2, although this variant contains two nonsynonymous nucleotide polymorphisms. Another variant, IPI-O13 has a C-terminal extension of 10 amino acids compared to IPI-O3. *P. infestans* variant IPI-O12 and *P. phaseoli* IPI-Op1 have frameshift mutations that result in truncated proteins.

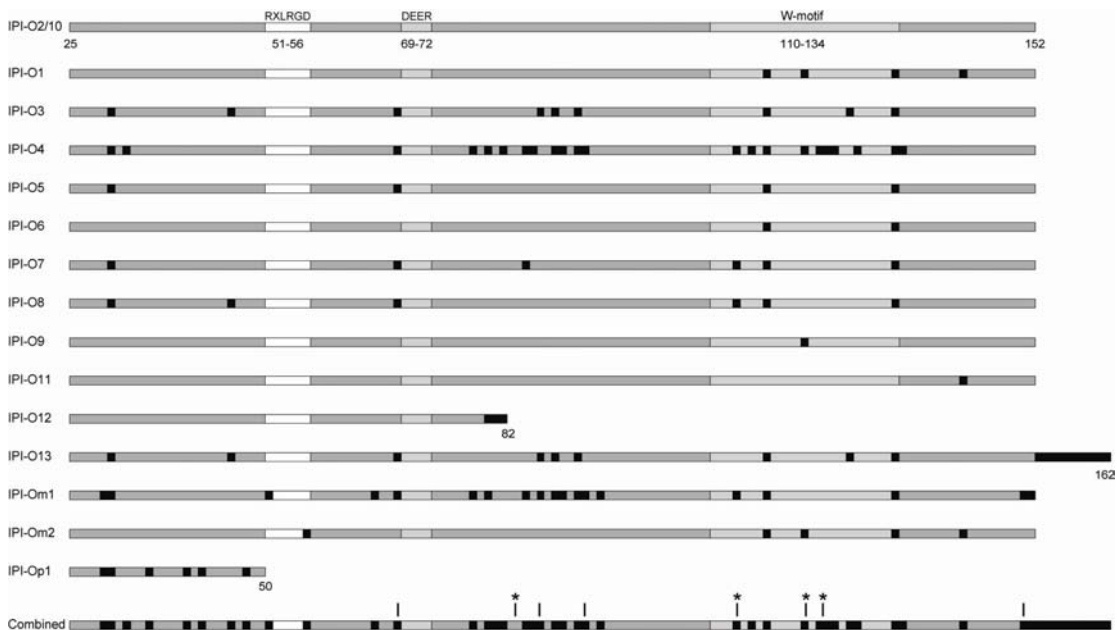**Figure 2. Thirteen variants of the mature IPI-O protein.** The motifs RXLR, RGD and DEER and a predicted W-motif are indicated. Numbers refer to the amino acid positions. Amino acid polymorphisms in IPI-O variants, as compared to IPI-O2, are depicted in black. In the lower bar (combined) all the amino acids that show polymorphism are indicated. The vertical lines refer to positively selected sites (see table 2). *= P<99%.

Table 2. Occurrence of *ipiO* variants and classes in isolates of *Phytophthora infestans* and related species

Phytophthora spp	Isolate	I											II		III		
		ipiO1	*ipiO2*	*ipiO5*	*ipiO6*	*ipiO7*	*ipiO8*	*ipiO9*	*ipiO10*	*ipiO11*	*ipiO12*	*ipiOm2*	*ipiO3*	*ipiO13*	*ipiO4*	*ipiOm1*	*ipiOp1*
P. infestans	F95573	x	x														
	89148-09	x															
	PIC99177												x				
	88069	x	x										x				
	PIC99189												x		x		
	90128	x	x							x							
	EC1	x	x							x			x				
	H30P04	x								x			x				
	USA618	x	x														
	IPO-0	x													x		
	IPO-C	x											x				
	PIC99183									x	x						
	NL01096	x	x										x				
	VK98014	x	x											x			
	IPO428-2	x	x							x							
	NL00228	x	x											x			
	DDR7704		x														
	UK7824		x												x		
	89094			x			x	x					x				
	91011	x			x	x			x								
	PIC97757	x			x	x			x								
	IPO98014			x			x	x					x				
	NL050105		x										x				
	NL05194		x										x				
	PRC505705		x														
	PRC506303		x														
P. infestans s.l. [a]	EC3260														x		
	EC3394														x		
	EC3364	x	x										x				
P. andina	EC3414														x		
P. ipomoeae	PIC99193											x					
P. phaseoli	CBS556.88																x
P. mirabilis	PIC99111											x					
	CBS150.88															x	

[a] s.l., sensu lato
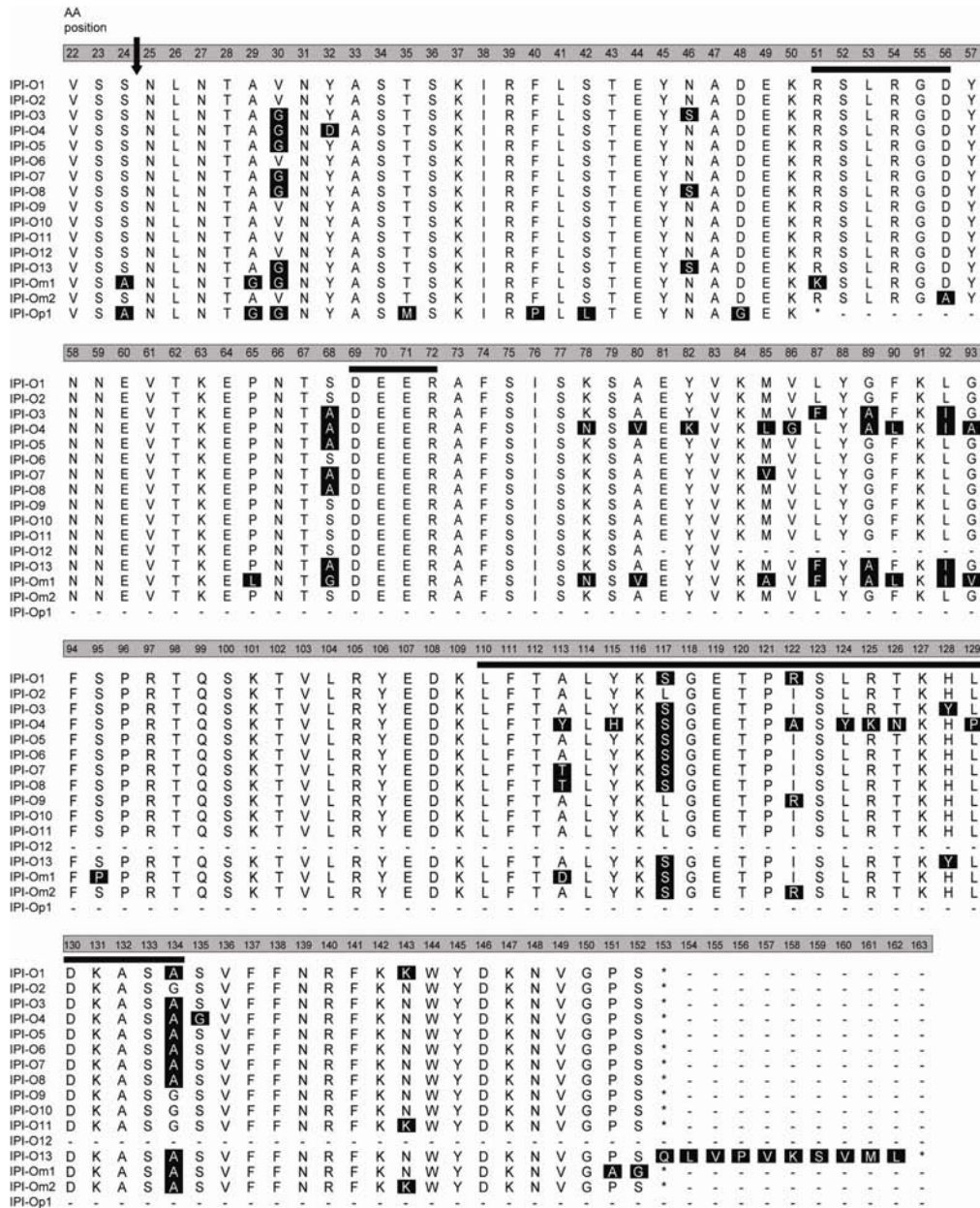
Figure 3. Amino acid alignment of *ipiO* variants. The motifs RXLR, RGD, DEER and a predicted W-motif are indicated. Numbers refer to the amino acid positions. The arrow depicts the predicted signal peptide cleavage site. Amino acid polymorphisms in IPI-O variants, as compared to IPI-O2, are depicted in black background.

Phylogenetic analyses on the protein alignment of the 16 IPI-O variants showed a grouping into three classes, which were designated as class I, II and III (Figure 4). Phylogenetic trees based on Neighbor-joining, minimal evolution or maximum parsimony algorithms resulted in similar clustering (not shown) and also analyses based on nucleotide alignments yielded comparable results. Most IPI-O variants group in class I, together with IPI-O1 and IPI-O2. Class II is significantly different from class I and includes IPI-O3 and IPI-O13. Class III comprises IPI-O4, which has 16 amino acid differences compared to the

class I and II IPI-O variants and is the most divergent IPI-O variant in *P. infestans*.
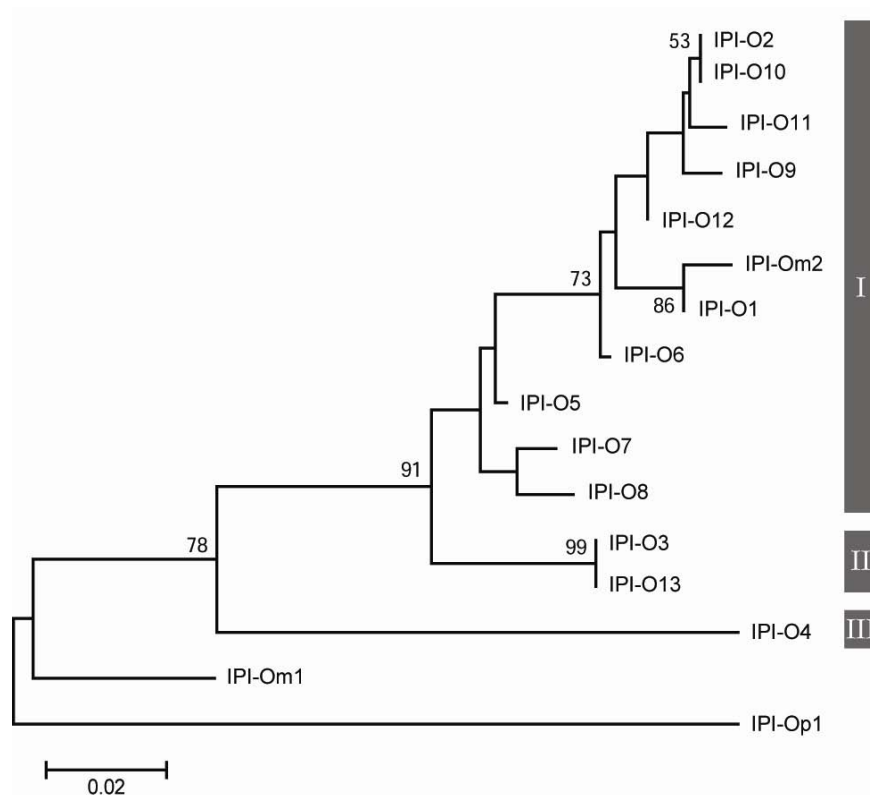
Figure 4. Phylogenetic relationship and clustering of the IPI-O variants. The minimum evolution (ME) tree was rooted with IPI-Op1. Rooting with IPI-Om1, IPI-O4, or *P. sojae* Avr1b resulted in similar clustering. Bootstrap values of 1000 replicates are indicated at the nodes; values less than 50% are omitted (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Class I, II and III IPI-O variants are indicated.

IPI-O has one W-motif with several positively selected sites

The majority of the RXLR-dEER effectors contain positively selected amino acid residues in the C-terminus (Jiang et al. 2008; Win et al. 2007). To investigate whether *ipiO* is also under diversifying selection, we assessed positive selection per residue on two sets of *ipiO* variants. The first set, called Pi, is composed of the 11 full length *ipiO* variants as found in *P. infestans*. The other set, Pi+Pm, includes the variants detected in the sibling species *P. mirabilis*, in addition to the Pi set. Of the different evolutionary models (Yang et al. 2005), model M2a for positive selection fits well on both data sets with ℓ values of -822.55 and -960.76 for Pi and Pi+Pm, respectively. With this model, several positively selected amino acid residues were identified, four of which overlap in the two data sets. Also the selection model M8 gave high log likelihood values for both sets. For the Pi set, M2a and M8 identified the same seven positively selected sites. For the Pi+Pm set, M8 identified the same set of four overlapping positively selected sites and four additional ones, one of which is also selected by the M2a model for the Pi+Pm set (Table 3).

Table 3. Evidence for positively selected sites in *ipiO*

	Model code	Parameters estimates	ℓ [a]	Positively diversified codons [b]
Pi [c]	M0: one ratio	ω=1.089	-839.78	Not allowed
	M1a: nearly neutral	ω0=0, ω1=1, p0=0.580, p1=0.420	-835.50	Not allowed
	M2a: positive selection	ω0=0, ω1=1, ω2=28.638, p0=0.353, p1=0.637, p2=0.010	**-822.55**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
	M7: beta	p=0.012, q=0.005	-837.03	Not allowed
	M8: beta & ω	p0=0.990, p1=0.010, p=0.008, q=0.005, ω=27.108	**-822.57**	46N, 82Y, 85M, 87L, 113A*, 122R*, 124L*
Pi + Pm [d]	M0: one ratio	ω=0.970	-980.05	Not allowed
	M1a: nearly neutral	ω0=0, ω1=1, p0=0.599, p1=0.401	-972.94	Not allowed
	M2a: positive selection	ω0=0.476, ω1=1, ω2=12.487, p0=0.971, p1=0, p2=0.028	**-960.76**	85M*, 93G, 113A*, 122R*, 124L
	M7: beta	p=0.005, q=0.007	-972.94	Not allowed
	M8: beta & ω	p0=0.983, p1=0.017, p=0.005, q=0.005, ω=14.665	**-960.34**	68S, 85M*, 87L, 93G, 113A*, 122R*, 124L*, 151P

[a] Log likelihood value
[b] Bayes Empirical Bayes (BEB) analysis (Yang et al. 2005); Positively selected sites (P>95%, *=P>99%)
[c] based on full length *P. infestans ipiO* variants, n=11
[d] based on full length *P. infestans* and *P. mirabilis ipiO* variants, n=13

In a recent study that used Hidden Markov Model (HHM) searches to find motifs in RXLR-dEER effectors, it was shown that many of the RXLR-dEER effectors contain conserved C-terminal motifs that may occur in repeated fashion (Jiang et al. 2008). These motifs were named W, Y and L after the amino acid at a fixed position in each motif. IPI-O contains a single W-motif with moderate to strong HMM scores ranging from 6.5 to 12.3 among the IPI-O variants (Figure 2 & 5). Interestingly, three of the four positively selected sites that overlap in the M2a and M8 model of the two sets have a high posterior probability (P>99%) and are located within the conserved W-motif (Figure 2).

The region comprising the W-motif is sufficient to trigger *Rpi-blb1* mediated cell death

To investigate whether or not the W-motif is involved in triggering *Rpi-blb1*-mediated cell death we analysed several deletion mutants of *ipiO2* (Figure 6A). In co-agroinfiltration assays in *N. benthamiana* IPI-O2 – with or without its signal peptide – triggers cell death in the presence of Rpi-blb1. Deleting the domain comprising the RXLR, RGD and dEER motifs did not abolish recognition and even an additional deletion of the first 26 amino acids of the C-terminal domain did not change the cell death response. Agroinfection and rub-inoculation assays on *S. stoloniferum* accession sto17605-4, which harbors the *Rpi-blb1* homologue *Rpi-sto1*, resulted in similar responses and showed that recognition of IPI-O by *Rpi-sto1* follows

the same pattern. The results show that the region spanning the last 54 amino acids of IPI-O and comprising the W-motif is sufficient for recognition by Rpi-blb1 and Rpi-sto1. Since the only mutations that are consistent between, on the one hand, the class I and II variants and, on the other hand, the class III variant are located within the W-motif it is conceivable that this motif plays a role in recognition of IPI-O by Rpi-blb1.

IPI-O variants of class I and II, but not class III, trigger *Rpi-blb1*-mediated cell death

In a previous study, we showed that the class I *ipiO* variants, *ipiO1* and *ipiO2*, trigger *Rpi-blb1*-mediated cell death. To assess whether the newly identified *ipiO* variants are also recognized by *Rpi-blb1* we used agroinfiltration in *Nicotiana benthamiana* to reconstruct the interaction between the *ipiO* variants and *Rpi-blb1*. Coinfiltration of *N. benthamiana* leaves with an *A. tumefaciens* strain carrying a construct expressing *Rpi-blb1* as well as a strain carrying a construct expressing either class I *ipiO* genes (*ipiO1*, *ipiO2*, *ipiO5*, *ipiO7*, and *ipiO8*), or class II *ipiO* genes (*ipiO3*) resulted in a confluent cell death response (Figure 6B). Also class I *ipiO* variant *ipiOm2* of *P. mirabilis* co-infiltrated with *Rpi-blb1* resulted in cell death (data not shown). In contrast, co-expression of *Rpi-blb1* with the class III *ipiO4* gene did not elicit a *Rpi-blb1*-mediated response, as no visible cell death was observed in the infiltrated leaves.

AA position	110	111	112	113*	114	115	116	117	118	119	120	121	122*	123	124*	125	126	127	128	129	130	131	132	133	134	HMM score
IPI-O1	L	F	T	A	L	Y	K	S	G	E	T	P	R	S	L	R	T	K	H	L	D	K	A	S	A	12.3
IPI-O2	L	F	T	A	L	Y	K	L	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	G	8.8
IPI-O3	L	F	T	A	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	Y	L	D	K	A	S	A	10.0
IPI-O4	L	F	T	Y	L	H	K	S	G	E	T	P	A	S	Y	K	N	K	H	P	D	K	A	S	A	6.5
IPI-O5	L	F	T	A	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	A	10.6
IPI-O6	L	F	T	A	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	A	10.6
IPI-O7	L	F	T	T	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	A	10.4
IPI-O8	L	F	T	T	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	A	10.4
IPI-O9	L	F	T	A	L	Y	K	L	G	E	T	P	R	S	L	R	T	K	H	L	D	K	A	S	G	10.5
IPI-O10	L	F	T	A	L	Y	K	L	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	G	8.8
IPI-O11	L	F	T	A	L	Y	K	L	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	G	8.8
IPI-O12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	–
IPI-O13	L	F	T	A	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	Y	L	D	K	A	S	A	10.0
IPI-Om1	L	F	T	D	L	Y	K	S	G	E	T	P	I	S	L	R	T	K	H	L	D	K	A	S	A	10.5
IPI-Om2	L	F	T	A	L	Y	K	S	G	E	T	P	R	S	L	R	T	K	H	L	D	K	A	S	A	12.3
IPI-Op1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	–

Figure 5. Hidden Markov model (HMM) scores of the conserved W-motif in the IPI-O variants. HMM scores >10 are considered as strong. The vertical lines refer to positively selected sites (Table 2 and Fig. 1). *= $P < 99\%$.
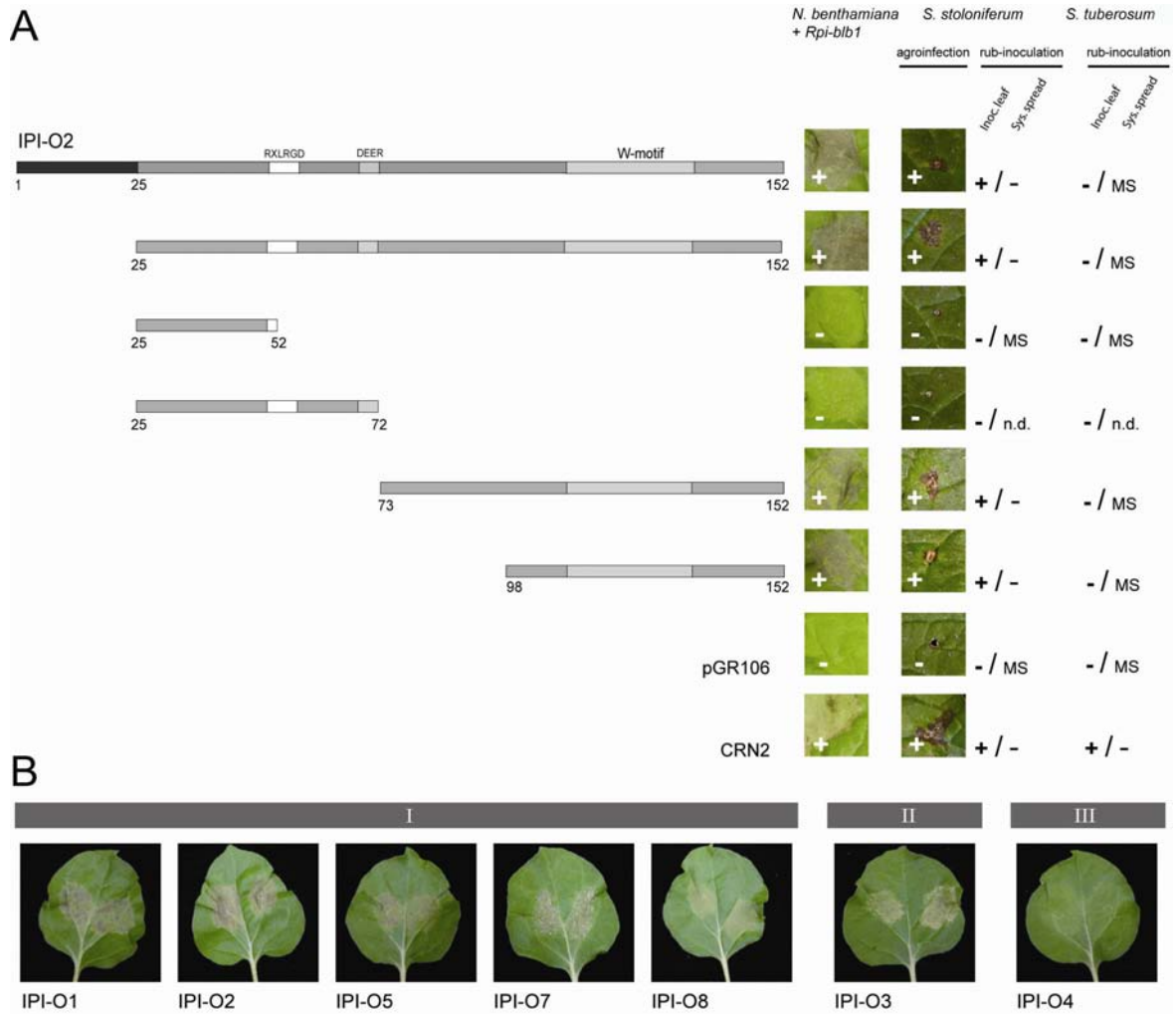
Figure 6. (A) The C-terminus of IPI-O comprising the W-motif is sufficient for recognition by Rpi-blb1. Deletion mutants of *ipiO*2 were co-agroinfiltrated with *Rpi-blb1* in *N. benthamiana* or were agroinfected or rub-inoculated with PVX particles carrying the deletion mutants on *S. stoloniferum* accession 17605-4. Potato cultivar Bintje was used as control. Mosaic symptoms (MS) indicate virus spread. Pictures were taken at 5 days after infiltration or agroinfection. (B) Class I and II IPI-O variants are recognized by Rpi-blb1. *N. benthamiana* leaves were agroinfiltrated – at both sides of the leaf midrib – with *Rpi-blb1* in combination with *ipiO* variants of class I, II or III. Pictures were taken at 5 days after infiltration.

IpiO variants are expressed *in planta*

To enable expression analyses of *ipiO* variants belonging to the three classes, class specific primers were designed that were tested for specificity on genomic DNA (Figure 7). Subsequently, RNA isolated from potato leaves infected with *P. infestans* isolates PIC99183, PIC99189 and PIC99177 was analyzed by semi-quantitative RT-PCR. As shown in Figure 7, mRNA derived from class I, class II and class III *ipiO* genes is present demonstrating that all three classes comprise functional genes that are expressed during *in planta* growth.
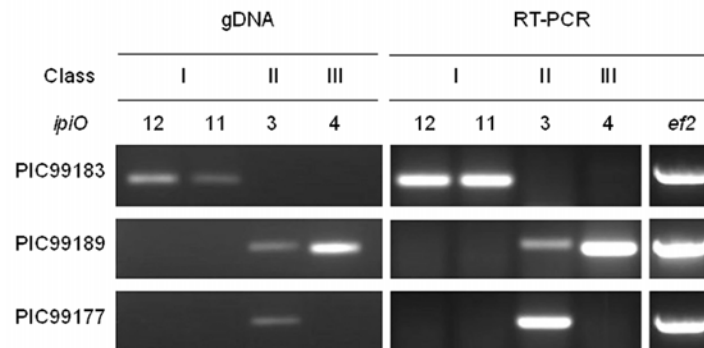

Figure 7. Expression of *ipiO* in infected potato leaves. *IpiO* class specific primers were used for semi-quantitative RT-PCR on RNA isolated from infected leaf tissue of potato cultivar Bintje at 6 dpi. To demonstrate that the primers are specific for each of the three *ipiO* classes, genomic DNA of *P. infestans* isolates PIC99183, PIC99189 and PIC99177 was used as template and detection of specific *ipiO* variants was confirmed. The *P. infestans* elongation factor 2 gene (*ef2*) was used as a control to determine the integrity of the RNA.

Isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* plants

To test whether the identified *ipiO* variants determine *Rpi-blb1*-mediated cell death, we performed infection assays using *P. infestans* isolates that are genetically diverse and carry different classes of *ipiO* variants (Figure 2, Table 2). To allow a correct interpretation of the virulence phenotypes of the isolates on *Rpi-blb1* plants we first tested 16 selected isolates for their infection capabilities on potato. Detached leaves of universal susceptible potato cultivar Désirée were inoculated and at 6 days post inoculation (dpi) lesion diameters were measured. Based on the lesion size (LS) the isolates were grouped in three classes of aggressiveness (Table 4). To investigate the specificity spectrum of *Rpi-blb1*, we inoculated the 16 isolates on the *S. bulbocastanum* accession blb8005-8, which is the genotype from which *Rpi-blb1* was isolated, and included Désirée and *S. bulbocastanum* blb2002 – containing *Rpi-blb2* – as susceptible and resistant controls, respectively (Table 5). Lesion diameters were measured at 4, 5, and 6 dpi and LS, lesion growth rates (LGR) and infection efficiency (IE) were calculated. Large lesions exceeding 25 mm^2 always coincided with massive sporulation and were scored as compatible interactions. In contrast, smaller lesions typically did not sporulate or showed a 'black-spot' phenotype, indicating a HR. As expected for the so-called 'broad-spectrum' *R* gene *Rpi-blb1*, blb8005-8 was incompatible with nearly

all isolates. Two Mexican isolates PIC99177 and PIC99189 however, were clearly compatible with blb8005-8 and both developed sporulating lesions on blb8005-8 leaves.

Table 4. Aggressiveness of *P. infestans* isolates on potato cultivar Désirée

P. infestans isolate	LS [a]	Scale of aggressiveness [b]
PIC99177	123.0	
F95573	146.2	
89148-09	151.3	moderately aggressive
88069	248.7	
PIC99189	332.6	
90128	370.7	
EC1	391.7	
IPO-0	399.3	
PIC99183	425.7	aggressive
H30P04	431.6	
IPO-C	488.9	
USA618	518.6	
NL01096	572.4	
VK98014	603.8	
IPO428-2	655.2	highly aggressive
NL00228	721.4	

[a] The lesion size (LS) at 6 dpi determined in two independent experiments, the mean LS are presented.
[b] Grouping is based on significant differences between LS (ANOVA, $P < 0.05$).

To investigate the correlation between compatibility or incompatibility, and *ipiO* variants, we compared the virulence phenotypes of the isolates on *Rpi-blb1*-containing host plants with the occurrence or the absence of specific *ipiO* variants. All avirulent isolates contained at least one class I *ipiO* variant (Table 2 & 5). In contrast, no class I *ipiO* variants were found in the two virulent isolates PIC99189 and PIC99177; only class II and/or III *ipiO* variants were detected. These results suggest that class I *ipiO* variants determine avirulence of *P. infestans* isolates on *Rpi-blb1* plants.

To verify these findings, we tested the *P. infestans* isolates on transgenic lines of cultivar Impala and cultivar Désirée containing *Rpi-blb1* as transgene. In general, *Rpi-blb1*-mediated resistance levels in the potato transgenic lines were lower than in its wild *Solanum* background, blb8005-8 (Table 5). In accordance with the previous experiment, isolate PIC99189 was able to establish sporulating lesions on transgenic Impala RGC-2A9 expressing *Rpi-blb1*, whereas isolates IPO-C and 90128 displayed a HR (Figure 8). On five Désirée *Rpi-blb1* transformants including A01-20, we quantitatively assessed the resistance

Table 5. *P. infestans* isolates virulent on *Solanum* plants containing *Rpi-blb1* lack class I *ipiO* variants

P. infestans isolate [a]	blb8005-8		sto17605-4		pta17831-8		A01-20		Désirée		blb2002		*[c]	ipiO class		
	IE [b]	LGR [b]	IE	LGR	IE	LGR	IE	LGR	IE	LGR	IE	LGR		I	II	III
F95573	0	0	0	0	0	0	3	1.4	69	4.4	0	0	A	x		
89148-09	0	0	0	0	0	0	16	1.1	69	2.7	0	0	A	x		
88069	0	0	0	0	0	0	22	0.6	100	3.2	0	0	A	x	x	
90128	0	0	0	0	0	0	50	3.7	78	3.4	0	0	A	x		
EC1	0	0	0	0	0	0	31	2.5	84	3.7	0	0	A	x	x	
IPO-0	0	0	0	0	0	0	78	3.4	91	3.8	0	0	A	x		x
PIC99183	0	0	50	0.5	63	1.2	88	3.6	91	3.6	0	0	A	x		
H30P04	0	0	0	0	0	0	9	2.4	100	4.4	0	0	A	x	x	
IPO-C	0	0	0	0	0	0	50	2.9	100	3.9	0	0	A	x	x	
USA618	0	0	0	0	0	0	19	3.5	100	3.4	0	0	A	x		
NL01096	0	0	0	0	0	0	66	3.4	100	3.8	0	0	A	x	x	
VK98014	0	0	0	0	0	0	66	1.8	100	4.7	0	0	A	x	x	
IPO428-2	0	0	0	0	0	0	38	1.9	100	4.2	0	0	A	x		
NL00228	0	0	0	0	0	0	47	4.6	100	4.8	0	0	A	x	x	
PIC99177	72	1.1	75	2.6	81	2.9	50	2.8	88	3.0	0	0	V		x	
PIC99189	34	1.1	75	0.9	100	4.3	91	4.5	91	3.6	0	0	V		x	x

[a] Additional information about the aggressiveness of P. infestans isolates on potato cultivar Désirée can be found in Table 4.
[b] Isolates were inoculated on different *Solanum* plants containing *Rpi-blb1* or its homologues (Table 7) and mean infection efficiency (IE) and lesion growth rate (LGR) were determined.
[c] *Phenotype on *Rpi-blb1* plants. A = avirulent; V = virulent.

levels. The virulent isolates PIC99177 and PIC99189 infected A01-20 (Table 5) and the other four transgenic lines (data not shown) equally well as the Désirée control plants (ANOVA, P<0.05). To the other 14 isolates, the Rpi-blb1 transgene conferred enhanced resistance at various levels, and generally the level of resistance negatively correlated with the aggressiveness of the isolates. The moderately aggressive isolates F95573, 89148-09 and 88069 reached only low levels of IE and LGR on A01-20, and *Rpi-blb1* clearly conferred a high level of resistance. Highly aggressive isolates, however, achieved slightly reduced or similar IE and LGR on A01-20 as compared to Désirée control plants, and were often able to establish high percentages of fast growing lesions on A01-20, despite the fact that these isolates contain a class I *ipiO* variant. Obviously the aggressiveness of the isolates overrules the recognition by *Rpi-blb1* in the transgenic potato background. The observation that the level of resistance conferred by *R* genes is influenced by the genetic background in which they reside, is in line with previous studies. For example, it was shown that expression of *Rpi-blb1* in *S. bulbocastanum* is dramatically higher than in potato lines carrying *Rpi-blb1* as a transgene (Bradeen et al. 2009; Kramer et al. 2009).

Recognition specificity of *ipiO* variants by *Rpi-blb1* is conserved in *Rpi-sto1* and *Rpi-pta1*

Recently, we described the identification and cloning of functional homologues of *Rpi-blb1* in the distantly related *Solanum* species *S. stoloniferum* (Vleeshouwers et al. 2008). The homologues *Rpi-sto1* and *Rpi-pta1* are nearly identical to *Rpi-blb1*; they only differ in 3 and 5 non-synonymous nucleotide substitutions, respectively. To test these *R* genes for *ipiO* specificity, we co-infiltrated *N. benthamiana* leaves with *A. tumefaciens* strains carrying constructs expressing either *Rpi-sto1* or *Rpi-pta1* combined with *A. tumefaciens* strains expressing *ipiO* variants of class I, II or III. Leaves co-infiltrated with the *Rpi-blb1* homologues and class I or class II *ipiO* variants showed a confluent cell death response, but leaf panels co-infiltrated with class III *ipiO* did not show cell death (Figure 9). These results demonstrate that similar to *Rpi-blb1*, *Rpi-sto1* or *Rpi-pta1* display differential specificity towards the different classes of *ipiO* variants.

We also investigated how *S. stoloniferum* sto17605-4 and pta17831-8 responded to infection with the 16 *P. infestans* isolates that posses different variants of *ipiO*. In detached leaf assays, infection of sto17605-4 and pta17831-8 with the isolates PIC99189 and PIC99177 resulted in sporulating lesions, in accordance with the results obtained with blb8005-8. Of the other 14 isolates only PIC99183 formed a few lesions on sto17605-4 and pta17831-8. There was, however, no biotrophic growth nor sporulation and the lesions expanded very slowly. Since the LGR and IE parameters were much lower than expected for such highly aggressive isolate we suspect that the necrotic spots are due to a trailing HR following invasion attempts, but not to specific virulence of PIC99183 on *S. stoloniferum* sto17605-4 and pta17831-8.
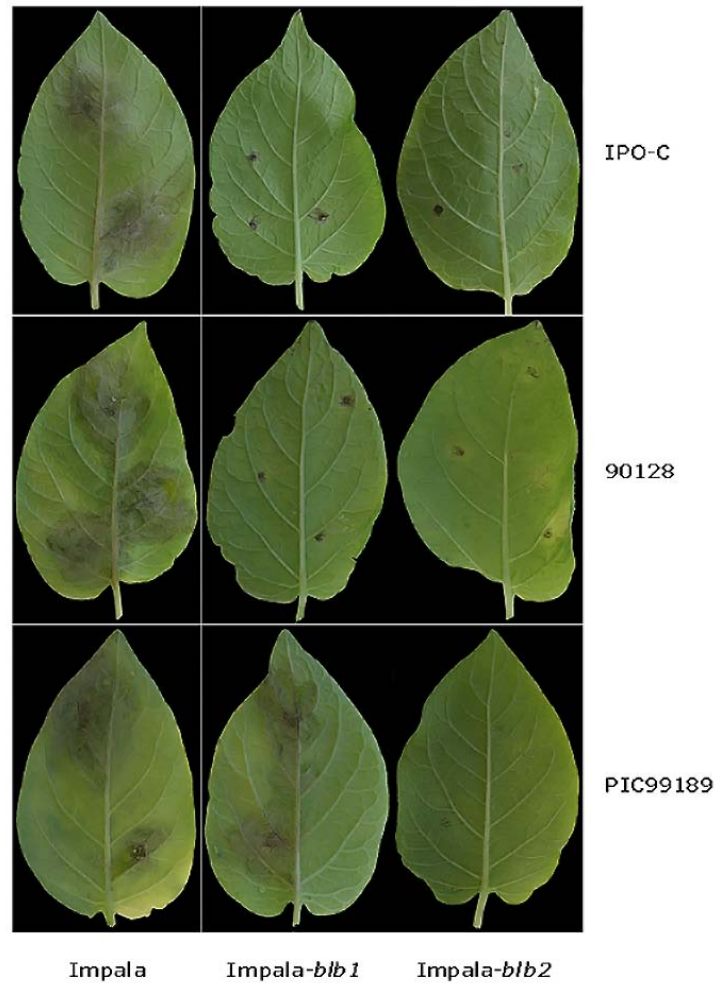
Figure 8. *Rpi-blb1* transgenic potato lines are resistant to *P. infestans* isolates carrying *ipiO* class I and/or II variants. Pictures were taken at 6 dpi.

Discussion

Rpi-blb1 is classified as a so-called broad-spectrum *R* gene that confers resistance to a broad range of *P. infestans* isolates (Song et al. 2003; van der Vossen et al. 2003). In a recent study we identified the RXLR-dEER effector IPI-O as the candidate for the cognate Avr factor of Rpi-blb1 (Vleeshouwers et al. 2008). In this study, we detected isolates that are virulent on Rpi-blb1 plants, and showed that *Rpi-blb1 – ipiO* is an *R-Avr* pair that basically interacts according to the gene-for-gene model (Flor 1971). IPI-O is the effector that triggers ETI in plants carrying *Rpi-blb1* whereas changes in the IPI-O effector repertoire result in loss of ETI.

The *ipiO* gene family is highly diverse but restricted to *Phytophthora* species that belong to clade 1c. Profiling 34 isolates of *P. infestans* and its close relatives revealed 16 *ipiO* variants, 14 of which were grouped in three distinct classes. In *P. infestans* sensu latu, *P. andina,*
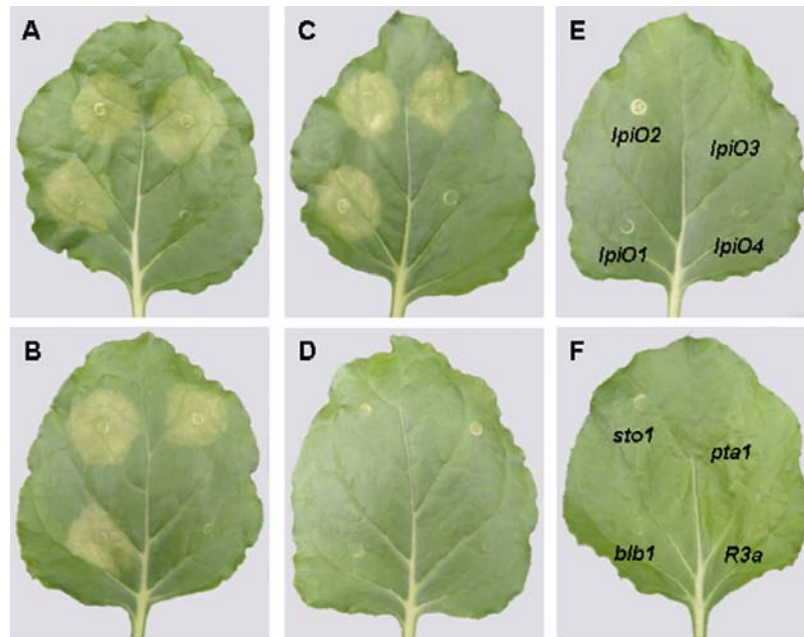
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Figure 9. The homologues *Rpi-blb1* (A), *Rpi-sto1* (B) and *Rpi-pta1* (C) induce cell death when co-agoinfiltrated with *ipiO* variants of class I and II. In negative control *R3a* (D), and single infiltration of effectors (E) or *R* genes (F) no cell death is observed. Pictures were taken at 8 days after infiltration. Infiltrated leaf panels in A–D are as indicated in E.

P. ipomoeae, P. phaseoli, and *P. mirabilis*, which all evolved on host species distant from *Solanum* section *Petota*, a more or less equal distribution of class I, II, and III variants was found and, in addition, two more distant *ipiO* variants were detected. In *P. infestans* however, class I was significantly expanded, with one to four class I variants in most isolates, of which the majority also has one class II and/or one class III variant. Class I includes *ipiO1* and *ipiO2*, the variants that were already isolated in the early 1990s (Pieterse et al. 1994), and more recently identified as *Avr-blb1* (Vleeshouwers et al. 2008). Disease testing on *Solanum* species showed that all isolates with class I *ipiO* variants were avirulent on *Rpi-blb1* plants, which is in line with the previously reported broad-spectrum character of this *R* gene (Song et al. 2003; van der Vossen et al. 2003). In contrast, two Mexican *P. infestans* isolates, PIC99189 and PIC99177, that both lack class I *ipiO* variants appeared to be virulent on *Rpi-blb1* plants. These data suggest that absence of class I *ipiO* genes is correlated with virulence on *Rpi-blb1* plants, and this is supported by the observation that co-infiltration of class I *ipiO* variants and *Rpi-blb1* in *N. benthamiana* leaves results in cell death. Apparently, class I IPI-O variants elicit HR in *Rpi-blb1* plants and this arrests pathogen invasion. Class III *ipiO* appeared unable to induce cell death when co-infiltrated with *Rpi-blb1*, thus strongly suggesting that the presence of class III *ipiO* in *P. infestans* strains is indeed unlikely to confer avirulence. It should be noted, though, that we have not used epitope tagged-constructs in our *in planta* expression assays to monitor the stability of the various IPI-O variants and, hence, can not exclude the possibility that class III IPI-O is less stable than class I or II IPI-O.

