Dissection of

the major late blight resistance cluster

on potato linkage group IV

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Content

Chapter 1

General introduction

General introduction

Potato (*Solanum tuberosum*) history and importance

The commercial potato derived from the wild species *Solanum tuberosum* originates from the Andes in South America. Originally it was first cultivated next to the present border separating Peru and Bolivia some 8,000 years ago. The Spanish took the potato from Latin America to Europe in the 16th century. Potato was first admired for its flowers before being appreciated for its tubers and since then potato became a major carbohydrate source in human and animal diets around the world. Adaptation to long days (Brown 1990; Hawkes 1978; 1994) and generations of breeding led to a panel of potato varieties differing in taste, skin color, shape, starch content, cooking type, etc… The fast growing characteristic of potato allowed poor families to cultivate it on small plots and break the circle of poverty. Hundred millions of people around the world depend on potato to survive. Potato is grown in more than 100 countries, under temperate, subtropical and tropical conditions and ranks as the world's third most important food crop, after rice and wheat. China is now the largest potato producer followed by India, Russian Federation and USA.

Late Blight and *Phytophthora infestans*

Monoculture and the lack of genetic diversity helped the emergence and dominance of the most significant threat to potato production worldwide: the Late Blight (LB) disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems and tubers. One memorable event happened in the 19th century, when Ireland was cultivating a few potato varieties sharing the same genetic background which were devastated by a Late Blight epidemic. Widespread famine followed with the death of millions of people and the migration of more than a million of Irish to the USA.

Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control and yield loss amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. To prevent spray of too large quantities of fungicides, the focus of different scientific groups is to find an efficient decision support system for sustainable cultivation of potato (Savenkov et al. 2003). The purpose is to gather information about the emergence and the progress of *Phytophtora infestans* in the field, weather forecast, cultivars planted, date of fungicide spreads and spatial epidemiology (spore release, escape,

transport, deposition and survival), with the aim to give a prediction about the risk of regional spore dispersal and survival helping the potato growers in an online service for decision making about when, which and how much fungicide to spray (Midilis program; Simcast (Skelsey et al. 2009).

Introgression of Late Blight Resistance by traditional breeding

Early in the 20th century the potential of the wild Mexican hexaploid species *Solanum demissum* was recognized for conferring extreme levels of Late Blight resistance and hybrids with *S. tuberosum* were generated. Subsequently, 11 major dominant resistance (*R*) genes were identified, and introgressed into potato cultivars by interspecific hybridization and back crosses with cultivated potato (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). The set of eleven genes (*R1* to *R11*) was exploited in monoculture, which besides providing an efficient crop protection, was exerting a strong selection pressure on the pathogen resulting in its rapid adaptation and consequently the defeat of those *R* genes (Fry 2008; McDonald and Linde 2002);. Even, the combination of different R genes in one cultivar did not always extend the life time of the cultivar in the field. The Scottish cultivar Pentland Dell containing three R genes, *R1, R2* and *R3* was infected six years after being released (Hein et al. 2009a). There are examples of cultivars, like cv Escort, with at least 3 combined R-genes which are most of the years showing a good protection against Late Blight (Dr. R. Hutten, Wageningen UR Plant Breeding pers. comm.).

Horizontal resistance

An alternative strategy to control Late Blight is to identify and combine loci harboring genes responsible for quantitative or partial resistance traits. Quantitative resistance loci (QRL) have been largely studied and are believed to provide a more stable durable resistance (Gebhardt 1994; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999); so called horizontal resistance, provided by partial resistance genes in contrast to the vertical resistance provided by major R-genes. However, Vleeshouwers et al. (2000)) showed that in backgrounds with partial resistance also HR reactions are found, suggesting the presence of R-genes with a partial effect. Late Blight QRL have been identified in many wild *Solanum* species. The Argentinean wild diploid species *S. microdontum* harbors PiQRL (Bisognin et al. 2005). *S. paucissectum* has PiQRL identified on linkage group X, XI and XII (Villamon et al. 2005). Ghislain et al (2001) mapped PiQRLs on linkage group VII and XII of *S. phureja*. Linkage group VII contains a PiQRL, identified in a population derived, from *S. demissum* (Meyer et al. 1998). Sarah Danan (2009) describes the identification in cross populations derived from *S. sparsipulim* and *S. spegazzinii*, a total of 30 Late Blight QRL present on 13 distinct genomic regions, confirming previous studies (Leonards-Schippers et al. 1992; Oberhagemann et al. 1999).

Pyramiding of R-genes and Cisgenesis

Another way to control Late Blight disease is to learn from local farmers in the Andean region who have domesticated thousands of varieties during generations. Nowadays, over 50 varieties are cultivated on their farms using the polyculture method acting as a genetic resistance shield against Late Blight. In modern potato culture, pyramiding is pictured in mixing individual *R*-gene containing cultivars or creating a cultivar containing stacked *R*-genes.

Conventional introgression of resistance often involves inter-specific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. The result is introgression of a piece of DNA with the gene of interest, surrounded by many other alleles of the donor species. This is called linkage drag and is almost always connected with linked alleles coding for traits with a negative impact (McGillivary and Clemente 1956). For durable Late Blight resistance strategies, efficient stacking of *Rpi* genes from one or several species is essential, but in practice this will enlarge the linkage drag problems considerably. Their introduction by genetic modification (GM) is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing varieties with a long history of safe use. Currently, *Rpi* genes of natural origin, so called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008) can be introduced using marker-free transformation systems leading to cisgenic plants with only the gene(s) of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date many *Rpi* genes have been cloned: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005a), *RB/Rpi-blb1, Rpi-blb2* and *Rpi-bt1* from *S. bulbocastanum* (Oosumi et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005), *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* (Vleeshouwers et al. 2008) and *Rpi-vnt1.1, Rp-vnti1.2* and *Rpi-vnt1.3* from *S. venturii* (Foster et al. 2009; Pel et al. 2009). All belong to the NBS-LRR class of plant *R* genes, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Additional *Rpi* genes that have been identified and genetically mapped, are *Rpi-blb3, Rpi-abpt, R2, R2-like* and *Rpi-mcd1* on chromosome 4 (Li et al. 1998; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c; Tan et al. 2008) but also *Rpi1*from *S. pinnatissectum* on chromosome 7 (Kuhl and Hanneman 2001); *Rpi-phu1* and *Rpi-mcq1* on chromosome 9 (Sliwka et al. 2006; Smilde and Brigneti 2005) R_{ber} on chromosome 10 (Ewing and Simko 2000; Rauscher et al. 2006) and *R3b, R5, R6, R7, R8, R9, R10* and *R11* on chromosome 11 (Bradshaw et al. 2006; Huang et al. 2005b; Huang et al. 2004).

The resistance pathway

Phytophtora infestans is an obligate biotroph which infects potato by an invaginating feeding structure (haustoria) into the host cell plasma membrane, establishing an intimate contact with this host, and delivering effector molecules (virulence effectors) into the plant cell to reprogram the plant cell in order to enhance microbial fitness. The plant reacts to this invasion by recognizing the avirulent effectors and activating the innate immunity via complex pathways that arrest proliferation of the pathogen, thus conferring disease resistance (McHale et al. 2006). The ZIGZAG model (Fig 1) in the oomycete-plant interaction (Hein et al. 2009b; Jones and Dangl 2006), nicely describes most important potato- *P-infestans* interactions. In its infection process, *P.infestans* first secretes the so-called pathogen-associated molecular patterns (PAMPs) like CBEL (the cellulose binding elicitor lectin) (Dumas et al. 2008; Gaulin et al. 2006) or elicitors like INF1 (Kamoun 2006) to trigger immunity (PTI). This process can be suppressed by secretion of effectors like the glucanase or protease inhibitor GIP2 (Damasceno et al. 2008) or EPI1 (Tian et al. 2004) which act either outside or inside host cells and re-program host metabolism to promote 'effector-triggered susceptibility' (ETS). When a successful *P. infestans* isolate has suppressed PTI, the products of plant, *Rpi* genes, as a second 'layer' of defense, can directly or indirectly detect avirulence proteins like AVR3a (Armstrong et al. 2005; Bos et al. 2006) or AVR1b (Dou et al. 2008; Shan et al. 2004). *Rpi* proteins subsequently activate resistance responses (effector-triggered immunity; ETI), often including programmed cell death during the hypersensitive response (Jones and Dangl 2006).