A more puzzling issue is the finding that class II variants elicit cell death when co-infiltrated with *Rpi-blb1* in *N. benthamiana* leaves. The class II variant *ipiO3* was found to be expressed *in planta* in the virulent strain and one would expect that the presence of a class II variant together with the Rpi-blb1 resistance protein in one cell would lead to HR. This is not the case and raises the question how the numerous RXLR effectors that are predicted to be targeted to the host cell interact with each other. In reconstruction experiments, for example, some RXLR effectors suppress cell death induced by other effectors such as elicitins or BAX (Bos et al. 2009; Bouwmeester et al. 2008; Dou et al. 2008; Whisson et al. 2007). *In vivo*, there could well be a kind of synergism between class I and class II IPI-O variants or even other RXLR effectors. For example, when class I is lacking the class II variant might not be potent enough to act as Avr factor by itself. Alternatively, the presence of a class I variant could suppress the virulence function of a class II variant by competition for the same virulence target. One can also not rule out the possibility that the class II variants are less stable *in planta* than the class I variants. The finding of a class III variant that is not recognized by *Rpi-blb1* but is expressed *in planta* makes the situation even more complex.

Nevertheless, the clear distinction between the three classes helped us to define which parts of the protein are involved in recognition by Rpi-blb1. Deletion analysis showed that the C-terminal part of the IPI-O effector protein is required for recognition, which is in line with other studies (Bos et al. 2006; Dou et al. 2008). The smallest fragment that we tested is 54 amino acids in length and comprises the single W-motif that is present in IPI-O. For *Phytophthora sojae* Avr1b, specific amino acid residues in the W- and Y-motifs in Avr1b are responsible for recognition by the RPS1 protein as well as suppressor activity of cell death. In IPI-O, the W-motif is the region that is the most divergent in the class III variants and the three amino acids that show positive selection are all located within the W-motif. More detailed analysis of the role of each individual amino acid residue in the W-motif will reveal the exact determinants of recognition by Rpi-blb1.

In recent years, insights into *R* gene-based resistance in potato and the role of cognate *Avr* genes from *P. infestans* have increased. For example, many studies were performed for the *R3a-Avr3a* model system. The *R3a*-harboring species *S. demissum* coexists with *P. infestans* in the cool and humid mountain forests in Toluca Valley, a perfect condition for a tight co-evolution between *R3a* and *Avr3a*. *R3a* is a typical fast evolving type I *R* gene (Huang et al. 2005; Kuang et al. 2005) resulting in numerous *R3a*-like genes (Friedman and Baker 2007; Huang 2005). For Avr3a, only two alleles have been detected; $Avr3a^{EM}$ is present in most *P. infestans* isolates world-wide (Armstrong et al. 2005; Rivera-Pena 1990b), whereas $Avr3a^{KI}$ is much less abundant. Avr3a^{KI} but not Avr3a^{EM} is recognized by R3a thus resulting in defeat of *R3a* by most *P. infestans* isolates. *R1, R2,* and *R4* also originate from *S. demissum* and similar to *R3a*, these *R* genes were quickly defeated in the field (Fry 2008). Similar to *Avr3a, Avr4* alleles in field isolates show very little variation and in all virulent strains, the *Avr4* gene is out of frame due to a one base pair deletion (van Poppel et al. 2008).

Preliminary analysis of *Avr1* and *Avr2* indicate that also these *Avr* genes are represented by only a few alleles (Francine Govers, Paul Birch and Eleanor Gilroy, personal communication). A completely different scenario exists for the *Rpi-blb* and *ipiO* interaction. *Rpi-blb1* originates from *S. bulbocastanum*, which occurs in more arid climates and most likely has less intensive encounters with *P. infestans*. Presence of *Rpi-blb1* homologues in other Mexican species such as *S. stoloniferum* (Wang et al. 2008) that partly grow in *P. infestans* conducive climates, creates the opportunity for virulent strains to evolve on these species. Indeed, the virulent strains PIC99189 and PIC99177 described in this study were collected from *S. stoloniferum* host plants in Mexico (Flier et al. 2002). Both *Rpi-sto1* and *Rpi-pta1* are almost identical to *Rpi-blb1*, and *Rpi-blb1* fulfills the criteria of a type II *R* gene with only little diversifying selection that is typically slow evolving (Kuang et al. 2005; Liu and Halterman 2006). Thus, for the *Rpi-blb1-ipiO* interaction, not the *R* gene, but the *Avr* gene is represented by a highly diverse and extensive gene family and the notable expansion of class I *ipiO* in *P. infestans,* but not in related *Phytophthora* species that host on other plant species, might be due to a certain degree of co-evolution between *Avr-blb1* and *Rpi-blb1* homologues in *Solanum* host plants. Also *Rpi-blb2*, another broad-spectrum *S. bulbocastanum R* gene that is not (yet) defeated, is interacting with a highly diverse *Avr* gene family, and perhaps such *Avr* genes may be less easy to overcome (Kamoun, personal communication).

In the wild *Solanum* species that contain *Rpi-blb1* or a functional homologue, full resistance was conferred to all tested avirulent *P. infestans* isolates, with the exception of the two Mexican strains. The transfer of *Rpi-blb1* into potato cultivars resulted in improved protection to *P. infestans* isolates. The enhanced resistance however, could only arrest the growth of mild isolates. Aggressive isolates were not blocked and could still cause high percentages of lesions expanding at high rates. The influence of the genetic background on the performance of *Rpi-blb1* was also reported in other studies, in which the basal *Rpi-blb1* expression levels were found dramatically higher in its wild *S. bulbocastanum* origin compared to transgenic potato (Bradeen et al. 2009; Kramer et al. 2009). Also the increase in expression after *P. infestans* inoculation was higher. These studies suggest that expression of *Rpi-blb1* as transgene in potato is not high enough to provide satisfactory resistance in the field, and this implies that stacking with other R genes, or engineering enhanced transgene expression in cultivars, is recommended.

The future of late blight resistance breeding is controlled application of well-studied *R* genes in high quality potato cultivars, and devising inherent durability predictions based on the interacting *Avr* gene. Potato cultivars engineered with *Rpi-blb1* and *Rpi-blb2* are expected to be the first GM potatoes to be cultivated for consumption purposes in Europe (Application GM field trial 2005). *Rpi-blb1* is still effective to a broad range of isolates; virulent isolates similar to PIC99189 and PIC99177 have not (yet) been detected in the Netherlands and neighboring countries and class I *ipiO* is well-represented in the *P. infestans*

isolates analyzed thus far. Future large-scale monitoring aimed at diagnosing the ipiO classes in the *P. infestans* population can help answering the question whether virulence to *Rpi-blb1* is evolving in commercial potato growing areas, or whether accidental introduction of potentially virulent *P. infestans* has occurred. When the first virulent isolates are detected, selection pressure towards losing class I *ipiO* might be avoided by omitting cultivars with *Rpi-blb1* for a certain period of time and apply other *R* genes in stead.

Materials and Methods

Phytophthora isolates, culture conditions and inoculum preparation

The *Phytophthora* isolates used in this study are listed were retrieved from our in-house collection or kindly provided by colleagues. *Phytophthora* isolates were routinely grown in the dark at 15°C in liquid Plich medium (Lee et al. 1997) prior to DNA extraction (Lees et al. 2006), or on solid rye sucrose medium (Caten and Jinks 1968) prior to disease tests. To isolate zoospores for plant inoculations, sporulating mycelium was flooded with cold water and incubated at 4°C for 1-3 h.

Cloning of *ipiO* variants

Primers (Table 6) and *Pfu* DNA polymerase (Promega) were used to amplifiy *ipiO* on genomic DNA. After 30 cycles Super*Taq* polymerase (HT Biotechnology) and its buffer were added followed by 15 minutes at 72°C. The obtained amplicons were cloned into pGEM®-T Easy vector (Promega) and transformed in DH5α competent cells (Invitrogen). Sequencing was performed using universal M13 primers and DNA sequences were analysed using DNAstar v6, Chromas 2.3 (Technelysium) and Vector NTI software.

SSR genotyping

SSR genotyping was performed using two multiplex set of four SSR markers each (SSR1, SSR3, SSR7, SSR11 and SSR2, SSR4, SSR6, SSR8). Experimental details can be found at the Eucablight site (http://www.eucablight.org/EucaBlight.asp).

Phylogenetic data analyses

The SSR data were analyzed by the phylogenetic software package TREECON® for Windows Version 1.3b (Van de Peer and De Wachter 1994). The evolutionary distance estimation was performed according to Nei and Li (1979) and clustering was performed using the Neighbor Joining algorithm. The tree was rooted using isolate IPO-0. Bootstrap values in percentage (>60) from 1000 replicate trees are shown at the nodes. The scale bar shows genotype divergence in percentage. Phylogenetic analyses of *ipiO* sequences were conducted

using the Minimum Evolution (ME) method (Rzhetsky and Nei 1992) in MEGA version 4 (Tamura et al. 2007).

Positive selection analysis

To test for amino acids under purifying or diversifying selection we used codon-based analysis (Codeml) implemented in PAML v.4 package (Yang 2007). Maximum-likelihood codon substitution models M0, M1a, M2a, M7, and M8 were used for analysis. Models M2a and M8 are capable to detect sites under positive selection. Bayes Empirical Bayes statistics was used to calculate positively selected sites with high posterior probability (Yang et al. 2005).

IpiO **expression analysis**

The zones surrounding the water-soaked lesions, where *ipiO1* is known to be highly expressed (van West et al. 1998), were cut from infected leaves of cultivar Bintje at 6 dpi. RNA was isolated using the RNeasy Mini Kit (QIAGEN), incubated with DNase and purified with the RNA clean-up protocol. The purity of the RNA was confirmed on gel. Semi-quantitative RT-PCR was performed with the OneStep RT-PCR kit (QIAGEN) using the specific primers RT-ipiO-I-F and RT-ipiO-I12-R for *ipiO12* (class I), RT-ipiO-I-F and RT-ipiO-I11-R for *ipiO11* (class I), RT-ipiO-II-F and RT-ipiO-II-R for *ipiO3* (class II), and RT-ipiO-III-F and RT-ipiO-III-R for *ipiO4* (class III). *P. infestans* elongation factor 2 gene (*ef2*) was used as a control (Torto et al. 2002).

Table 7. *Solanum* plant material used in this study

Solanum species	Abb.[a]	Genotype	*R* gene	Background	Reference
S. bulbocastanum	blb	8008-5	*Rpi-blb1*		(van der Vossen et al. 2003)
S. bulbocastanum	blb	2002	*Rpi-blb2*		(van der Vossen et al. 2005)
S. stoloniferum	sto	17605-4	*Rpi-sto1*		(Vleeshouwers et al. 2008)
S. stoloniferum[b]	pta	17831-8	*Rpi-pta1*		(Vleeshouwers et al. 2008)
Potato cultivars					
S. tuberosum	tbr	Désirée	-		(van Berloo and Hutten 2005)
S. tuberosum	tbr	Impala	-		(van Berloo and Hutten 2005)
S. tuberosum	tbr	Bintje	-		(van Berloo and Hutten 2005)
Potato transformants					
S. tuberosum	tbr	A01-20	*Rpi-blb1*	Désirée	this study
S. tuberosum	tbr	RGC-2A9	*Rpi-blb1*	Impala	(van der Vossen et al. 2003)
S. tuberosum	tbr	T5/7	*Rpi-blb2*	Impala	(van der Vossen et al. 2005)

[a] Abbreviation of the *Solanum* species
[b] Previously classified as *S. papita*

Table 6. Primers used in this study

Target	Primer[a]	Primer sequence (5'-3')
SSR fingerprinting	SSR1-F	GGCGCCCTACCCACCGTC
	SSR1-R	GTTTGCGCCTCTTCGCGGACGC
	SSR2-F	CGACTTCTACATCAACCGGC
	SSR2-R	GTTTGCTTGGACTGCGTCTTTAGC
	SSR3-F	ACTTGCAGAACTACCGCCC
	SSR3-R	GTTTGACCACTTTCCTCGGTTC
	SSR4-F	TCTTGTTCGAGTATGCGACG
	SSR4-R	GTTTCACTTCGGGAGAAAGGCTTC
	SSR6-R	TCGCCACAAGATTTATTCCG
	SSR6-F	GTTTCATCATGGAGCGTAGGATGG
	SSR7-F	GTCCTCGGCGTTCTATGAC
	SSR7-R	GTTTCCGAGTACCGAATGAGGC
	SSR8-F	AATCTGATCGCAACTGAGGG
	SSR8-R	GTTTACAAGATACACACGTCGCTCC
	SSR11-F	TTAAGCCACGACATGAGCTG
	SSR11-R	GTTTAGACAATTGTTTTGTGGTCGC
ipiO variants	ipiO-F	ATGGTTTCATCCAATCTCAACACC
	ipiO-R	CTATACGATGTCATAGCATGACAC
	F-dT-IpiOwoL	CACCATGGTTTCATCCAATCTCAACACC
	R-dT-IpiOwoL+	CTAGCTAGGGCCAACGTTTTTATC
	PIET10	AAGGCTACGACATGTCC
	ipiO-R-long	GAATTAGAAAAAGACACGTGG
	Cla1-PR1a-ipiOF	CATCGATATGGGATTTGTTCTCTTTTCAC
	Cla1-IPIOF	CATCGATGGTTTCATCCAATCTCAACACCG
	Cla1-IPIO1/3F	CATCGATGGCGTTTTCTATCTCAAA
	Cla1-IPIO2/3F	CATCGATGCGCACTCAGTCCAAGACG
	StopoIPIO1/3R	CACCCTATGAGATAGAAAACGCCCGC
	StopoIPIO2/3R	CACCCTACTTGGACTGAGTGCG
	Not1IPIOR	CGCGGCCGCCTAGCTAGGGCCAACGTTT
Rpi-blb1	AL57-Rpi-blb1	TCGGCTATGACTGGGCACAACAGACA
	AL58- Rpi-blb1	AAGAAGGCGATAGAAGGCGATGCG
RT-PCR	RT-ipiO-I-F	GATGGTACTTTATGGATTCAAAC
	RT-ipiO-I11-R	CTTCTCGGCGTCTCTCCTA
	RT-ipiO-I12-R	CTTCTCGGCGTCTCTCCGG
	RT-ipiO-II-F	GATGGTATTTTATGCATTCAAAA
	RT-ipiO-II-R	GCGGAAGCCTTATCGAGATA
	RT-ipiO-III-F	GTTGGGATTGTATGCATTAAAGA
	RT-ipiO-III-R	ATCGGGATGCTTGTTCTTGTA

[a] F is forward primer, R is reverse primer

Plant material and generation of *Rpi-blb1* transgenic potato plants

Solanum plant material used is this study is listed in Table 7. Potato cultivars and wild *Solanum* accessions were obtained from our-in-house collection and the Center of Genetic Resources CGN, Wageningen, The Netherlands, respectively (http://www.cgn.wur.nl/uk). *Solanum* plants were maintained *in vitro* on Murashige and Skoog medium (Duchefa) supplemented with 20% sucrose (MS20) in climate chambers at 18°C with a 16h

photoperiod. Top shoots were transferred to fresh medium and 1-2 weeks later rooted plantlets were transferred to the soil and grown under greenhouse conditions. *N. benthamiana* plants used for agroinfiltration were grown in climate chambers at 22-25°C and high light intensity. The generation of the *Rpi-blb1* transformant in cultivar Impala has been described previously (van der Vossen et al. 2003), and the transformant A01-20 of cultivar Désirée was generated using identical procedures. Briefly, the binary vector pBINPLUS containing *Rpi-blb1* under control of its native promoter and terminator (van der Vossen et al. 2003) was transformed to *A. tumefaciens* strain COR308 and introduced into cultivar Désirée according to standard protocols (Visser et al. 1991). Regenerative shoots were transferred on solid selective medium Zcvk (MS20 with zeatine 1 mg l^{-1}, claforan 200 mg l^{-1}, vancomycine 200 mg l^{-1} and kanamycine 100 mg l^{-1}) and transformants to solid MS30 with 100 mg l^{-1} of kanamycine.

Disease tests

Leaves from 6-8 week old plants were detached and placed in water-saturated oasis in trays, according to (Vleeshouwers et al. 1999). Leaves were spot-inoculated at the abaxial leaf side with 10 µl droplets containing $5*10^4$ zoospores ml^{-1} and incubated in a climate chamber at 15°C with a 16h photoperiod. Lesion diameters were measured at 4, 5, and 6 dpi. The area of the lesions (LS), the infection efficiency (IE) representing the percentage of successful infections were calculated, and the lesion growth rate (LGR) was estimated using linear regression in GenStat 10.

***In planta* expression assays**

IpiO variants without signal peptide were introduced in pK7WG2 and pGR106 (Karimi et al. 2002). *R3a*, *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1* with their native expression elements were introduced into the pBINPLUS binary vector (Engelen et al. 1995). *Agrobacterium tumefaciens* strain GV3101, and AGL1 (Lazo et al. 1991) in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Fits et al. 2000), were used for transformation. For agroinfiltration, *A. tumefaciens* strains were grown as described previously (van der Hoorn et al. 2000) to a final OD600 of 0.4. Leaves of 4-5 week old *N. benthamiana* plants were infiltrated with the *A. tumefaciens* suspensions (at a 1:1 ratio for co-infiltration) in MMA induction buffer (1 l MMA: 5 g MS salts, 1.95 g MES, 20 g sucrose, 200 µM acetosyringone, pH 5.6), and responses were scored from 3-8 days post-infiltration. To obtain PVX particles for PVX agroinfection, *Agrobacterium* strains containing the various recombinant pGR106-*ipiO* plasmids were agroinfected on *N. clevelandii* plants. After appearance of mosaic symptoms leaves were grinded in 50 mM potassium phosphate buffer (pH 7.0). Potato plants were rub-inoculated with the obtained homogenate after light dusting with carborundum powder.

Acknowledgements

Author recommended Internet Resources

http://www.eucablight.org/EucaBlight.asp

http://www.cgn.wur.nl/UK/CGN+Plant+Genetic+Resources/Collections/Potato/-+Species/

Data deposition

DNA sequences were deposited in NCBI Genbank under the accession numbers GQ371190 - GQ371203.

CHAPTER 3

Evolutionary and Functional Analyses Reveal a Diverse Family of *R2* Late Blight Resistance Genes in Mexican *Solanum* Species

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Evolutionary and Functional Analyses Reveal a Diverse Family of *R2* Late Blight Resistance Genes in Mexican *Solanum* Species

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Abstract

Potato, the third most important mankind-feeding crop, is prone to huge annual losses due to late blight, the disease caused by the oomycete pathogen *Phytophthora infestans*. Modern management of late blight necessitates the use of multiple resistance (*R*) genes, which requires efficient pipelines for identification and characterization of *R* genes. In this study, we used 55 RXLR effectors of *P. infestans* to screen and probe 24 resistant *Solanum* genotypes for late blight *R* genes. One effector, PEXRD11, was recognized by two resistant genotypes. We examined PEXRD11 recognition on a further 100 genotypes representing the broad *Petota* genepool and detected specific cell death responses in the phylogenetically diverse species *S. edinense*, *S. schenckii* and *S. hjertingii*, which all originate from Mexico. The *R* genes responding to PEXRD11 were localized in the *R2* cluster. Allele-mining for *R2* yielded 17 new variants. We identified positively selected amino acid sites predicted to be functionally important in *R2*, principally in the LRR domain but also in the leucine-zipper and the NB domain. Association between phylogenetic relatedness and functional activity was evident. Six new *R2* homologues, i.e. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3*, recognised *PexRD11* and *PiAvr2*, a previously described related RXLR effector gene, and conferred resistance to *P. infestans*. We conclude that *R2* family has evolved in Mexico, the centre of origin of the pathogen, and provide an overview of the evolution and functional diversification of this *R* gene family in its natural environment. In addition, we demonstrate that effectoromics is a fast approach to identify functional *R* gene homologs in phylogenetically diverse *Solanum* and to sort out their functional redundancy. These results highlight the high degree of natural variation in functional late blight resistance genes, and offer to potato breeders, a number of potential important and R genes that can be translated to agriculture.

Introduction

Potato (*Solanum tuberosum*) is currently, one of the three most consumed crops worldwide in addition to wheat and rice (Haverkort et al. 2009). Potato is susceptible to many pathogens, and the most devastating disease hampering potato production is late blight, caused by the oomycete *Phytophthora infestans*, which destroys leaves, stems, and tubers. Late blight causes global yield losses of 16% of the potato crop, representing an annual loss of € 5.2 billion (Haverkort et al. 2009). To manage the disease, early potato breeding efforts focussed on characterisation of resistance spectrum and introgression of 11 resistance (*R*) genes obtained from *Solanum demissum*, a wild relative of potato. The exploitation of new cultivars containing these *R* genes proved to be successful (Black et al. 1953; Malcolmson and Black 1966). Rapidly changing populations of *P. infestans*, however, overcame the deployment of *R1-R11* genes, even after pyramiding (Hein et al. 2009). Contemporary potato breeding for *P. infestans* resistance is exploring the wealth of *R* gene diversity in a broad range of botanical *Solanum* section *Petota* species, to build up a collection of diverse *Rpi* (*Resistance* to *P. infestans*) genes available for stacking in existing or new cultivars (Jacobsen and Schouten 2007). To this end, we initiated an extensive survey of about 1,000 wild *Solanum* accessions for resistance to *P. infestans,* and established a useful resource for breeding and cloning *Rpi* genes (*SolR*gene database). A number of new *Rpi* genes have been cloned recently from Mexican *Solanum* species, e.g. *R1*, *R2*, and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009); *Rpi-blb1/RB* (Song et al. 2003; van der Vossen et al. 2003), *Rpi-blb2* (van der Vossen et al. 2005), and *Rpi-blb3* from *S. bulbocastanum* (Lokossou et al. 2009); *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008); and *Rpi-abpt / R2-like* from unknown species used in a pre-breeding program (Lokossou et al. 2009). The first *Rpi*-genes of South American origin also are being cloned, such as *Rpi-vnt1.1*, *Rpi-vnt1.2*, and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009). Many more *Rpi* genes are expected to be cloned in the future, so that the set of cloned *R* genes against late blight is rapidly growing (*SolR*gene database ; Kuhl et al. 2001; Rauscher et al. 2006; Sandbrink et al. 2000; Smilde et al. 2005; Villamon et al. 2005).

The co-evolution of *R-Avr* gene pairs is driven by a molecular arms race between the pathogen and its host (Flier et al. 2003; Flor 1971; Jones and Dangl 2006; Thompson and Burdon 1992). The central highlands of Mexico and, particularly, the Toluca valley are considered to be a center of genetic diversity for the potato late blight pathogen (Flier et al. 2003; Fry et al. 1993; Goodwin et al. 1992; Grünwald et al. 2001) and for tuber-bearing *Solanum* spp. (Hawkes 1990; Hijmans and Spooner 2001; Spooner et al. 2004). *R* genes for resistance to potato late blight were first discovered in endemic *Solanum* species, such as *S. demissum* (Black and Gallegly 1957; Black et al. 1953; Malcolmson and Black 1966). The revelation of *P. infestans* genome plasticity, and its abundance of putative effectors (Haas et

al. 2009), shows that this oomycete remains a formidable foe. Not only is the discovery of new *R* genes important, but the identification of functional *R* gene homologs (*RGH*) from a family could also provide a first hint about co-evolution within this pathosystem.

R genes typically occur in clusters in various linkage groups (LG). A particularly interesting family of *P. infestans R* genes is located in a Major Late Blight (MLB) locus in LG IV (Park et al. 2005b). Of this MLB locus, *R2*, *R2-like*, *Rpi-blb3*, and *Rpi-abpt* were cloned recently. Up to 16 *R2* variants, or *R2GH*, furthermore, were observed in the Mastenbroek *R2* differential (Lokossou et al. 2009). These so-called *R2*-family genes have the closest homology to *RPP13* of *Arabidopsis thaliana,* i.e. 39.4% identity at the amino acid level (Bittner-Eddy et al. 2000; Lokossou et al. 2009), but no other homologs in *Solanaceae* have been identified. Structural analyses of the *R2*-family members revealed clear blocks of sequence exchange between paralogs (Lokossou et al. 2009). This family has evolved, therefore, as a type I *R* gene (Friedman and Baker 2007; Kuang et al. 2005). Another MLB cluster is located in LG XI, which is highly extended and harbours up to 45 type I *R3a* homologs per haplotype (Huang et al. 2005; Kuang et al. 2005). Various *R* genes acting against *P. infestans* have been shown to occur in this MLB, namely *R3a*, *R3b*, and *R5 - R11* (Bradshaw et al. 2006b; El-Kharbotly et al. 1996; Huang et al. 2005; Huang et al. 2004). Homologues of *Rpi-blb1* reside in LG VIII (Vleeshouwers et al. 2008; Wang et al. 2008); these type II *R* genes are thought to rarely experience sequence exchanges between paralogs and maintain orthologous relationships (Friedman and Baker 2007; Liu and Halterman 2006). Finally in LG V, a diversity of *RGH* have been characterized, *R1* confers resistance to *P. infestans*, *Rx2* to potato virus X and *Gpa2* to potato cyst-nematodes (Kreike et al. 1994; Ritter et al. 1991).

Similar to other plant pathogens *P. infestans* translocates effector proteins inside host cells (Birch et al. 2006; Dou et al. 2008; Rehmany et al. 2005; Whisson et al. 2007). The primary activity of these effectors is to promote virulence, for instance by suppressing plant immunity (Schornack et al. 2009b). A number of these effectors, namely members of the RXLR family, however, trigger hypersensitive cell death and resistance responses by activating particular NBS-LRR immune receptors, generically known as CC-NBS-LRR (Dangl and Jones 2001). Six *Avr* genes were characterized recently for the potato – *P. infestans* pathosystem, i.e. *Avr1*, *PiAvr2, Avr3a, Avr4, Avr-blb1 (ipiO),* and *Avr-blb2* (Armstrong et al. 2005; Champouret et al. 2009; Lokossou et al. 2009; Oh et al. 2009; Tyler 2009; van Poppel et al. 2008; van Poppel et al. 2009; Vleeshouwers et al. 2008). Except for *Avr4*, the corresponding *R* genes to the other five *Avr* genes have been cloned (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). Two of these *Avr* genes, occur as one or two copies in the genome and show limited allelic diversity: *Avr3a* which is known to have two major alleles *Avr3a^{KI}* and *Avr3a^{EM}* in *P. infestans* populations (Armstrong et al. 2005), and *Avr4* of which the avirulent form are the full length protein with few a.a. changes and the virulent form has a

disrupted ORF (van Poppel et al. 2008). Others of these *Avr* genes occur in multiple copies in the genome and are highly diverse: *Avr-blb1*, which is represented by up to four paralogous copies per genotype, with 13 allelic variants identified in *P. infestans* isolates (Champouret et al. 2009), and *Avr-blb2*, which is diverse and occurs as seven paralogues in the *P. infestans* genome (Oh et al. 2009). Identification of allelic diversity of an *Avr* in *P. infestans* populations, as well as detection of paralogous copies in a genome, therefore, are two factors to consider to categorize *R* genes that could stand in agriculture, if matching *R* variants can be found and combined to recognise natural variation in the pathogen population. It was discovered recently, that effectors can be useful to facilitate breeding, cloning, and deployment of *R* genes by screening plant germplasm for response to candidate effectors (Schornack et al. 2009b). This approach has been used to identify novel R proteins and to classify their activities into discrete recognition specificities (Ellis et al. 2009; Oh et al. 2009; Vleeshouwers et al. 2008). Effectoromics can accelerate the cloning of functional *R* genes. The screen and probe of multiple *Solanum* germplasm for late blight *R* genes can provide a pattern of responses to a specific effector from a group of *Solanum* species, and therefore, can help to avoid redundant cloning efforts. For these reasons, we have based our approach to identify the diversity of one *R* gene in the wild *Solanum* population not on the resistance spectrum to *P. infestans* isolates but rather on the responses to a specific effector.

Allele-mining strategy is emerging as an efficient approach to identify genetic variation of *R* genes, especially now that sequence and positional information is expanding in the genome era (Leister et al. 1996; Lokossou et al. 2009; Pel et al. 2009; Wang et al. 2008). After genetic mapping of *Rpi-vnt1* in *S. venturii* at the locus of *Tm-2²*, a Tomato mosaic virus resistance gene (Foster et al. 2009; Pel et al. 2009), allele-mining methods quickly led to full-length *Tms-GHs* that confer resistance to *P. infestans* isolates (Pel et al. 2009). In some cases, however, numerous *Rpi*-candidate genes are amplified and the challenge is to identify efficiently the functional *RGH* among these. We argue that functional allele-mining, by using the effector, is a new way to delimit the number of *RGH* to analyse for functional *R* genes.

In this study, we functionally profiled *Solanum* species with specific RXLR effectors of *P. infestans*. Based on observed responses, we focussed on the recognition of one effector, PEXRD11, an RXLR effector related to the previously described PiAVR2 (Lokossou et al. 2009). Exploring the stretch of specific recognition to PEXRD11 by functional allele-mining on a large set of *Solanum* section *Petota* species yielded three species from phylogenetically diverse series (Jacobs et al. 2008) and originating from Mexico. Genetic approaches located the *Rpi* genes responsive to PEXRD11 in the *R2* cluster. PCR allele-mining on the three genotypes led to the identification of 17 *R2* homologues (*R2GH*), divided in six clades. Diversifying selection in the R2-GHs was confirmed by positive selection analyses, and could be the result of a molecular arms race between wild populations of *P. infestans* and the three *Solanum* species in Mexico. Combination of transient assays for specific effector recognition and transient complementation in *Nicotiana benthamiana* resulted in the

identification of six new *R2* homologues that confer resistance to *P. infestans* and recognized PEXRD11 and PiAVR2 with varying degrees of intensity.

Results

Effectoromics screens reveal specific recognition of PEXRD11 in *Solanum* spp.

A broad diversity of the *Solanum* section *Petota* gene pool was subjected to disease assays with *P. infestans* isolate 90128, and 24 resistant *Solanum* genotypes that belong to 18 wild species within eight taxonomic series were selected (*SolR*gene database ; Jacobs et al. 2008). Three susceptible cultivars and one transgenic plant, Impala-*Rpi-blb1*, were added as controls. To functionally assess these 28 genotypes for their recognition spectra towards *P. infestans* effectors, we assayed them for response to 55 putative RXLR effectors, previously described by Oh et al. (2009) and Vleeshouwers et al. (2008), using an expression system based on potato virus X (PVX) and *Agrobacterium tumefaciens* and known as PVX agroinfection (Oh et al. 2009; Vleeshouwers et al. 2008; Vleeshouwers et al. 2006). Based on observed responses, the effectors were classified in two groups: specific and non-specific responses (Table 1). The non-specific group contained effectors that activated responses in resistant and susceptible genotypes. Our study priority is the identification of new specific interactions; therefore, we did not pursue further with this group. The specific group, represented by 41 effectors, contained 86 specific genotype-effector combinations, where effectors triggered cell death in resistant but not in susceptible plants (Table 1), which suggests that these effectors might exhibit avirulence activity and might activate *R* genes that are absent in susceptible *Solanum* plants (Vleeshouwers et al. 2008). Eight specific combinations correspond to the known R-AVR interaction of *Rpi-blb1* homologues with *Avr-blb1* (*IpiO*): namely *S. bulbocastanum* (8005-8) carrying *Rpi-blb1*, *S. stoloniferum* (CGN 17605-4) carrying *Rpi-sto1*, *S. papita* (CGN 17831-8-1) carrying *Rpi-pta1* and Impala-*Rpi-blb1* transformants carrying *Rpi-blb1*. These responses showed a localized cell death to *IpiO1* and *piO2* (see [1] Table 1), as described in previous studies (Champouret et al. 2009; Vleeshouwers et al. 2008), which indicates that the screen was relevant.

To determine the extent to which the remaining 78 specific combinations correspond to bona fide *R* gene-mediated recognition, we used a genetic approach. We crossed resistant *S. polyadenium* (pld17749-4), *S. stoloniferum* (sto17605-4), and two *S. microdontum* (mcd23050-1 and mcd18200-6) with susceptible *Solanum* genotypes, and assessed the populations for segregation of cell-death response to candidate effectors and for resistance to *P. infestans* isolates. Segregation of response to candidate effectors at different ratios was observed in 11 combinations: with the genotype pld17749-4, mcd23050-1, and sto17605-4 (see [3] Table 1). Because there was no co-segregation between effector response and resistance to *P. infestans*, we did not pursue this further.

Table 1. Functional profiling of RXLR effectors in *Solanum*.

Origin	Series according to Jacobs et al. (2008)	*Solanum* species	Genebank[*]	Resistance[†] *P. infestans* 90128	Names of Genotypes
Bolivia	Circaeifolia Piurana Megistacroloba Tuberosa	*S. capsicibaccatum*	CGN 22388-1	R	cap22388-1
Bolivia	Circaeifolia Piurana Megistacroloba Tuberosa	*S. circaefolium subsp. quimense*	CGN 18158-1	R	qum18158-1
Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. polyadenium*	CGN 17749-4	R	pol17749-4
USA	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. jamesii*	CGN 18349-1	R	jam18349-1
Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. pinnatisectum*	CGN 17743-4	R	pnt17743-4
Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. pinnatisectum*	CGN 23011-1	R	pnt23011-1
	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. bulbocastanum*	Blb99-256-3	R	blb99-256-3
	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. bulbocastanum*	2002	R	blb2002
	Polyadenia Pinnatisecta Bulbocastana Morelliformia	*S. bulbocastanum*	8005-8	R	blb8005-8
Mexico	Longipedicellata	*S. stoloniferum*	CGN 17605-4	R	sto17605-4
Mexico	Longipedicellata	*S. papita*	CGN 18309-1	R	pat18309-1
Mexico	Longipedicellata	*S. stoloniferum*	CGN 17606-2	R	sto17606-2
Peru	Longipedicellata	*S. huancabambense*	CGN 17719-1	R	hcb17719-1
Mexico	*S. verrucosum*	*S. verrucosum*	CGN 17768-10	R	ver17768-10
Mexico	Demissa Conicibaccata	*S. schenckii*	PI 498042-1	R	snk498042-1
		dihaploid clone from AM78-3778	AM3778-16	R	AM3778-16
Peru	Cultivated Tuberosa and Tuberosa from Peru	*S. mochiquense*	GLKS 2319-2	R	moc2319-2
Bolivia	Tuberosa from Bolovia, Argentina and Chile Yungasensa	*S. avilesii*	CGN 18256-2	R	avl18256-2
Bolivia	Tuberosa from Bolovia, Argentina and Chile Yungasensa	*S. chacoense*	CGN 962709-5	R	cha962709-5
Bolivia	Tuberosa from Bolovia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 23050-1	R	mcd23050-1
Argentina	Tuberosa from Bolovia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 17596-8	R	mcd17596-8
Bolivia	Tuberosa from Bolovia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 18200-6	R	mcd18200-6
Bolivia	Tuberosa from Bolovia, Argentina and Chile Yungasensa	*S. microdontum*	CGN 21342-1	R	mcd21342-1
Argentina	Tuberosa Megistacroloba	*S. okadae*	CGN 18000-1	R	oka18000-1
		S. tuberosum cv.Impala-*Rpi-blb1*		R	Impala-*Rpi-blb1*
		S. tuberosum cv. Impala		S	Impala
		S. tuberosum cv. Désirée		S	Désirée
		S. tuberosum cv. Bintje		S	Bintje

Table 1. (*Continued*) Functional profiling of RXLR effectors in *Solanum*.

Names of Genotypes	PexRD6				PexRD11		PexRD1	PexRD3	PexRD4	PexRD10	PexRD7			PexRD13		PexRD14		PexRD16		PexRD17		PexRD21	PexRD28	PexRD22		PexRD26	PexRD24	
Specific	1	2	3	4	1	2	1	1	1	1	1	2	3	1	2	1	2	1	2	1	2	1	1	1	2	2	1	2
	ipiO1	ipiO2	ipiO3	ipiO4	21-1	43-1	195-2	12-9	53-1	96-1	Avr3a-KI	Avr3a-EM	Pex147-2	98-3	98-4	99-4	99-5	56-2	59-1	MKA-1	MKB-4	64-2	176-2	68-2	66-1	118-1	113-1	116-1
cap22388-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
qum18158-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
pol17749-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
jam18349-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
pnt17743-4	-	-	nd[2]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
pnt23011-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
blb99-256-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
blb2002	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
blb8005-8	+[1]	+[1]	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+
sto17605-4	+[1]	+[1]	-	+[5]	-	-	-	-	-	-	-	-	-	+[5]	-	-	-	-	-	+[5]	-	-	-	+[3]	-	+[5]	-	-
pat18309-1	+[1]	+[1]	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
sto17606-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
hcb17719-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	-	+
ver17768-10	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
snk498042-1	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AM3778-16	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
moc2319-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
avl18256-2	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
cha962709-5	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
mcd23050-1	-	-	nd	-	-	-	+[3]	-	-	-	+[3]	-	-	+[3]	-	-	-	+[3]	-	+[3]	-	+[3]	-	-	-	-	+[3]	+[3]
mcd17596-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
mcd18200-6	-	-	-	+	-	-	-	+[4]	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
mcd21342-1	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
oka18000-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Impala-*Rpi-blb1*	+[1]	+[1]	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Impala	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Désirée	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bintje	-	-	nd	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Names of Genotypes	Specific													Non-specific														Controls	
	PexRD31	PexRD36		PexRD41			PexRD45		PexRD46			PexRD50	PexRD51	PexRD2	PexRD7	PexRD8	PexRD12		PexRD26	PexRD27	PexRD39/40				PexRD44	PexRD49	PexRD50	neg	pos
	1	1	2	1	2	3	1	2	1	2	3	1	1	1	4	1	1	2	1	1	1	2	3	4	1	1	2		
	120-1	45-1	45-10	91-3	91-5	91-7	184-2	215-3	92-4	92-2	92-12	191-1	T304-4	11-8	Pex147-3	95-1	101-2	103-2	119-1	143-2	169-6	170-1	89-2	89-7	183-1	186-2	191-6	pGR106	Cm2
cap22388-1	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	-	+	-	-	-	-	+
qum18158-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+
pol17749-4	-	-	-	-	-	-	-	-	-	-	$+^{3}$	-	-	+	-	+	-	-	-	+	+	+	+	+	+	-	-	-	+
jam18349-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	-	+
pnt17743-4	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	nd	+	-	-	-	+
pnt23011-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+
blb99-256-3	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	+
blb2002	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+
blb8005-8	-	-	-	-	+	+	-	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
sto17605-4	-	-	-	-	-	-	-	+	+	+	$+^{3}$	$+^{5}$	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+
pat18309-1	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	-	+	+	+	+	+	-	-	-	-	+
sto17606-2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	+	+	nd	-	-	-	-	+
hcb17719-1	-	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
ver17768-10	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
snk498042-1	-	-	-	-	-	-	-	-	nd	nd	nd	nd	-	+	+	+	-	-	-	+	+	+	+	+	+	nd	nd	-	+
AM3778-16	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	+	+	+	+	+	+	-	-	-	+
moc2319-2	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-	-	+
avl18256-2	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	+	nd	-	-	-	-	+
cha962709-5	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	nd	+	-	-	-	+
mcd23050-1	-	-	-	$+^{3}$	$+^{3}$	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	nd	-	-	-	-	+
mcd17596-8	-	-	-	+	-	+	-	+	+	+	-	-	-	+	-	+	+	-	-	+	+	+	+	+	-	-	-	-	+
mcd18200-6	-	-	-	+	-	-	-	-	-	+	+	-	-	+	-	+	+	+	-	+	+	+	+	+	+	-	-	-	+
mcd21342-1	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	+	+	+	-	+	-	+	-	nd	-	-	-	-	+
oka18000-1	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	+	+	+	nd	+	-	-	-	+
Impala-*Rpi-blb1*	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	+
Impala	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
Désirée	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	+
Bintje	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-	+	-	nd	-	-	-	-	+

Table 1. Legend

A set of 55 RXLR effectors were inoculated by PVX agroinfection on 24 wild *Solanum* genotypes (corresponding to eight taxonomic series), three susceptible cultivars (Impala, Désirée and Bintje), and one resistant control, the transformant Impala-*Rpi-blb1*. Eight to sixteen replicates were inoculated, and necrotic responses occurring at frequencies higher (+) or lower (--) than 40% of the inoculated sites are presented. Candidate effectors are classified in specific vs. non-specific, representing candidates that showed response specifically in resistant plants or in both resistant and susceptible plants, respectively.

*Abbreviations for genebank source codes: CGN: Centre for genetic Resources, the Netherlands; PI: NRSP-6 - United States potato Genebank; GLKS: Gross Lusewitz, Germany.

[†] Resistant (R) or susceptible (S) to *P. infestans* isolate 90128.

[1] Specific *Rpi-blb1*, *Rpi-sto1*, *Rpi-pta1*/*IpiO1&2* interactions

[2] nd, not determined

[3] Segregation of effector response in population, but not linked to resistance

[4] Effector response detected in all progeny plants

[5] No effector response detected in progeny plants

Another tested combination, with mcd18200-6, resulted in the detection of the candidate effector in all progeny plants (see [4] Table 1). This phenotype might be explained by homozygous presence of corresponding *R* gene(s) in the resistant parent. In five other combinations, the response to the candidate effector was not detected in progeny plants. These combinations represent perhaps 'false positives' due to over- expression of the effector by PVX in the high throughput screening of the parents (see [5] Table 1) (*SolR*gene database). In summary, we tested 13 PEXRD families of the 16 showing a response, and the number of effector candidates was reduced to three by genetic analysis: PEXRD11, PEXRD45, and PEXRD51.

We decided to focus on PEXRD11 (PITG_13930), because it caused cell death to snk498042-1, and to breeding clone AM3778-16 (Lokossou et al. 2009; Park et al. 2005c) the donor plant of *R2*-like gene that was reported to recognize PiAVR2 (Lokossou et al. 2009). PEXRD11 is represented by a functional clone 21-1 (51 amino acids (a.a.) after the EER motif), and by clone 43-1 that has an early stop codon in the putative functional domain (8 a.a. after the EER motif) (Oh et al. 2009). In the T30-4 reference genome of *P. infestans* (Haas et al. 2009), *PexRD11* is located in a gene-sparse, repeat-rich region that interrupts a region of colinearity between *P. infestans*, *P. sojae*, and *P. ramorum* (Figure 1A). This region is a typical genomic localization for RXLR-type and for other classes of effectors in *P. infestans* (Haas et al. 2009). PEXRD11 belongs to *P. infestans* RXLRfam32, which includes eight members, and is related to PiAVR2 (PITG_22870) of RXLRfam7, which includes an additional 18 members. Similar to *PiAvr2*, *PexRD11* is expressed at an early stage of infection in potato, i.e. at 16 hours post infection (Figure 1B), which is in line with previous studies (Haas et al. 2009; Oh et al. 2009; Whisson et al. 2007) and is consistent with features as an avirulence effector.
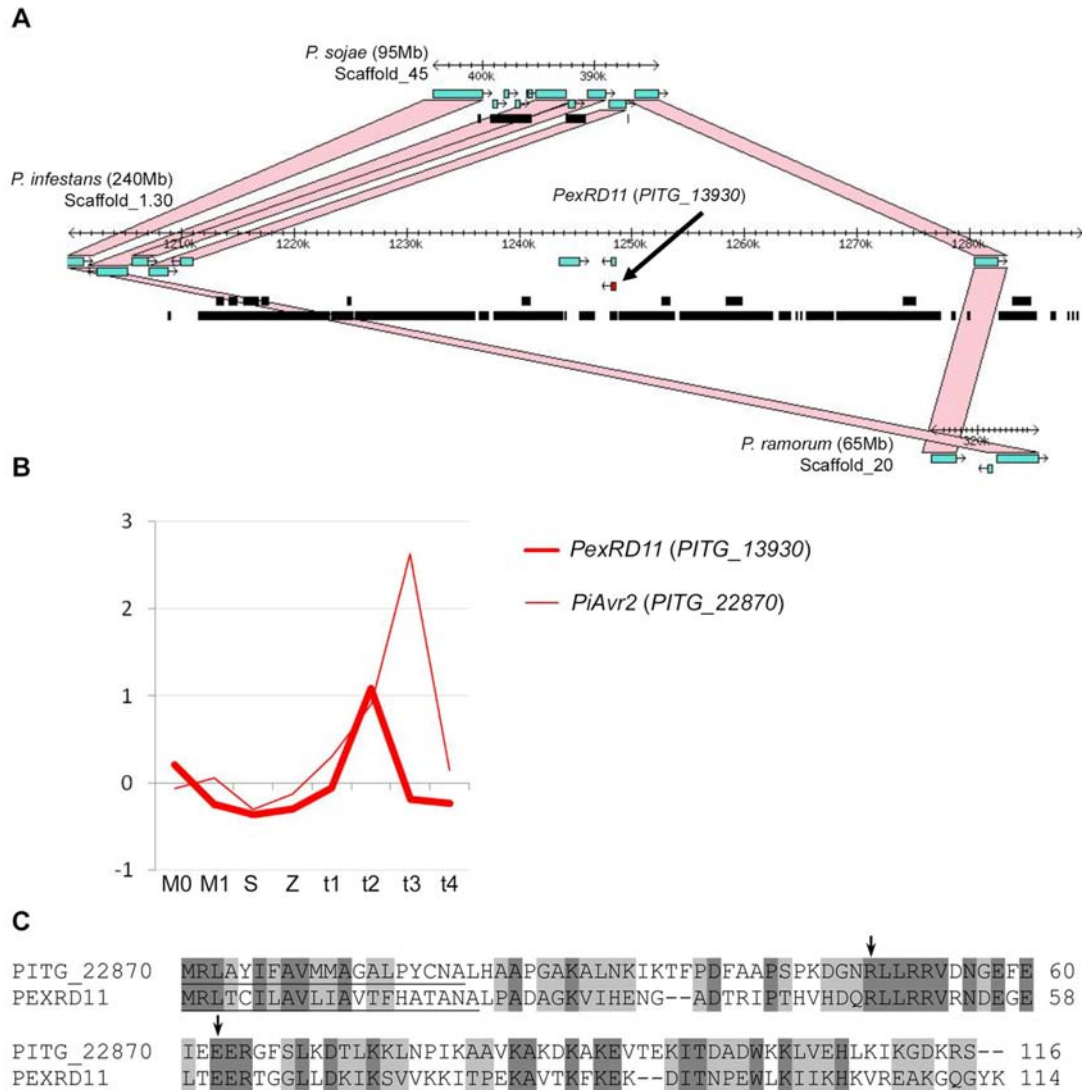
Figure 1. *PexRD11* has a similar pattern of expression and a similar protein structure compare to *PiAvr2*.

(A) *PexRD11* occurs in a gene-sparse, repeat-rich region flanked by regions of conserved colinearity between *Phytophthora sojae, P. infestans* and *P. ramorum*. Scaffold number from each genome is indicated. Genes are shown as turquoise boxes, *PexRD11* as red box, repeats as black boxes. Colinear orthologous gene pairs are connected by pink bands. (B) *PexRD11* is induced *in planta* at early stage of infection. Log2 normalization of *PexRD11* and *PiAvr2* expression profile from microarrays data is presented. Total RNA, of *P. infestans* T30-4 genome strain, isolated from mycelium grown, on Rye Sucrose Agar meduim (M0), on V8 medium (M1), from sporangia (S), zoospores (Z), infected leaves of potato, 6, 16 hours post inoculation, 2 and 5 days post inoculation (t1, t2, t3, and t4, respectively) were used. (C) Deduced amino acid sequences of PiAVR2 (PITG_08943 and PITG_22870) and PEXRD11 (PITG_13930) from *P. infestans* genome sequences database were aligned using the ClustalW software. Identical residues between PiAVR2 and PEXRD11 are shaded in dark grey, and conserved and semi-conserved residues are shaded in grey. Arrows point at RXLR and EER motif typical for cytoplasmic effectors. Underlined sequences are predicted signal peptide from SignalP 3.0 Server.

Table 2. Functional allele-mining for response to PexRD11 in *Solanum*.

Series[†]	Origin	Solanum species	Gene bank *	Resistance [§] *P. infestans* 90128	Agroinfection 21-1 pGR106:PEXRD11	43-1 pGR106:PEXRD11	neg. pGR106:empty	pos. pGR106:CRN2
Acaulia *S. demissum*	Bolivia	*S. acaule*	CGN 20620-4	9	-	-	-	+
	Argentina	*S. acaule*	CGN 17924-2	7	-	-	-	+
	Mexico	*S. acaule aemulans*	CGN 20562-4	7	-	-	-	+
	Peru	*S. albicans*	CGN 20667-3	5	-	-	-	+
	Mexico	*S. demissum*	CGN 17820-1	9	-	-	-	+
	Mexico	*S. demissum*	CGN 17829-2	9	-	-	-	+
	Mexico	*S. edinense*	PI 607474-3	9	+	-	-	+
Tuberosa Megistacroloba	Peru	*S. humectophilum*	GLKS 2829-2	1	-	-	-	+
	Unknown	*S. kurtzianum*	CPC 5889-3	9	-	-	-	+
	Bolivia	*S. megistacrolobum*	CGN 22347-1	7	-	-	-	+
	Bolivia	*S. megistacrolobum toralapanum*	CGN 18146-4	9	-	-	-	+
	Bolivia	*S. megistacrolobum toralapanum*	CGN 18146-5	9	-	-	-	+
	Bolivia	*S. megistacrolobum toralapanum*	CGN 18147-2	9	-	-	-	+
	Peru	*S. mochiquense*	GLKS 2319-1	9	-	-	-	+
	Peru	*S. multidissectum*	CGN 17840-22	9	-	-	-	+
	Argentina	*S. species*	GLKS 919-23	3	-	-	-	+
Cultivated Tuberosa and Tuberosa from Peru	Bolivia	*S. candolleanum*	CGN 18132-3	3	-	-	-	+
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. aracc-papa*	GLKS 82-1	7	-	-	-	+
	Bolivia	*S. astleyi*	CGN 18211-4	3	-	-	-	+
	Bolivia	*S. avilesii*	CGN 18256-2	6	-	-	-	+
	Bolivia	*S. berthaultii*	CGN 18190-3	9	-	-	-	+
	Bolivia	*S. chacoense*	CGN 18365-2	9	-	-	-	+
	Bolivia	*S. doddsii*	PI 545854-5	6	-	-	-	+
	Bolivia	*S. doddsii*	PI 545854-3	6	-	-	-	+
	Bolivia	*S. gourlayi pachytrichum*	CGN 18188-2	1	-	-	-	+
	Argentina	*S. gourlayi vidaurrei*	CGN 23045-2	8	-	-	-	+
	Argentina	*S. hawkesianum*	CGN 17888-4	7	-	-	-	+
	Argentina	*S. incamayoense*	CGN 21320-1	7	-	-	-	+
	Argentina	*S. incamayoense*	CGN 17875-5	7	-	-	-	+
	Argentina	*S. incamayoense*	CGN 17968-4	6	-	-	-	+
	Bolivia	*S. leptophyes*	CGN 18173-5	7	-	-	-	+
	Argentina	*S. microdontum*	CGN 17596-8	7	-	-	-	+
	Argentina	*S. microdontum*	CGN 17596-1	3	-	-	-	+
	Argentina	*S. microdontum*	CGN 21342-1	7	-	-	-	+
	Argentina	*S. neorossii*	CGN 18280-1	2	-	-	-	+
	Peru	*S. raphanifolium*	CGN 18089-5	9	-	-	-	+
	Peru	*S. ruiz-lealii*	CGN 18117-1	4	-	-	-	+
	Peru	*S. species*	CGN 20580-1	5	-	-	-	+
	Bolivia	*S. sucrense*	CGN 18026-1	6	-	-	-	+
	Bolivia	*S. sucrense*	CGN 18026-2	5	-	-	-	+
	Bolivia	*S. tarijense*	CGN 22729-5	8	-	-	-	+
	Argentina	*S. vernei*	CGN 18112-6	9	-	-	-	+
	Bolivia	*S. virgultorum*	CGN 17775-3	8	-	-	-	+
S. verrucosum	Mexico	*S. brachycarpum*	CGN 18347-8	9	-	-	-	+
	Mexico	*S. verrucosum*	CGN 22374-2	9	-	-	-	+
Demissa Conicibaccata	Mexico	*S. agrimonifolium*	PI 545748-1	9	-	-	-	+
	Mexico	*S. brachycarpum*	CGN 17721-2	8	-	-	-	+
	Mexico	*S. brachycarpum*	CGN 17721-3	8	-	-	-	+
	Colombia	*S. colombianum*	CGN 18289-3	9	-	-	-	+
	Mexico	*S. hougasii*	CPC 7050-1	9	-	-	-	+
	Mexico	*S. hougasii*	CGN 18339-2	9	-	-	-	+
	Unknown	*S. iopetalum*	CPC 2922-1	9	-	-	-	+
	Costa Rica	*S. longiconicum*	PI 310994-3	7	-	-	-	+
	Mexico	*S. schenckii*	PI 498042-1	9	+	-	-	+
	Venezuela	*S. subpanduratum*	PI 498289-3	4	-	-	-	+
	Ecuador	*S. tundalomense*	PI 473476	3	-	-	-	+

Table 2. (*Continued*) Functional allele-mining for response to PexRD11 in *Solanum*.

Series[†]	Origin	Solanum species	Gene bank *	Resistance [§]	Agroinfection			
					21-1	43-1	neg.	pos.
				P. infestans 90128	pGR106:PEXRD11	pGR106:PEXRD11	pGR106:empty	pGR106:CRN2
Longipedicellata	Argentina	*S. gourlayi*	CGN 22380-1	9	-	-	-	+
	Mexico	*S. hjertingii*	CGN 17717-3	9	+	-	-	+
	Mexico	*S. papita*	CGN 17830-1	9	-	-	-	+
	Mexico	*S. papita*	CGN 17832-2	9	-	-	-	+
	Mexico	*S. papita*	CGN 17831-1	9	-	-	-	+
	Mexico	*S. papita*	CGN 17831-8	9	-	-	-	+
	Mexico	*S. papita*	CGN 18319-1	4	-	-	-	+
	Mexico	*S. papita*	CGN 18319-4	6	-	-	-	+
	Mexico	*S. polytrichon*	CGN 17750-1	6	-	-	-	+
	Mexico	*S. polytrichon*	CGN 17750-2	7	-	-	-	+
	Mexico	*S. polytrichon*	CGN 17750-4	7	-	-	-	+
	Chile	*S. species*	CPC 7211-4	9	-	-	-	+
	Unknown	*S. species*	CPC 7328-2	9	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 17605-4	7	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 18332-1	8	-	-	-	+
	Peru	*S. stoloniferum*	CGN 22718-2	9	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 23591-3	8	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 18348-5	7	-	-	-	+
	Mexico	*S. stoloniferum*	CGN 18333-6	7	-	-	-	+
Circaeifolia Piurana Megistacroloba Tuberosa	Ecuador	*S. albornozii*	PI 561635-2	9	-	-	-	+
	Bolivia	*S. capsicibaccatum*	CGN 22388-1	9	-	-	-	+
	Peru	*S. chancayense*	CGN 18356-2	4	-	-	-	+
	Bolivia	*S. circaeifolium*	CGN 18133-3	2	-	-	-	+
	Bolivia	*S. circaeifolium quimense*	CGN 18127-6	3	-	-	-	+
	Peru	*S. dolichocremastrum*	PI 498236-1	9	-	-	-	+
	Peru	*S. huancabambense*	CGN 17719-1	9	-	-	-	+
	Peru	*S. huancabambense*	CGN 17719-2	1	-	-	-	+
	Peru	*S. immite*	PI 365330-4	9	-	-	-	+
	Peru	*S. mochiquense*	CGN 18263-3	7	-	-	-	+
	Peru	*S. piurana*	PI 473501-1	9	-	-	-	+
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. bulbocastanum*	CGN 21306-10	8	-	-	-	+
	Mexico	*S. bulbocastanum*	CGN 17693-2	9	-	-	-	+
	Guatemala	*S. bulbocastanum*	CGN 23075-1	9	-	-	-	+
	Guatemala	*S. bulbocastanum partitum*	PI 275200-2	4	-	-	-	+
	Mexico	*S. cardiophyllum*	CGN 22387-2	9	-	-	-	+
	United States	*S. jamesii*	CGN 18349-1	9	-	-	-	+
	United States	*S. jamesii*	CGN 18349-10	9	-	-	-	+
	Mexico	*S. michoacanum*	GLKS 2346-2	5	-	-	-	+
	Bolivia	*S. neocardenasii*	CGN 18217-2	9	-	-	-	+
	Mexico	*S. pinnatisectum*	CGN 23011-1	5	-	-	-	+
	Mexico	*S. polyadenium*	CGN 17749-4	7	-	-	-	+
	Mexico	*S. tarnii*	PI 545742-3	7	-	-	-	+
Out group	Bolivia	*S. chaparense*	CGN 18060-1	9	-	-	-	+
	Costa Rica	*S. nigrum*	CGN 21367-1	9	-	-	-	+

100 *Solanum* genotypes belonging to various phylogenetic series (Jacobs et al. 2008) and from different countries were tested by PVX agroinfection for response to pGR106::PEXRD11 clone 21-1, pGR106::PEXRD11 clone 43-1, pGR106::empty vector and pGR106::CRN2. At least 16 replicates were inoculated, and necrotic responses occurring at frequencies higher (+) or lower (--) than 40% of the inoculated sites are presented.

n.d. not determined

[†] Series according to Jacobs (2008)

*Abbreviations for genebank source codes: see Table 1.

[§] Resistance evaluated in field and/or detached leaf assay (*Sol*Rgene database)

Functional allele mining reveals specific response to PEXRD11 in *S. edinense*, and *S. hjertingii*.

To identify additional *Solanum* species responding to PEXRD11, we selected a set of 144 genotypes: 142 genotypes of *Solanum* species section *Petota* and two genotypes outside the *Petota* as controls: *S. chaparense* and *S. nigrum*. These 144 plants represents genotypes from North, Central, and South America, covering all taxonomic series, as described by Jacobs et al. (2008), and covering different degrees of resistance to *P. infestans* isolate 90128 (Table 2), as obtained in a high throughput screen of germplasm (*SolR*gene database). From each genotype, we performed a PVX agroinfection with PEXRD11 clones 21-1 and 43-1, the negative control pGR106::empty, and the positive control pGR106::CRN2. Forty-four genotypes showed either response to pGR106::empty, or no response to pGR106::CRN2, and were removed from the analyses. Within the remaining 100 *Solanum* genotypes, we identified a specific response to pGR106::PEXRD11 clone 21-1 in *S. edinense* (edn) 607474-3 and *S. hjertingii* (hjt) 17717-3, in addition to snk498042-1 control (Table 2 and Figure 2). The identified species belong to one of the three phylogenetic series: Acaulia, Demissa/Conicibaccata, and Longipedicellata (Jacobs et al. 2008), which suggests that recognition of PEXRD11 is dispersed over polyploid *Petota* species (Table 2).

Response to PEXRD11 in edn607474-3 maps to the *R2* cluster.

In addition to edn607474-3, hjt17717-3, and snk498042-1, the breeding clone AM3778-16 also responds to PEXRD11 by PVX agroinfection (Table 1). The *R2*-like gene from AM3778-16 was located previously on an MLB locus on LG IV (Lokossou et al. 2009; Park et al. 2005b; Park et al. 2005c), where *R2, Rpi-abpt* and *Rpi-blb3* also reside. To investigate whether this locus also contains a functional *Rpi* gene in edn607474-3, we generated a population of 190 F1 genotypes and inoculated them with *P. infestans* isolate 90128. A 1:1 segregation for resistance was evident and pointed to presence of a dominant *R* gene, designated *Rpi-edn1*. We tested eight resistant and eight susceptible F1 genotypes and their parents for response to pGR106::PEXRD11, and found a 100% correlation between resistance to *P. infestans* isolate 90128 and response to PEXRD11 (21-1) (Table 3). This correlation leads to the hypothesis that *Rpi-edn1* might be responsible for the response to PEXRD11 and might be located in the *R2* cluster of LG IV. To test this hypothesis, a set of PCR markers mapping on the short arm of LG IV were examined on the population. SSR marker RGH-SSR51 showed linkage with resistance to *P. infestans*, with 26 recombinants out of 142 F1 individuals (18cM), and was present in the eight resistant genotypes responding to PEXRD11 (21-1), but not in the eight susceptible, non-responding plants (Table 3). This finding indicates that *Rpi-edn1* is located on LG IV, possibly in the *R2* cluster. To identify more closely linked markers, we designed primers corresponding to the NBS region of *R2*, and we identified one marker (R2-2350-Rsa) linked with resistance to *P. infestans* with 2 recombinants out of 93 F1 individuals (2cM). The Th21 marker, which is located in the *R2*

Table 3. Co-segregation for resistance, PexRD11 response and *R2* genetic markers in the edn607474-3 population.

Genotype	A Resistance test *P. infestans* inoculation 90128	B PVX Agroinfection PEXRD11 pGR106 ::21-1	pGR106 ::43-1	negative pGR106 ::empty	positive pGR106 ::CRN2	C Markers HRM Th21	SSR RGH-SSR51	NBS R2-2350-Rsa
edn607474-3	R	+	-	-	+	+	+	+
Concurrent	S	-	-	-	+	-	-	-
Offspring								
104	S	-	-	-	+	-	-	n.d.
108	S	-	-	-	+	-	-	-
111	R	+	-	-	+	+	+	+
112	R	+	-	-	+	+	+	+
114	R	+	-	-	+	+	+	+
115	R	+	-	-	+	+	+	+
116	S	-	-	-	+	-	-	-
117	S	-	-	-	+	n.d	-	-
119	S	-	-	-	+	-	-	-
120	R	+	-	-	+	+	+	+
122	R	+	-	-	+	+	+	+
123	R	+	-	-	+	+	+	+
124	R	+	-	-	+	+	+	+
125	S	-	-	-	+	-	-	-
126	S	-	-	-	+	-	-	-
128	S	-	-	-	+	-	-	-

(A) Resistance (R) or susceptibility (S) against *P. infestans* isolate 90128. (B) Presence (+) or absence (-) of cell death around the inoculation site with *A. tumefaciens* clones expressing pGR106::PEXRD11 clones 21-1 and 43-1, pGR106::CRN2 (positive control) and the pGR106::empty vector (negative control) at 14 days post inoculation. (C) Presence (+) or absence (-) of the polymorphic band of the genetic HRM marker TH21 (Park et al. 2005b), the SSR marker RGH-SSR51 and profiling marker R2-2350-Rsa. n.d. not determined.

cluster (Park et al. 2005b), also showed linkage with resistance to *P. infestans*, with 6 recombinants out of F1 126 individuals (5cM). R2-2350-Rsa and Th21 fully co-segregated with resistance and effector recognition in the eight resistant genotypes tested with PEXRD11 (Table 3), suggesting that *Rpi-edn1* is located in the *R2* cluster and may be an *R2* homologue.

Snk498042-1 has been shown to contain *Rpi-snk1* on LG IV and an *R* gene in a population that was derived from Snk498042-1 and *S. brachycarpum* (CGN: 18347-8) (Jacobs et al. 2010). *Rpi-snk1* was linked to the CAPS marker Th21, and, was present in 2/3 of resistant offspring (Jacobs et al. 2010). Because bcp18347-8 was resistant to PVX, we used another population, derived from a cross with *S. stoloniferum* (sto0012-1), to investigate segregation of response to PEXRD11 effector. We found again 2/3 of resistant offspring responding to PEXRD11 (21-1), which led us to postulate that *Rpi-snk1* is also potentially an *R2* homologue that responds to PEXRD11.

Crosses with resistant parent hjt17717-3 were not successful, and therefore, genetic mapping of *Rpi-hjt1* was not further pursued. Nevertheless, we showed that edn607474-3 contained *Rpi-edn1* and snk498042-1 contained *Rpi-snk1,* and that both are located on the short arm of LG IV, in the same locus as *R2-like* (AM3778-16). Both *R* genes are potentially

homologues of *R2* and respond to pGR106::PEXRD11. We postulate, therefore, that hjt17717-3 might also contain an *R2* homologue, because it also responds to PEXRD11.
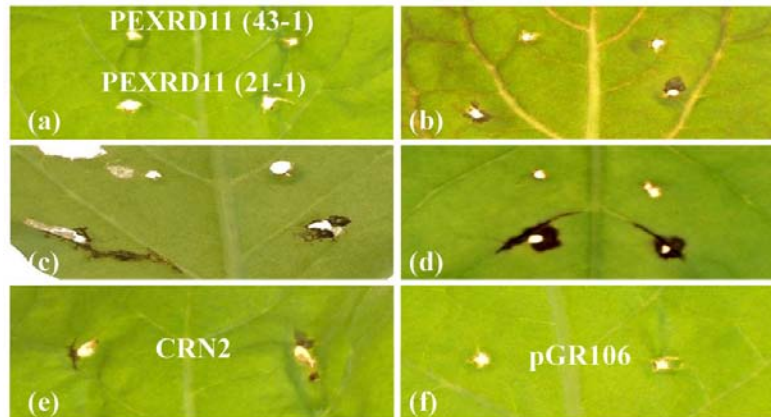
Figure 2. PEXRD11 induces cell death in *S. edinense* 607474-3, *S. schenckii* 498042-1 and *S. hjertingii* 17717-3.
PVX agroinfection was conducted on leaves of *S. tuberosum cv. Concurrent* (a), edn607474-3 (b), snk498042-1 (c) and hjt17717-3 (d) by wound-inoculation with *A. tumefaciens* strains carrying pGR106::PEXRD11 (21-1), and pGR106::PEXRD11 (43-1). Positive and negative controls, pGR106::CRN2 and pGR106::empty respectively, are shown on potato cultivar Concurrent (e and f). Pictures were taken at 12 and 16 days post inoculation.
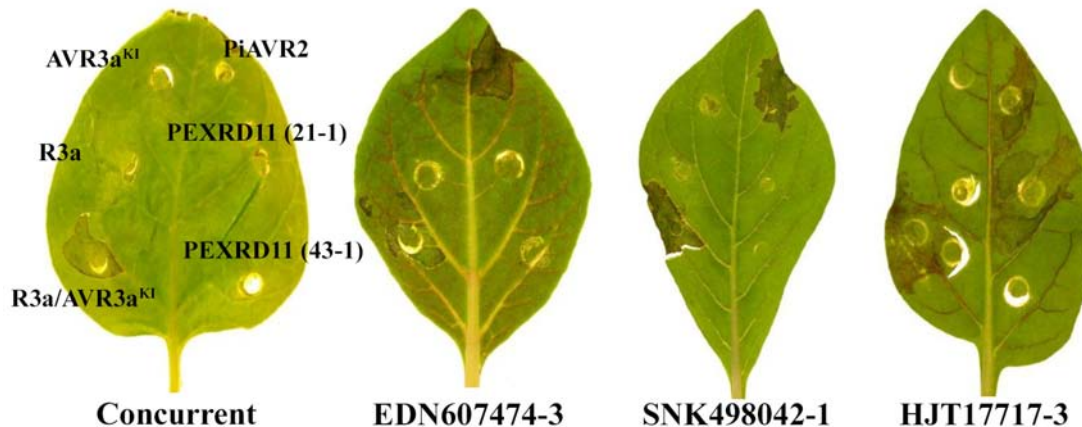
Figure 3. PiAVR2 induces cell death in *S. edinense* 607474-3, *S. schenckii* 498042-1 and *S. hjertingii* 17717-3.
Agroinfiltration using *A. tumefaciens* strain carrying, *PiAvr2*, *PexRD11* (21-1) and *PexRD11* (43-1) independently, was performed in *Solanum tuberosum cv. Concurrent*, edn607474-3, snk498042-1 and hjt17717-3. *R3a/Avr3a^{KI}* co-infiltration, and *R3a* and *Avr3a^{KI}* single infiltrations were included as positive and negative controls, respectively. Photographs of symptoms were taken at four dpi.

Hjt17717-3, edn607474-3 and snk498042-1 respond to PiAVR2.

The *R2*, *R2-like*, *Rpi-abpt*, and *Rpi-blb3* genes from the *R2* cluster specifically induce cell death when coinfiltrated with PiAVR2 from *P. infestans* (Lokossou et al. 2009). PEXRD11 and PiAVR2 proteins showed a high level of protein-sequence similarity, with 63% of similarity based on identical, conserved and semi-conserved amino acid substitutions (Figure 1C). To test the presence of a functional *R2* homologue in hjt17717-3, edn607474-3, and snk498042-1, we infiltrated *A. tumefaciens* strains expressing PiAVR2 in *Solanum* leaves. In the three wild *Solanum* genotypes, agroinfiltration with *PiAvr2* resulted in a specific cell death response at four days post infiltration (Figure 3).

In the same experiment, we included PEXRD11 clone 21-1 and 43-1 to test whether the results obtained with the PVX agroinfection could be confirmed by agroinfiltration (Figure 2 and 3). In hjt17717-3, PVX agroinfiltration of PEXRD11 (21-1) resulted in cell death. In edn607474-3 and snk498042-1, however, agroinfiltration with PEXRD11 (21-1) was not sensitive enough to result in cell death (Figure 3). In summary, the agroinfiltration experiments showed that specific cell death was always induced with *PiAvr2*, which indicates presence of functional *R2* homologues in hjt17717-3, edn607474-3, and snk498042-1, and might suggest some degree of functional redundancy between PiAVR2 and PEXRD11.

Allele mining in edn607474-3, snk498042-1 and hjt17717-3 reveals new variants of the *R2* family.

To identify which *R* genes in edn607474-3, snk498042-1, and hjt17717-3 were responsible for response to PiAVR2 or PEXRD11 (21-1) or both, we followed an allele-mining approach. PCR with conserved *R2* primers on genomic DNA of the three wild *Solanum* plants yielded amplicons of 2500bp, which is in the range of the expected size of an *R2* homologue. From hjt17717-3, additional amplicons of 3200bp were obtained that seemed to contain a hypothetical intron of about 750bp in the NBS region (data not shown). To determine the presence or absence of the intron, as well as the expression of these potential genes, new PCR amplifications were made on cDNA for this genotype, and amplicons were cloned and sequenced; each sequence was about 2500bp. Sequence analysis of the amplicons of the three *Solanum* species revealed eight unique *RGH* sequences from edn607474-3 (*ednR2GH*), seven from snk498042-1 (*snkR2GH*), and six from hjt17717-3 (*hjtR2GH*), with amino acid identities from 82.4% to 100% with *Rpi-blb3*, *Rpi-abpt*, *R2*, and *R2-like* (Table 4). Nucleotide sequences identical to *R2*-like were retrieved from edn607474-3 (*ednR2GH2*) and from hjt17717-3 (*hjtR2GH6*). *hjtR2GH1*, furthermore, is identical to *snkR2GH1* and *hjtR2GH4* is identical to *ednR2GH6,* which illustrates a degree of redundancy and origin of the *R2* homologues in *Solanum*.

The phylogenetic relationship between the R2 variants was examined by constructing a neighborjoining (NJ) tree (Figure 4). The NJ tree rooted with R3a, included RPP13 sequence from *Arabidopsis thaliana*, the closest known homologue of R2 in plants (Bittner-

Eddy et al. 2000; Lokossou et al. 2009). The R2 variants were grouped in six clades: clade I to clade VI. Clade I contains the four known functional R2 homologues, R2, Rpi-blb3, Rpi-abpt, and R2-like (ednR2GH2, hjtR2GH6), plus three new variants, ednR2GH1, hjtR2GH3, and snkR2GH3. Clade II contains hjtR2GH2, –GH5, and snkR2GH4; clade III contains the two identical snkR2GH1 and hjtR2GH1, as well as snkR2GH2; and clade IV contains snkR2GH5, -GH6, -GH7, and ednR2GH4. EdnR2GH-3 from edn607474-3 is different from any other variant, and represents clade V. Clade VI is the most distant from the original R2, and it contains ednR2GH5, -GH6, -GH7, -GH8, and hjtR2GH4. In summary, the three wild *Solanum* species contain an extended and diverse number of R2 variants, some of which are close to the known R2 family genes. In addition, five new clades representing 14 additional variants were identified.
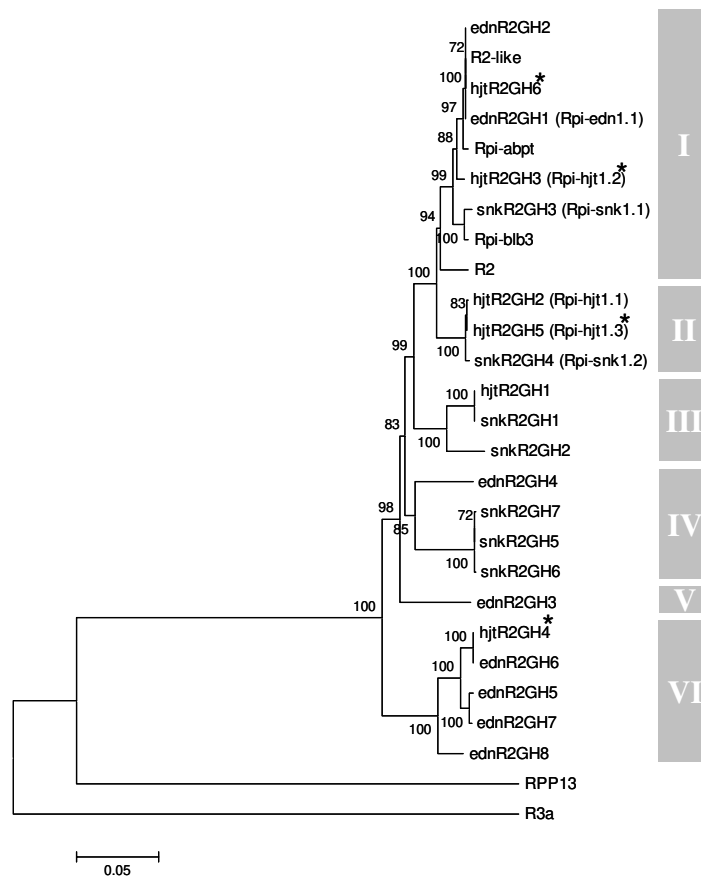
Figure 4. The *R2* family is composed of a high number of diverse of variants.
The neighbor-joining tree is illustrating phylogenetic relationship at the amino acid level between R2 variants from edn607474-3, snk498042-1 and hjt17717-3 with R2, R2-like, Rpi-abpt Rpi-blb3 and RPP13 vs. R3a as out group. Sequences are grouped in six clades. Sequences with a * were amplified from cDNA. Bootstrap values greater than 60% (based on 1000 resample) are indicated at the nodes.

Table 4. Percentage similarity between all RGH sequences used in the phylogenetic tree in Figure 4.

	ednR2GH1	ednR2GH2	ednR2GH3	ednR2GH4	ednR2GH5	ednR2GH6	ednR2GH7	ednR2GH8	hjtR2GH1	hjtR2GH2	hjtR2GH3	hjtR2GH4	hjtR2GH5	hjtR2GH6	snkR2GH1	snkR2GH2	snkR2GH3	snkR2GH4	snkR2GH5	snkR2GH6	snkR2GH7	Rpi-blb3	R2	R2-like	Rpi-abpt	RPP13	R3a
ednR2GH1	***	99.9	85.1	85.6	83.1	82.9	83.2	83.2	87.7	94.6	98.7	82.9	94.7	99.9	87.7	87.2	96.1	94.4	86.9	86.7	86.6	97.2	94.6	99.9	98.8	36	27.3
ednR2GH2	0.1	***	85	85.8	83	82.8	83.1	83.1	87.9	94.7	98.8	82.8	94.8	100	87.9	87.3	96.2	94.6	87	86.9	86.7	97.3	94.4	100	98.9	36	27.1
ednR2GH3	16.7	16.8	***	84.7	80.6	80.2	80.7	85.7	84.5	85.4	85.2	80.2	85.4	85	84.5	84.3	85.7	85.3	86.1	86	85.9	86.2	84.1	85	84.3	35.4	26.9
ednR2GH4	16	15.8	17.2	***	79.8	79.8	80	80.8	84.2	86.2	85.6	79.8	86.4	85.8	84.2	84.4	84.3	86.1	88.2	88.1	88	84.9	87.9	85.8	85.1	36.8	27
ednR2GH5	19.2	19.3	22.5	23.5	***	97.1	99.6	92.8	81.1	83	82.9	97.1	83.1	83	81.1	80.2	82.4	83.1	81	81	80.7	82.5	83.4	83	83.6	36.7	27
ednR2GH6	19.5	19.6	23	23.5	2.9	***	97.5	94.5	81	82.9	82.6	100	83	82.8	81	80.2	82.3	82.9	81.5	81.5	81.3	82.4	83	82.8	83.4	36.3	27.4
ednR2GH7	19	19.2	22.4	23.4	0.4	2.5	***	92.9	81.2	83.1	83	97.5	83.2	83.1	81.2	80.3	82.5	83.2	81.1	81.1	80.8	82.6	83.5	83.1	83.7	36.6	27
ednR2GH8	19	19.2	15.9	22.3	7.6	5.7	7.5	***	81.2	82.8	83	94.5	82.9	83.1	81.2	80.8	83.8	82.6	82.4	82.4	82.1	84.2	83.5	83.1	83.7	35.7	27.2
hjtR2GH1	13.4	13.3	17.4	17.8	21.8	22	21.7	21.7	***	87.5	87.7	81	87.6	87.9	100	91.6	86.6	87.1	85.1	85	84.9	87	85.7	87.9	87.1	36.2	26.3
hjtR2GH2	5.6	5.5	16.2	15.2	19.3	19.5	19.2	19.6	13.7	***	95.9	82.9	99.9	94.7	87.5	87.2	93.8	99.4	86.3	86.2	86	94.1	92.7	94.7	93.6	36.2	27.5
hjtR2GH3	1.3	1.2	16.5	16	19.5	19.8	19.3	19.3	13.4	4.3	***	82.6	96	98.8	87.7	87.4	95.8	95.5	87.1	87	86.9	96.6	93.3	98.8	97.8	35.8	27
hjtR2GH4	19.5	19.6	23	23.5	2.9	0	2.5	5.7	22	19.5	19.8	***	83	82.8	81	80.2	82.3	82.9	81.5	81.5	81.3	82.4	83	82.8	83.4	36.3	27.4
hjtR2GH5	5.5	5.4	16.2	15.1	19.2	19.3	19	19.5	13.6	0.1	4.1	19.3	***	94.8	87.6	87.3	93.9	99.3	86.3	86.2	86	94.2	92.8	94.8	93.7	36.2	27.5
hjtR2GH6	0.1	0	16.8	15.8	19.3	19.6	19.2	19.2	13.3	5.5	1.2	19.6	5.4	***	87.9	87.3	96.2	94.6	87	86.9	86.7	97.3	94.4	100	98.9	36	27.1
snkR2GH1	13.4	13.3	17.4	17.8	21.8	22	21.7	21.7	0	13.7	13.4	22	13.6	13.3	***	91.6	86.6	87.1	85.1	85	84.9	87	85.7	87.9	87.1	36.2	26.3
snkR2GH2	14.1	13.9	17.7	17.6	23.1	23.1	22.9	22.3	8.9	14.1	13.8	23.1	13.9	13.9	8.9	***	85.8	87	85.6	85.5	85.4	86.3	86.2	87.3	86.8	36.4	27
snkR2GH3	4	3.9	15.9	17.6	20.1	20.3	20	18.3	14.9	6.5	4.4	20.3	6.4	3.9	14.9	15.8	***	93.6	85.6	85.4	85.3	98.8	92.1	96.2	95.1	36	27.4
snkR2GH4	5.8	5.7	16.4	15.5	19.3	19.4	19.1	19.9	14.2	0.6	4.6	19.4	0.7	5.7	14.2	14.4	6.7	***	86.5	86.3	86.2	94	92.5	94.6	93.5	36.1	27.5
snkR2GH5	14.5	14.3	15.4	12.8	22	21.2	21.9	20.1	16.7	15.2	14.2	21.2	15.2	14.3	16.7	16	16.1	15	***	99.9	99.8	86	84.9	87	86.1	35.2	26.4
snkR2GH6	14.6	14.5	15.5	13	22	21.2	21.9	20.1	16.8	15.3	14.3	21.2	15.3	14.5	16.8	16.2	16.2	15.1	0.1	***	99.6	85.9	84.8	86.9	86	35.2	26.4
snkR2GH7	14.8	14.6	15.7	13.1	22.4	21.6	22.2	20.5	17	15.5	14.5	21.6	15.5	14.6	17	16.3	16.4	15.3	0.2	0.4	***	85.8	84.7	86.7	85.9	35	26.1
Rpi-blb3	2.9	2.8	15.3	16.9	20	20.1	19.8	17.8	14.3	6.2	3.5	20.1	6	2.8	14.3	15.2	1.2	6.3	15.5	15.6	15.8	***	92.9	97.3	96.2	36.3	27.1
R2	5.7	5.8	17.9	13.3	18.9	19.3	18.7	18.7	15.9	7.7	7.1	19.3	7.6	5.8	15.9	15.3	8.4	7.9	16.9	17	17.2	7.5	***	94.4	95.5	36.6	27.3
R2-like	0.1	0	16.8	15.8	19.3	19.6	19.2	19.2	13.3	5.5	1.2	19.6	5.4	0	13.3	13.9	3.9	5.7	14.3	14.5	14.6	2.8	5.8	***	98.9	36	27.1
Rpi-abpt	1.2	1.1	17.6	16.6	18.5	18.9	18.4	18.4	14.2	6.7	2.3	18.9	6.6	1.1	14.2	14.6	5	6.8	15.4	15.6	15.7	3.9	4.6	1.1	***	36.1	27
RPP13	127.8	127.8	130.6	124.5	124.8	126.5	125.4	129.2	127.1	127.2	128.9	126.5	127.2	127.8	127.1	126.3	127.8	127.7	131.5	131.5	132.6	126.7	125.2	127.8	127.4	***	22.6
R3a	179.2	180.1	182.2	181	181.4	177.7	181.4	179.5	187.2	177.3	181.1	177.7	177.3	180.1	187.2	181	178.3	177.5	186.4	186.4	188.4	180.1	178.7	180.1	181.5	217	***

Amino acid sequence distances of the 21 R2 variants of edn607474-3, snk498042-1 and hjt17717-3 with R2, R2-like, Rpi-abpt, Rpi-blb3 compared to RPP13 and R3a. The percentage of similarity is in the upper triangle, the percentage of divergence is in the lower triangle.

R2 is under diversifying selection.

Alignment of amino acids sequences of the 17 new *R2* variants with *R2*, *R2-like*, *Rpi-abpt*, and *Rpi-blb3* revealed two groups of sequence affiliation (Figure 5). All R2 variants in clade I and II had a mostly conserved LRR domain; only LRR repeats 3, 6, and 14 displayed one single nucleotide polymorphism (SNP) each (Figure 5). In contrast, the 11 other variants contained multiple SNPs throughout the entire LRR domain. To investigate which region of R2 was under diversifying selection, we applied the PAML method (Yang et al. 2005) and identified several positively selected amino acid residues (posterior probability, P>95%), of which the majority were in the LRR domain (Figure 6). Model M2 identified 19 amino acid residues under positive selection (Table 5), 15 of which were present in the LRR domain. The selection model M8 gave the same log likelihood value as M2 and identified the same 19 positively selected residues plus 22 additional a.a. of which 12 were in the LRR domain (Figure 6), for a total of 27 in the LRR domain. Most positively selected amino acid residues with a high posterior probability (P>99%) in the LRR domain were in LRR repeats 8, 10, 11, 12, and 13; on the border of LRR repeats 5 and 9; and in LRR subdomains 4/5, 5/6, and 6/7. In addition to the 27 sites in the LRR domain, six sites in the Leucine Zipper and eight in the NB-ARC domain, with one amino acid in the Kinase 2, were also under positive selection.

Table 5: Positive selection analysis among R2 variants.