AVR protein detection is possible in a direct way or indirectly by the resistance genes harboring key domains for pathogen recognition, the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006). Although, the N terminal domain of NBS-LRR protein has been demonstrated to play a crucial role in AVR protein recognition (Moffett et al. 2009), multiple studies showed that the LRR domain is the region of the protein that confers recognition specificity (Hulbert et al. 2001; Meyers et al. 1998; Mondragon Palomino and Meyers 2002; Noel et al. 1999). In silico secondary structure of the NBS-LRR protein shows that the LRR repeats with the motif xxLxLxx form a β-strand/β-turn structure in which the x residues are exposed to solvent and are available for interactions with potential ligands (Kobe and Deisenhofer 1995). These solvent exposed residues are hyper variable and subject to diversifying selection, suggesting that these residues have evolved to detect variation in pathogen-derived ligands (Michelmore and Meyers 1998). So far five matching pairs of *PiAVR* and potato *R*-gene have been identified, *PiAVR3a/R3a* (Armstrong et al. 2005), *AVR4/ R4* (van Poppel et al. 2008), *AVR-blb1/Rpi-blb1* (Vleeshouwers et al. 2008), *AVR-blb2/ Rpi-blb2* (Oh et

al. 2009), and *AVRvent1 /Rpi-vent1-1* (Pel 2010). Detailed investigation of the PiAVR/R interaction was so far orientated to the effector side to identify the crucial domain necessary for the activation of the hypersensitive reaction. The C terminal domains were found indispensable for PiAVR3a and AVR4 recognition (Bos et al. 2006; Van Poppel et al. 2009).