Model of selection	Estimated parameters	l^a	Model comparison	2Dl $(df,p)^b$	Positively diversified codons[c]
M0: one ratio	w = 0.637	-9429.41			Not allowed
M1: neutral	r_0 = 0.619; r_1 = 0.381 w_0 = 0.034; w_1 = 1 (fixed)	-9194.64			Not allowed
M2: positive selection	r_0 = 0.582; r_1 = 0.320; r_2 = 0.097 w_0 = 0.040; w_1 = 1.000; w_2 = 4.015	-9124.45	M1 vs. M2	140.4 (2, <0.001)	**32R 134N 254G** 267R 598H **620T 621F** 622F 627C 648C **681S 683D** 684R 686R 710R 751S **752F** 776G 799R
M7: beta	r = 0.018; q = 0.025	-9196.00			Not allowed
M8: beta & w	r_0 = 0.897; r = 0.080; q = 0.138 r_1= 0.103; w = 3.931	-9124.53	M7 vs. M8	142.9 (2, <0.001)	25K 29D **32R** 107A **134N** 136A 145T 204T 209L 210N 240R **254G 267R** 341A 550R 553S 557L **598H 620T 621F** 622F 624E **627C** 643T **648C** 662A **681S 683D 684R 686R 710R** 715S 731Q **751S 752F** 754E 773K 775D **776G 799R** 802W

[a] Log likelihood value

[b] Likelihood ratio test: 2Δl = 2(lnl $_{alternative\ hypothesis}$-lnl $_{null\ hypothesis}$), with significance evaluated from χ^2 distribution; df is degree of freedom and p is the probability

[c] Bayes Empirical Bayes (BEB) analysis (Yang et al. 2005); Amino acid sites, based on *R2-like* sequence, inferred to be under diversifying selection with probability >95% and >99% in bold
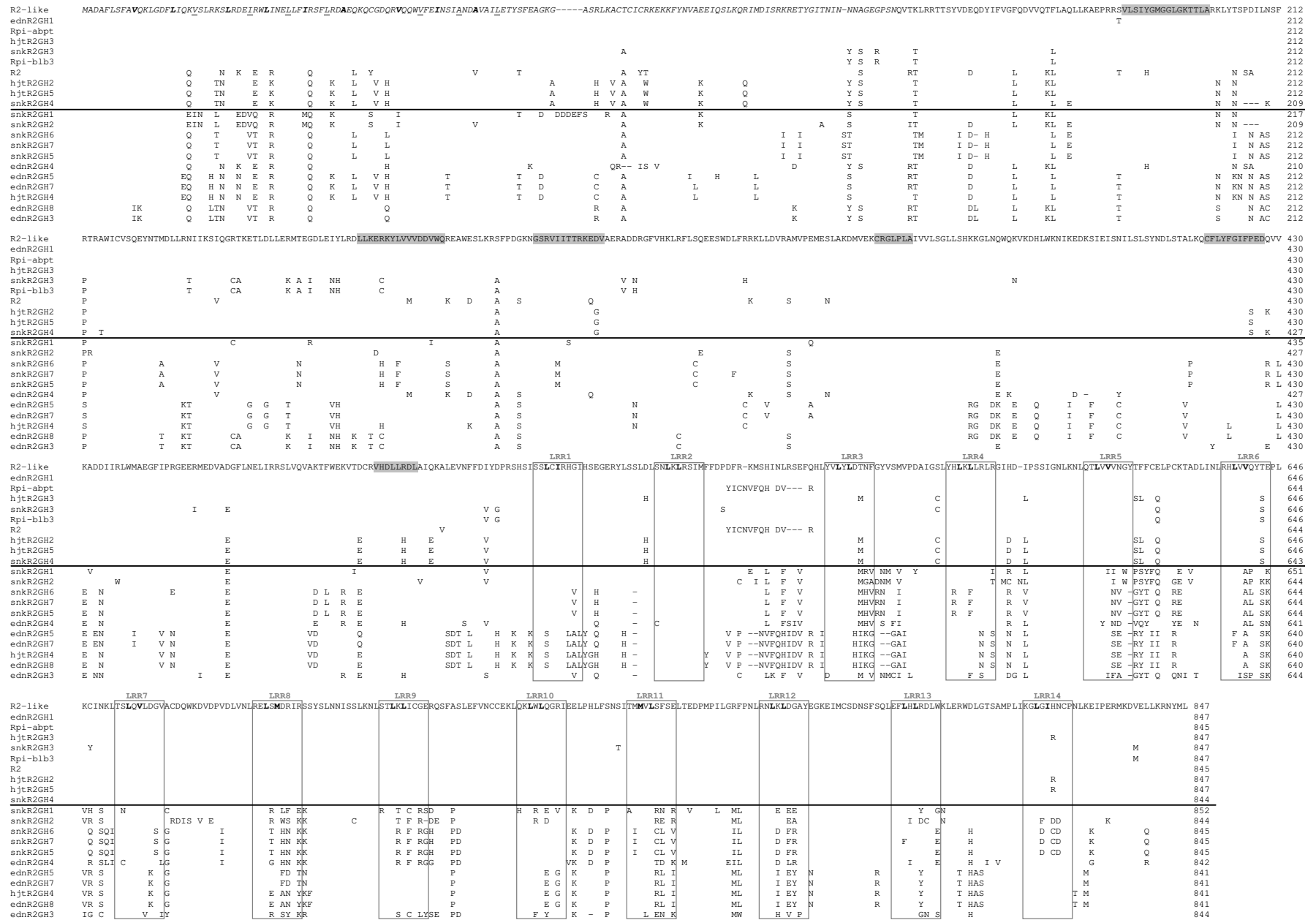
Figure 5. Protein alignment of R2 variants reveals a mainly conserved LRR domain in clade I/II R2 variants.
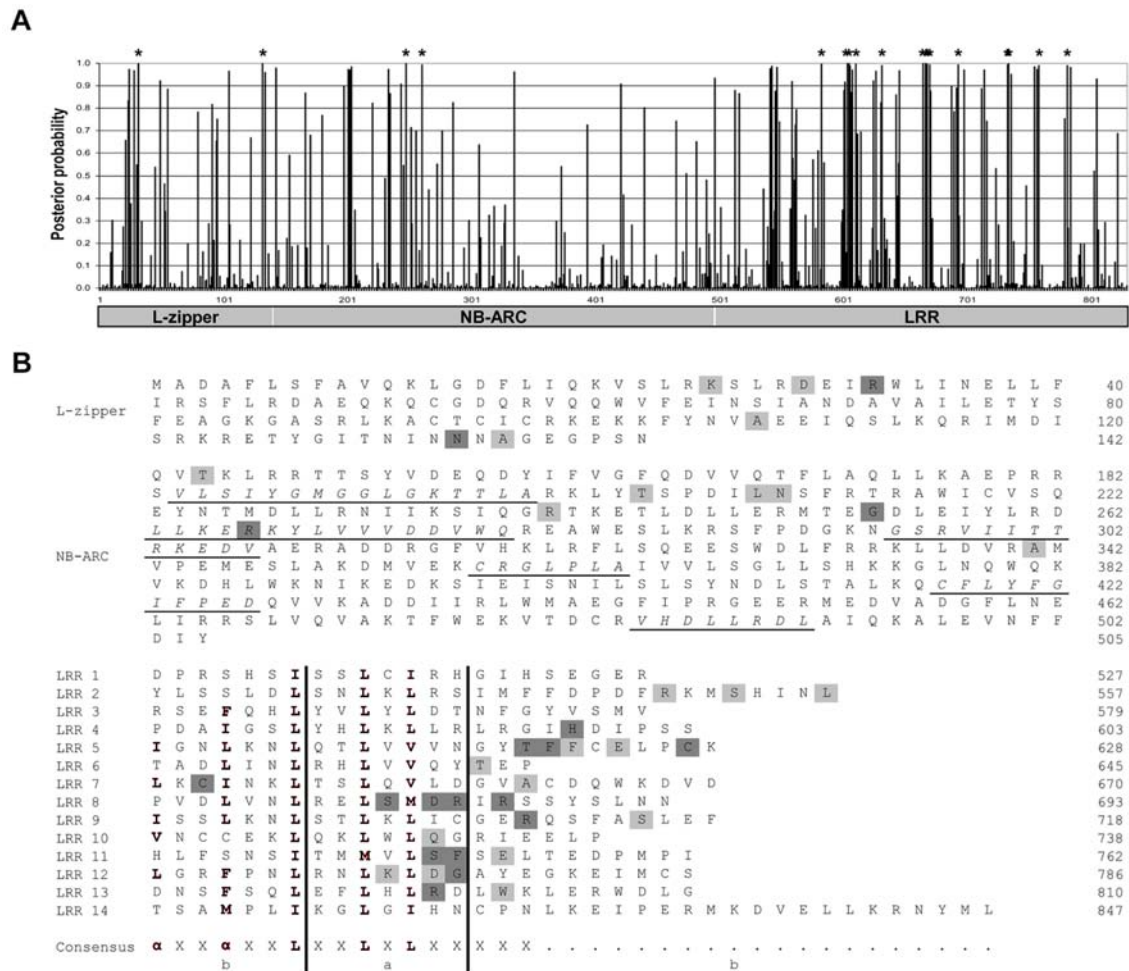
Figure 6. Positive selection in *R2* variants has mostly targeted the LRR domain.
(A) Graphical representation of posterior probability of diversifying selection based on model M8 at each site in R2 variants. The * indicates a posterior probability greater than 99% of having ω > 1. The *x* axis denotes codon position in the alignment of R2 variants made from codeml and removing all gaps. (B) Amino acids with posterior probability of diversifying selection greater than 95% (highlight in clear-grey) or greater than 99% (highlight in dark-grey) are presented on the sequence of R2-like. In the NB-ARC domain, characteristic conserved domains i.e. kinase 1, kinase 2, kinase 3, GLPL, RNBS-D and VHD motifs in that order, are underlined and in italic. The leucine-rich repeat domain is composed of 14 repeats, with the putative β-strand/β-turn motif, xxLxxLxx, in which the x residues are solvent-exposed residues available for interactions with potential ligands (Zhou et al. 2006). In the consensus, L. represents any one of five hydrophobic amino acids (F, I, L, M or V), X represents any amino acid, α. indicates sites with mostly hydrophobic amino acids (F, I, L, M, V, Y, T, H or C), a. designate the LRR repeats motif enclosed between the two vertical lines, and b. point the subdomains which may form a potential connecting βα loop (Jones et al. 1997).

Figure 5. (*Previous page*) **Protein alignment of R2 variants reveals a mainly conserved LRR domain in clade I/II R2 variants.**
The R2-like amino acid sequence is used as reference and only positional a.a. changes are noted for the other sequences. The leucine zipper domain is in italics with the first and the fifth hydrophobic residues from the heptads in bold and underlined, respectively (Lokossou et al. 2009). The NB-ARC domain includes the P-loop (kinase 1), kinase 2, and kinase 3 motifs of the NBS and GLPL, RNBS-D and MHDV motifs underlined that are conserved in NBS-LRR proteins (Bendahmane et al. 2002; Meyers et al. 1999; van der Biezen and Jones 1998). The 14 LRR repeats are presented in a grey box. A black line separates sequences in clade I and II from those in clade III to VI.

Six new R2 homologues respond to PiAVR2/PEXRD11 and confer resistance to *P. infestans*.

To test the function of the 17 new *R2* variants, we cloned them under *Rpi-blb3* promoter and terminator, and transferred them to *Agrobacterium tumefaciens* strain AGL1. We coinfiltrated *N. benthamiana* leaves with mixtures of the *A. tumefaciens* strains expressing the *R2* variants with either *PexRD11* (21-1), *PexRD11* (43-1), or *PiAvr2*, and we visually scored the symptoms at six days post infiltration. Six new variants (ednR2GH1, snkR2GH3, snkR2GH4, hjtR2GH2, hjtR2GH3, and hjtR2GH5) induced cell death when co-expressed with PiAVR2 and PEXRD11 (21-1) (Figure 7). These six R2 homologues shared between 92.1 and 99.9% identity, at the amino acid level, with R2, R2-like, Rpi-abpt, or Rpi-blb3 (Table 4), and the six clustered in either clade I or II. No cell death was observed for any member of clades III to VI. Again, cell death with all clade I and II members was more severe when triggered by PiAVR2 than by PEXRD11 (21-1). HjtR2GH5 showed a severe response to PEXRD11 (21-1). A low degree of auto-necrosis induction, however, was visible when hjtR2GH5 was expressed alone (Figure 7).

In order to test the extent to which the *R2* variants conferred resistance to *P. infestans,* we conducted transient complementation assays in *N. benthamiana* (Lokossou et al. 2009). The same six *R2* homologues (*ednR2GH1*, *snkR2GH3*, *snkR2GH4*, *hjtR2GH2*, *hjtR2GH3* and *hjtR2GH5*) that responded to the effectors also conferred specific resistance to *P. infestans* isolate IPO-0 with visible HR (Figure 8) but not to IPO-C, and the six were designated *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*. As expected, based on the effector infiltrations, *snkR2GH2* belonging to clade III, *ednR2GH3* belonging to clade V*,* and *hjtR2GH4* belonging to clade VI, were non-functional, and large expanding necrotic lesions surrounded by a sporulation zone were observed on inoculated leaves with the virulent (IPO-C) and avirulent (IPO-0) isolates (Figure 8).

In summary, we showed that the polyploid Mexican *Solanum* species contained multiple *R2* homologues that conferred *R2*-specific resistance to *P. infestans*. Hjt17717-3 contains four functional homologues, *R2-like*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*; the last three are expressed in leaves because they were cloned from a leaf tissue cDNA. Snk498042-1 and edn607474-3 each contain two functional *R2* homologues, *Rpi-snk1.1* and *Rpi-snk1.2* for snk498042-1, and *R2-like* and *Rpi-edn1.1* for edn607474-3.
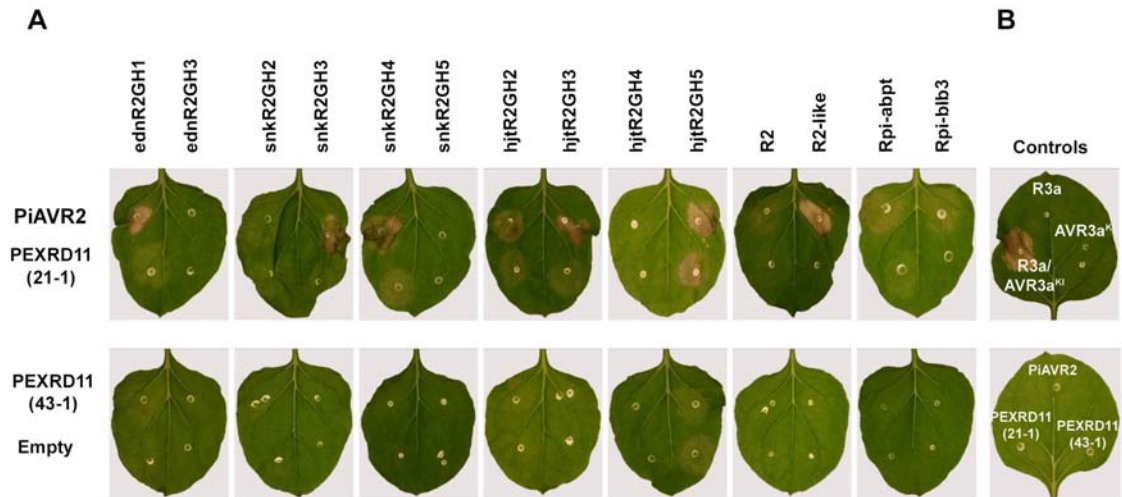
Figure 7. PiAvr2 and PexRD11 induce cell death with the six new *R2* variants.
(A) *Agrobacterium*-mediated expression of PiAvr2, PexRD11 (21-1) and PexRD11 (43-1) with the ten class I and II R2 variants and one representative of the class III to VI in *N. benthamiana*. Infiltration of each *R* gene alone was performed to test the R protein stability. (B) Other controls include the co-infiltration of *R3a/Avr3a^{KI}* as positive control and single infiltration of all effector clones used as negative controls.
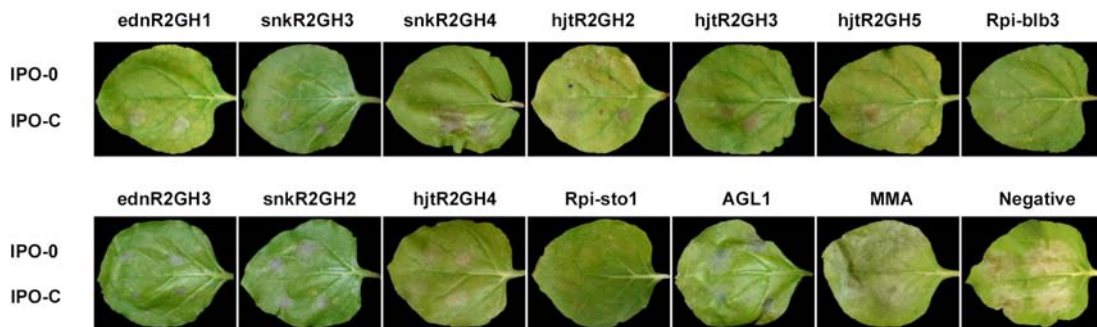
Figure 8. The six new *R2* variants confer resistance to *Phytophthora infestans*.
Transient expression of *R2* variants in *N. benthamiana*, followed by inoculation with the avirulent *P. infestans* isolate IPO-0, and the virulent isolate IPO-C on R2-plants. The six new *R2* homologues, *Rpi-blb3*, and three *R2* variants (*ednR2GH3*, *snkR2GH2* and *hjtR2GH4*) from clade III, V and VI are presented. *Rpi-sto1* from *S. stoloniferum* was included as a resistant control (Vleeshouwers et al. 2008). *N. benthamiana* leaves infiltrated with medium (MMA), with *A tumefaciens* strain AGL1, or not infiltrated were used as negative controls. HR was observed only with all six new *R2* homologues and *Rpi-sto1* when challenged with IPO-0.

Discussion

With the availability of genome sequences and genetic mapping information for numerous resistance loci in plants, the main challenge now is to identify functional *R* genes among candidate RGHs. In this paper, we rapidly and drastically reduced the number of R2GH to analyze by combining *R2* allele-mining with PEXRD11 effector assays. From a *Petota* selection-wide pool of 100 species originating from North, Central, and South America, we found that R2 activity was geographically restricted to Mexican species. We pinpointed three species (*S. edinense*, *S. schenckii* and *S. hjertingii*) bearing six *R2* homologues (*Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2*, and *Rpi-hjt1.3*) that induced cell death with PiAVR2 (Lokossou et al. 2009) and PEXRD11, and conferred resistance against *P. infestans.* We demonstrated that *R2* family is under diversifying selection and identified putative *R* gene domains that might be the target for adaptive evolution and recognition with cognate effectors PiAVR2 and PEXRD11, or their effector target.

Genetic mapping of the gene responding to PEXRD11 in *S. edinense* and *S. schenckii* led us to the *R2* cluster on potato LG IV. From this locus, *Rpi-blb3, Rpi-abpt, R2*, and *R2-like* were recently cloned and the four gene products recognized the RXLR effector PiAVR2 (Lokossou et al. 2009). These R2 homologues originate from various *Solanum* germplasms, including breeding line AM3778-16. In our study, AM3778-16 showed cell death response to PEXRD11 and PiAVR2, and we demonstrated that *S. edinense*, *S. schenckii*, and *S. hjertingii* also responded to PiAVR2. Thus, similar to some other R genes, such as *Rpi-blb1*, *Rpi-blb2* or *Gpa2* which recognize multiple effector variants (Champouret et al. 2009; Oh et al. 2009; Sacco et al. 2009), *R2* homologues responded to at least two effectors, *PiAvr2* and *PexRD11*. Although the overall sequence similarity between PiAVR2 and PEXRD11 was low (23%), a protein structure of 63% similarity emerged in the C-terminal domain, which is often dedicated to effector function and is a target of positive selection (Win et al. 2007). These observations suggest that interaction between PiAVR2/PEXRD11 effectors of *P. infestans* with similar motifs in their C-terminal domain might occur with *R2* homologues and follow the gene-for-gene or the guard hypothesis model (Flor 1971; Jones and Dangl 2006).

Evolutionary analysis of the *R2* sequences suggested that 41 residues are under selective pressure and distributed in LRR domains, particularly in repeats 8, 10, 11, 12 and 13, in L-zipper, and NB domain, but not in the ARC domains. These results are in line with the different current models of R/AVR interaction (Collier and Moffett 2009; Moffett 2009; Takken and Tameling 2009). The R protein activation model of Takken and Tameling (2009) characterizes the activation for signal transduction of an R protein by ATP/ADP binding in the NB-ARC domain, and more precisely in the MHDV motif, which is thought to be analogous to the sensor II motif of AAA+ ATPases (Takken et al. 2006). This activation model would explain why positive selection was not observed in ARC domains. Collier and

Moffett (Collier and Moffett 2009) proposed a two-step recognition process involving interactions with both cellular cofactors (guardees) and the LRR domain, which in turn activates the molecular "switch" leading to resistance. This process of recognition would explain the identification of residues under positive selection in the L-zipper and NB domains of *R2*, perhaps for the guard function of the cofactor, as well as in the LRR domains to function in effector recognition. There are examples that support this model, including the ternary interactions of p50/NRIP1/N and Gp-RBP-1/RanGAP2/Gpa2. Here different variants of the effectors interact with corresponding cofactors that are guarded by R proteins. The region that determines whether resistance response is executed, however, is under control of LRR domains (Caplan et al. 2008; Sacco et al. 2009; Ueda et al. 2006). Regarding the R2 family, ten of the 27 residues identified as under positive selection pressure were solvent-exposed residues within the LRR repeats 8, 10, 11, 12 and 13 (Figure 6), consistent with their putative function for interaction with potential ligands (Wulff et al. 2009; Zhou et al. 2006). Domain swapping between LRR repeats 11, 12, and 13 of functional and non-functional variants of R2 from Ma-R2 plant has shown the importance of these three repeats in PiAVR2 recognition (Lokossou 2010). Subsequent domain swapping or point mutations in the L-zipper, NB, and more LRR domains of *R2* genes promises to identify which sites are involved in effector recognition, guarding function, or molecular "switch".

Solanum section *Petota* is known to represent a great and diverse resource of *R* genes to *P. infestans,* and functional studies with the cognate *Avr* genes shed light on the evolution of the *R2* gene family. Most *Petota* species occur in the Andes of South America, and a secondary centre of diversity exists in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004). Hawkes (1990), postulated that the ancestral potato species originated from Central America, then migrated to South America, and returned to Mexico. This migration was followed by hybridization and allo-polyploidisations within Central American taxa, which then led to species with various ploidy levels. We found the functional activity to PEXRD11 and PiAVR2 to be geographically restricted to Mexico, which is an eminent source of *R* genes against *P. infestans* (Ballvora et al. 2002; Black and Gallegly 1957; Huang et al. 2005; Lokossou et al. 2009; Malcolmson and Black 1966; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005; Vleeshouwers et al. 2008).

Regarding the broader family of *R2,* we identified a diverse set of 17 new variants and observed a robust association between phylogenetic relatedness and biological function, because cell-death inducing activity with *PiAvr2* and *R2*-specific resistance to *P. infestans* was restricted to clade I and II. The 11 variants of *R2* from clade III to VI showed more divergence compared with *R2* homologues. Because some of these *R2* homologues are transcribed, we can postulate that they are functional genes against other effectors of *P. infestans* or other pathogens. All species exhibiting functional *R2* homologues, i.e. *S. edinense*, *S. schenckii*, *S. hjertingii, S. bulbocastanum*, and *S. demissum* (Lokossou et al. 2009), are from Mexico, therefore, we postulate that the *R2* potato blight resistance family

emerged in Mexcico, one of the centres of origin of the pathogen (Grünwald and Flier 2005). The cloning of a QTL *Rpi-mcd1* in the short arm of LG IV of the South American species *S. microdontum* (Lokossou 2010; Tan et al. 2008) might provide further information on functional aspects of the diverse *R2* clades related to geographical co-evolution with local *P. infestans* genotypes.

The origin of *R2-like* in the AM3778-16 breeding material (Lokossou et al. 2009; Park et al. 2005c) is elucidated in this study, because *R2-like* was amplified from *S. edinense*, which is one of the progenitors in the AM breeding material. Occurrence of identical sequences between species was identified in three cases, which could be due to interspecific hybridization events rather than to ancestral origin of these phylogenetically distant species (Jacobs et al. 2008). That *S. edinense* is a hybrid between *S. demissum* and *S. tuberosum,* and that *R2* originates from *S. demissum*, supports this hypothesis (Li et al. 1998; Serquen and Hanneman 2002).

Recent studies on *Rpi-blb1* have shown that increasing the copy number or transcript level of an *R* gene in transgenic plants improves level of resistance against *P. infestans* (Bradeen et al. 2009; Kramer et al. 2009; Kuhl et al. 2007). There is, however, contradictory data on fitness costs of *R* genes in transgenic plants (Bergelson et al. 2001; Goss and Bergelson 2007; Kniskern and Rausher 2006; Korves and Bergelson 2004; Tian et al. 2003), and recent study revising the work of Tian et al. (2003) demonstrates that their observation on *Arabidopsis thaliana* remained inconclusive (Schwachtje et al. 2008). In our study on natural germplasm, we found two copies of functional *R2* homologues present in *S. edinense*, two in *S. schenckii*, and four in *S. hjertingii*, most likely due to their penta-, hexa-, and tetraploid background; no aberrant phenotypes were observed on any of the three wild species. Our work highlights the importance of late blight resistance in natural populations of *Solanum*. These *Solanum* genotypes are naturally exposed to a high degree of genetic diversity of *P. infestans* population originating, from the commercial potato-growing fields, and the native *Solanum* spp. on the forested slopes in the centre of origin of the pathogen (Flier et al. 2003). The high degree of natural diversity in functional late blight resistance genes is a boon for potato breeders, and offers a number of new genes and traits that can be translated to commercial agriculture.

In summary, the identification of large numbers of *R2* variants in Lokossou et al. (2009) and in this study illustrates that the *R2* family is highly diverse in *Solanum*. The demonstrated *R* gene diversity, such as in the flax rust *R* genes (Dodds et al. 2006) and the *Arabidopsis RPP* genes (Allen et al. 2008; Hall et al. 2009; Rehmany et al. 2005), matches a "use-it-and-diversify-it" R-AVR co-evolutionary dynamics model, in which both R and AVR display high diversification and differential recognition. It will be of interest to identify additional *PiAvr2* family members within the *P. infestans* genome, and in contemporary populations, and test their functionality for different effector targets in the host, to gain

further insight in recognition events that test the guard or decoy models (Dangl and Jones 2001; van der Hoorn and Kamoun 2008).

Materials and Methods

Plant material

The wild *Solanum* plant material used for effector screening and functional allele-mining is listed in Table 1 and Table 2, respectively. The accessions were retrieved from Centre for Genetic Resources, the Netherlands (CGN), from PI: NRSP-6 – United States Potato Genebank), and from CPC: Commonwealth Potato Collection, UK.). Two populations were generated for genetic studies. Edn607474-3 population was originated from the cross between *S. edinense* (PI: 607474-3) and potato cultivar Concurrent. Snk498042-1 was originated from *S. schenckii* (PI: 498042-1) with *S. stoloniferum* (CPC: 0012-1). Plant genotypes were maintained *in vitro* on MS medium supplemented with 20% sucrose (Murashige and Skoog 1962) at 18 °C. Top shoots were transferred to fresh medium; two weeks later, rooted plantlets were transferred to the greenhouse. *Nicotiana benthamiana* plants were grown from seeds in the greenhouse and subjected to agroinfiltration experiments in climate chambers at an ambient temperature of 22–25 °C and high light intensity.

Phytophthora infestans isolates and disease tests

The *P. infestans* isolates IPO-0 (race 3b.4.7.10.11), 90128 (race 1.3.4.7.8.10.11) and IPO-C (race 1.2.3.4.5.6.7.10.11) (Champouret et al. 2009) were cultured on rye sucrose agar medium in the dark at 15 °C for 1 to 2 weeks (Caten and Jinks 1968). Sporulating mycelium was flooded with cold water, and the sporangiospore suspension was incubated at 4 °C for 3 h. After the release of the zoospores, the inoculum was adjusted to a concentration of $5*10^4$ spores/ml. Leaves from 6 to 8 week-old plants were detached and placed in water-saturated oasis in humid trays; leaflets were spot-inoculated with the spore suspension. The trays were incubated in a climate chamber with a 16h light and 8h dark cycle at 15 °C. At six days post inoculation, lesion sizes (LS) were measured. Sporulating lesions exceeding 15 mm were scored as susceptible (S), whereas lesions displaying localized hypersensitive response (HR) smaller than 5 mm at the inoculation sites were scored as resistant (R). For high throughput resistance screening in *Solanum* germplasm, disease tests were performed in field trials, in detached leaf, and in vitro assays. Scores were assessed and converted to a relative scale of 9 to 1. Nine corresponds to no symptoms or very small LS due to localized HR, 2 is set to the susceptible standard Bintje, 1 is massively sporulating and large lesions exceeding those of Bintje, and the other ratings correspond to intermediate classes, as shown on the *Sol*R*gene* database website (*Sol*Rgene database).

PCR allele mining

R2 variants from wild *Solanum* species were cloned by PCR amplification on genomic DNA, using the conserved start and stop primer pair blb3-F-Start-GW and blb3-R-stop-GW (Table 6) containing AttB1 and AttB2 sequences for Gateway cloning purposes. Amplicons were cloned into pDON221. For amplification on *S. hjertingii*, mRNA was extracted from hjt17717-3 leaves using RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN), and cDNA was made using iScript™ cDNA synthesis kit (Biorad). Amplification was made using Phusion High-Fidelity DNA Polymerase (FINNZYMES). PCR fragments were purified on 1% TBE gel using Min Elute™ Gel Extraction Kit (QIAGEN) and were cloned in pDONR221 (INVITROGEN) using BP clonase (INVITROGEN). Primers were designed on *R2* (Table 6) to sequence clones using Greenomics facilities (Wageningen, The Netherlands), and sequences were analyzed using DNASTAR Lasergene v8. Clones containing a *R2* variant in frame were cloned under *Rpi-blb3* promoter and terminator using multisite Gateway (Lokossou et al. 2009).

Table 6: Primers list.

Primer name	Primer sequence 5'- 3'	Comments
RGH-SSR51F	CGCAGTATACGCTGTCCAAC	SSR marker RGH-
RGH-SSR51R	GTTTGCAGTGTGAGTTGGTTGGTG	SSR51
Th21F	AGCATCCGGAGGCAAATC	HRM marker
Th21R	TAGGCTTAACTGTCAAATGG	
R2ch4F	TGTGCAGTGATAACAGCTTCA	R2 NBS marker
blb3-F-start-GW	GGGGACAAGTTTgtacaaaaaagcaggctATGGCTGATGCCTTTCTATCATTTG	R2 variants amplification
blb3-R-stop-GW	GGGGACCACTTTGTACAAGaaagctgggtTCACAACATATAATTCCGCTTCAAC	
R2F	ATGGCTGATGCCTTTCTATCATTTGC	
SpF2	GAAGGAGAAGAAATTCTACAATG	
Glo2F	GTGTCTCTCAAGAGTACAACAC	
SpF4	TAATACGGTTGTGGATGGCG	
abptF2	CAAGAAGCCACTCCATATCC	
Lrr-F1	CTCTTTATGTATCAGACATGGC	
SpF11	CCGTCTTCCATTGGCAACC	
SpR1b	CATTGTAGAATTTCTTCTCCTTC	
SpblbR	ACTTTTTCCCAAAATGTTTTAGC	
SpR3	AGCTCCTGATACGATCCATG	
LrrR3	CAACATCTTTCCACTGATCAC	Sequencing
abptR3	TCAACAGGGTCAACATCTTTC	
LrrR2	GAGGCATGGCACTTGTGCC	
R2R	TCACAACATATAATTCCGCTTC	
R2-HJT	GGAAACTACTTGATGTTCGAG	
R2-HJT-NC57_17F532	AAATCTCTTCAGGCTGAGGC	
R2-HJT-NC57_17R1	TGCCATACATCATCAACCAC	
R2-HJT-NC57_17F682	CTCTGGACAAGAGTTGAGTC	
R2-HJT-NC57_17R2	CTTGGCGCTGCCTTGGATG	

Agroinfection and Agroinfiltration

A collection of 55 non-redundant RXLR effectors was identified in previous studies, and primer pairs based on the mature region of candidate effectors were designed and used to amplify total DNA from a panel of nine *P. infestans* isolates (Oh et al. 2009; Vleeshouwers et al. 2008). Amplicons were cloned into the pGR106 (Lu et al. 2003) for occurrence of cell death responses on *Solanum* plants using PVX agroinfection, as described previously (Oh et al. 2009; Vleeshouwers et al. 2008; Vleeshouwers and Rietman 2009), and pGR106::empty vector and pGR106::CRN2 were included as negative and positive controls in that order. Each individual effector was tested twice on six leaves over two plants in two independent experiments. Leaves were monitored for symptoms over 16 days.

PexRD11 clone 21-1 (NCBI Genbank code GQ869426) and clone 43-1 (NCBI Genbank code GQ869427), without signal peptide, were introduced in pK7WG2 (Karimi et al. 2002) by gateway technology (see primers used in Table 6). *Avr3a^{KI}* and *PiAvr2* (Lokossou et al. 2009), without signal peptide, were cloned in pGRAB, as was *R3a* (kindly provided by S.N. Chapman). *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) was transformed with individual binary vectors in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Fits et al. 2000). *Solanum* plants infiltration with *A. tumefaciens* was performed as described (Vleeshouwers and Rietman 2009). The *A. tumefaciens* strains were grown as described (van der Hoorn et al. 2000), except that culturing steps were performed in LB media supplemented with 50 µg/ml kanamycin. Strains were grown to desired optical density at 600 nm (OD_{600}) of 0.2, although the same results were observed with OD_{600} between 0.1 to 0.4, *A. tumefaciens* solutions were mixed in a 1:1 ratio for co-infiltration. Leaf panels of middle age leaves on *Solanum* plants beyond nine-leaf stage, or four- to five-week-old *Nicotiana benthamiana* plants were infiltrated with the *A. tumefaciens* suspensions, and symptoms were monitored from two to six days post infiltration (dpi).

Genetic mapping

The SRR marker RGH-SSR51 (Table 6) was developed by Dr. A. Goverse (Laboratory of Nematology, Wageningen University, The Netherlands). PCRs were performed using the following cycle profile: an initial cycle at 95 °C for 2 min; then 30 cycles of (i) 95 °C for 30s sec, (ii) 56 °C for 30 sec, using a ramp of 1 °C/min, and (iii) 72 °C for 45 sec, using a ramp of 1 °C/min; and a final step at 72 °C for 3 min. PCR products were analysed by electrophoresis acrylamide gels on the LI-COR sequencer (Lincoln, Nebrasca, U.S.A.). SCAR marker Th21 primer (Table 6) combination (Park et al. 2005b) was tested with the High Resolution Melting curve analysis (HRM) on the LightScanner (Idaho Technology). PCRs were performed using the following cycle profile: an initial cycle at 98 °C for 30 sec then 40 cycles of three steps, (i) 98 °C for 5 sec, (ii) 60 °C for 5 sec, and (iii) 72 °C for 15 sec; followed by three final steps of 72 °C for 30 sec, 94 °C for 30 sec, and 25 °C for 30 sec. The *R2* PCR marker R2-2350-Rsa was developed by NBS profiling (van der Linden et al.

2004), and resulted from the primer R2ch4F4 (Table 6) in combination with the restriction enzyme RsaI.

Transient complementation in *N. benthamiana*

Four-week-old *N. benthamiana* plants were infiltrated using an OD_{600} of 0.2 with a solution of *A. tumefaciens* AGL1 (Lazo et al. 1991) harbouring the putative *R* gene candidates. Two days after infiltration, a detached leaf assay was performed with *P. infestans* isolates IPO-0 and IPO-C, symptoms were scored (Lokossou et al. 2009) between four and eight days post inoculation.

Positive selection analysis

To test for amino acids under purifying or diversifying selection, we used codon-based analysis (Codeml) implemented in PAML v.4.2 package (Yang 2007). Maximum-likelihood codon substitution models M0, M1, M2, M7, and M8 were used for analysis (Win et al. 2007). Positively selected sites detected by, models M2 and M8 were identified using Bayes Empirical Bayes statistics (Yang et al. 2005).

Phylogenetic data analyses

Phylogenetic analyses of the new R2 variants from the wild species were conducted using the Neighbor-joining method (Nei and Li 1979) in MEGA version 4 (Tamura et al. 2007). R2, R2-like, Rpi-abpt, Rpi-blb3, and RPP13 protein sequences were used as closest known homologue genes, and the tree was rooted using R3a. Bootstrap values greater than 60% from 1000 replicate trees are shown at the nodes. Horizontal branch lengths and scale bar correspond to evolutionary distances assigned by MEGA. The evolutionary distances are measured as the proportion of nucleotide substitutions between sequences (Tamura et al. 2007).

CHAPTER 4

Diversity of *PiAvr2/PexRD11* and *R2* gene families underpins co-evolution between *Phytophthora infestans* and Mexican *Solanum* species

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Diversity of *PiAvr2/PexRD11* and *R2* gene families underpins co-evolution between *Phytophthora infestans* and Mexican *Solanum* species

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Abstract

The interaction between potato and the late blight pathogen *Phytophthora infestans* is controlled in a gene-for-gene manner by cytoplasmic resistance (*R*) genes of the CC-NBS-LRR group, which confer recognition of their cognate pathogen avirulence (*Avr*) proteins. R2 is one of these *R* genes. It recognizes two effector proteins of *P. infestans* PiAVR2 and PEXRD11. In this study, we focussed on the identification and characterization of *PiAvr2/PexRD11* family members within the *P. infestans* genome. We performed BLAST similarity searches of the genome sequence of *P. infestans* with the C-terminal effector domain of PiAVR2 and PexRD11 and identified 11 independent RXLR effectors that were grouped in classes I, II and III. Robust association between phylogenetic classes of the *PiAvr2/PexRD11* effector family and *R2* family with recognition leading to cell death was uncovered. The ten known functional R2 homologues recognised three new effectors with different specificities. One effector protein, PITG_13940 induced cell death with the six R2 homologues, i.e. Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 and Rpi-hjt1.3, but not with R2, R2-like, Rpi-abpt and Rpi-blb3 which were used in breeding material for the last decades. Interestingly escape of recognition of PITG_13940 is linked to two amino acids in the C-terminal domain. Two other effectors, PITG_14936 and PITG_05121, from the class I and II did not induce hypersensitive response (HR) with R2 homologues, and different hypotheses based on secondary structure, presence of a KIT/KIK/PIK motif and folding protein arrangement are presented. Also, we predicted that PiAVR2/PEXRD11 family members of class I and II contain a Nuclear Export Signal (NES) and that the presence of the

putative NES sequence is linked to the induction of cell death with R2 homologues. All together, these results highlight the first functional support for the arms-race model in the *Solanum*/*P. infestans* pathosystem based on the R2-PiAVR2/PEXRD11 interaction. Moreover using effectoromics we could characterize six new *R2* homologues variants with potentially broader resistance spectrum. This study, therefore, provides more *R* gene diversity and specificity, valuable for potato breeders.

Introduction

Plant diseases impose a major constraint on food production worldwide. They are often combated by chemical, genetic or biological methods. Nowadays, the management of plant diseases by chemical approaches is becoming more and more unpopular even if in general it is a reliable process. An emerging interest for a more economical and sustainable crop production system in concert with the protection of the environment renders genetic methods highly desirable. Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is a huge threat for potato and tomato farming. Chemical management of the disease has been relatively successful, however, developed resistance to the main fungicide, the phenylamide metalaxyl (Cohen and Reuveni 1983; Cooke 1981; Davidse et al. 1981; Deahl et al. 2002; Deahl et al. 1993; Miller et al. 1997), has led to new interest in exploring the wealth of Resistance (*R*) gene diversity in tuber-bearing *Solanum* species of the section *Petota*. In those plants, new <u>*Resistance*</u> genes to <u>*P*</u>. <u>*infestans*</u> (*Rpi*), with different spectra of resistance were discovered and cloned (Foster et al. 2009; Kuhl et al. 2001; Lokossou et al. 2009; Pel et al. 2009; Sandbrink et al. 2000; Smilde et al. 2005; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005; Villamon et al. 2005; Vleeshouwers et al. 2008). Interestingly all these studies have brought more insight in *R* gene diversity from different *R* gene clusters, by identifying several *R* genes homologues (*RGH*) in various wild *Solanum* species (Lokossou et al. 2009; Pel et al. 2009); This thesis Chapter 3). From the R2 cluster, which was studied by map based cloning and functional allele mining, up to 50 non-redundant *R2* variants were isolated from *S. demissum*, *S. bulbocastanum*, *S. edinense*, *S. schenckii*, and *S. hjertingii*, with ten of them being functionally active against late blight (Lokossou et al. 2009; Chapter 3). Interestingly, these *R2* homologues are responding to two RXLR effectors of *P. infestans*, *PiAvr2* and *PexRD11*, which have a low percentage of identity sensu stricto but a 63% of similarity based on identical, conserved and semi-conserved amino acid (a.a.) substitutions (Chapter 3).

In a plant-pathogen co-evolutionary state, the extreme intimacy between plant and pathogen drives a complex and dynamic selection pressure on the genes involved in the interaction. Adaptation of the host by gain of resistance (via an *R* gene), changes the selection pressure on the pathogen, giving rise to a counter-adaptation. If this occurs reciprocally

(escape of recognition by mutation or silencing of the avirulence gene and therefore gain of virulence on the host), an unstable runaway escalation or "arms-race" occurs. These produce an up/down effect in the interaction, visible by a cycle of resistance followed by susceptibility, which is described as a Zig-Zag model by Jones and Dangl (2006). Effectors in that case act as avirulent factors (*Avr*) and their recognition by *R* genes is based on the gene-for-gene model (Flor 1971). Examples of such direct interaction are relatively rare (Deslandes et al. 2003; Dodds et al. 2006; Ellis et al. 2007a; Jia et al. 2000). Van der Biezen and Jones (1998), have come up with a new model, the guard model, in which the R proteins do not directly detect the presence of the pathogen avirulence protein, but perceive alterations of the virulence target protein (guardee) by guarding it for interaction with the effector. This model is well illustrated with effector proteins from *Pseudomonas syringae* and R proteins of *Arabidopsis thaliana* (Axtell and Staskawicz 2003; Mackey et al. 2003; Mackey et al. 2002; Shao et al. 2003; van der Linden et al. 2004). Yet recognition specificity does not always correlate with virulence activity. van der Hoorn and Kamoun (2008), therefore, came with a decoy model. In this model of evolution, guardees are subject to alternating selective pressures depending on the presence or absence of their guarding R protein. In the presence of the R protein, the guardee would be optimized for Avr interaction, and hence detection. In the absence of the R protein, the guardee would be under pressure to evade interaction with pathogen effectors in order to reduce pathogen virulence. The recent completion of the *P. infestans* genome (Haas et al. 2009) brings new opportunities to study the evolution of gene families of this pathogen. In a previous study we have postulated that the *R2* gene family emerged in Mexico (Chapter 3), one of the centres of origin of the pathogen (Grünwald and Flier 2005). In this respect, the interaction between Mexican *Solanum* species and *P. infestans* provides an excellent opportunity for functional and evolutionary studies of *R2 – PiAvr2/PexRD11* family interaction.

In this chapter, we examine the pattern of gene diversity of the *PiAvr2/PexRD11* family using the recently sequenced *P. infestans* genome of strain T30-4 (Haas et al. 2009). In total 13 different RXLR secreted proteins of the *PiAvr2/PexRD11* family were identified, and we tested their avirulence against 21 R2 variants originating from five Mexican *Solanum* species (Chapter 3). Our results show a robust association, with some differential specificity, between phylogenetic classification of the *R2* variants and members of the *PiAvr2/PexRD11* family. We also provide functional evidence of the co-evolution between the *R2* family and the cognate *PiAvr2/PexRD11* family.

Results

PiAvr2/PexRD11 effector family is highly diverse.

To identify specificity of recognition between *P. infestans* effectors and *R2* homologues, we first mined the *P. infestans* T30-4 genome for effectors with homology to *PiAvr2* and *PexRD11*. In a previous study we demonstrated that *PexRD11* has a functional redundancy with *PiAvr2* by being recognized by functional *R2* homologues of different *Solanum* genotypes. Although the overall sequence similarity between PiAVR2 and PEXRD11 is low (23%), a protein structure similarity of 63% emerged in the C-terminal domain based on identical, conserved and semi-conserved amino acids substitutions. A BLASTp search of the *P. infestans* T30-4 genome sequence database with the C-terminal domain of PiAVR2 and PEXRD11 resulted in the identification of 21 independent gene models, 13 out of 21 come from a BLASTp search using PiAVR2 as query and eight from BLASTp search using PEXRD11 as query. In total, 18 out of the 21 gene models when translated to proteins annotated as secreted RXLRs (Table 1). Gene sequence analyses of the 18 RXLR effectors revealed that *PiAvr2* and *PITG_22870*, *PITG_08278* and *PITG_20025, PITG21645* and *PITG_13956, PITG_23009* and *PITG_23008* are present as double copies in the T30-4 genome. PITG_21645 and PITG_23008, which have three amino acid differences between the signal peptide and the EER motif, are fully identical in their C-terminal domain, and therefore represent only one variant in this selection. The two BLASTp searches show very different homologous proteins with only one protein hit in common PITG_21949. Finally, we identified 11 non-redundant effectors in *P. infestans* T30-4 genome having some degree of similarity with the C-terminal domain of PiAVR2 and PEXRD11 (Table 2).

Neighbour-joining phylogenetic clustering of the C-terminal domain of 13 PiAVR2/PEXRD11 family members resulted in three classes that were designated with the suffix I, II, and III (Figure 1). Class I contains PEXRD11 and four other effectors (PITG_13940, _21949, _21645 and _13936) which are all from the RxLRfam7 previously described by Haas et al. (2009), and have a 60 to 62.6% similarity with PEXRD11 (Table 2). Class II contains PiAVR2 and PITG_05121 with 43% of similarity. Class III contains the six remaining effectors which are more divergent with only 30.4 to 41.3% of similarity to PEXRD11 and PiAVR2 (Table 2).

Specificities of recognition of R2 homologues with class I and II PiAVR2/PEXRD11 family effectors.

In previous studies, we showed that *PexRD11* and *PiAvr2* specifically trigger an *R2*-mediated cell death with ten homologues from *S. edinense*, *S. schenckii*, *S. hjertingii*, *S. demissum*, *S. bulbocastanum* and two breeding clones, but not with 11 other *R2* variants (Chapter 3). To assess whether the newly identified *PiAvr2/PexRD11* family members are also recognized by *R2* homologues, their C-terminal domain was synthesized and inserted in

Table 1: List of genes in *Phytophthora infestans* genome T30-4 with sequence similarity to the C-terminal effector domain of PiAVR2 and PEXRD11.

A

Gene Name	Genome location	Gene description	Score (Bits)	E-value	Alignment length	Identities in percentage (%)	Positives
PITG_22870	Supercontig 16: 1903493-1903843	Secreted RxLR effector peptide, putative	117.6	$4.99e^{-28}$	100	100	100
PiAvr2 (PITG_08943)	Supercontig 16: 1901141-1901491	Secreted RxLR effector peptide, putative	117.6	$4.99e^{-28}$	100	100	100
PITG_05121	Supercontig 7: 459447-459788	Secreted RxLR effector peptide, putative	43.1	$1.3e^{-5}$	80.4	41.2	51
PITG_19617	Supercontig 99: 380489-380845	Secreted RxLR effector peptide, putative	40	$1.13e^{-4}$	64.7	33.3	47.1
PITG_07500	Supercontig 11: 1294260-1294616	Secreted RxLR effector peptide, putative	38.1	$2.1e^{-4}$	64.7	31.4	49
PITG_07499	Supercontig 11: 1290401-1290757	Secreted RxLR effector peptide, putative	38.1	$2.1e^{-4}$	64.7	31.4	49
PITG_08278	Supercontig 13: 612373-612729	Secreted RxLR effector peptide, putative	37.3	$7.23e^{-4}$	64.7	31.4	45.1
PITG_20025	Supercontig 137: 149824-150180	Secreted RxLR effector peptide, putative	37.3	$7.23e^{-4}$	64.7	31.4	45.1
PITG_15972	Supercontig 39: 1376315-1376599	Secreted RxLR effector peptide, putative	36.9	$9.84e^{-4}$	51	33.3	39.2
PITG_16221	Supercontig 43: 623613-625594	Crinkler (CRN) family	29.7	0.138	64.7	27.5	29.4
PITG_00323	Supercontig 1: 1810206-1813748	Conserved hypothetical protein	29.3	0.188	78.4	25.5	47.1
PITG_21949[*]	Supercontig 811: 3517-3861	Secreted RxLR effector peptide, putative	21.3	0.263	23.5	15.7	17.6
PITG_06077	Supercontig 9: 3123662-3124018	Secreted RxLR effector peptide, putative	28.4	0.349	49	21.6	35.3

B

Gene Name	Genome location	Gene description	Score (Bits)	E-value	Alignment length	Identities in percentage (%)	Positives
PexRD11 (PITG_13930)	Supercontig 30: 1248179-1248523	Secreted RxLR effector peptide, putative	121	$4.46e^{-29}$	100	100	100
PITG_21949[*]	Supercontig 811: 3517-3861	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	100	51	74.5
PITG_21645	Supercontig 559: 43867-44211	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_23009	Supercontig 30: 1532184-1532528	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_23008	Supercontig 30: 1436877-1437221	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_13956	Supercontig 30: 1470702-1471046	Secreted RxLR effector peptide, putative	66.2	$1.4e^{-12}$	96.1	51	70.6
PITG_13940	Supercontig 30: 1349582-1349926	Secreted RxLR effector peptide, putative	63.6	$9.06e^{-12}$	100	47.1	74.5
PITG_13936	Supercontig 30: 1300754-1301044	Secreted RxLR effector peptide, putative	49.2	$1.92e^{-7}$	64.7	39.2	47.1

A BLASTp search using C-terminal effector domain of PiAvr2 (A) and PexRD11 (B) was done on the Broad Institute website (*SolR*gene database). ([*]) Indicate genes found in both BLASTp search.

Table 2. Percentage similarity on amino acids level between the 11 new PiAVR2/PEXRD11 family members vs. PiAVR2 and PEXRD11.

Percent of Similarity at the Amino Acid Level		Phylogenetic Class
	PiAVR2	PEXRD11	
PITG_13940	30	60	
PITG_21949	30	62.6	
PITG_21645	29.1	60	I
PITG_13936	34.8	62.9	
PEXRD11	33.6	100	
PITG_05121	43	28.7	II
PiAVR2	100	33.6	
PITG_06077	34.5	30.4	
PITG_08278	33.6	33.9	
PITG_19617	33.6	33.9	III
PITG_15972	41.3	39.1	
PITG_07499	34.5	34.8	
PITG_07500	34.5	35.7	

Percentages of similarity are based on the C-terminal domain of the effector proteins. The three classes described in the phylogenetic tree (Figure 1) are represented on the right.
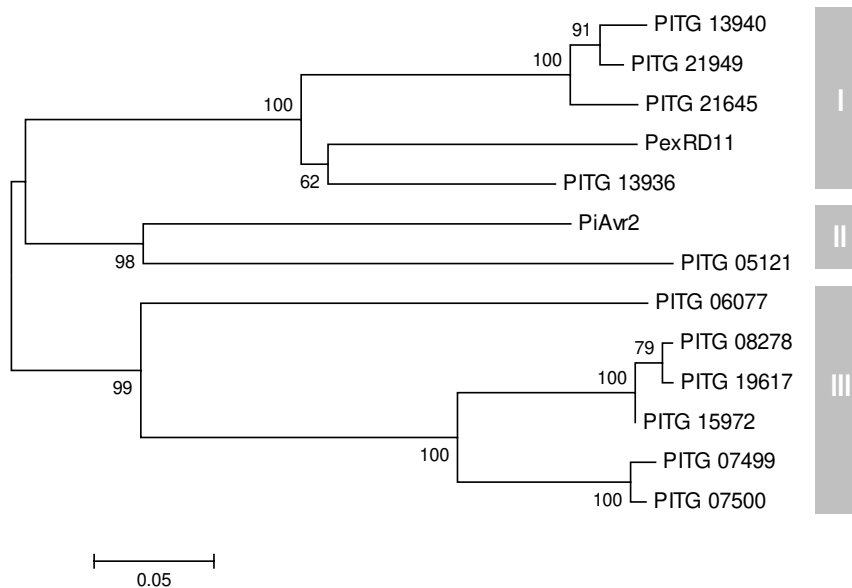
Figure 1. Phylogenetic relationship and clustering of *PiAvr2/PexRD11 family* members resulting in three classes I, II and III.
Neighbour-joining tree illustrating phylogenetic relationship of *PiAvr2/PexRD11 family* members in *P. infestans* strain T30-4. Amino acids sequences are grouped in three classes. Bootstrap values greater than 60% (based on 1000 resample) are indicated at the nodes.

plasmid pJL-TRBO vector (Lindbo 2007). *Agrobacterium tumefaciens* cultures expressing individual PiAVR2/PEXRD11 members were coinfiltrated with *Agrobacterium* cultures expressing R2 variants in *N. benthamiana* leaves. Four types of responses were observed. The first type of response, observed with PITG_21949 and PITG_21645 (Figure 2), is the typical PiAVR2 avirulence activity that show induced cell death specificity to ten *R2* homologues (Chapter 3). In the second type of response we observed a differential specificity of recognition to PITG_13940. Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 and Rpi-hjt1.3 triggered HR when coinfiltrated with the C-terminal domain of PITG_13940, but no cell death was induced with R2, R2-like, Rpi-abpt and Rpi-blb3. In the third type of response, no cell death with any R2 variant was detected, this correspond to all class III PiAVR2/PEXRD11 members, in addition to PITG_13936 of class I, and PITG_05121 of class II. PITG_13936 is a slightly mutated and truncated form of PEXRD11 and it is present in the PEXRD11 locus (Table 1 and Figure 3). PITG_05121 belongs to the same class as PiAVR2 (Figure 1), and it is located in a different locus (Table 1). Interestingly, PITG_05121 has a unique three amino acid deletion starting after a.a. position 79 which seems relatively conserved among the five functional PiAVR2/PEXRD11 family members and the truncated PITG_13936, a KIT/KIK/PIK motif (Figure 3). The last type of response is described as a high and low degree of auto-necrosis induction, detected for PITG_07499 and PITG_06077 that belong to class III (Figure 2). In conclusion, five members of the PiAVR2/PEXRD11 family were identified to display PiAVR2 activity, of which PITG_13940 showed a differential recognition among *R2* homologues.
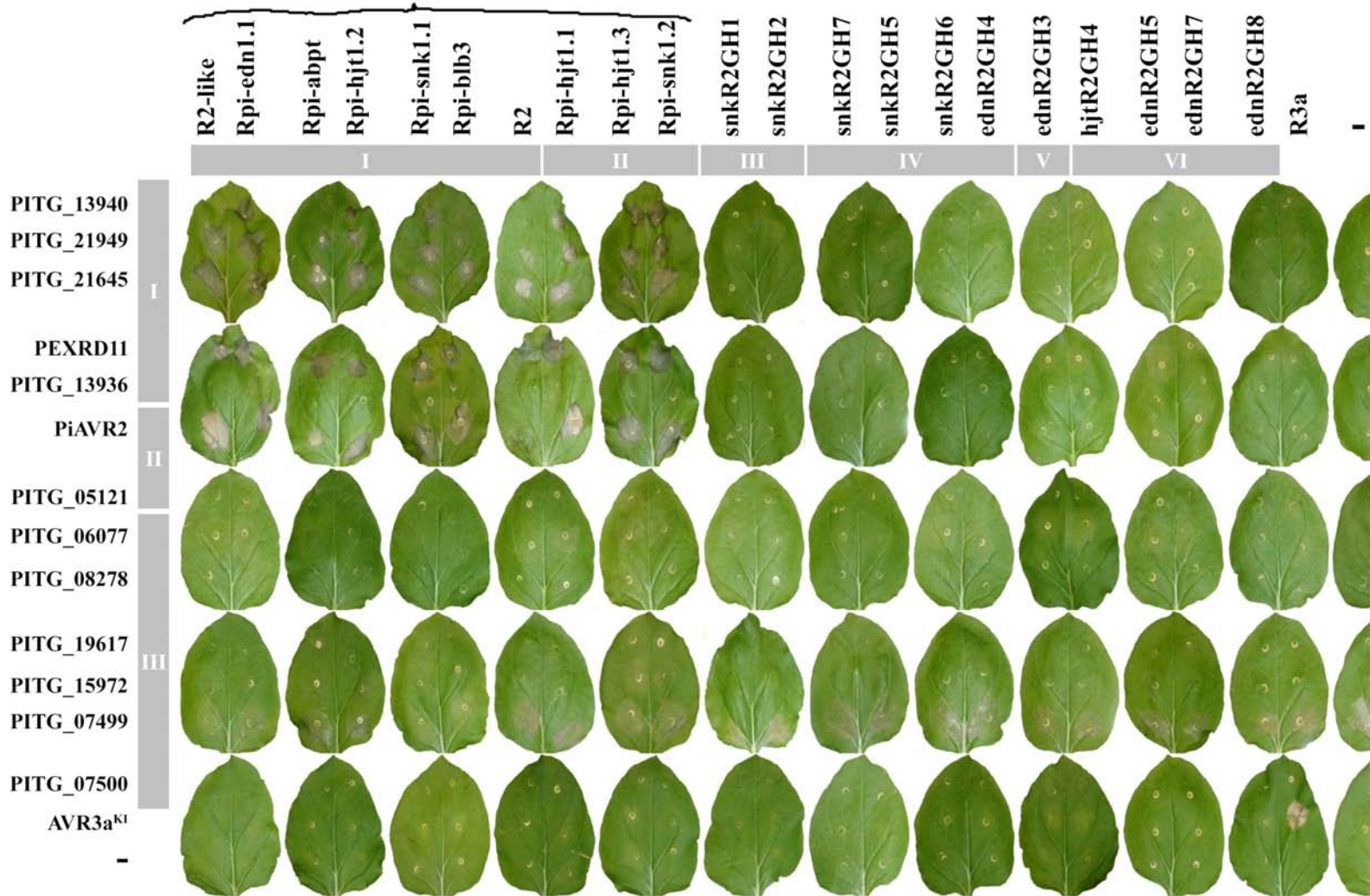
Figure 2. Specificity of recognition between R2 homologues and C-terminal effector domain of the PiAVR2/PEXRD11 family.
Co-infiltration between C-terminal effector domain of PiAVR2/PEXRD11 family members and AVR3a[KI] versus R2 variants and R3a. The grey blocks on the top of the figure indicate the R2 variants classes as described in Chapter 3, with the ten functional R2 homologues of class I and II. The grey blocks on the left of the figure indicate the PiAVR2/PEXRD11 family member classes as described in Figure 1. (*) Functionality against *P. infestans* demonstrated in Chapter 3. R3a and AVR3a[KI] were included as controls and each construct was infiltrated alone to detect any auto-necrosis function. Photographs of symptoms were taken at four days post infiltration (dpi).

```
PITG_13940  ⁶⁴TKGFKEKLKAVLEKITPKKSLDKLKETVVTTLDWVRIVKHKTDEAKRLGFK----¹¹⁴
PITG_21949  ⁶⁴TKGFKEKLKAVVEKITPKKSLDKLKETVVTTLDWVKIVKHKTDEAKRLGFK----¹¹⁴
PITG_21645  ⁶⁴AKGFKEKLKAVVEKITPKKSLEKLKETVISTPDWAKIVKHKTDEAMRLGYK----¹¹⁴
PexRD11     ⁶⁴TGGLLDKIKSVVKKITPEKAVTKFKEKDITNPEWLKIIKHKVREAKGQGYK----¹¹⁴
PITG_13936  ⁶⁴TGGFMDKLKSVAEKIKPSKAVEKVKEMVVTKPE--------------------⁹⁶
PiAvr2      ⁶⁶GFSLKDTLKKLNPIKAAVKAKDKAKEVTEKITD--ADWKKLVEHLKIKGDKRS--¹¹⁶
PITG_05121  ⁶⁷IVSWPEVLKKVT---AARSAKMKVRKVTEKFED--PVSKKLVEHLRVYRDKGS--¹¹⁴
PITG_06077  ⁶⁷--TIGNAILEAAKKLDPVEAVKKAKE-AAKRKKSILETMKLQAWLEKMRETIMKD¹¹⁸
PITG_08278  ⁶⁶--GFGGALADGLKKPNPAKAAKKAKEKAAKIKQDLKEIGEHAAWLEKMRETIGKD¹¹⁸
PITG_19617  ⁶⁶--GFGGALADGLKKLNPAKAAKKAKEKAAKIKQDLKEIGEHAAWLEKMRETIGKD¹¹⁸
PITG_15972  ⁶⁶--GFGGALADGLKKLNPAKAAKKAKEKSCEN-----------------------⁹⁴
PITG_07499  ⁶⁶--GLGGALVDGVKKLNPITAAKKAKEKAEKIKQHVKELGKYEDWLEEVREAIDKD¹¹⁸
PITG_07500  ⁶⁶--GFGGALVDGVKKLNPITAAKKAKEKAEKIKQHVKELGKYEDWLKEVREAIDKD¹¹⁸
                       :         .   :   *  ::
```

Figure 3. Effectors of PiAVR2/PEXRD11 family recognized by R2 homologues contain a NES motif.
ClustalW alignment of C-terminal effector domain of the 13 effectors from the PiAVR2/PEXRD11 family. (*) Residues in that column are identical in all sequences in the alignment, (:) conserved and (.) semi-conserved substitutions have been observed. Highlighted in grey are the Nuclear Export Signal (NES) predicted by NetNES 1.1 webserver. The Box highlights the KIT/KIK/PIK motif. Arrows pinpoint the difference in two amino acids between PITG_13940 and PITG_21949.

PiAVR2/PEXRD11 proteins recognised by R2 homologues contain a putative Nuclear Export Signal motif (NES)

To investigate the differential response of PITG_13940 among the ten R2 homologues, we compared the C-terminal domain of PITG_13940 and the closest related effector in the PiAVR2/PEXRD11 family, PITG_21949. Only two amino acid changes were identified (Figure 3), i.e. in position 75 a valine is replaced by a leucine residue and in position 99 a lysine is replaced by an arginine in PITG_13940. These amino acid changes could be responsible for structural changes and explain the differential responses between the six Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 and Rpi-hjt1.3 versus R2, R2-like, Rpi-blb3 and Rpi-abpt to recognize PITG_13940.

To determine if the specificity of cell death induced by PITG_21949, PITG_21645, PITG_13940, PEXRD11 and PiAVR2 with the ten R2 homologues was due to the presence of a common motif recognized by the R genes, we aligned the C-terminal domain of all effectors of PiAVR2/PEXRD11 family using ClustalW and searched for any known motif present in each member of the family. No W, Y or L motif typical to *Phytophthora* effectors were identified (Jiang et al. 2008), nor a new motif specific to this family (Figure 3). Interestingly, we identified sequences with similarity to the Nuclear Export Signal motif (NES) in all five effectors with PiAVR2 activity and also in the PITG_05121, which did not trigger HR with any of the R2 variants. Based on this result, we speculated that NES motif might be important for the effector activity of PiAVR2. Since none of the point mutations in PITG_13940 were identified in this NES motif, the escape of recognition of this effector to R2, R2-like, Rpi-abpt and Rpi-blb3 blb3 is probably due to another mechanism, such as

secondary structure changes which affect its interaction with these R2 homologues or their guardee.

Discussion

With the release of *P. infestans* T30-4 annotated genome (Haas et al. 2009), a wealth of effector genes with positional information became available. In this study, we identified 11 new effectors from *P. infestans* with a C-terminal domain structure related to the recently identified avirulent effectors PiAVR2 and PEXRD11 against *R2* homologues (Lokossou et al. 2009; Chapter 3). Our approach was based on the use of the C-terminal domain of these effectors because it is often dedicated to the effector function and is the target of positive selection (Win et al. 2007). The PiAvr2/PexRD11 variants were grouped in three classes. Interestingly all effectors from the class I and II, except PITG_13936 and PITG_05121, induced HR with different specificity when coinfiltrated with R2 variants from class I and II (Chapter 3). We uncovered, therefore, the first robust association between phylogenetic classes of the *PiAvr2/PexRD11* effector family and *R2* family with recognition leading to HR cell death. These data provide a first functional support for the arms-race model in the *Solanum*/*P. infestans* pathosystem for the R2-PiAVR2 interaction.

Here we have identified three classes of the PiAVR2/PEXRD11 family, where members of class I/II are triggering cell death in the presence of R2 homologues with different specificity. For three members of class I/II, recognition is partly or fully avoided, for which several evolutionary mechanisms could be involved. (i) Mutation of two amino acids already led to specific escape of recognition of PITG_13940, with the R2, R2-like, Rpi-abpt and Rpi-blb3 proteins. Similar findings were reported for AVR3a of *P. infestans* where the AVR3a[KI] form is recognised by the resistance gene R3a and the AVR3a[EM] form is not (Armstrong et al. 2005), but both effector variants can still interact with the same effector target protein, CMPG1, an ubiquitin E3 ligase required for INF1-mediated cell death (Birch et al. 2009; Gonzalez-Lamothe et al. 2006). From a co-evolutionary point of view, this strategy is partially successful for *P. infestans*, since there are only six R2 homologues that recognise PITG_13940. (ii) Truncation of the *Avr* gene leading to recognition avoidance was found for PITG_13936. This mechanism is also described for the *Avr4* effector of *P. infestans,* of which the 'virulent' allele present at the *Avr4* locus encodes a truncated protein, and therefore isolates with the virulent form only can infect Ma-R4 plants (van Poppel et al. 2008; van Poppel et al. 2009). (iii) The third loss of recognition, by PITG_05121 is more puzzling, but perhaps it could be due to a three amino acid deletion, which then has the same mechanism as (i), or the accumulation of 24 mutations (51%) compared to PiAVR2 sequence, has created a new effector with different function for the pathogen. In conclusion, we have identified ten R2 homologues, present in multiple copies in wild *Solanum* species,

responding to five effectors of the PiAVR2/PEXRD11 family, present in the genome of *P. infestans*, which represent a high number of R/AVR combinations within host/pathogen interaction events, and therefore, demonstrates a real arms-race (Bergelson et al. 2001; Dawkins and Krebs 1979) between potato and *P. infestans*.

At the biochemical/structural level, the specificity of PITG_13940 that caused cell death when co-expressed with six R2 homologues (Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2, and Rpi-hjt1.3), but not with the four other R2 homologues (R2, R2-like, Rpi-abpt and Rpi-blb3) can have two origins. The absence of HR can either be due to a fast recognition inducing Extreme Resistance (ER) response, as between Potato Virus X and Rx (Baurès et al. 2008; Bendahmane et al. 2002; Kang et al. 2005), or to the lack of interaction between PITG_13940 and these four R2 homologues. The failure of HR induction with the four R2 homologues can be explained by various hypotheses. Structural changes of PITG_13940 may prevent direct interaction with the four R2 homologues, or interaction with the guardee or decoy protein, as required for the gene-for-gene (Flor 1971), guard, and decoy models (van der Biezen and Jones 1998; van der Hoorn and Kamoun 2008). An alternative hypothesis is based on the bait and switch model (Collier and Moffett 2009). In that case PITG_13940 can interact with the effector target, but there is no transduction of signal response because the LRR domain of R2, R2-like, Rpi-abpt and Rpi-blb3 do not interact with this effector. On the side of the host R genes, however, we could not identify obvious SNPs that discriminate the six active R2 homologues from the four R2 homologues that did not induce cell death with PITG_13940. Therefore, recognition of PITG_13940 without transduction of resistance signal may be less supported. Future studies on protein folding structure differences between the ten R2 homologues may provide more information. On the side of the effector genes supplied by the pathogen, we identified only two a.a. difference between PITG_13940 and PITG_21949 (Figure 3), and since especially the K at position 99 seems rather conserved within the functional PiAVR2/PEXRD11 members, these amino acids may play an important role in PiAVR2 effector activity. Further studies, regarding protein stability, protein-protein interactions by coimmunoprecipitation and protein localisation in the host cell (Schornack et al. 2009a) will provide clues to unravel the mechanism of this specificity.

The identification of a NES motif on all effectors with PiAVR2 activity provides new insights in effector biology. We cannot yet conclude that the NES motif is required to trigger a programmed cell death in the presence of R2 homologues, however, we postulate that it has an important function in avirulence activity, for example by targeting proteins in the cytoplasm of host cells from *Solanum*. We hypothesize that R2 homologues - PiAVR2/PEXRD11 effector family recognition is due to the constrained function of PITG_13940, PITG_21949, PITG_21645, PiAVR2 and PEXRD11, which are cytoplasmatically localized *in planta* due to their NES motif, and we postulate that PITG_05121 escape of recognition is either due to its secondary structure or instability *in*

planta. The five functional PiAVR2/PEXRD11 variants are then exposed to the R2 proteins that belong to the cytoplasmic resistance gene family, and onset of defence triggering can occur. Protein stability studies and future comparisons with other *Avr* genes carrying RXLR motif could restrain the quest of new *Avr* genes of *P. infestans* to the RXLR effectors expressed at early stages of infection and possessing a NES motif.

Our findings provide new support for the co-evolutionary R2-PiAVR2/PEXRD11 arms-race model in the potato/*P. infestans* pathosystem. The wild *Solanum* species *S. edinense*, and *S. hjertingii* contain a mix of R2 homologues, with two and four functional variants in their genome respectively (Chapter 3), that are or not able to detect PITG_13940. Perhaps, we are facing an illustration of diversifying selection in *Solanum* evolution. In addition to adding copy numbers of an *R* gene family to increase the transcript level (Bradeen et al. 2009; Kramer et al. 2009) wild *Solanum* species keep different copies with broader recognition with an effector family to optimise the resistance to late blight.

In conclusion, our study reveals a gain of function for the six R2 homologues, Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2, and Rpi-hjt1.3, which are able to detect PITG_13940, while R2, R2-like, Rpi-abpt and Rpi-blb3 can not. Therefore it would be of interest to study the expression level of PITG_13940 in *P. infestans* isolates from potato growing areas and Mexico. Interestingly the four R2 homologues used in potato breeding programs in the last 60 years (Lokossou et al. 2009; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c) are those which do not detect the PITG_13940 effector of the PEXRD11 family. Specific functional characterisation of *R2* homologues using *PiAvr2/PexRD11* effector family has brought six new *R2* homologues variants with broader resistance spectrum, which provide more diversity and specificity for potato breeding.

Material and Methods

Microbial Strains, Plants, and Culture Conditions

Escherichia coli DH5α and DH10B and *Agrobacterium tumefaciens* strain GV3101 and AGL1 (Hellens et al. 2000; Lazo et al. 1991) were routinely grown in Luria-Bertani (LB) media (Sambrook and Russell 2001) with appropriate antibiotics at 37 and 28 ˚C, respectively. All bacterial DNA transformations were conducted by electroporation using standard protocols (Sambrook and Russell 2001). *A. tumefaciens* strain AGL1 (Lazo et al. 1991) was transformed with individual binary vectors in combination with the ternary plasmid pBBR1MCS-5.*virGN54D* (Fits et al. 2000). *Nicotiana benthamiana* plants were grown and maintained at 22 to 25 ˚C in controlled greenhouse under 16/8-h light-dark photoperiod.

Gene mining and cloning of *PiAvr2/PexRD11* effector family

The *PiAvr2/PexRD11* family genes were mined from the *Phytophthora infestans* T30-4 genome annotation (Haas et al. 2009) available through the Broad Institute website at http://www.broad.mit.edu/annotation/genome/phytophthora_infestans. BLASTp search was run with the C-terminal of PiAVR2 (a.a. 66 to 116) and PEXRD11 (a.a. 64 to 114). To perform Blast searches we used as criteria an E-value cutoff at 10 using a BLOSUM62 matrix, no filter, and an ungapped alignment. To the DNA fragments of the C-terminal domain a sequence corresponding to the FLAG tag "MDYKDDDDKVKLLENS" was added on the N-terminus, in respect of future protein stability studies. Constructs were synthesized using GenScript USA Inc. Generated DNA fragments were inserted into the PacI and NotI cloning sites of Tobacco mosaic virus binary vector pJL-TRBO (Lindbo 2007). All R2 variants used came from earlier studies (Lokossou et al. 2009) and constructs were in the pKGW-MG vector (kindly provided by Dr. Andreas Untergasser, Laboratory of Molecular Biology, Wageningen, The Netherlands) in between *Rpi-blb3* regulatory elements. *Avr3a^{KI}*, and *R3a* used as positive controls were inserted in pGRAB plasmid (kindly provided by Dr. Sean Chapman, SCRI, Dundee, UK) under control of the 35S promoter and terminator.

Phylogenetic analysis of *PiAvr2/PexRD11* effector family

Phylogenetic analyses of the PiAVR2/PEXRD11 variants from *P. infestans* genome T30-4 was conducted using the Neighbour-joining method (Nei and Li 1979) in MEGA version 4 (Tamura et al. 2007). Bootstrap values greater than 60% from 1000 replicate trees are shown at the nodes. Horizontal branch lengths and scale bar correspond to evolutionary distances assigned by MEGA. The evolutionary distances are measured as the proportion of nucleotide substitutions between sequences (Tamura et al. 2007). Percentages of similarity in Table 2 were calculated using using DNASTAR Lasergene v8. ClustalW analyses were performed on the EMBL-EBI website: http://www.ebi.ac.uk/Tools/ using ClustalW and protein on the CBS (Center for Biological Sequence Analysis) website: http://www.cbs.dtu.dk/ using the NetNES 1.1 webserver predicts leucine-rich nuclear export signals (NES) in eukaryotic proteins using a combination of neural networks and Hidden Markov models.

Transient expression in planta

Agroinfiltration (*A. tumefaciens* infiltration) experiments were performed on four- to five-weeks old *N. benthamiana* plants using previously described methods (Champouret et al. 2009). The cells were collected by centrifugation (2,500 r.p.m., 15 min, and 16°C). *A. tumefaciens* strains carrying the respective constructs were mixed in a 1:1 ratio in inducing media (10 mM MgCl2, 10 mM MES, pH 5.6, and 200 mM acetosyringon), and then incubated at room temperature in the dark for 2 to 3 hours before infiltration. *A. tumefaciens* solutions were infiltrated at an OD600 of 0.2. All clones were inoculated on six leaves

divided over two plants and two repeat experiments were made. Symptoms were monitored at three to six days after infiltration (dai).

CHAPTER 5

Functional allele-mining with *Avr3a* reveals active *R3a* in *S. stoloniferum* and hints at two centres of *R3a* evolution in Mexico and Argentina

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Functional allele-mining with *Avr3a* reveals active *R3a* in *S. stoloniferum* and hints at two centres of *R3a* evolution in Mexico and Argentina

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Abstract

Functional allele mining with effectors facilitates the discovery of novel active resistance gene homologues (*RGH*) and sheds light on the evolution of resistance (*R*) genes. *R3a* from *Solanum demissum* is known to interact with the avirulence gene $Avr3a^{KI}$ of *P. infestans*. Here we used the two known allelic variants $Avr3a^{KI}$, and $Avr3a^{EM}$, and the paralogue *Pex147-3* to probe 177 *Solanum* genotypes representing the broad *Petota* genepool, for *R3aGH*. We identified 73 genotypes responding with different patterns of responses to one or more AVR3a variants. Further functional studies by agroinfiltration and disease test using specific *P. infestans* isolates, led to nine *Solanum* genotypes containing putative functional *R3aGH*. All these plants were originating from two different centres of diversity, Mexico and Argentina. Allele mining on the Mexican *S. stoloniferum* 554-2 revealed 29 independent *R3aGH* sequences. Ten *R3aGH* were complete, and 19 appeared to be pseudo genes. Sequence analysis revealed multiple recombination sites that suggest high rates of evolution. One R3aGH, *Rpi-sto2,* triggered cell death when coinfiltrated with $AVR3a^{KI}$ on *N. benthamiana*, and therefore, is a functional *R3a* against *P. infestans* isolates. Monitoring the genetic diversity of *Avr3a* in *P. infestans* isolates revealed an over-representation of the virulence allele $Avr3a^{EM}$, and a new allele, $Avr3a^{KIL}$ was revealed. This study shows that *R3aGH* are under an evolutionary pressure and potentially co-evolved under pressure of two geographically distinct populations of *P. infestans*. In addition, we offer to potato breeder, new *R3aGHs* that can be used in future breeding programs or in molecular engineering to develop *R* genes with broader spectrum of effector recognition.

Introduction

Over the last century, potato breeders have introduced at least 11 late blight resistance (*R*) genes, designated *R1-R11*, from the Mexican hexaploid species *Solanum demissum* (Black et al. 1953; Malcolmson and Black 1966). In the last decade, multiple studies on other wild *Solanum* species has resulted in the localisation or cloning of 19 new <u>R</u>esistance genes against <u>*Phytophthora infestans*</u> (*Rpi*-genes) (Foster et al. 2009; Hein et al. 2009; Hein et al. 2007; Jacobs et al. 2010; Kuhl et al. 2001; Lokossou et al. 2009; Pel et al. 2009; Rauscher et al. 2006; Sandbrink et al. 2000; Smilde et al. 2005; Song et al. 2003; Tan et al. 2008; van der Vossen et al. 2003; van der Vossen et al. 2005; Villamon et al. 2005; Vleeshouwers et al. 2008; Wang et al. 2008). The interaction between potato and *P. infestans* follows the gene-for-gene model (Flor 1971). In this model, resistance is dependent on the presence of a gene in the pathogen that makes the pathogen avirulent, but only with the host genotype possessing the corresponding *R* gene. This gene from the pathogen is called avirulence gene (*Avr*). In the presence of a corresponding R protein, an Avr protein triggers a localized programmed cell death called the hypersensitive response (HR) culminating in disease resistance. One of the best studied *R* genes thus far is *R3a* (Huang et al. 2005), which occurs at the potato *R3* locus on Linkage Group (LG) XI, (Huang et al. 2004). Comparative genomic studies of the complex *R3* locus with the corresponding *I2* locus in tomato, harbouring major genes encoding resistance to the fungus *Fusarium oxysporum* (Ori et al. 1997; Simons et al. 1998), enabled the isolation of the cytoplasmic coiled-coil nucleotide binding site leucine-rich repeat (CC-NBS-LRR) protein *R3a* (Huang et al. 2004). *R3a* is located in a Major Late Blight (MLB) locus, where *R3b*, *R5 – R11* were also mapped, and is thought to be a hot spot of *Rpi* genes (Bradshaw et al. 2006b; El-Kharbotly et al. 1996; Huang et al. 2005; Huang et al. 2004; Pel et al. 2009). This locus is highly extended in *S. demissum* and harbours up to 45 type I *R3a* homologs per haplotype (Friedman and Baker 2007). Type I *R* genes show high rates of sequence exchange and correspondingly may have high homology between paralogs and high haplotypic diversity (variable gene copy number, disruption of synteny) (Kuang et al. 2005).

Recent studies have demonstrated that *P. infestans* is predicted to secrete hundreds of effector proteins that target two distinct sites in the host plant; the extracellular space with the apoplastic effectors and intracellular compartments with the cytoplasmic effectors (Bhattacharjee et al. 2006; Dou et al. 2008; Grouffaud et al. 2008; Haas et al. 2009; Kamoun 2006; Whisson et al. 2007). Oomycete cytoplasmic effectors are characterized by their N-terminal signal peptides followed by conserved motifs, RXLR, LXLFLAK, etc. (Birch et al. 2008; Birch et al. 2006; Kamoun 2006; Morgan and Kamoun 2007; Tyler 2009; Whisson et al. 2007). One of the best-studied oomycete RXLR effectors is *P. infestans Avr3a* (Armstrong et al. 2005). In early studies on a set of *P. infestans* isolates from all around the world, only two allelic variants of AVR3a were identified, named AVR3a^{KI} and AVR3a^{EM}, which differ by their amino acid (a.a.) in positions 80 and 103, respectively. In addition, two paralogues

called PEX147-3 and PEX147-2 were identified at the *Avr3a* locus (Armstrong et al. 2005). Among these AVR3a variants, only AVR3a^{KI} has been proven to have an avirulence activity in the presence of R3a (Armstrong et al. 2005). Structure-function studies by Bos et al. (2006) have demonstrated that the C-terminal half of AVR3a^{KI} is sufficient to trigger R3a-mediated hypersensitivity. In addition to its avirulence activity, a virulence function of AVR3a^{KI} was determined, namely to suppress the cell death induced by INF1 elicitin, an apoplastic protein of *P. infestans* with features of pathogen-associated molecular patterns (PAMPs) (Bos et al. 2006; Bos et al. 2009). Deletion of the C-terminal tyrosine of AVR3a^{KI} at position 147 abolishes its ability to suppress INF1-mediated cell death whilst not affecting R3a recognition (Bos et al. 2009). Recently, the CMPG1 protein, an ubiquitin E3 ligase required for INF1-mediated cell death (Gonzalez-Lamothe et al. 2006), has been identified as one of the interactors with both AVR3a^{KI} and AVR3a^{EM} (Birch et al. 2009). Since a virulence function seems concrete for both AVR3a variants, whereas avirulence is only reserved for AVR3a^{KI}, identification of an *R* gene responding to AVR3a^{EM} became an important challenge for potato breeders. Stacking such R3a^{EM} with R3a is expected to confer a broad-spectrum resistance to different *P. infestans* isolates.

Solanum section *Petota* species occur in the Andes of South America and a secondary centre of diversity exists in the central Mexican highlands (Hawkes 1990; Spooner et al. 2004). *Solanum* species sometimes easily hybridize with each other (Hawkes 1990; Jacobs et al. 2008; Spooner and Hijmans 2001), and therefore diploid, triploid, tetraploid, pentaploid and hexaploid species exist. Polyploid species can contain genes with high homology to genes in one of its progenitors, as for example found for the diploid *S. bulbocastanum* carrying *Rpi-blb1* and the polyploid *S. stoloniferum* containing the functional homologue *Rpi-sto1* and *Rpi-pta1* (Vleeshouwers et al. 2008; Wang et al. 2008).

For *P. infestans*, also two geographic centres of diversity have been described. First, the Toluca Valley in Mexico, where a sexual population with both the A1 and A2 mating types is present in similar frequencies and a high degree of molecular genetic diversity occurs (Flier et al. 2003; Fry et al. 1992; Goodwin and Fry 1994; Grünwald and Flier 2005). Second, South America based on endemic coexistence of distinct lineages as well as on mitochondrial and nuclear gene genealogies studies (Gomez-Alpizar et al. 2007). These studies suggest that endemic *P. infestans* isolates may have adapted to different host species prevalent in either centre. As a consequence, *R* loci that co-evolved with *P. infestans* in South American *Solanum* species are not necessarily expected to display functional similarity to those from the Central American species, and vice versa. Recently, we could determine that *R2* was functionally restricted to Mexican *Solanum* species (Chapter 3). From an evolutionary standpoint it would be of great interest to identify functional homologues which are derived from the two different centres of diversity.

In this study we decided to screen wild *Solanum* species from North, Central and South America and identified nine genotypes with putative functional *R3a* homologues (*R3aGH*)

originating from South (two *S. microdontum*) as well as Central America (three *S. demissum*, one *S. stoloniferum*, one *S. cardiophyllum* and one *S. ehrenbergii*). The last genotype identified is one *S. semidemissum* from unknown origin. This is the first recorded opportunity to study an *R* gene that evolved in both main centres of origin of *Solanum* species. Allele mining and sequence analyses on one selected *S. stoloniferum* genotype from Mexico revealed a great diversity of type I *R3aGH* and confirmed the presence of a functional *R3a* gene in this species.

Results

R3a/AVR3a-mediated cell death differ in agro-infection or -infiltration

To assess the spectrum and intensity level of R3a responses to the *Phytophthora infestans* effector AVR3a and its closest paralogue *Pex147-3,* we performed a coinfiltration using an *A. tumefaciens* strain containing *R3a* under its native expression elements, with *A. tumefaciens* strains containing *Avr3a^{KI}*, *Avr3a^{EM}* and *Pex147-3* independently in *Nicotiana benthamiana* leaves. After five days, a confluent cell death was observed in leaf panels that were co-infiltrated with AVR3a^{KI} but also with the paralogue PEX147-3 (Figure 1A). Also AVR3a^{EM} induced some necroses in one out of four co-infiltrated sites, but not as confluent as with AVR3a^{KI}, which is all similar to observations by Bos et al. (2006). Based on the agro-infiltration assays, we conclude that R3a quickly induces cell death at high frequencies when co-expressed with AVR3a^{KI} as well as Pex147-3, and interpret that as a strong or real HR whereas less frequent and less confluent necrosis of AVR3a^{EM} was interpreted as weak or back ground response.

Agroinfection, based on Potato Virus X system (PVX) is another *in planta* expression system which is generally more sensitive and can be used in a slightly higher throughput fashion. PVX agroinfection on *Solanum tuberosum* cultivar Desiree and *N. benthamiana* plants transformed with *R3a* expressed under native elements and wild type control plants with the two different forms of AVR and two controls (respectively pGR106::AVR3a^{KI}, pGR106::AVR3a^{EM}, pGR106::CRN2 and pGR106::empty). At 14 days post inoculation no HR was visible with the pGR106::empty and with pGR106::CRN2 HR was induced with all four genotypes, indicating that the experiment was successful (Torto et al. 2003). No visible HR was observed on *R3a* plants with pGR106::AVR3a^{KI}, but cell death induction was evident with pGR106::AVR3a^{EM} on the same plants (Figure 1B). These results are in line with the observation of Extreme Resistance (ER) induced with the Chilean *S. tuberosum* resistance protein Rx (Ross 1986) which recognizes PVX. Extreme resistance is seen with a number of *R* genes conferring resistance to viruses; it is manifested as a complete lack of both macroscopic and microscopic HR lesions and is accompanied by the absence of detectable virus accumulation (Bendahmane et al. 2002; Kang et al. 2005; Kohm et al. 1993). We

interpreted the absence of cell death from pGR106::AVR3a^{KI} as ER in *R3a* plants. In contrast, the generally weak HR described with AVR3a^{EM} on the *R3a* plants was now observed as a localized cell death, a typical HR, and we argue that this can happen due to extremely high expression levels inherent to the virus system used. We conclude that including *Pex147-3* paralogue and *Avr3a^{EM}* to *AVR3a^{KI}* would increases the chance to detect a functional *R3aGH* when using a PVX agroinfection with *Avr3a*.
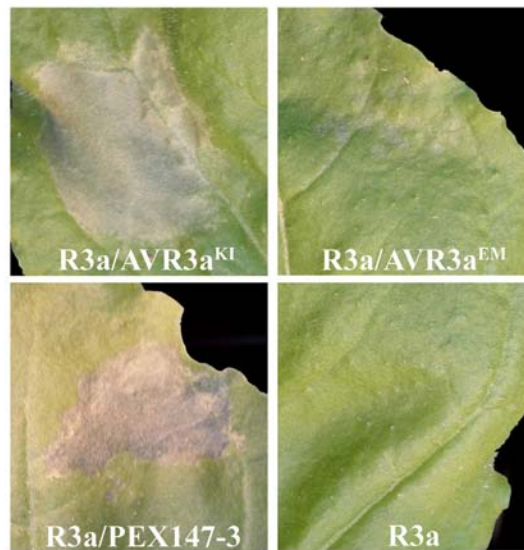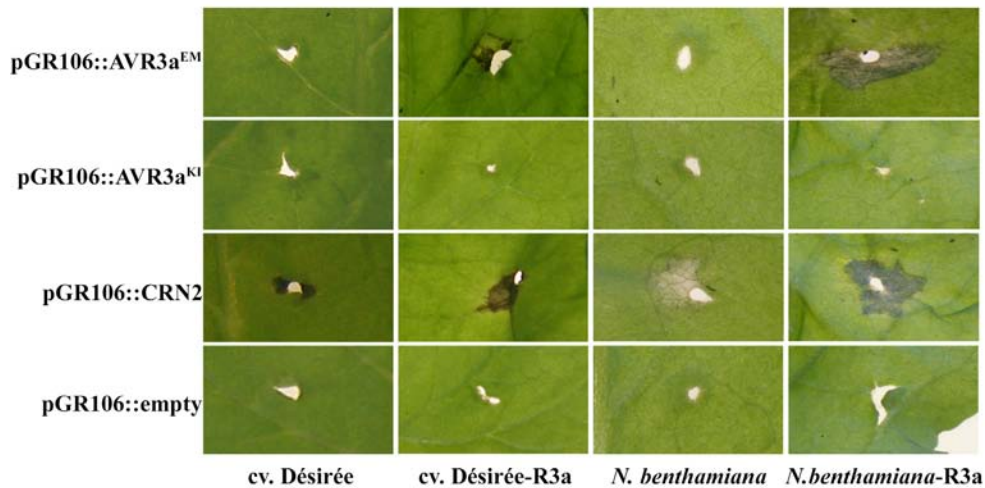
Figure 1. AVR3a triggers Hypersensitive Response or Extreme Resistance with R3a.
A) Leaves of *N. benthamiana* plants were infiltrated with *A. tumefaciens* strain COR308 carrying pBINplus::R3a alone or mixed with *A. tumefaciens* GV3101 carrying pGR106::AVR3a^{KI}, pGR106::AVR3a^{EM} or pGR106::PEX147-3. Photographs of symptoms were taken five days post infiltration. These are a representative infiltrated spots on leaves from multiple assays and experiments. B) Leaves of *S. tuberosum* cultivar Désirée, *N. benthamiana* and R3a-transformants were wound-inoculated with *A. tumefaciens* strainsGV3101 carrying pGR106::AVR3a^{EM}, pGR106::AVR3a^{KI}, and pGR106::CRN2 and pGR106::empty vector as positive and negative controls, respectively. No visible HR is observed with pGR106::AVR3a^{KI} on R3a-transformants due to ER responses. Local cell death to pGR106::AVR3a^{EM} was observed in both R3a-transformants plants but not on *S. tuberosum* cultivar Désirée and *N. benthamiana*.

Table 1. Functional allele-mining in *Solanum* for response to AVR3a[KI], AVR3a[EM] and PEX147-3.

Series [a]	Country of origin	Solanum species	CBSG genotype code	AVR3a[KI]	AVR3a[EM]	PEX147-3	neg. pGR106	pos. CRN2	Group
		S. tuberosum clone	SH83-92-488	+	+	+	-	+	
	Argentina	*S. acaule*	861-2	+	+	+	-	+	
	Bolivia	*S. acaule*	681-2	+	+	+	-	+	
	Mexico	*S. demissum*	343-1	+	+	+	-	+	
	Mexico	*S. acaule aemulans*	669-4	+	+	+	-	+	
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	299-2	+	+	+	-	+	
	Mexico [b]	*S. demissum* [c]	264-1	+	+	+	-	+	
	Mexico [b]	*S. demissum* [c]	364-2	+	+	+	-	+	
	Peru	*S. acaule punae*	441-4	+	+	+	-	+	
	Peru	*S. acaule*	513-5	+	+	+	-	+	
	Unknown	*S. semidemissum*	295-1	+	+	+	-	+	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. dolichocremastrum*	148-1	+	+	+	-	+	
	Peru	*S. immite*	172-4	+	+	+	-	+	
Cultivated Tuberosa and Tuberosa from Peru	Peru	*S. coelestipetalum*	135-1	+	+	+	-	+	
Demissa Conicibaccata	Colombia	*S. moscopanum*	720-1	+	+	+	-	+	
	Mexico	*S. schenckii*	293-2	+	+	+	-	+	1
Longipedicellata	United States	*S. fendleri*	596-2	+	+	+	-	+	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. lesteri*	358-4	+	+	+	-	+	
	Argentina	*S. microdontum*	715-1	+	+	+	-	+	
	Argentina	*S. microdontum*	360-1	+	+	+	-	+	
	Argentina [b]	*S. tarijense* [c]	280-4	+	+	+	-	+	
	Bolivia	*Unknown species* [c]	891-1	+	+	+	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. sparsipilum*	383-5	+	+	+	-	+	
	Bolivia	*S. sparsipilum*	383-4	+	+	+	-	+	
	Peru	*S. aracc-papa*	110-4	+	+	+	-	+	
	Peru	*S. aracc-papa*	109-1	+	+	+	-	+	
	Peru	*S. raphanifolium*	793-5	+	+	+	-	+	
	Argentina	*S. venturii*	250-2	+	+	+	-	+	
Tuberosa Megistacroloba	Argentina	*S. okadae*	367-1	+	+	+	-	+	
	Argentina	*S. okadae*	366-8	+	+	+	-	+	
	Peru	*Unknown species* [c]	253-1	+	+	+	-	+	
		S. tuberosum clone	Pentland Ace	+	+	-	-	+	2
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	488-1	+	+	-	-	+	
		S. tuberosum clone	Ma-R3	+	-	+	-	+	3
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	714-1	+	-	+	-	+	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. huacabambense*	354-1	-	+	+	-	+	
Longipedicellata	Mexico [b]	*S. stoloniferum* [c]	554-2	-	+	+	-	+	
	Unknown	*Unknown species* [c]	287-2	-	+	+	-	+	
	Guatemala	*S. clarum*	133-1	-	+	+	-	+	4
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Guatemala	*S. bulbocastanum partitum*	120-2	-	+	+	-	+	
	Mexico	*S. ehrenbergii*	153-3	-	+	+	-	+	
	Mexico	*S. michoacanum*	279-1	-	+	+	-	+	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Unknown	*S. neorossii*	281-2	-	+	+	-	+	
Tuberosa Megistacroloba	Argentina	*S. okadae*	366-2	-	+	-	-	+	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. cardiophyllum*	541-2	-	+	-	-	+	5
S. verrucosum	Mexico	*S. brachycarpum*	504-8	-	+	-	-	+	
Acaulia *S. demissum*	Bolivia	*S. acaule*	318-4	-	-	+	-	+	
	Peru	*S. albicans*	461-3	-	-	+	-	+	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. huacabambense*	354-2	-	-	+	-	+	
	Colombia	*S.colombianum*	574-3	-	-	+	-	+	
Demissa Conicibaccata	Costa Rica	*S. longiconicum*	180-3	-	-	+	-	+	
	Ecuador	*S. tundalomense*	247-1	-	-	+	-	+	
	Venezuela	*S. subpanduratum*	223-5	-	-	+	-	+	
Longipedicellata	Mexico	*S. stoloniferum*	835-3	-	-	+	-	+	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Bolivia	*S. neocardenasii*	734-2	-	-	+	-	+	
	Bolivia	*S. neocardenasii*	734-1	-	-	+	-	+	
	Argentina	*Unknown species* [c]	210-5	-	-	+	-	+	
	Argentina	*S. ruiz-leaii hybrid*	802-1	-	-	+	-	+	
	Argentina	*S. microdontum*	714-3	-	-	+	-	+	
	Argentina	*S. microdontum*	360-5	-	-	+	-	+	
	Argentina	*S. gourlayi vidaurrei*	626-2	-	-	+	-	+	
	Argentina [b]	*S. tarijense* [c]	280-1	-	-	+	-	+	6
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. gourlayi pachytrichum*	616-2	-	-	+	-	+	
	Bolivia	*S avilesii*	477-1	-	-	+	-	+	
	Bolivia	*S. doddsii*	144-3	-	-	+	-	+	
	Bolivia	*S. microdontum*	361-2	-	-	+	-	+	
	Bolivia	*S. chacoense*	544-2	-	-	+	-	+	
	Bolivia	*S. berthaultii*	481-3	-	-	+	-	+	
	Bolivia	*S. arnezii*	471-1	-	-	+	-	+	
	Argentina	*Unknown species* [c]	194-23	-	-	+	-	+	
	Bolivia	*S. megistacrolobum*	699-1	-	-	+	-	+	
	Peru	*S. mochiquense*	186-1	-	-	+	-	+	
Tuberosa Megistacroloba	Peru	*S. medians*	183-4	-	-	+	-	+	
	Peru	*S. multidissectum*	731-22	-	-	+	-	+	
	Peru	*S. humectophilum*	171-2	-	-	+	-	+	
	Unknown	*S. commersonii malmeanum*	266-2	-	-	+	-	+	

Table 2. *R3a* functional profiling by Agroinfiltration on the 73 wild *Solanum* genotypes.

Series [a]	Country of origin	Solanum species	CBSG genotype code	R3a/AVR3a[KI]	AVR3a[KI]	AVR3a[EM]	R3a	Group
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. tuberosum clone*	Ma-R3	+++	+++	+	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*Unknown species* [c]	891-1	++	++	++	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Guatemala	*S. microdontum*	715-1	++	++	++	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. clarum*	133-1	++	+	+	-	
		S. ehrenbergii	153-3	++	++	+	-	I
Acaulia *S. demissum*	Mexico [b]	*S. tuberosum clone*	Pentland Ace	+	+	+	-	
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	299-2	+	+	+	-	
Acaulia *S. demissum*	Unknown	*S. demissum* [c]	264-1	+	+	+	-	
Acaulia *S. demissum*	Mexico	*S. semidemissum*	295-1	++	+/-	+/-	-	
		S. demissum	343-1	+/-	+/-	+/-	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. tuberosum clone*	SH83-92-488	+++	+++	-	-	
Longipedicellata	Mexico [b]	*S. cardiophyllum*	541-2	+++	+++	-	-	II
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Bolivia	*S. stoloniferum* [c]	554-2	+++	+++	-	-	
Demissa Conicibaccata	Colombia	*S. neocardenasii*	734-2	+++	-	-	-	
Demissa Conicibaccata	Ecuador	*S. moscopanum*	720-1	+++	-	-	-	
Demissa Conicibaccata	Mexico	*S. tundalomense*	247-1	+++	-	-	-	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. schenckii*	293-2	+++	-	-	-	
Acaulia *S. demissum*	Peru	*S. dolichocremastrum*	148-1	+++	-	-	-	
Tuberosa Megistacroloba	Argentina	*S. acaule punae*	441-4	+++	-	-	-	
Tuberosa Megistacroloba	Argentina	*S. venturii*	250-2	++	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. okadae*	366-2	++	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Guatemala	*S. gourlayi pachytrichum*	616-2	++	-	-	-	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. bulbocastanum partitum*	120-2	++	-	-	-	
Tuberosa Megistacroloba	Peru	*S. immite*	172-4	++	-	-	-	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*Unknown species* [c]	253-1	++	-	-	-	
Acaulia *S. demissum*	Peru	*S. huacabambense*	354-1	++	-	-	-	III
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. albicans*	461-3	++	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Bolivia	*Unknown species* [c]	210-5	+	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. neocardenasii*	734-1	+	-	-	-	
Demissa Conicibaccata	Colombia	*S avilesii*	477-1	+	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S.colombianum*	574-3	+	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. lesteri*	358-4	+	-	-	-	
Tuberosa Megistacroloba	Peru	*S. aracc-papa*	110-4	+	-	-	-	
Circaeifolia Piurana Megistacroloba Tuberosa	Peru	*S. mochiquense*	186-1	+	-	-	-	
Longipedicellata	United States	*S. huacabambense*	354-2	+	-	-	-	
Longipedicellata	Unknown	*S. fendleri*	596-2	+	-	-	-	
Tuberosa Megistacroloba	Unknown	*Unknown species* [c]	287-2	+	-	-	-	
Demissa Conicibaccata	Venezuela	*S. commersonii malmeanum*	266-2	+	-	-	-	
		S. subpanduratum	223-5	+	-	-	-	
Acaulia *S. demissum*	Argentina	*S. acaule*	861-2	-	-	-	-	
Tuberosa Megistacroloba	Argentina	*Unknown species* [c]	194-23	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. ruiz-leaii hybrid*	802-1	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. sparsipilum*	383-5	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. sparsipilum*	383-4	-	-	-	-	
Acaulia *S. demissum*	Bolivia	*S. acaule*	681-2	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. doddsii*	144-3	-	-	-	-	
Tuberosa Megistacroloba but not clear due to polytomy	Bolivia	*S. megistacrolobum*	699-1	-	-	-	-	IV
Demissa Conicibaccata	Costa Rica	*S. longiconicum*	180-3	-	-	-	-	
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. michoacanum*	279-1	-	-	-	-	
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	364-2	-	-	-	-	
Cultivated Tuberosa and Tuberosa from Peru	Peru	*S. coelestipetalum*	135-1	-	-	-	-	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. aracc-papa*	109-1	-	-	-	-	
Tuberosa Megistacroloba	Peru	*S. medians*	183-4	-	-	-	-	
Tuberosa Megistacroloba	Peru	*S. multidissectum*	731-22	-	-	-	-	
Tuberosa Megistacroloba	Argentina	*S. okadae*	367-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa Megistacroloba	Argentina	*S. okadae*	366-8	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	360-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	360-5	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. gourlayi vidaurrei*	626-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	280-4	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	280-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	488-1	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	BOLIVIA	*S. microdontum*	361-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. chacoense*	544-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	481-3	Agro.	Agro.	Agro.	Agro.	V
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. arnezii*	471-1	Agro.	Agro.	Agro.	Agro.	
Acaulia *S. demissum*	Bolivia	*S. acaule*	318-4	Agro.	Agro.	Agro.	Agro.	
Acaulia *S. demissum*	Mexico	*S. acaule aemulans*	669-4	Agro.	Agro.	Agro.	Agro.	
S. verrucosum	Mexico	*S. brachycarpum*	504-8	Agro.	Agro.	Agro.	Agro.	
Longipedicellata	Mexico	*S. stoloniferum*	835-3	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Peru	*S. raphanifolium*	793-5	Agro.	Agro.	Agro.	Agro.	
Acaulia *S. demissum*	Peru	*S. acaule*	513-5	Agro.	Agro.	Agro.	Agro.	
Tuberosa Megistacroloba	Peru	*S. humectophilum*	171-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Unknown	*S. neorossii*	281-2	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	714-3	Agro.	Agro.	Agro.	Agro.	
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	714-1	i	i	i	i	

Table 1. (page 84) **Functional allele-mining in *Solanum* for response to AVR3a[KI], AVR3a[EM] and PEX147-3.**

73 *Solanum* genotypes belonging to various phylogenetic series (Jacobs et al. 2008) and from different countries, and three genotypes containing *R3a* (SH83-92-488, Pentland Ace, Ma-R3), were tested by PVX agroinfection for response to *A. tumefaciens* GV3101 containing, pGR106::AVR3a[KI], pGR106::AVR3a[EM], pGR106::PEX147-3, pGR106::empty vector and pGR106::CRN2. At least 16 replicates were inoculated, and (+) or (-) correspond to necrotic responses occurring or not on the inoculated sites. Leaves were monitored for developing symptoms during 16 days. Group I to VI were defined based on their pattern of response to each of the three effectors tested.
[a] Series according Jacobs et al. (2008)
[b] Deduced from Hawkes (1990)
[c] the label of this genotype was changed based on AFLP's data of Jacobs et al. (2008)

Table 2. (page 85) ***R3a* functional profiling by Agroinfiltration on the 73 wild *Solanum* genotypes.**

73 *Solanum* genotypes belonging to various phylogenetic series selected from the agroinfection screen, and three genotypes containing *R3a* (SH83-92-488, Pentland Ace, Ma-R3), were tested by agroinfiltration for response to pGRAB::AVR3a[KI], and pGRAB::AVR3a[EM]. The transient co-infiltration, used as positive control, of *A. tumefaciens* strains carrying pGRAB::R3a and pGRAB:Avr3a[KI] were performed. At least 2 leaves per plant and 3 plants per genotypes were tested. Scoring is represented by the following symbols, (-) no response, (+/-) small response visible on 15-30% of the infiltrated spots, (+) small, (++) clear, (+++) very clear responses on 100% of the infiltrated spots, (Agro.) background responses to *A. tumefaciens*. (i) inconclusive data.
[a] Series according Jacobs et al. (2008), [b] Deduced from Hawkes (1990), [c] the label of this genotype was changed based on AFLP's data of Jacobs et al. (2008)

Specific responses to PVX-AVR3a detected in various wild *Solanum* species

To identify wild *Solanum* species containing functional *R3a* homologues, we initiated a functional allele-mining approach with AVR3a[KI], AVR3a[EM] and PEX147-3. The pGR106::empty and pGR106::CRN2 were included as negative and positive controls, respectively. As positive control on the plant side, *S. tuberosum* clone Pentland Ace, SH83-92-488, and Mastenbroek R3 (Ma-R3) were included, since they contain a functional *R3a* (Armstrong et al. 2005; Huang et al. 2005; Huang et al. 2004). We selected a phylogenetically diverse set of 177 *Solanum* genotypes originating from North, Central to South America (Jacobs et al. 2008). In total, 73 wild *Solanum* genotypes showed a response to one of the three effectors, and six different patterns of responses (group 1-6) were observed (Table 1). Group 1 contains 30 wild *Solanum* species and the positive control SH83-92-488, and all these plants display an HR upon agroinfection with pGR106::AVR3a[KI], ::AVR3a[EM] and ::PEX147-3. The second group consists of *S. berthaultii* 488-1 and Pentland Ace, which both show specific cell death to pGR106::AVR3a[KI] and ::AVR3a[EM] but not to pGR106::PEX147-3. Group 3 consists of *S. microdontum* 714-1 and Ma-R3, which both show specific response to AVR3a[KI] and PEX147-3 but not to AVR3a[EM]. Since our three control plants arise in group 1-3, the identified 32 wild *Solanum* genotypes species might contain a functional R3aGH. Group 4, 5, and 6 consists of eight, three or 30 genotypes exhibiting responses to both AVR3a[EM] and PEX147-3, AVR3a[EM] only, or PEX147-3 only, respectively, but not to pGR106::AVR3a[KI]. These last 41 genotypes may either contain

R3aGH that caused ER to pGR106::AVR3a^{KI}, or they may contain a specific functional *R* gene interacting with *Avr3^{EM}*.

Response to AVR3a confirmed by agroinfiltration in 13 *Solanum* genotypes.

To validate the PVX agroinfection screen we performed an agroinfiltration on the 73 genotypes identified with pGRAB::AVR3a^{KI} and pGRAB::AVR3a^{EM}, and included SH83-92-488, Pentland Ace, Ma-R3 genotypes as positive controls. On each genotype, the R3a/AVR3a^{KI} co-infiltration was included as a positive control. Five patterns of responses were observed, represented by group I - V (Table 2). Group I contains eight wild *Solanum* genotypes, Pentland Ace and Ma-R3, which trigger HR upon infiltration with pGRAB::AVR3a^{KI} and pGRAB::AVR3a^{EM} at different levels of intensity. Group II, consists of two wild *Solanum* genotypes and SH83-92-488, show strong response to pGRAB::AVR3a^{KI} only. Since our three *R3a*-genotypes are in these first two groups, we postulate that the 12 *Solanum* genotypes in group I and II either contain an *R3aGH* responding to one or both allelic versions of *Avr3a*, or a second *R* gene interacting with *Avr3a^{EM}* in the case of genotypes from group I. Group III contains 26 genotypes all showing HR only to the positive control, and we conclude that these plants do not show any presence of functional *R3a*. Group IV and group V do not show any HR to the positive control in the first group, or show a response to the *A. tumefaciens* negative controls, respectively, indicating that the agroinfiltration on these 35 genotypes is inconclusive. *S. microdontum* 714-1 was also included in group V since results were inconclusive due to non-reproducible outcome between repeats.

Nine genotypes from Central and South America may contain a functional R3a homologue

To further study whether the responses to *AVR3a* alleles and *PEX147-3* is based on the presence of a functional *R3a* or another *R* gene, we continued with *P. infestans* isolates. First we determined the genetic variation of *Avr3a* and *PEX147-3* for a collection of 15 *P. infestans* isolates, that originated from European potato growing areas and the Central Highlands of Mexico (Champouret et al. 2009). PCR with conserved *Avr3a* primers (Table S1), on genomic DNA yielded amplicons of expected size which were cloned and a subset of 48 individual clones were sequenced. Sequence analysis revealed an over-representation of *Avr3a^{EM}* in *P. infestans* population, with ten isolates out of the 15 tested being homozygous for the virulent allele of *Avr3a* (Table 3). Also, one new variant of *Avr3a* in isolate PIC99189 and IPO-0 was discovered. This new variant has one amino acid difference compared to *Avr3a^{KI}* in position 139 where a methionine (M) is replaced by a leucine (L) and therefore we designated it *Avr3a^{KIL}* (Table 3).

Table 3. Occurrence of *Avr3a* variants and *Pex147-3* in different isolates of *Phytophthora infestans*.

P. infestans Isolate	Country of origin	$E^{80}M^{103}$	$K^{80}I^{103}$	$K^{80}I^{103}L^{139}$	Pex147-3
F95573	The Netherlands	x			x
PIC99177	Mexico	x			x
88069	The Netherlands	x			x
PIC99189	Mexico	x		x	x
90128	The Netherlands	x			x
EC1	Ecuador	x			x
H30P04	The Netherlands	x			x
USA618	Mexico	x			x
IPO-0	Unknown		x	x	x
IPO-C	Belgium	x	x		x
PIC99183	Mexico	x			x
NL01096	The Netherlands	x			x
VK98014	The Netherlands		x		x
IPO428-2	The Netherlands	x			x
NL00228	The Netherlands		x		x

To test specific resistance responses to the three AVR3a variants, we produced zoospore suspensions of IPO-0 (*Avr3a*^{KI} - *Avr3a*^{KIL}), PIC99189 (*Avr3a*^{EM} -*Avr3a*^{KIL}) and PIC99183 (*Avr3a*^{EM} homozygote) and inoculated the 73 selected genotypes from the agroinfection screen (Table 1) in addition to three *R3a* control genotypes (data not shown). All three *R3a* controls were resistant to both isolates IPO-0 and PIC99189, and susceptible to PIC99183, as expected. Overall susceptibility to all three *P. infestans* isolates was found for 41 wild *Solanum* genotypes, which were classified in group 1,2,4,5,6 (by agroinfection) and III – V (by agroinfiltration), and these plants were discarded from further analysis. Eleven more genotypes were susceptible to one of both isolates containing an *Avr3a*^{KI} form; as a result they were removed from the genotype pool studied. Twelve other genotypes were fully resistant to all three isolates; seven of them displayed no AVR3a response and showed adequate responses to the agroinfiltration controls (Table 2), and five showed responses to the *A. tumefaciens* negative controls (Table 2). In the first seven cases we expect the resistance to be due to another *R* gene, different from *R3aGH*, and did not include them for further study. In the second five cases we could not confirm R3a activity by agroinfiltration and set aside these genotypes for future studies (Table 4B). In total nine genotypes remained (Table 4A). Seven of these, i.e. *S. demissum* (dms) 299-2, *S. semidemissum* (sem) 295-1, *S. microdontum* (mcd) 714-1, *S. microdontum* (mcd) 715-1, *S. stoloniferum* (sto) 554-2, *S. cardiophyllum* (cph) 542-2 and *S. ehrenbergii* (ehr) 153-3 show an R3a-specific resistance to the *P. infestans* isolates, and similar responses to AVR3a as the R3a-genotypes, suggesting that these plants might contain functional R3a homologues. Two other genotypes dms343-1, and sem264-1 also displayed a similar pattern of responses to the *Avr3a* variants, and showed resistance even to *P. infestans* isolate PIC99183, suggesting presence of a functional *R3a* homologue that also responds to *Avr3a*^{EM} or another *R* gene responding to another effector in PIC99183.

Table 4. Functional profiling reveals the presence of *R3a* homologues in diverse *Solanum* species from Central and South America.

Series [a]	Country of origin	Solanum species	Species abbreviation	CBSG genotype code	Agroinfection					Agroinfiltration				*P. infestans* isolates		
					AVR3aKI	AVR3aEM	Pex147-3	pGR106 (neg.)	Crn2 (pos.)	R3a/AVR3aKI	AVR3aKI	AVR3aEM	R3a	IPO-0 (*Avr3a^KI* - *Avr3a^KII*)	PIC99189 (*Avr3a^KII* - *Avr3a^EM*)	PIC99183 (*Avr3a^EM*)
		S. tuberosum clone		SH83-92-488	+	+	+	-	+	+++	+++	-	-	R	R	S
		S. tuberosum clone		*Pentland Ace*	+	+	-	-	+	+	+	+	-	RQ	R	S
		S. tuberosum clone		Ma-R3	+	-	+	-	+	+++	+++	+	-	R	RQ	S
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	dms	299-2	+	+	+	-	+	+	+	+	-	R	R	S
Acaulia *S. demissum*	Unknown	*S. semidemissum*	sem	295-1	+	+	+	-	+	++	+/-	+/-	-	R	R	S
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	mcd	714-1	+	-	+	-	+	i	i	i	i	R	R	S
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina	*S. microdontum*	mcd	715-1	+	+	+	-	+	++	++	++	-	R	R	S
Longipedicellata	Mexico [b]	*S. stoloniferum* [c]	sto	554-2	-	+	+	-	+	+++	+++	-	-	R	R	S
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. cardiophyllum*	cph	541-2	-	+	-	-	+	+++	+++	-	-	R	R	S
Polyadenia Pinnatisecta Bulbocastana Morelliformia	Mexico	*S. ehrenbergii*	ehr	153-3	-	+	+	-	+	++	++	+	-	R	R	S
Acaulia *S. demissum*	Mexico	*S. demissum*	dms	343-1	+	+	+	-	+	+/-	+/-	+/-	-	R	R	R
Acaulia *S. demissum*	Mexico [b]	*S. demissum* [c]	dms	264-1	+	+	+	-	+	+	+	+	-	R	R	R
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	tar	280-4	+	+	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Tuberosa Megistacroloba	Argentina	*S. okadae*	oka	367-1	+	+	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Argentina [b]	*S. tarijense* [c]	tar	280-1	-	-	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Tuberosa from Bolivia, Argentina and Chile Yungasensa	Bolivia	*S. berthaultii*	ber	481-3	-	-	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R
Longipedicellata	Mexico	*S. stoloniferum*	sto	835-3	-	-	+	-	+	Agro.	Agro.	Agro.	Agro.	R	R	R

A) Solanum genotypes with confirmed *R3aGH* functional content due to the different phenotypes on the three biological assays. B) *Solanum* genotypes with *R3aGH* functional content inaccurate due to the results of agroinfiltration assays. Agroinfection and Agroinfiltration scoring are as shown in Table 1 and 2. Detached leaf assay: *Solanum* genotypes were challenged by three *P. infestans* isolates and scored for their Resistance (R), partial resistance (RQ) or susceptibility (S).

[a] Series according Jacobs et al. (2008)
[b] Deduced from Hawkes (1990)
[c] the label of this genotype was changed based on AFLP's data of Jacobs et al. (2008)

In summary, our functional profiling approach combined with resistance tests lead to the identification of nine diverse *Solanum* genotypes which are likely to contain a functional *R3a* homologue. These plants originate from four different phylogenetic series, i.e. Acaulia S. Demissum; Tuberosa from Bolivia, Argentina and Chile Yungasensa; Longipedicellata; and Polyadenia Pinnatisecta Bulbocastana Morelliformia; and two different main centres of origin, Mexico and Argentina.

Genetic and pathogenic studies support presence of a functional *R3a* gene in *S. stoloniferum* 22718-2

To genetically test the presence of a functional *R3a* homologue, *S. stoloniferum* 554-2 was crossed with the susceptible *S. stoloniferum* 297-1, which did not respond to $Avr3a^{KI}$ or $Avr3a^{EM}$ (Table 5A). We examined 50 individuals of the F1 population for response to *Avr3a* variants by agroinfiltration and observed a HR to $AVR3a^{KI}$ by each individual of the population. We also assayed all the individuals of the F1 progeny for segregation of resistance to two isolates of *P. infestans*, IPO-0 and 90128, which are avirulent and virulent, respectively, on R3a-plants. All individuals in the F1 population and sto554-2 were resistant to IPO-0, and all genotypes were susceptible to 90128 (Table 5B). Since no segregation in the progeny was detected, the responsible *R* gene is most likely present in a homozygous state in sto554-2, and conclusions on co-segregation for effector response and *P. infestans* resistance could not be drawn. However, the results were still in line with the hypothesis, that a functional *R3a* might be present in sto554-2.

Table 5. Reaction pattern and population study of *S. stoloniferum* 554-2 reveals the presence of a functional and homozygous *R3a* homologue in parent 554-2.

Solanum species	Genotype code	A Agroinfiltration				B *P. infestans* isolates	
		R3a/AVR3aKI	AVR3aKI	AVR3aEM	R3a	IPO-0 ($Avr3a^{KI}$ - $Avr3a^{KIL}$)	90128 ($Avr3a^{EM}$)
S. stoloniferum	554-2	+++	+++	-	-	R	S
S. stoloniferum	297-1	+++	-	-	-	S	S
Offspring							
Genotypes 1 to 50		+++	+++	-	-	R	S

A) Agroinfiltration: At least two leaves per plant and three plants per genotypes were tested. Scoring is represented by the following symbols, no response (-), (+++) very clear response on 100% of the infiltrated leaf panel. B) Detached leaf assay: *Solanum* genotypes were challenged by five *P. infestans* isolates and scored for their Resistance (R), or Susceptibility (S).

We then tested whether R3a-specific resistance was associated with specific Avr3a variants in the *P. infestans* isolates. We inoculated sto554-2 with ten *P. infestans* isolates that carry diverse *Avr3a* variants in homozygous or heterozygous state (Armstrong et al. 2005). Cultivar *S. tuberosum* cv. Impala and *S. bulbocastanum* 2002 containing the broad-spectrum *Rpi-blb2* gene were susceptible and resistant respectively to all isolates tested, which indicates that the assay was reliable. Sto554-2 was specifically resistant to the four isolates containing an *Avr3a^{KI}* or *Avr3a^{KIL}* and susceptible to the six isolates homozygous for *Avr3a^{EM}* (Table 6). We conclude that the resistance spectrum of sto554-2 supports the presumed presence of an *R3a* homologue.

Allele mining reveals a high diversity of *R3a* homologues in *S. stoloniferum*

To assess the diversity of *R3a* in sto554-2, we performed an allele mining approach. PCR analyses, using a combination of conserved primers for *R3a* and *R3a* paralogues (*I2GA-SH194-2*, *I2GA-SH23-1*, *I2GA-SH23-3*), on genomic DNA of genotype sto554-2 yielded amplicons of 3200 to 4000bp. *R3a* and *R3a* paralogues described by Huang et al. (2005) range from 3798 to 3984bp. In total 29 non-redundant sequences were cloned from this genotype and designated *stoR3aGH1* to 29. Sequence analysis revealed that two sequences did not have a stop codon (*stoR3aGH28* and *29*) and seventeen sequences possessed an additional early stop codon on their Coiled-coil (*stoR3aGH22* to *27*), NBS domains (*stoR3aGH15* to *21*) or first half of the LRR (*stoR3aGH11* to *14*) domains. In total ten full length sequences, from 3513 to 3984bp, with an *R* gene structure were identified (*stoR3aGH1* to *10*), with nucleotide identities from 87.2% to 99.9% compared to *R3a* and *R3a* paralogues (Table 7). StoR3aGH5 is most similar to R3a and has only four amino acids different, i.e. one in the CC domain, one in subdomain LRR18, and two in subdomain LRR21 (Figure 2). Another clone, stoR3aGH4, has only 89.2% of a.a. similarity with R3a (Table 7) and shows only three different a.a. compared to I2GA-SH23-3, a non-functional R3a paralogue described by Huang et al. (2005). Two sequences, stoR3aGH3 and stoR3aGH10 are synonymous and have only one nucleotide difference in their NBS domain (data not shown). Sequence alignment shows that recombination events have occurred in the past, i.e. stoR3aGH8 has a first half of its CC domain identical to stoR3aGH9, and then from this point to the LRR13 subdomain, stoR3aGH8 is similar to stoR3aGH5, with only one a.a. change in LRR4 subdomain. In the LRR13 subdomain a new recombination event occurs and the stoR3aGH8 got the C-terminal domain of stoR3aGH7, with only two a.a. changes in the LRR28 subdomain of R3a alignment (Figure 2). The phylogenetic relationship between the *R3a* variants was examined by constructing a Neighbour-joining (NJ) tree (Figure 3). The tree was rooted with *I2* from *Solanum lycopersicum*, the closest known homologue of *R3a* in plants (Huang et al. 2005). The phylogenetic tree shows that the *R3a* gene family is highly diverse and extended in *S. stoloniferum*.

Table 6. Pathogenic assay supports the presumed presence of an *R3a* homologue in *S. stoloniferum* 554-2.

	P. infestans isolates		Genotypes			
	Origin	*Avr3a* content	*S. tuberosum* cv. Impala	*S. stoloniferum* 554-2	Ma-*R3a*	*S. bulbocastanum* 2002 (*Rpi-blb2*)
IPO-0	The Netherlands	Heterozygote $Avr3a^{KI}$ - $Avr3a^{KIL}$	S	R	R	R
PIC99189	Mexico	Heterozygote $Avr3a^{KIL}$ - $Avr3a^{EM}$	S	R	R	R
Sc95.17.3.2	Scotland	Heterozygote $Avr3a^{KI}$ - $Avr3a^{EM\,*}$	S	R	R	R
CA-65	U.S.A.	Homozygote $Avr3a^{KI\,*}$	S	R	R	R
H3OPO4	The Netherlands	Homozygote $Avr3a^{EM}$	S	S	S	R
90128	The Netherlands	Homozygote $Avr3a^{EM}$	S	S	S	R
PIC99177	Mexico	Homozygote $Avr3a^{EM}$	S	S	S	R
PIC99183	Mexico	Homozygote $Avr3a^{EM}$	S	S	S	R
88133	The Netherlands	Homozygote $Avr3a^{EM\,*}$	S	S	S	R
Sc96.9.5.1	Scotland	Homozygote $Avr3a^{EM\,*}$	S	S	S	R

Detached leaf assay: *Solanum* genotypes were challenged by ten *P. infestans* isolates and scored for their Resistance (R), or Susceptibility (S).
* *Avr3a* content according to (Armstrong et al. 2005).

Table 7: Percentage similarity between the ten *StoR3aGH* genes and *R3a*.

	stoR3aGH1	stoR3aGH2	stoR3aGH3	stoR3aGH10	stoR3aGH4	stoR3aGH5	stoR3aGH6	stoR3aGH7	stoR3aGH8	stoR3aGH9	R3a	I2
stoR3aGH1	***	88.3	89	89.1	88.3	94.8	94.9	89.4	94.5	90	94.8	87.3
stoR3aGH2	12.7	***	96.6	96.6	94.9	88.4	89.4	94.6	89.3	89.9	88.4	87.7
stoR3aGH3	11.9	3.5	***	99.9	91.3	89	90.1	93.2	89.9	90.4	89.1	87.6
stoR3aGH10	11.8	3.5	0.1	***	91.4	89	90.1	93.3	90	90.4	89.1	87.7
stoR3aGH4	12.8	5.3	9.2	9.2	***	89.1	89	93	88.6	90.5	89.2	86.9
stoR3aGH5	5.4	12.6	11.9	11.9	11.8	***	98.1	89.3	96.2	92.3	99.9	88.1
stoR3aGH6	5.3	11.4	10.7	10.6	12	1.9	***	90.2	96.6	92.6	98.1	88
stoR3aGH7	11.4	5.6	7.1	7.1	7.4	11.5	10.5	***	92.8	90.5	89.4	88.2
stoR3aGH8	5.7	11.6	10.8	10.8	12.3	3.9	3.5	7.6	***	92.6	96.2	87.6
stoR3aGH9	10.7	10.9	10.3	10.2	10.2	8.1	7.8	10.2	7.9	***	92.4	87.5
R3a	5.4	12.6	11.9	11.8	11.7	0.1	1.9	11.4	3.9	8.1	***	88.2
I2	13.9	13.4	13.5	13.5	14.4	12.9	13.1	12.8	13.6	13.7	12.8	***

Nucleotide sequence distances of the ten *R3a* variants of *S. stoloniferum* 554-2 compared to *R3a* and *I2*. The percentage of similarity in the upper triangle, the percentage of divergence in the lower triangle.

Figure 2. **Protein alignment of R3a variants reveals a diverse set of R3a homologues in *S. stoloniferum* 554-2.**

Figure 2. (Previous page) **Protein alignment of R3a variants reveals a diverse set of R3a homologues in *S. stoloniferum* 554-2.**
The R3a amino acid sequence is used as reference and only positional amino acids changes are noted for the other sequences. Positions of Coiled coil domain, the nucleotide binding (NB)-ARC and leucine-rich repeat are indicated by arrows. The coiled coils described by Huang et al., (2005) are underlined. The NB-ARC domain includes the Kinase 1a, Switch, Kinase 2, Kinase 3a motifs of the NBS and RNBS-C, GLPL, RNBS-D and MHDV motifs underlined in that order (Huang et al. 2005). The leucine-rich repeat domain is composed of 30 LRR repeats, in bold, with the motif xxLxLxx. New LRR repeats from new R3aGH are bold and underlined. Cross represent putative recombination site, the first cross between StoR3aGH8 and StoR3aGH5, and the second cross between StoR3aGH8 and StoR3aGH7.
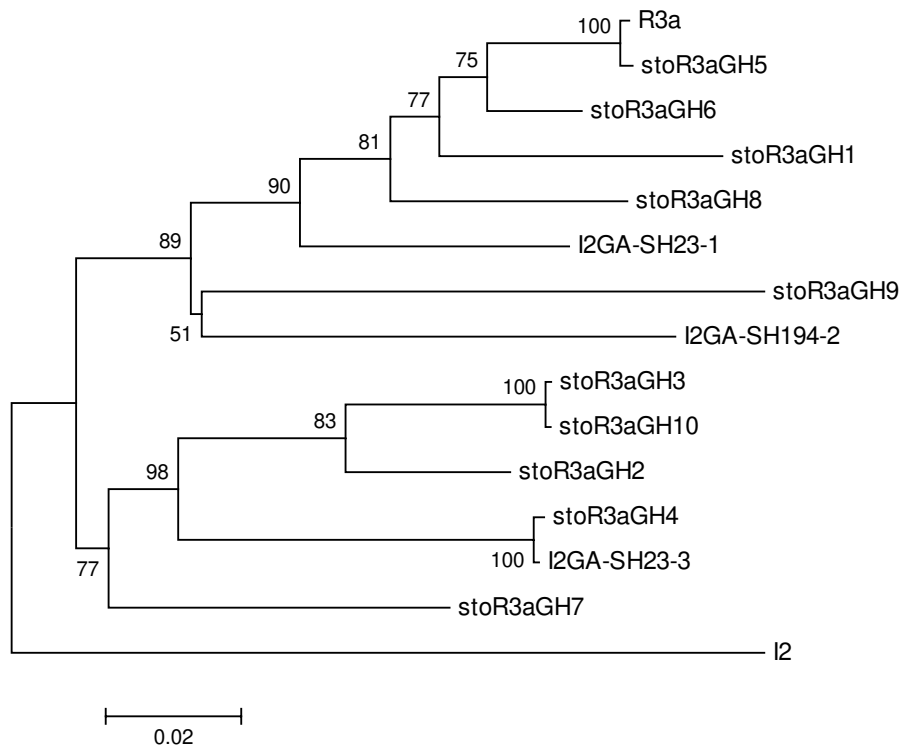
Figure 3. The R3a family is composed of highly diverse variants.
Neighbour-joining tree illustrating phylogenetic relationship of R3a variants from *S. stoloniferum* 554-2 with I2 vs. Rpi-blb1 as out group. Sequences are grouped in two clades. Bootstrap values greater than 50% (based on 1000 resample) are indicated at the nodes.

R3a homologue Rpi-sto2 from *S. stoloniferum* 554-2 is responding to AVR3a[KI]

To evaluate the functionality of the 26 clone *R3a* homologues from sto554-2 we co-infiltrated R3a and stoR3aGH1 to 29 in *N. benthamiana* leaves with AVR3a[KI] and AVR3a[EM] respectively (Figure 4). StoR3aGH5 specifically induced cell death when co-expressed with AVR3a[KI], and slight necroses with AVR3a[EM] like for R3a, whereas all other stoR3aGH did not (Figure 4). This shows that stoR3aGH5 is a functional *R3a*, and according to the common Rpi gene nomenclature, it was designated *Rpi-sto2*. A high degree of auto-necrosis induction was visible with three other R3a homologues, i.e. stoR3aGH4, stoR3aGH14 and

stoR3aGH22 (Figure 4). StoR3aGH22 has an early stop codon at the end of the coiled coil domain. StoR3aGH4 has only three a.a. difference compared to I2GA-SH194-2, in LRR4, between LRR 25 and 26, and stoR3aGH14 is more diverse than any known R3a homologue. These results show that Rpi-sto2 recognizes AVR3a^{KI}, which explains the resistance of *S. stoloniferum* 554-2 to *P. infestans* isolates expressing any form of AVR3a^{KI}.
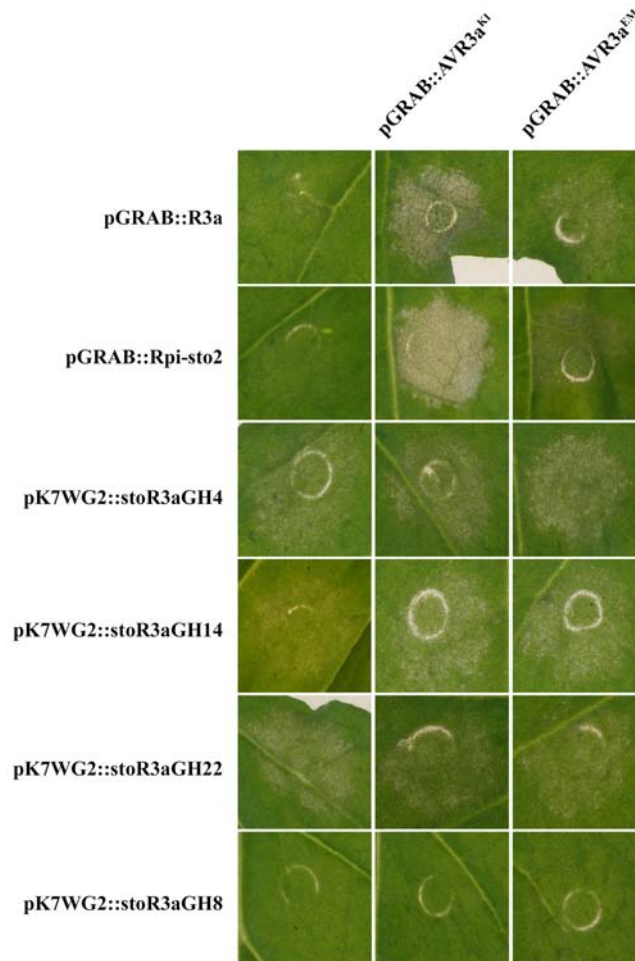
Figure 4. StoR3aGH5, but no other R3a homologue of *S. stoloniferum* 554-2, specifically recognizes AVR3a^{KI}.
Leaves of *Nicotiana benthamiana* were infiltrated with *Agrobacterium tumefaciens* strain AGL1 to co-express the R3a and R3a homologues of *S. stoloniferum* 554-2 with AVR3a^{KI} and AVR3a^{EM}. The R3a and StoR3aGH5 with AVR3a^{KI} combination were the only one to induce the hypersensitive response (HR) starting from three days post-infiltration (dpi). StoR3aGH4, 14 and 22 shows an auto-necrosis symptom and all other R3a homologues from *S. stoloniferum* 554-2, like StoR3aGH8, did not trigger any cell death. Photographs were taken at five dpi.

Discussion

Identification of geographical origin of an *R* gene is valuable information for breeders, since the natural *R* gene diversity, due to its co-evolution with the pathogen, can be used to get a broader resistance. In recent studies, we determined that the *R2* late blight resistance gene was functionally restricted to *Solanum* species from Mexico (Chapter 3) and identified a centre of co-evolution between *R2* and *PiAvr2/PexRD11* effector family members (Chapter 4). In this study, we focus on *R3a,* a constitutively expressed potato gene that confers race-specific resistance to the late blight pathogen *P. infestans. R3a* was introgressed from the Mexican *Solanum* species, *S. demissum* (Black et al. 1953; Malcolmson and Black 1966). Previous studies have mentioned co-existence of the *R3a*-harboring species *S. demissum* with *P. infestans* in the cool and humid mountain forests in Toluca Valley of Mexico, to be a perfect condition for a tight co-evolution between *R3a* and *Avr3a* (Armstrong et al. 2005; Huang et al. 2005). These conditions, therefore, could be considered as a main driving force for tight co-evolution between *R3a* and *Avr3a*. However it was never demonstrated that other species than *S. demissum* and *Solanum* species outside Mexico could also harbour a functional *R3aGH*. Here we identified nine genotypes containing a putative functional *R3aGH*, which were originating from two different centres of diversity, Mexico and Argentina. Moreover we also identified a functional R3aGH from *S. stoloniferum*, Rpi-sto2, which interact with AVR3a^{KI}. Sequence analysis of the *R3aGH* from both centres will reveal more information of the evolution of *R3a* under two different *P. infestans* populations' pressure.

R3a originate from a Major Late Blight (MLB) locus on LG XI which is highly extended in *S. demissum* (Friedman and Baker 2007). In the tetraploid sto554-2 we identified 29 independent sequences, with some potential recombination sites like in R-stoR3aGH8, which confirm the potentially high rate of evolution in this cluster. However, our finding also suggests that the number of *R3aGH* per haplotype is much less than in *S. demissum* (Friedman and Baker 2007). This difference of *R3aGH* amplified might be linked to our approach using specific primers for *R3a* and *R3a* paralogues instead of sequenced BACs (Bacterial Artificial Chromosome) from this locus in *S. stoloniferum*. However the difference is striking and it perhaps represents a difference of evolutionary rate of the *R3a* locus between *S. demissum* and *S. stoloniferum*. In addition, among all identified *stoR3aGH* sequences, only 14 out 29 were coding for a full length *R* gene, and only one *Rpi-sto2* was functional for AVR3a activity. Since all stoR3aGH have still a high homology to R3a and its paralogues, and therefore are likely to be used for further sequence exchange in this hot-spot, this is in line with the Trench-Warfare-model (Stahl et al. 1999).

Avr3a allele-mining on the 15 *P. infestans* isolates showed that *Avr3a* contains only little diversity, and is mostly represented by *Avr3a^{KI}* and *Avr3a^{EM}*, in line with previous work (Armstrong et al. 2005). A newly detected variant *Avr3a^{KIL}*, which thus far seems to have

similar specificity as Avr3a[KI] because it did not evade *R3a* recognition based on disease test, appeared in the Mexican isolate PIC99189 and IPO-0, from unknown origin. Interestingly, *Avr3a[EM]* was found predominantly present in almost all isolates. In ten out of 15 isolates *Avr3a[EM]* was even the only Avr3a variant, which is in line with a generally defeated *R3a* in the potato fields (Black et al. 1953; Malcolmson and Black 1966). The abundance of *Avr3a[EM]* vs. *Avr3a[KI]* suggests that *P. infestans* isolates evolved to evade *R3a* recognition. On the other hand, wild *Solanum* species have maintained *R3a* in the population and most likely even in two different geographical regions. Therefore we hypothesise that despite the effort of the pathogen to evade Avr3a[KI] recognition, the virulence function of AVR3a[KI], such as suppression of INF1-mediated cell death through CMPG1 protein (Birch et al. 2009; Bos et al. 2009), is important enough to maintain it, and resulted in the sustainability of *R3a* in wild *Solanum* species. In conclusion, for the quest of durable resistance against late blight, it would be of interest to identify, or create by shuffling or swapping approaches, a R3a variant which responds to AVR3a[EM] at similar levels as Avr3a[KI]

     R gene allele mining is a powerful approach, but can also be inefficient since it does not make any distinction between genotypes containing functional or non-functional RGH. Use of gene-specific molecular markers (diagnostic marker) to identify functional *RGH* in *Solanum* still leads to diverse rates of efficiencies. For example 98% accuracy was obtained for *I2* tomato blight resistance gene (Arens et al. 2010), but only 27% for the *Rpi-blb3* potato blight resistance gene (Lokossou et al. submitted), mainly due to the high recombination events present in this *R* gene cluster (Lokossou 2010). Moreover, the number of *RGH* per genotype can depend on ploidy level, and localization in MLBs (Friedman and Baker 2007; Lokossou et al. 2009; Pel et al. 2009). Here we have combined three different techniques to gradually reduce the number of genotypes which potentially contained a functional R3aGH. PVX agroinfection is the most facile assay available for *in planta* testing of an effector on a large scale of *Solanum* genotypes (Vleeshouwers and Rietman 2009). However, interpretation of results can be difficult since many *R* genes that induce ER, including *Rx*, also induce HRs depending on the strain of the virus, host genetic background and environmental conditions (Baurès et al. 2008; Bendahmane et al. 1999; Kang et al. 2005). We anticipated on this by including other *Avr3a* variants with slightly altered levels of sensitivity, and use PVX agroinfection with Avr3a mainly as a high-trough-put pre-screening. The 73 identified genotypes, which gave responses to any of the two Avr3a alleles and/or to the paralogue Pex147-3, where than further studied by agroinfiltration, the currently best developed and most reliable method for reconstructing the R-AVR interaction in plants (Armstrong et al. 2005; Bos et al. 2006; Champouret et al. 2009; Lokossou et al. 2009), and 12 were confirmed to show response to AVR3a[KI]. However, because also *A. tumefaciens* is a plant pathogen, various defence responses from the host can be initiated (Vleeshouwers and Rietman 2009), and in our case, only 54% of tested *Solanum* genotypes could reliably be tested. Another factor that contributes to the deviation of efficiency is explained by the

absence of PEX147-3 in the agroinfiltration test. The high number of genotypes showing responses to this paralogue of *Avr3a* remains questionable, and we postulate an ancient recognition of this effector by most of the wild *Solanum* species, which would explain why almost no isolates of *P. infestans* express this effector (Haas et al. 2009; Whisson et al. 2007). In conclusion, this study suggests that the sensitive agroinfection technique in combination with agroinfiltration and disease assays provide an efficient platform for *in planta* testing of large numbers of genotypes containing *RGH* candidates of extended gene families, like *R3a* of the MLB on LG XI.

Future late blight resistance breeding can explore *RGH* with broader specificity, and deploy *R* genes that recognize the full spectrum of Avr alleles. Also molecular engineering of *R* gene could be a solution to late blight management using the *R3aGH* diversity information. Here we propose four domains to investigate for future researches. First, towards our results on R3a response to AVR3a^{EM} in transient assays, it would be of interest to transform potato cultivars with R3a under a highly constitutive promoter or with multiple copies inserted, to increase the transcription level *in planta*, and therefore, obtain broader resistance like for the enzyme resistance (*eR*) genes At1 and At2 from wild Melon (Taler et al. 2004) or with the RB/Rpi-blb1 against potato late blight (Kramer et al. 2009). A second approach would be to shuffle *R3a* to synthetically evolve this resistance gene against AVR3a^{EM}. This approach proved to be successful with Rx, which by shuffling could respond to different variants of PVX but also to a distantly related poplar mosaic virus (Farnham and Baulcombe 2006). Therefore the amplification of *R3aGH* on the remaining nine genotypes identified in our study and particularly on the Argentinean *S. microdontum*, would provide even more diversity of sequences to start with. The third opportunity is to screen additional wild *Solanum* species to find a natural genotype responding to AVR3a^{EM}. The two dms343-1 and dms264-1 as well as in the unclassified *Solanum* genotype 287-2 are the three first candidates to test for functional *R3a* homologue responding to the virulent variant *Avr3a^{EM}*. The fourth domain of investigation is based on the diversity of *R3aGH* we have uncovered. We can expect that the high diversity of *R3aGH* sequences in *S. stoloniferum* could bring recognition against other effectors than the Avr3a effector family. Therefore screening of these *R3aGH* genes against more RXLR effectors of *P. infestans* or effectors from other pathogen of potato could reveal new specificities and more understanding on R/AVR interactions and *R* gene cluster evolution.

Materials and Methods

Plant materials

The wild *Solanum* plant material used for effector screening and functional allele mining is listed in Table 1. The genotypes were retrieved from the accessions in the Centre for Genetic

Resources, the Netherlands (CGN), and information on species, origin, collection site, GPS coordinates and other genbank code are accessible on the Sol*R*gene database (*SolR*gene database). SH83-92-488 (Huang et al. 2005; Huang et al. 2004), Pentland Ace (provided by Paul Birch, SCRI, Scotland, U.K.), Ma-R3 (Mastenbroek 1953) genotypes, were used as R3a control plants in agroninfection and agroinfiltration assays. *S. tuberosum* cultivar Désirée and *N. benthamiana* and there respective transformants *S. tuberosum* cultivar Désirée-R3a and *N. benthamiana*-R3a transformed with R3a under its native expression elements in pBINPlus (van Engelen et al. 1995) were used in Figure 1B. Taxonomic series information was extracted from the data in Jacobs et al., (2008) paper. The population generated for genetic studies originated from the *P. infestans* resistant *S. stoloniferum* (CBSG: 554-2) crossed with the susceptible *S. stoloniferum* (CBSG: 297-1). Plant genotypes were maintained *in vitro* on MS medium supplemented with 20% sucrose (Murashige and Skoog 1962) at 18 °C. Top shoots were transferred to fresh medium, and two weeks later, rooted plantlets were transferred to the greenhouse. *Nicotiana benthamiana* plants were grown from seeds in the greenhouse and subjected to agroinfiltration experiments in climate chambers at an ambient temperature of 22–25 °C and high light intensity.

Microbial strains and growth conditions

Agrobacterium tumefaciens strains GV3101 (Hellens et al. 2000), COR 308 (Hamilton et al. 1996), and AGL1 (Lazo et al. 1991) were used in molecular cloning experiments and were routinely cultured at 28 °C in Luria–Bertani (LB) media using appropriate antibiotics (Sambrook and Russell 2001). All bacterial DNA transformations were conducted by electroporation in DH10B cells (INVITROGEN) or heat shock in DH5α cells (INVITROGEN) using the supplier recommendations.

Phytophthora infestans isolates and disease tests

The *P. infestans* isolates F95573, PIC99177, 88069, PIC99189, 90128, EC1, H3OPO4, USA618, IPO-0, IPO-C, PIC99183, NL01096 and VK98014 (Champouret et al. 2009) were cultured on rye sucrose agar medium in the dark at 15 °C for 1–2 weeks (Caten and Jinks 1968). Sporulating mycelium was flooded with cold water and the sporangiospore suspension was incubated at 4 °C for 3 h. After the release of the zoospores, the inoculum was adjusted to a concentration of $5*10^4$ spores/ml. Leaves from 6-8 week old plants were detached, placed in water-saturated oasis in humid trays, and leaflets were spot-inoculated with the spore suspension. The trays were incubated in a climate chamber with a 16/8h light/dark regime at 15 °C. At six days post inoculation (dpi), the lesion sizes (LS) were determined. Sporulating lesions exceeding 15 mm were scored as susceptible (S), whereas spots displaying localized HR smaller than 5 mm at the inoculation sites were scored as resistant (R).

PCR allele mining

All primers and plasmids used in this study are described in Table S1. To clones *Avr3a* variants from the different *P. infestans* isolates we performed PCR amplification with Phusion High-Fidelity DNA Polymerase (FINNZYMES) on genomic DNA extracted from fresh mycelium tissue of each isolate using the primers PEX147-F and PEX147-R. After 30 cycles, Super*Taq* polymerase (HT Biotechnology, Cambridge) and its buffer were added, followed by 15 min at 72 °C. The yielded amplicons of 450bp, in range for *Avr3a* homologue, were ready to be cloned into pGEM-T Easy vector (Promega). *R3aGH* variants from *S. stoloniferum* 554-2 were cloned by PCR amplification on genomic DNA using Phusion High-Fidelity DNA Polymerase (FINNZYMES). Two amplifications were made to amplify all R3a and R3a paralogues, using the conserved start primer R3a-GatBP-F2 containing the attB1 gateway, in combination with the stop primers R3a-GatBP-R2 and SH194-2-GatBP-R2 containing the attB2. All PCR fragments were purified on 1% TBE gel using Min Elute^TM Gel Extraction Kit (QIAGEN). Sequencing was performed using universal M13 primers and specific primers for R3aGH (Table S1). DNA sequences were analyzed using DNASTAR Lasergene v8 and ClustalW.

Plasmid constructs

All effectors in the potato virus X (PVX) binary vectors, i.e. pGR106::empty, pGR106::*Crn2*, pGR106::*Avr3a^KI*, pGR106::*Avr3a^EM* and pGR106::*Pex147-3* (kindly supplied by Dr. S. Kamoun and Dr. J. I. B. Bos, OARDC, Wooster, USA) used in agroinfection test were without signal peptide and without RXLR motif. Agroinfiltration screens were made with *Avr3a^KI*, *Avr3a^EM* and *R3a* in pGRAB plasmid (kindly provided by Dr. S. Chapman, SCRI, Scotland, U.K.) under 35S promoter and terminator. Effectors were cloned without their signal peptide. pBINplus::*R3a* was provided by Huang et al. (2005). *Avr3a* variants amplicons from the different *P. infestans* isolates tested were cloned in pGEM®-T Easy Vector Systems (Promega) following the supplier recommendations. The purified *R3aGH* amplicons from *S. stoloniferum* 554-2 were used in a BP reaction (Gateway® INVITROGEN) together with the donor plasmid pDONR221. After sequencing *R3aGH* ORF variants were introduced in pK7WG2 (Karimi et al. 2002) by LR reaction (Gateway® INVITROGEN).

Agroinfiltration and PVX agroinfection assays

Recombinant *A. tumefaciens* strains were grown as described previously (Champouret et al. 2009), except that culturing steps were performed in LB media supplemented with 100 µg/ml kanamycin for the pGR106 and pGRAB constructs, and 100 µg/ml spectinomycin for the pK7WG2 constructs. Agroinfiltrations were performed on the middle section of leaf panels of *Solanum* plants beyond nine-leaf stage, or four to five week old *Nicotiana benthamiana*. Plants were infiltrated with the *A. tumefaciens* suspensions, and symptoms were monitored at two to six day after infiltration (dai). Plants were grown and maintained throughout the

experiments in a greenhouse with an ambient temperature of 22–25 °C and high light intensity. For transient co-expressions *A. tumefaciens* solution were mixed in a 1:1 ratio before infiltration. PVX agroinfection was performed as described in previous studies (Oh et al. 2009; Vleeshouwers et al. 2008; Vleeshouwers et al. 2006; Vleeshouwers and Rietman 2009).

Table S1: List of used primers.

Primer name	Primer sequence 5'- 3'	Comments
Pex147F	CCATGCGTCTGGCAATTATGCT	Avr3a variants amplification
Pex147R	CTAATATCCAGTGAGCCCCAGGTG	
R3a-GatBP-F2	GGGGACAAGTTTGTACAAAAAGCAGGCTATG GAGATTGGCTTAGCAGTTGGTG	R3a gene homologues amplification
R3a-GatBP-R2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCA CATGCATTCCCTATCGATCTT	
SH194-2-GatBP-R2	GGGGACCACTTTGTACAAGAAAGCTGGGTTCA GATTGGATATGGCAAGTGTTC	
R3a-Gat-F	CACCATGGAGATTGGCTTAGCAGTTGGTG	R3a gene homologues sequencing
R3a-Gat-R	TCACATGCATTCCCTATCGATCTT	
SH194-2-GatBP-R2-G	GCTGGGTTCAGATTGGATATGGCAAGTGTTC	
R3aH-307-29F	CAGCATCAAAATCTTGCAGAAAC	
R3aH-607-28F	GTAGTTCCTATTGTTGGAATGG	
R3aH-961-82F	GAGAGTGTTGCCTTGATGATGG	
R3aH-1202-23F	GAAGTGAAATATGGGAGCTGCC	
R3aH-1501-22F	CATGACCTTGTCAATGATTTAG	
R3aH-1934-54F	TCTTGAGGAGCTACCGCTGCA	
R3aH-2244-65F	CAATTCACAAACAGAAAGAGAC	
R3aH-2592-12F	GGAGAGTTCCCTATACTTGAG	
R3aH-2913-37F	CAACCTTACTAGGTTTTTGATTCC	
R3aH-3446-70F	ACCTCACTTCGCTTCAAAGTCTAC	

Sequences underlined correspond to Gateway® (INVITROGEN) attB1 and attB2 sites.

Phylogenetic data analyses

Phylogenetic analyses of the R3a gene homologues in *S. stoloniferum* 554-2 (StoR3aGH) were conducted using the Neighbour-joining method (Nei and Li 1979) in MEGA version 4 (Tamura et al. 2007). R3a, I2GA-SH23-1, I2GA-SH23-3, I2GA-SH194-2 from SH83-92-488 *S. tuberosum* and I2 from tomato were used as closest known sequences and the tree was rooted using Rpi-BLB1. Bootstrap values greater than 50% from 1000 replicate trees are shown at the nodes. Horizontal branch lengths and scale bar correspond to evolutionary distances assigned by mega. The evolutionary distances are measured as the proportion of nucleotide substitutions between sequences.

CHAPTER 6

General Discussion
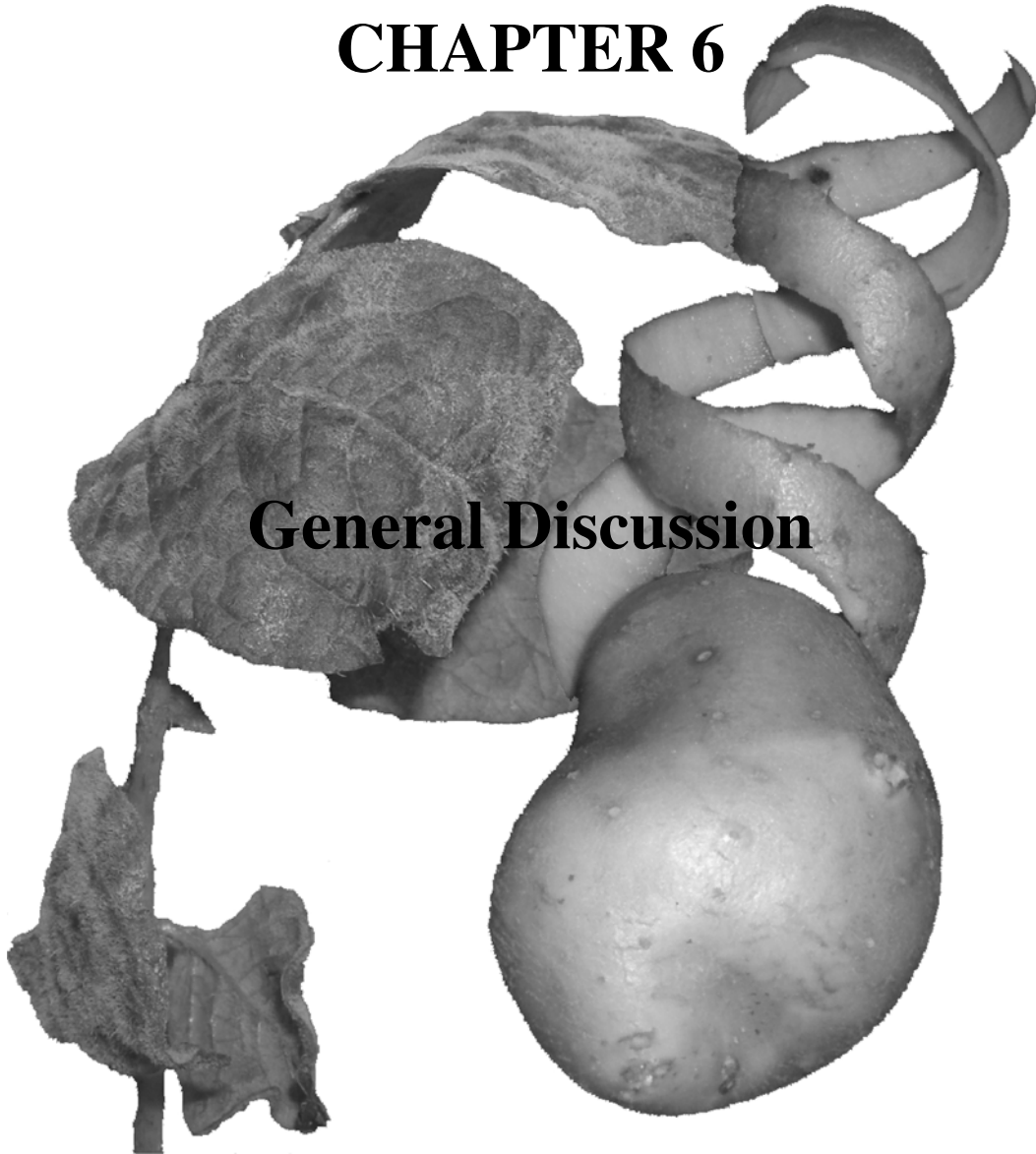
Introduction

This thesis, entitled "Functional Genomics of *Phytophthora infestans* Effectors and *Solanum* Resistance Genes" was aimed to identify new R/AVR pairs and functionally characterize the *R* gene as well as the cognate *Avr* gene. Knowledge of *R* and *Avr* as they evolved under natural conditions can then be applied to explain or predict the performance of *R* genes in natural and practical situations. To answer such questions we have based our approaches on effector genomics, allele-mining of *Avr* and cognate *R* genes pairs, accurate disease testing with well-characterized *Solanum* genotypes, transient complementation assays, and a diverse set of *P. infestans* isolates from different geographic origin and with distinct lineage based on SSR markers. This discussion will reflect on the experimental findings described in this thesis, their impact for future management of late blight and new questions.

Effectoromics potential

Oomycete plant pathogens, such as *P. infestans*, secrete an arsenal of effector proteins that modulate host innate immunity and enable parasitic infection (Birch et al. 2009; Birch et al. 2008; Haas et al. 2009; Kamoun 2006; Schornack et al. 2009b). Host immunity responses are initiated by a number of these effectors, namely members of the RXLR family, by triggering hypersensitive cell death and resistance responses through activating particular NBS-LRR immune receptors or resistance (*R*) genes, generically known as CC-NBS-LRR in the cytoplasm (Dangl and Jones 2001).The transient assay based on *Agrobacterium tumefaciens* carrying a binary vector made of Potato Virus X of which the virulent gene is replaced by *P. infestans* C-terminal domain of an effector (PVX agroinfection) proved to be a useful high throughput system to screen for resistance responses in wild *Solanum* species (Vleeshouwers and Rietman 2009). In Chapter 5, the agroinfection approach in combination with agroinfiltration and disease assays provided an efficient platform for *in planta* testing of large numbers of genotypes with *R3a* gene homologue (*R3aGH*) candidates and led to the identification of one functional *R3aGH* in *S. stoloniferum*. In addition, we identified nine other genotypes from Mexico and Argentina that contain a putative functional *R3aGH*. In Chapter 3, we have identified three wild *Solanum* species (*S. edinense*, *S. schenckii* and *S. hjertingii*) responding to the RXLR effector PEXRD11, which is related to a previously described RXLR effector gene *PiAvr2* (Lokossou et al. 2009). Genetic studies demonstrated that the new *Rpi*-candidates responding to PEXRD11 in *S. edinense* and *S. schenckii* was located in the *R2* locus. Using the effector again we could rapidly test a number of *R2* gene homologues (*R2GH*) for cell death activity with PiAVR2/PEXRD11 in agroinfiltration assays. We efficiently identified six new R2GH that induced cell death with PEXRD11 and PiAVR2, and all six R2GH confer resistance to *P. infestans* isolate avirulent on R2-plants.

The *R* genes were designated *Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2,* and *Rpi-hjt1.3*. In Chapter 4, we continued on the same R-AVR interaction and mined 11 related effectors of the *PiAvr2/PexRD11* effector family from the *P. infestans* T30-4 genome (Haas et al. 2009). This observation of high diversity of genes in this effector family is explained by the highly accelerated rate of evolution of effectors (Hogenhout et al. 2009). Challenging independently the 13 effectors of the PiAVR2/PEXRD11 family with the R2GH cloned in chapter 3 and the already known functional resistance genes *R2, R2-like, Rpi-abpt* and *Rpi-blb3* (Lokossou et al. 2009), resulted in the identification of two effectors, PITG_21949 and PITG_21645, with avirulence activity against the ten functional R2GH. Furthermore, we identified a differential recognition between the six new R2GH of chapter 3 and the four known R2GH for PITG_13940. In all these studies, effectors are the key to characterise the R2GH response spectrum.

One difficulty we encountered with the PVX agroinfection technique is the extreme resistance (ER) phenotype (Chapter 5). Extreme resistance is seen with a number of *R* genes conferring resistance to viruses; it is manifested as a complete lack of both macroscopic and microscopic HR lesions and is accompanied by the absence of detectable virus accumulation (Bendahmane et al. 1999; Kang et al. 2005; Kohm et al. 1993). However interpretation of results can be difficult since many *R* genes that induce ER, including *Rx*, also induce HR depending on the strain of virus, host genetic background and environmental conditions (Baurès et al. 2008; Bendahmane et al. 1999; Kang et al. 2005). We observed these HR and ER responses and this posed a few questions. The response pathway in R/AVR interaction between R3a and AVR3a^{KI} has always been observed by HR responses with agroinfiltration assay, but in this study we have observed ER on R3a plants when infected with PVX-AVR3a^{KI}. Recently, Liu et al. (2010) demonstrated that HR is dispensable for R protein-mediated induction of systemic acquired resistance (SAR), and that Rx-induced SAR is mediated by the same salicylate-dependent pathway induced by other R proteins, even if it results in ER response. Therefore, future studies to understand the switch of phenotype observed for R3a/AVR3a^{KI} interaction could bring new solutions for resistance breeding by providing *R* genes with a fast resistance response induction which would stop infection not only from avirulent isolates but also from avirulent and aggressive isolates, which sometime escape from the HR response (Chapter 2).

In conclusion, effectoromics has been a successful approach to identify R-AVR interactions with new RGHs and AVRs. Moreover we have proven that in combination with accurate disease tests based on well-characterized *P. infestans* strains in *Solanum* and *N. benthamiana* we could functionally characterise the RGHs. These results, therefore, offer a great future for effectoromic studies in fundamental and applied sciences (Schornack et al. 2009b).

Functional characterisation of *R* genes

Since the discovery that single *R* genes in potato were quickly defeated by virulent *P. infestans* isolates in the field, potato breeders focussed their search for resistance on *R* genes that are effective against a broad range of *P. infestans* isolates, preferably against all isolates. Two *R* genes against late blight, *Rpi-blb1* and *Rpi-blb2*, from the Mexican *S. bulbocastanum*, were originally described as broad spectrum *R* genes, since there were no reports of defeat by highly virulent *P. infestans* isolates (Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). Interestingly, the qualification of broad spectrum was defined by only three and two "avirulent" isolates from The Netherlands and Belgium in the case of *Rpi-blb1,* and *Rpi-blb2,* respectively. In Chapter 2 we reassessed the resistance spectrum of *Rpi-blb1* using a broad diversity of *P. infestans* strains from Europe and Mexico, including isolates that were isolated from *S. stoloniferum* of which the *Rpi-blb1* homologue *Rpi-sto1* was recently isolated (Vleeshouwers et al. 2008; Wang et al. 2008). Two isolates, PIC99189 and PIC99177 collected in 1999 from the Toluca Valley in Mexico were infecting *Rpi-blb1*-plants, and the myth of *Rpi-blb1* being a broad-spectrum *R* gene to all *P. infestans* isolates, was defeated. Studying the genetic diversity of *Avr-blb1* (*ipiO*) (Pieterse et al. 1994; Vleeshouwers et al. 2008) revealed that *ipiO* is highly diverse, and that class I ipiO was the most abundant class in most European isolates. This explains why *Rpi-blb1* had been performing well against most isolates in Europe. Interestingly, we found that virulent isolates on *Rpi-blb1*-plants are lacking *Avr-blb1* Class I ipiO, and these evolved in Mexico (Chapter 2). These findings suggest that migration of isolates from Mexico, where *S. stoloniferum* occurs, to European farming areas, will probably lead to defeat of *Rpi-blb1* in Europe. Similar phenomena already happened with A2 mating type of *P. infestans* isolates was found in Europe in the 1980s (Goodwin and Drenth 1997; Hohl and Iselin 1984; Spielman et al. 1991). Also, directional selection for virulence is superimposed when race-specific resistance is introduced (Montarry et al. 2006), and therefore, spread of virulence against *Rpi-blb1* is just a matter of time. In addition, deployment of *Rpi-blb1* as a single *R* gene in potato is expected to even accelerate the process.

In another effector genomics study, the cognate *Avr* gene matching *Rpi-blb2* was identified. Phylogenetic analysis by maximum likelihood showed that *Avr-blb2* (*PexRD39/40*) is highly diverse in the *P. infestans* population. One variant, named PEXRD40^{V69F}, with a.a. in position 69 being a phenylalamine, was identified to be not recognized by *Rpi-blb2* (Oh et al. 2009). Since the *P. infestans* genome showed presence of 7 copies of *Avr-blb2* this may decrease the possibility to have this virulent variant in all seven positions, and that could explain to some extent the degree of the durability of this resistance gene in the field. Still, virulent isolates on *Rpi-blb2* plants were discovered recently in potato trap fields (Dr. G. Kessel, Biosciences, Wageningen University and Research centre, personal communication), and these isolates are currently being studied to understand how virulence

on *Rpi-blb2* plants can occur. One hypothesis is perhaps a combination of presence of virulent variants of Avr-blb2 and silenced avirulent variants as observed for *Avr-vnt1* in a *P. infestans* isolate that is virulent on *Rpi-vnt1* plants (Pel 2010). The flexibility of the *P. infestans* genome can result in various ways to escape recognition by resistant plants, and characterisation of each individual R/AVR is important prior release of new resistance genes in the field.

Another important aspect to take into consideration regarding the description of an *R* gene against late blight is the efficiency of the *R* gene when exposed to aggressive isolates. Frequently, aggressiveness is interpreted as virulence (Suassuna et al. 2004). Niks (2001) highlighted the discrepancies between terminologies of virulence in a review. De Wit (2000) described virulence based on a functional gene that determines quantitatively or qualitatively the disease development, and therefore the term virulence described as the loss of avirulence should be avoided. However, Boller and Keen refer in the same multi-author book virulence as to the loss of avirulence, based on the gene-for-gene model (Boller and Keen 2000). The first description of virulence, as a determinant of quantitative or qualitative disease development, does not make any difference between effector genes that have a virulence function to the pathogen by suppressing the host defence system, for example, AvrPto and AvrPtoB which target the kinase domains of the receptor-like kinases CERK1, BAK1, EFR1 and FLS2 in *P. syringae* (Gimenez-Ibanez et al. 2009; Göhre et al. 2008; Shan et al. 2008; Xiang et al. 2008), and metabolic genes involved in growth development or life cycles of the pathogen, such as a transcription factor with a highly conserved APSES domain of *Ustilago maydis* which regulates dimorphism, sporulation, and pathogenic development (2010). Here, we follow the definition of aggressiveness as the overall ability of a pathogen to infect its host plant (Chacon et al. 2007; Cooke and Deahl 1998), irrespective of specific virulence or avirulence determinants and more based on physiological and metabolic pathway fitness. In Chapter 2 we have demonstrated that an aggressive isolate of *P. infestans*, PIC99183, could overrule the recognition by *Rpi-blb1*-plants, with 50% infection efficiency but with a low lesion growth rate, despite the fact that this isolate contains a class I *ipiO* variant. Aggressive isolates were also able to infect transgenic *Rpi-blb1*-plants which had one or two copies of the resistance gene, even if these isolates were expressing avirulence alleles. In contrast, aggressive isolates could not infect wild *S. bulbocastanum* that express *Rpi-blb1* in their own genetic context at higher levels, compared to transgenic potatoes. Our data are all in line with recent studies that suggested a positive correlation between resistance level of *Rpi-blb1* plants and *Rpi-blb1* expression level (Bradeen et al. 2009; Kramer et al. 2009). Therefore, insufficient expression levels of *R* genes in the field can also lead to susceptible phenotypes when challenged with aggressive isolates of *P. infestans*.

In conclusion, to describe and predict the range of resistance of *R* genes against late blight, characterisation of genetic diversity and expression of the corresponding Avr gene in *P. infestans* isolates is essential. In addition, the level of *R* gene transcript that is required to

confer resistance when challenged by aggressive and contemporary isolates of *P. infestans* has to be characterized.

Co-evolution of R loci and their corresponding AVR loci

R genes are classified in two rates of evolution. Type I *R* genes are characterized by frequent sequence exchanges between paralogs that obscure orthologous relationships, whereas type II resistance genes rarely undergo sequence exchanges with paralogs and maintain orthologous relationships (Friedman and Baker 2007; Kuang et al. 2004). This thesis presents three different R-AVR pairs involved in the co-evolution between the *Solanum* section *Petota* host species and *P. infestans*, namely R3a - AVR3a (Chapter 5), Rpi-blb1 - AVR-blb1 (Chapter 2) and R2 -PiAVR2/PEXRD11 (Chapter 3 and 4).

R3a is located in loci known for hot spot recombination (Huang 2005; Huang et al. 2005) and fits perfectly to the Type I *R* gene definition. In Chapter 5, we detected 29 R3aGH among which one was functional with AVR3a^{KI} in *S. stoloniferum*. However, all *stoR3aGH* still have a high homology to *R3a* and its paralogues, and therefore are likely to evolve by sequences exchanges in this hot-spot, in line with the Trench-Warfare-model (Stahl et al. 1999). On the pathogen side there are only three variants of the effector AVR3a detected thus far (Armstrong et al. 2005). In the co-evolution dynamics, this high diversity on the resistance gene side combined with the low diversity on the effector side could fit to an effector-triggered immunity (ETI) step in the Zig-Zag model of Dangle and Jones (2006), where one effector is recognised and host develops a high diversity of RGHs in an attempt to recognise all variants of the effector.

In Chapter 3 we discovered that the R2 cluster is highly diverse and with other studies (Lokossou et al. 2009), we defined the R2 cluster also as a Type I R gene. Interestingly, effectors recognized by functional R2 homologues are allelic variants that are present as multiple copies in the genome and belong to two different gene structures, PiAVR2 and PEXRD11, based on a.a. alignment (Chapter 4). In the case of *S. hjertingii* we demonstrated the presence of four functional R2GH with different spectrum of recognition to five PiAVR2/PEXRD11 members (Chapter 3 and 4). Bergelson et al. (2001)} and Dawkins and Krebs (1979) refer to a classical arms race as a series of selective sweeps as novel *R*-gene alleles, capable of recognizing pathogenicity determinants (*Avr*) that previously avoided detection in a plant population, spread to high frequency. The longevity and high allelic diversity in some cases suggest a micro-evolutionary mechanism that promotes the maintenance of stable polymorphism (Stahl et al. 1999) until the pathogen overcomes the resistance (Jones and Dangl 2006). Therefore, we argue that *R2-PiAvr2/PexRD11* with multiple copies of each gene family in the genome of the host and in the genome of the pathogen fits to the arms-race model (Bergelson et al. 2001; Dawkins and Krebs 1979). This

diversity on the *R* loci and the *Avr* loci could represent an advanced stage of co-evolution between the host and the pathogen, where multiple cycles of resistance (ETI) followed by susceptibility (ETS) have occurred in the Zig-Zag model.

In Chapter 2 we worked with a type II *R* gene (*Rpi-blb1*) which is present in resistant genotypes and just absent from its locus in susceptible genotypes (van der Vossen et al. 2003). Moreover little genetic diversity has been found in wild *Solanum* species for *Rpi-blb1* and only three functional variants of Rpi-blb1 have been identified so far (Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008; Wang et al. 2008). The low diversity of Rpi-blb1 can be explained by having another role than just late blight resistance (van der Vossen et al. 2003), or by representing a recent *R* gene which has not (long) been under co-evolutionary pressure yet. The latter could explain why no susceptible variants of *Rpi-blb1* were found in this locus (van der Vossen et al. 2003) or only one form with 18bp deletion (Song et al. 2003) scattered around in *S. bulbocastanum* species only in Central America (Lokossou et al. submitted; Vleeshouwers et al. 2008; Wang et al. 2008). In contrast, on the effector side we identified up to 13 variants of *Avr-blb1* in *P. infestans*. This might reflect a selection pressure to escape recognition by *Rpi-blb1*. Therefore, we postulate that the natural co-evolution described by this interaction might be in the first transition between effector-triggered immunity (ETI) and effector-triggered susceptibility (ETS) (Jones and Dangl 2006).

In conclusion, the different R/AVR pairs presented in this thesis seem to reflect a variety of co-evolutionary stages and together provide more insight in the genetic coexistence mechanism of potato and *P. infestans.*

The future of resistance breeding to late blight

Potato breeding for late blight resistance was one of the earliest practices mankind used to combat plant pathogens by means of genetic improvement. Introgression of resistance genes from wild *Solanum* species into potato cultivars in the past has only provided temporary solutions and durable resistance based on one single R gene appears nowadays more and more an illusive theory. The genome flexibility of *P. infestans* as well as its fast evolving abilities (Fry 2008; Haas et al. 2009) are two of the reasons that explain the fast defeat of *R* genes in agricultural practice (Hein et al. 2009). The recent molecular insight in biology of the pathogen (Birch et al. 2009; Birch et al. 2008; Birch et al. 2006; Kamoun 2007; Morgan and Kamoun 2007; Tyler 2009; Whisson et al. 2007; Win et al. 2007), the better understanding of the interaction with the host based on co-evolutionary principles (Armstrong et al. 2005; Lokossou et al. 2009; Oh et al. 2009; Pel 2010; Vleeshouwers et al. 2008; Chapter 3 and 4), the newly uncovered annotated *P. infestans* genome (Haas et al. 2009), and the development of new techniques to functionally characterise the gene pool of

effectors of the pathogen (Oh et al. 2009; Vleeshouwers et al. 2008), provide new genetic tools to manage the disease. In this thesis on effectoromics we have characterized new RGHs, some of which with new functionality, and we have uncovered the diversity of *Avr-blb1* in *P. infestans* population. Below we present the utilities of these results in future potato breeding programs, and discuss some new research areas of interest for future *R* gene characterisation.

We demonstrated in Chapter 3 that effectoromics accelerates the cloning of functional *R* genes. The screening of *Solanum* germplasm with effectors can provide a pattern of responses to a specific effector from a group of *Solanum* species, and therefore, can help to avoid redundant *R* gene cloning efforts. For these reasons, future studies to identify the recognition spectrum of one *R* gene in the wild *Solanum* population should not only be based on the resistance spectrum to *P. infestans* isolates but preferably on the responses to the cognate effector.

Effectoromics also accelerates the characterization of functional *R* genes, and identification of additional RGH can lead to a broader resistance. For example, six functional R2GH respond to PITG_13940 of *P. infestans*, whereas the four already known R2GH do not. Interestingly in *S. hjertingii* we have found that three R2GH are expressed, i.e. *R2-like* responding to PiAVR2, PEXRD11, PITG_21949 and PITG_21645, and *Rpi-hjt1.2* and *Rpi-hjt1.3* with additional response to PITG_13940. This supplementary specificity of recognition can be valuable for potato breeding and by combining R2GH get a broader spectrum of resistance against *P. infestans*. However before using these new R2GH in breeding programs it would be of interest to study the expression level of these five effectors from the *PiAvr2/PexRD11* family in contemporary *P. infestans* populations.

Knowledge on R/AVR pair diversity, like in the Rpi-blb1/AVR-blb1 interaction provides new handles for late blight management. Durability of an *R* gene is based on its application. Therefore the large-scale monitoring aimed at diagnosing the *Avr-blb1* classes in the *P. infestans* population on commercial potato growing areas can be applied to detect (evolution and migration of) virulence against *Rpi-blb1*. When virulence is detected selection pressure towards losing class I *ipiO* might be avoided by omitting cultivars with *Rpi-blb1* for a certain period of time and applying other *R* genes instead (Chapter 2). In conclusion, one way to manage the selection pressure on the pathogen is by large scale effector monitoring for virulence frequencies in the *P. infestans* population

Mapping and cloning of new *Rpi* genes is mainly done on wild *Solanum* genotypes collected in Central and South America 50 or more years ago (*SolR*gene database). However, the high genetic variability in the tetraploid potato genome (D'Hoop et al. 2010; Simko et al. 2006), which is even more important in *R* gene loci, shows that genetic changes occur at high rates. For example, a SNP every 15 bp in the region of the S*tVe1* resistance gene against *Verticillium albo-atrum* was identified by Simko et al. (2004). Also, *R* loci contain multiple transposon families which are though to be involved in the rapid diversification (Song et al. 1997). Recent evidence that infection may stimulate host recombination rates in subsequent

generations via induction of transposable elements (Mirouze et al. 2009; Molinier et al. 2006), and demonstration of instability of an epigenome (Reinders et al. 2009), allows us to extrapolate that contemporary wild *Solanum* species may display new resistance gene that are more adapted to the contemporary *P. infestans* population. As a result, the continuation of collecting wild *Solanum* species in centers of co-evolution such as the Toluca Valley and South America is of great interest for future breeding programs.

To conclude, intensive farming aims to create a crop without any diseases. In the natural environment this is an utopia, all plants coexist with their pathogens and a co-evolutionary balance has been created which allows both partners to be still alive even up to this date. New insight in this co-evolution mechanism show that presence of both partners is needed to create new natural *R* genes and matching *Avr* effectors. As a result and due to the recent constrain to reduce use of pesticides, modern potato farming should aim more for (i) integrating pest management by monitoring virulence of *P. infestans* contemporary population, (ii) continue screening contemporary wild *Solanum* species for new resistance genes that have co-evolved with the pathogen, also in the last decades, and (iii) integrate the natural high degree of diversity of functional *Rpi* genes in stacking approaches or in *in vitro* evolution approaches like shuffling techniques (Farnham and Baulcombe 2006), to create new *R* genes with altered specificities.

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Summary

Potato (*Solanum tuberosum L.*) is nowadays the most important non-cereal food crop in the world. It is prone to huge annual losses due to late blight, the disease caused by the oomycete pathogen *Phytophthora infestans*. Modern management of late blight necessitates the use of multiple resistance (*R*) genes, which requires efficient pipelines for identification, isolation and characterization of *R* genes. Since the genomics era, the *P. infestans* genome is disclosing numerous effector genes. Effectors are molecules that manipulate the host to facilitate infection and/or trigger defence responses. In the presence of a matching *R* gene, effectors cause a hypersensitive response (HR) that often leads to avirulence, and in such case they are designated avirulence (*Avr*) genes. One group of cytoplasmic effectors of *P. infestans* is characterised by an RXLR motif which allow these proteins to be translocated in the host cytoplasm. This thesis employs effectoromics, use of effectors to probe resistance in a host plant, by using RXLR effectors of *P. infestans* to probe resistant *Solanum* germplasm for late blight *R* genes and sort out their functional redundancy and specificity. Research was focused on the genomic diversity and (co-)evolution of three R/AVR interactions: (i) the *Rpi-blb1/ipiO* pair by exploring the genetic diversity of the *ipiO* effector family in a large set of *P. infestans* isolates, (ii) the *R3a/Avr3a* pair, with the identification of various *R3a* gene homologues (*R3aGH*) and a functional *R* gene (*Rpi-sto2*), and (iii) the *R2-PiAvr2/PexRD11* interaction, where we identified and characterized genetic diversity at both *R* and AVR side, and describe their co-evolution.

In the last half century, most late blight *R* genes that were introgressed in potato were quickly defeated. In the early XXI century, a <u>R</u>esistance gene against *<u>P</u>hytophthora <u>i</u>nfestans* (*Rpi*-gene) from *S. bulbocastanum* (*Rpi-blb1*) was characterized as an exclusive broad-spectrum *R* gene. Using the recently uncovered avirulence effector *Avr-blb1* (also known as *ipiO*) recognised by *Rpi-blb1*, we have screened and characterized a genetically diverse set of *P. infestans* isolates originating from different countries, for their *ipiO* diversity (**Chapter 2**). In total 16 *ipiO* variants grouped in three distinct classes by phylogeny were identified. Class I and II, but not class III *ipiO* variants induced cell death when co-infiltrated with *Rpi-blb1* in the model plant *Nicotiana benthamiana*. The major breakthrough came from the discovery of the two first *P. infestans* isolates that defeated *Rpi-blb1*. The virulence on *Rpi-blb1* potatoes correlated with the absence of the highly diverse class I *ipiO*. Both isolates were originating from Mexico, one of the centres of origin of the pathogen, and probably evolved due to the resistance pressure of the host in this region. Consequently, the expected broad-spectrum *Rpi-blb1* has shown weaknesses as any other *R* gene against late blight. This study showed that characterization of the genetic diversity of an *Avr* gene is essential to predict the effectiveness of an *R* gene in long-term disease management. Moreover, our findings provided a technical solution for potato farmers, large-scale monitoring aimed at diagnosing the *Avr-blb1* classes

in the *P. infestans* population on commercial potato growing areas can be applied to detect (evolution and migration of) virulence against *Rpi-blb1*. When virulence is detected, cultivars with *Rpi-blb1* should be omitted for a certain period of time and other *R* genes applied in stead, in order not to increase selection pressure towards losing class I *ipiO* in the pathogen.

In an attempt to identify new *Rpi* genes against late blight we have screened and probed resistant *Solanum* germplasm with cytoplasmic effectors of *P. infestans* using a transient assay based on Potato Virus X (PVX) agroinfection (**Chapter3**). This approach resulted in the identification of candidate *Avr* gene *PexRD11*. Further screening with PEXRD11 on a large set of genotypes representing the broad *Petota* genepool, followed by mapping studies, resulted in the identification of new *Rpi*-candidate genes against *PexRD11* in the *R2* cluster. Interestingly, only three species originating from Mexico responded to PEXRD11, i.e. *S. edinense*, *S. schenckii* and *S. hjertingii*. Allele mining on these genotypes yielded 17 new *R2* variants grouped in six classes. From these, six new *R2* homologues were characterized, i.e. *Rpi-edn1.1*, *Rpi-snk1.1*, *Rpi-snk1.2*, *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3*, recognising *PexRD11* and *PiAvr2*, a previously described related RXLR effector gene, and conferring resistance to *P. infestans*. PEXRD11 and PiAVR2 have a low percentage of similarity, however some degree of redundancy was observed because both are triggering cell death when co-infiltrated with R2 homologues. We concluded that the *R2* family has evolved in Mexico, one putative centre of origin of the pathogen, and provided an overview of the evolution and functional diversification of this *R* gene family in its natural environment.

The recent completion of the sequence and annotation of the *P. infestans* genome provides new opportunities to study the evolution of gene families of this pathogen and new avirulence functions for some of its effectors can be identified. Following the discovery of the effector PEXRD11 recognition by R2 in Chapter 3, research was focussed on the identification and characterization of *PiAvr2/PexRD11* family members within the *P. infestans* genome (**Chapter 4**). In addition of *PiAvr2* and *PexRD11,* 11 new independent RXLR effectors were identified that were grouped in three classes. A robust association was discovered between phylogenetic classes of the PiAvr2/PexRD11 effector family and the R2 resistance gene family with recognition leading to cell death. The ten known functional R2 homologues, i.e. the six found in Chapter 3 and the already cloned *R2, R2-like, Rpi-abpt* and *Rpi-blb3*, recognised three new effectors, i.e. PITG_13940, PITG_21949 and PITG_21645, with different specificity. The six R2 homologues responded to PITG_13940 but not the four previous known R2 homlogues which were used in breeding material for the last decades. Different hypotheses based on secondary structure, and presences of motifs between the PiAVR2/PEXRD11 effector family members have been discussed to explain induction or absence of cell death with the ten R2 homologues. All together, these results highlight the diversity of the R2-PiAVR2/PEXRD11 interaction resulting from a co-evolution of the *Solanum*/*P. infestans* pathosystem. Moreover by using effectoromics, six new *R2* homologues variants with potentially a broader resistance spectrum could be characterized.

This study therefore provides more diversity and specificity of *R* gene homologues, which may be valuable for potato breeding.

R3a from *Solanum demissum* is known to interact with the avirulent effector *Avr3a^{KI}* of *P. infestans*, resulting in the induction of cell death visible by necrotic symptoms on the interaction site. Screening wild *Solanum* species to identify R3aGH and new R genes responding to the virulent allele *Avr3a^{EM}* and/or avirulent allele *Avr3a^{KI}* is crucial to provide more *R* gene diversity, as we saw in Chapter 4. In **Chapter 5** we have combined three different techniques to gradually reduce the number of genotypes which potentially contained a functional R3aGH. On the 177 genotypes tested, we indentified nine genotypes with the same pattern of response to the effectors as three R3a-plants and with seven of them showing the same resistance spectrum as R3a plants. Interestingly these genotypes came from two different centres of diversity, Mexico and Argentina. Allele mining on one of the seven genotypes, *S. stoloniferum* from Mexico, revealed a high rate of evolution for *R3a*. We identified one functional *R3aGH*, designated *Rpi-sto2,* which triggered cell death when coinfiltrated with AVR3a^{KI} on *N. benthamiana*. This study shows that *R* genes in the *R3a* locus are under a tremendous evolutionary pressure from the pathogen with the presence of only one functional *R3aGH* out of 29 variants cloned in the Mexican *S. stoloniferum*, and that *R3a* has potentially evolved under pressure of two geographically distinct populations of *P. infestans*. To unravel the tight pressure from the pathogen on this resistance gene we monitored the *Avr3a* family allelic combination in a set of isolates of *P. infestans*. We observed an over-representation of isolates that only contained virulence allele *Avr3a^{EM}*, which suggests a selection pressure to avoid AVR3a^{KI} recognition in host plants. In addition we discovered a new allele, *Avr3a^{KIL}*, which thus far seems to have similar specificity as Avr3a^{KI}. Our results in this chapter offer new *R3aGHs* that can be used in future breeding programs or in molecular engineering of *R* genes with perhaps a broader spectrum of effector recognition.

This study has led to the development of genetic tools to identify and characterise new RGHs valuable for potato breeding. Moreover the functional genomics studies of specific R/AVR pairs of *Phytophthora infestans* effectors and *Solanum* resistance genes have brought more insight in the co-evolution of this pathosysthem. In conclusion, three major discoveries have been made, 1. Discovery of new *R* gene homologues for *R2* and *R3a*, 2. With the *R2* homologues displaying a broader spectrum of responses to the *PiAvr2/PexRD11* family members of *P. infestans*, and 3. Characterization of the diversity of *ipiO* variants in the *P. infestans* population, which can be used for monitoring and provides new perspectives for management of *Rpi-blb1* expressing cultivars in the field.

Samenvatting

De aardappel (*Solanum tuberosum* L.) is wereldwijd het belangrijkste voedselgewas, na tarwe en rijst. Echter, de aardappelteelt lijdt jaarlijks grote verliezen door de aardappelziekte, die veroorzaakt wordt door de oömyceet *Phytophthora infestans*. Voor het beheersen van de aardappelziekte tijdens de teelt zijn gestapelde resistentiegenen (*R* genen) nodig. Om deze te verkrijgen zijn nieuwe strategieën voor de identificatie, isolatie en karakterisering van R genen een vereiste, en hier worden nieuwe mogelijkheden beschreven die dit op een efficiënte manier mogelijk maken. Tijdens het huidige "genomics" tijdperk, zijn in het genoom van *P. infestans* vele effectorgenen ontdekt. Effectors zijn moleculen van *Phytophthora* die de waardplant manipuleren om infectie mogelijk te maken, en/of afweerreacties te initiëren. Als een effector en het bijbehorende *R* gen beide aanwezig zijn, wordt in de plant de overgevoeligheidsreactie (HR) geïnitieerd. Dit leidt vaak tot avirulentie (de plant is resistent), en in dit geval noemen we de effectors ook wel avirulentiegenen (Avr). Een groep van cytoplasmatische effectors van *P. infestans* bezit een zogenaamd RXLR motief, dat ervoor zorgt dat de effectors de plantencel binnen kunnen dringen om daar hun (a)virulentie rol te vervullen. Dit proefschrift beschrijft "effector genomics", het gebruik van effectors om resistentiegenen te identificeren in resistente planten. We testen RXLR effectors op wilde *Solanum* soorten voor het induceren van HR, om daarmee de aanwezigheid van *R* genen te ontdekken. Vervolgens karakteriseren we de *R* genen op hun werking met behulp van functionele assays. Ons onderzoek was gericht op genetische diversiteit en de (co-)evolutie van drie specifieke *R / Avr* interacties: (i) de *Rpi-blb1 / ipiO* interactie, waarbij de genetische variatie van de *ipiO* familie bestudeerd is in een uitgebreide set van *P. infestans* isolaten, (ii) de *R3a / Avr3a* interactie, waarbij een aantal *R3a* homologen (*R3aGH*) en een nieuw, functioneel, *R* gen (*Rpi-sto2*) geïdentificeerd zijn, en (iii) de *R2 / PiAvr2/PexRD11* interactie, waarbij de genetische diversiteit aan zowel *R* als *Avr* gene gekarakteriseerd is. Dit leidde tot een beschrijving van hun co-evolutie in Mexico.

In de tweede helft van de vorige eeuw, zijn ingekruiste *R* genen tegen *P. infestans* (*Rpi*) doorbroken waardoor resistente aardappelrassen toch weer vatbaar werden. In het begin van de 21[e] werd een nieuw *R* gen geïsoleerd uit *Solanum bulbocastanum*. Dit *Rpi-blb1* gen werd beschreven als een exclusief breed-spectrum *R* gen, dat duurzame resistentie zou geven. In **hoofdstuk 2** hebben we een diverse set van *P. infestans* isolaten gekarakteriseerd voor hun avirulentie eigenschappen op *Rpi-blb1*. We ontdekten twee *P. infestans* isolaten die *Rpi-blb1* konden doorbreken. Beide isolaten waren afkomstig uit Mexico, een gebied waar *P. infestans* oorspronkelijk geëvolueerd is, waarschijnlijk onder druk van resistente waardplanten uit die regio. Een studie naar de genetische variatie van *Avr-blb1* (ook bekend als *ipiO*) leverde 16 *ipiO* varianten. Deze konden, met behulp van een fylogenetische analyse, in drie klassen gegroepeerd worden. Klasse I en II *ipiO* bleken de HR te induceren na co-infiltratie met *Rpi-*

blb1 in de modelplant *Nicotiana benthamiana*, maar klasse III *ipiO* deed dit niet. De virulentie van de nieuwe isolaten op *Rpi-blb1* aardappels hing nauw samen met afwezigheid van de zeer diverse klasse I *ipiO*. Deze resultaten bewijzen dat het *Rpi-blb1* gen, waarvan een breed-spectrum resistentie verwacht werd, inmiddels ook doorbroken is, net als eerder gebruikte *R* genen tegen *P. infestans*. Onze studie toont aan dat de karakterisering van genetische variatie van een *Avr* gen een goed handvat kan bieden om de werkzaamheid van een *R* gen in de aardappelplant te voorspellen. Dit is van belang voor resistentiemanagement op de lange termijn. Daarnaast bieden onze bevindingen een technische oplossing voor aardappeltelers: het op grote schaal monitoren van *Avr-blb1* klassen in de *P. infestans* populatie in commerciële aardappel velden is bruikbaar om ontwikkeling van virulentie tegen *Rpi-blb1* snel te detecteren. Zodra virulentie wordt gedetecteerd, zouden rassen met *Rpi-blb1* resistentie voor een zekere periode niet geteeld moeten worden, om de selectiedruk voor verlies van klasse I *ipiO* niet te stimuleren. In plaats van *Rpi-blb1* zouden dan tijdelijk aardappelrassen met andere *R* genen gebruikt kunnen worden.

In een poging om nieuwe *Rpi* genen en bijbehorende *Avr* genen tegen de aardappelziekte te identificeren, werden resistente *Solanum* soorten op het induceren van de HR tegen RXLR effectors van *P. Infestans* gescreend. Dit werd gedaan met behulp van een transiënte expressiemethode gebaseerd op het aardappel X virus (PVX) (**hoofdstuk 3).** Deze benadering resulteerde in de identificatie van het kandidaat *Avr* gen *PexRD11*. Verdere screening met *PexRD11* op een grote set van aardappelgenotypen, die de brede "gene pool" van de *Solanum* sectie *Petota* (knoldragende wilde verwanten van de aardappel) representeren, resulteerde in de identificatie van nieuwe *R* gen kandidaten in het *R2* cluster. Slechts 3 *Solanum* soorten, allemaal afkomstig uit Mexico, reageerden op PEXRD11, t.w. *Solanum edinense, S. schenckii* en *S. hjertjingii*. "Allele mining" op deze genotypen leverde 17 nieuwe *R2* varianten op, die in 6 klassen werden gegroepeerd. Zes R2 homologen, t.w. *Rpi-edn1.1, Rpi-snk1.1, Rpi-snk1.2, Rpi-hjt1.1, Rpi-hjt1.2 en Rpi-hjt1.3*, allemaal behorend tot klasse I of II, herkenden PEXRD11 en PiAVR2 (een eerder beschreven RXLR effector die herkend wordt door R2). PEXRD11 en PiAVR2 hebben weinig homologie, echter een zekere mate van functionele redundantie werd gevonden, omdat beide genen celdood induceren wanneer ze gecoïnfiltreerd worden met *R2* homologen. We concluderen dat de *R2* familie geëvolueerd is in Mexico, een van de waarschijnlijke oorsprongsgebieden van *P. infestans*, en presenteren een overzicht van de evolutie en functionele diversificatie van de R2 gen familie in zijn natuurlijke omgeving.

De recent beschikbaar gekomen, geannoteerde, *P. infestans* genoomsequentie biedt nieuwe mogelijkheden om de evolutie van genenfamilies te bestuderen, en nieuwe virulentiefuncties voor een aantal effectors te kunnen identificeren. Na de ontdekking van de PEXRD11 effector herkenning door R2 in hoofdstuk 3, hebben we ons gericht op de

karakterisering van *PiAvr2 / PexRD11* genen families in het *P. infestans* genoom. Naast *PiAvr2* en *PexRD11* werden 11 nieuwe RXLR effectors geïdentificeerd en in 3 klassen gegroepeerd. Een robuuste associatie tussen het optreden van de HR en fylogenetische verwachtschap van de *Avr* en *R* familie werd gevonden. Tien bekende *R2* homologen, t.w. de 6 *R2* homologen beschreven in hoofdstuk 3 en de reeds eerder gekloneerde *R2, R2-like, Rpi-abpt* en *Rpi-blb3*, herkenden 3 nieuwe effectors, t.w. PITG_13940, PITG_21949 en PITG_21645, met verschillende specificiteit. De 6 R2 homologen gaven een HR na coinfiltratie met PITG_13940, maar de 4 reeds bekende R2 homologen die de afgelopen decennia in veredelingsprogramma's gebruikt zijn, deden dit niet. Verschillende hypothesen gebaseerd op de secundaire structuur van R2 genen, en aanwezigheid van bepaalde motieven in de PiAVR2/PEXRD11 varianten worden bediscusseerd om het al dan niet induceren van de HR met de 10 R2 homologen te verklaren. Samenvattend belichten onze resultaten de diversiteit van de R2 – AVR2/PEXRD11 interactie in het *Solanum – P. infestans* pathosysteem. Met effector "genomics" konden 6 nieuwe *R* gen homologen met een potentieel breder resistentie spectrum worden gekarakteriseerd. Deze studie laat additionele diversiteit en specificiteit van *R* gen homologen zien, die waardevol kan zijn voor resistentieveredeling van de aardappel.

Het R3a resistentie-eiwit uit *Solanum demissum* herkent het AVR3a[KI] avirulentie-eiwit van *P. infestans*, en dit resulteert in de inductie van de HR. Om een bredere diversiteit van *R3a* homologen te identificeren in wilde *Solanum* soorten kunnen deze gescreend worden voor het optreden van de celdoodreactie tegen het avirulente allel Avr3a[KI] en/of het virulente allel Avr3a[EM]. In hoofdstuk 5 werden drie verschillende technieken gecombineerd, om gradueel het aantal genotypen met een potentieel functioneel R3a gen te reduceren. Van de 177 geteste *Solanum* genotypen, konden uiteindelijk 9 genotypen geïdentificeerd worden die dezelfde reactiepatronen gaven tegen de *Avr3a* effectors als 3 *R3a* controle genotypen. Zeven van deze genotypen lieten ook hetzelfde resistentiepatroon tegen *P. infestans* isolaten zien. De geïdentificeerde *Solanum* genotypen zijn oorspronkelijk afkomstig van twee verschillende oorsprongsgebieden, Mexico en Argentinië. "Allele-mining" op één van deze genotypen, *S. stoloniferum* uit Mexico, liet zien dat *R3a* snel evolueert. Tussen 29 *R3a* varianten vonden we een functionele *R3a* homoloog, *Rpi-sto2*, die de HR induceert na co-infiltratie met *Avr3a[KI]* in *N. benthamiana*. Deze studie laat zien dat *R3a* onder hoge evolutionaire druk staat, en suggereert dat *R3a* mogelijk geëvolueerd is in twee geografisch verschillende gebieden met verschillende *P. infestans* populaties. Na bestudering van de allelische variatie van *Avr3a* in een aantal *P. infestans* isolaten vonden we dat isolaten met de *Avr3a[EM]* variant oververtegenwoordigd waren, mogelijk door een selectie druk om AVR3a[KI] herkenning in waardplanten te ontwijken. Naast de twee bekende allelen *Avr3a[KI]* en Avr3a[EM] vonden we nog een nieuw allel *Avr3a[KIL]*, dat dusver dezelfde specificiteit blijkt te hebben als *Avr3a[KI]*. Dit hoofdstuk biedt de ontdekking van nieuwe *R3a* homologen die direct gebruikt kunnen

worden in toekomstige veredelingsprogramma's, of die via toekomstig gerichte moleculaire aanpassingen van *R* genen kunnen leiden tot een breder herkenningsspectrum van effectors.

Deze studie heeft geleid tot de ontwikkeling van nieuwe genetische handvatten voor het identificeren en karakteriseren van nieuwe *R* gen homologen die waardevol zijn voor de aardappelveredeling. Daarnaast hebben de effector "genomics" studies van specifieke R / AVR combinaties van *P. infestans* effectors and *Solanum R* genen meer inzicht gegeven in de co-evolutie van dit pathosysteem. Samengevat zijn de volgende waarnemingen gedaan, 1.) De ontdekking van nieuwe *R* gen homologen van *R2* en *R3a*, 2.) de ontdekking van *R2* homologen met een breder spectrum van resistentiereacties tegen *PiAvr2 / PexRD11* familieleden, en 3.) de ontdekking dat genetische variatie van *ipiO* varianten gebruikt kan worden voor het monitoren van de *P. infestans* populatie en daarmee nieuwe perspectieven voor management van aardappelrassen met *Rpi-blb1* in de praktijk biedt.

Acknowledgements

At last, here we are. After a little more than four years in Wageningen, I have completed this PhD thesis. This thesis is not only the results of my scientific work from the past four years but the outcome of a long road and I am glad to acknowledge everyone who has contributed to the build-up of my scientific personality and therefore to the completion of this thesis.

As I like to say, it all started by "accident". In my case it was an advertisement, on a black board, for a training period in the Department of Biology at the University of Fribourg in Switzerland. This training period ended-up in an MSc. thesis under the supervision of **Prof. Felix Mauch** who taught me the art of thoroughness in science and **Dr. Lassaâd Belbahri** who gave me a liking to large scales experiments. I really appreciate the valuable time you have spent with me and I thank you both to have started off my scientific career.

Soon after my MSc. Thesis, I started to work has a Pre-Doc. in the research group of **Prof. Dr. Sophien Kamoun** at the Ohio State University, and that was definitively my key to science. I will never find enough words to thanks you for all what you have taught me. Science is not only an art of discovering but also an art of changing lives around you with simple things. I could not speak or write in English when we have met but by time you helped me through. I have always considered you more as a friend then a boss. Even during my PhD, you brought me lot of support and suggestions, and I thank you for that, as well as for your eternal scientific enthusiasm which is highly communicative.

All my work in Ohio and later in Basel brought me finally to Wageningen. I would like to thanks, therefore, my promoters, **Prof. Dr. Richard G.F. Visser** and **Prof. Dr. Evert Jacobsen** to have given me the opportunity to do my PhD in their department and under their promotion. I would also like to show my gratitude to the representative members of the **TQS** project in collaboration with **Avebe** who also contributed in this thesis. Thanks also to **Dr. Edwin A.G. van der Vossen** for your guidance for few months. It was a short but productive period at a key point of my PhD. And thanks to **Dr. Vivianne G.A.A. Vleeshouwers** for your supervision. **Vivianne**, I will never forget during my job interview your question. Why do you want to do science? I answered, because I always want to know "why". I think it is this same passion for "why" that has made us work together the last four years. You always gave me freedom to test all my crazy ideas and tried at the same time to curb my enthusiasm to large scale experiments. Looking at all the large tables in my thesis, I am not sure to have always listened at you. As we know, writing is not my favourite part in science, I much prefer the lab work, however you arrived to focus me on that task and to teach me all your knowledge about scientific writing and I am glad to have had the opportunity to be your student.

During my thesis I had the chance to supervise few students and I want to thank you all for your contribution in this thesis. **Anika**, and **Miranda**, your help in *ipiO* and *Avr3a* variants cloning has been so efficient and valuable that both of you ended up as co-author of a chapter. I wish you all the best in your scientific career. **Dennis**, my dear LD, you are what I call a character in your own. I have enjoyed a lot to work with you, and your contribution on the effectoromic screening is rewarded in one of this thesis chapter. But the most I enjoyed was to joke with you. I think for each experiment you have made, you have taught me a swearword in Dutch. Luckily you have spent only an MSc thesis with us!!! I wish you will be successful in your hunt of a PhD position; you have all the qualities to be a good PhD student. And finally **Tim**, we had only little interaction at the end of my PhD but your independence and knowledge in the laboratory, as well as the help from **Gerard** has brought the key to my Chapter 5, I will never thanks you both enough for that.

Our research group and department could never run smoothly without all the help we get from all technicians, and when you are a PhD student it is extremely appreciated. I would like to particularly thank **Maarten Nijenhius, Marian Oortwijn, Koen Pelgrom (Koentje), Annelies Loonen, Johan Bucher,** and **Gert van Arkel,** who were always ready to help on hunting for equipments or products and exchanging protocols tricks, without you guys, lab would be so boring and knowledge exchange so slow. Another big thank to all the vitro-team and principally to **Marcel Visser, Marjan Bergervoet, Bernadette Kronenburg, Greetje Kuiper-Groenwold, Isolde Pereira and Iris Tinnenbroek-Capel.** The vitro job is a repetitive boring work, but with you guys, it always ended-up to be a time of fun and productive work. Without your devotion in maintaining genetic material alive and producing transformants our work would be insignificant. As well, thanks to the secretaries, **Annie, Mariame, Letty** and **Janneke,** without your help the administrative work would have taken my full PhD time. I thank also people working at Unifarm for their assistance of taking care of our plants, especially to **Henk, Bert, and Rene Alles** but also **Dirkjan Huigen** who was my umbilical cord between Plant Breeding and Unifarm. Without your assistance, advice and expertise, most of my plants would have been dead before any experiments could be conducted.

My heartfelt thanks are also to **Dirk Budding**. My dear OR, you have been a great colleague and above all a very good friend. All the *P. infestans* disease test experiments we have conducted were more an enjoyment rather than work. You had the good intension to teach me Dutch language, and therefore I could learn the alphabet, how to count, to listen the time, and start a discussion with somebody. However this noble task turned rapidly in a game of swearwords, which I am sure we both enjoyed. Unfortunately you had to leave us much too early. I will always miss you my dear friend and this thesis is also in the loving memory of you.

I will now switch gear and I would like to thank **Chris Maliepaard** for his advises on statistics and on the microarrays design experiments which unfortunately could not be

In a big family you have brothers but also sisters and two of them are **Mariame Gada** and **Anoma Lokossou**. Thank you Mariame for your support and help in this jungle, and thank you Anoma for all the scientific discussion and for your friendship.

I want also to thanks the Thai community of Wageningen and particularly: **P'Voew and Kees, P'Rat and Marcel, P'Oh, P'Tan, P'Sayam, P'Pam, P'Aun, Book, Bright, Aum (Chang), Im, Mint, Mong, Pla, Earth, Mac, Off** and **Duang**. My stay in Wageningen would have never been the same without you guys.

To **Lykele** and **Mariane Aardema**, thanks for all your kindness and all the passionate discussion we had. My stay in Holland would never have been the same without you.

My former Ohio State Buckeyes friends, **Vero, Hedgar, Joe, Jorrun**, **Angel, Mizuho, Santiago ...** You all motivated me in one way or another to do a PhD and here I am. It took me a time but finally I made it. So, Go Buckeyes Go...

Finally I would like to thanks my family and particularly **my father** and **my mother** who always supported me in any decision I took, and **my little brother** who is so unique and special to me. Et oui c'est enfin finit, j'ais ma thèse et j'ai finit mes études ;-)

My last words go to my amazing wife, **Krissana**, and our daughter, **Phoebe**. I have always travelled where the wind blows me and my move to USA has not only been important in my professional life, but more significantly valuable in my private life. Without it I would have never met my lovely wife, Krissana. Thanks for all your support and love during these years. You always help me even when you had hard time in your own career you style had energy to motivate me. To my daughter, Phoebe, I want to just to say thanks as a proud daddy, thanks to bring so much love and spices in our lives. To my two girls: โปรดจงมั่นใจว่าฉันรักเธอมากมาย เกินกว่าจะบรรยายออกมาได้

About the author

Nicolas Champouret was born on November 15th, 1978 in Dijon, Côte d'Or, France. After completing his academic high school in 1997, he started a BSc (1997-2000) in Cell Biology and Physiology at the University of Burgundy followed by a first year of MSc. (2000-2001) in Plant Biotechnology at the same University. He completed his MSc (2001-2002) at the University of Victor Segalen Bordeaux II on Fungal Biotechnology. During his second year of MSc, he performed a seven-month internship at the Department of Biology at the University of Fribourg, Switzerland, where he developed gateway vectors and techniques to transform the Oomycete pathogen *Phytophthora brassicae*. From 2002 to 2004, he worked as a visiting scholar the Ohio State University where he developed a technique to transform the potato late blight pathogen *Phytophthora infestans*, and studied functional roles of extracellular protease inhibitors proteins from *P. infestans*. At the end of 2004 he moved to the University of Basel, Switzerland, where he started a PhD program on *P. infestans* transformation and microarrays genechip analyses.

From January 2006 to February 2010, he worked as a PhD student (AIO) in the Laboratory of Plant Breeding at Wageningen University, The Netherlands. During his PhD, he studied the *Functional Genomics of Phytophthora infestans Effectors and Solanum Resistance Genes* under the supervision of Dr. Vivianne G.A.A. Vleeshouwers. This thesis presents the outcome of his four years of PhD research. Since April 2010, he obtained a Post-Doc position at The Sainsbury Laboratory (Norwich, United Kingdom), where he works on *Identification, isolation and characterisation of new sources of stem rust resistance in diploid relatives of cultivated wheat*. This project is supported by the Two Blades Foundation.

List of Publications

Champouret N, Bouwmeester K, Rietman H, van der Lee T, Maliepaard C, Heupink A, van de Vondervoort PJ, Jacobsen E, Visser RG, van der Vossen EA, Govers F and Vleeshouwers VGAA (2009) *Phytophthora infestans* isolates lacking class I *ipiO* variants are virulent on *Rpi-blb1* potato. Mol Plant Microbe Interact **22**:1535-45

Vleeshouwers VGAA, Rietman H, Krenek P, Champouret N, Young C, Oh SK, Wang M, Bouwmeester K, Vosman B, Visser RG, Jacobsen E, Govers F, Kamoun S, Van der Vossen EA (2008) Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. PLoS One **3(8)**:e2875

Champouret N, Verzaux E, Lokossou AA, Rietman H, Win J, Cano LM, Meijer DA, Budding D, Pelgrom K, Yang M, van der Vossen EAG, Jacobsen E, Visser RGF, Birch PRJ, Kamoun S, Vleeshouwers VGAA. Evolutionary and Functional Analyses Reveal a Diverse Family of *R2* Late Blight Resistance Genes in Mexican *Solanum* Species. PLoS Pathogen (Submitted).

Rietman H, Champouret N, Hein I, Niks R and Vleeshouwers VGAA. Plants and oomycetes, an intimate relationship: Co-evolutionary principles and impact on agricultural practice. CAB Abstract (Submitted)

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School
EXPERIMENTAL PLANT SCIENCES
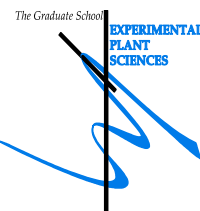
Issued to:	**Nicolas Champouret**	
Date:	**9 June 2010**	
Group:	**Laboratory of Plant Breeding, Wageningen University**	

1) Start-up phase	*date*
► **First presentation of your project**	
Identification and characterisation of Avr and associated R-genes: development of predicted durability resistance	Jun 2006
► **Writing or rewriting a project proposal**	
Identification and characterisation of Avr and associated R-genes: development of predicted durability resistance	Oct 2006
► **Writing a review or book chapter**	
Plants and oomycetes, an intimate relationship: Co-evolutionary principles and impact on agricultural practice. CAB reviews, in press (2010).	Oct 2009
► **MSc courses**	
► **Laboratory use of isotopes**	
Safe handling with radioactive materials and sources	Nov 2008
Subtotal Start-up Phase	*12.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days (Wageningen)	Sep 19, 2006
EPS PhD student days (Wageningen)	Sep 13, 2007
1st Joint Retreat of the PhD Students in Experimental Plant Sciences (IMPRS, SDV and EPS)	Oct 02-03, 2008
► **EPS theme symposia**	
Theme 2: Interactions between Plants and Biotic Agents, University of Amsterdam	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting Lunteren 2006: attendance	Apr 03-04, 2006
NWO-ALW meeting Lunteren 2007: attendance, poster	Apr 02-03, 2007
NWO-ALW meeting Lunteren 2008: attendance, presentation	Apr 07-08, 2008
NWO-ALW meeting Lunteren 2009: attendance	Apr 06-07, 2009
► **Seminars (series), workshops and symposia**	
Seminar Dr. Kurt H. Lamour	Nov 06, 2006
Seminar Prof. Andrew Bent	Jun 18, 2007
Plant Breeding research day	Sep 27, 2007
► **Seminar plus**	
► **International symposia and congresses**	
Oomycete Molecular Genetics Network (Wageningen, The Netherlands)	May 05-06, 2006
Oomycete Molecular Genetics Network (Asilomar, USA)	Mar 19-20, 2007
24th Fungal Genetics Conference (Asilomar, USA)	Mar 20-25, 2007
XIII International Congress on MPMI (Italy)	Jul 21-27, 2007
Oomycete Molecular Genetics Network (Birman, UK)	May 06-09, 2008
SOL Meeting 2008, Cologne (Germany)	Oct 12-16, 2008
XIV International Congress on MPMI (Québec, Canada)	Jul 19-23, 2009
► **Presentations**	
Oral presentation at international OMGN in Wageningen, 2006	May 05-06, 2006
Poster presentation at international OMGN in Wageningen, 2006	May 05-06, 2006
Poster presentation at Lunteren, OMGN and 24th FGC in Asilomar, 2007	Mar 19-25, 2007
Poster presentation at MPMI in Sorrento, 2007	Jul 21-27, 2007
Oral presentation at Plant Breeding research day, Wageningen, 2007	Sep 27, 2007
Oral presentation at Lunteren, OMGN in Birman and Summer school, 2008	Apr, May, Jun, 2008
Poster presentation at SOL meeting in Cologne, 2008	Oct 12-16, 2008
Poster presentation at MPMI in Québec, 2009	Jul 19-23, 2009
Oral presentation at TQS project (x9)	Apr 2006-Jun 2009
► **IAB interview**	Dec 2009
► **Excursions**	
Subtotal Scientific Exposure	*26.9 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Summerschool Signaling in plant development and defence:towards systems biology	Jun 19-21, 2006
Gateway technology (hands-on)	Nov 20-24, 2006
Molecular Phylogenies: Reconstruction and Interpretation	Oct 15-19, 2007
Summerschool: On the Evolution of Plant Pathogen Interactions: from Principles to Practice	Jun 18-20, 2008
► **Journal club**	
Literature discussion "Plant Breeding"	2006-2009
► **Individual research training**	
Collaboration on R3a gene shuffling, Scottish Crop Research Institute, Dundee, UK	Apr 18 – May 02, 2006
Subtotal In-Depth Studies	*10.6 credits**

4) Personal development	*date*
► **Skill training courses**	
Teaching and supervising Thesis students	Jun 13-14, 2006
Endnote 9 introduction and advance	Sep 26 and Oct 12, 2006
Basics Statistics	Dec 18-22, 2006
► **Organisation of PhD students day, course or conference**	
Organisation of the Lab-Trip of the Laboratory of Plant Breeding and PRI groups	Jun 01, 2006
Organisation of the 1st Joint Retreat of the PhD Students in Experimental Plant Sciences	Oct 02-03, 2008
► **Membership of Board, Committee or PhD council**	Nov 2007-Dec 2009
Member of EPS PhD student council	
Subtotal Personal Development	*6.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	56.3

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

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