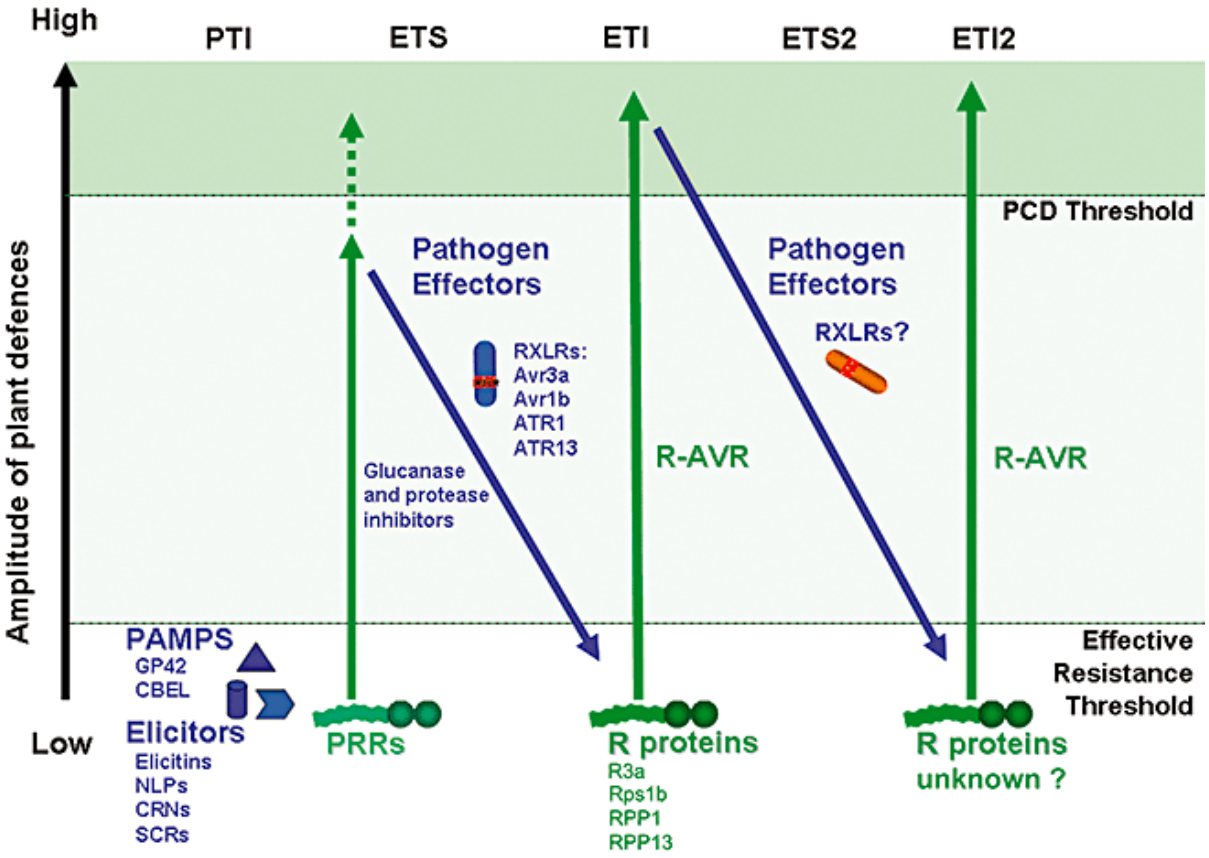

Fig. 1 The zig-zag-zig in oomycete–plant interactions. Published in Hein et al (2009b)

Outline of the thesis

The aim of the research described in this thesis was to study the major Late Blight resistance locus on linkage group IV, following the fine mapping and the characterization of the four orthologues resistance genes *Rpi-blb3, Rpi-abpt, R2* and *R2-like* (Park et al. 2005a, 2005b, 2005c; Li et al. 1998).

Cloning and functional characterization of the four genes is described in **Chapter 2,** using map based cloning and candidate gene allele mining approaches in combination with transient complementation in the heterologous *Nicotiana benthamiana* 'model' plant system. All 4 *Rpi* genes code for proteins of the NBS-LRR class of R proteins and share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete

H. arabidopsidis. Despite apparent differences between the four functional R proteins, all four induce a hypersensitive response upon co-expression with the *P. infestans* derived RxLR effector PiAVR2 (Gilroy et al., in preparation) in *N. benthamiana* leaves, indicating that they recognize the same effector protein

Solanum bulbocastanum is well known for its resistance to Late Blight (Niederhauser and Mills 1953), from which four Rpi-genes were so far cloned, *Rpi-blb1, Rpi-blb2, Rpi-blb3* and *Rpi-bt1* (Oosumi et al. 2009; Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005). As population genetic processes and dynamics might affect the evolution of *R*-genes, a better understanding of variation within and between populations is crucial. **Chapter 3** reports about the allelic frequencies of the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, Avr expression assays as well as detached leaf assays with key *P. infestans* isolates were utilized to strengthen the evidence for the presence of functional alleles. We related the occurrence of the genes to the geographical origin of the accessions.

Interestingly, several PiQRL mapped to the short arm of linkage group IV (Bradshaw et al. 2004; Bradshaw et al. 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000), on the R2 locus (Hein et al. 2009a; Hein et al. 2007). Exploring the putative homology with R2GHs we attempted, in **Chapter 4**, to molecularly identify the *Rpi* genes responsible of the field resistance evaluated in a F1 population with *S. microdontum* BGRC 18302-34 (CGN 21342) as source of resistance in the earlier parentage. Molecular and phenotypic characterization of the population permitted the identification of at least three genes segregating in the population. *Rpi-mcd1.1* and *Rpi-mcd1.2* were originating from *S. microdontum* with resistance to PIC99177 and IPO-0, respectively, and *Rpi-phu2* from *S. phureja* providing only resistance to PIC99177. The use of allelic mining strategies resulted in the identification of 27 *R2* homologues, among which *Rpi-mcd1.1* was identified to be functional by using a transient complementation assay in *Nicotiana benthamiana*.

In **Chapter 5** we explored the information provided by the alleles generated in different *Rpi-blb3* allele mining strategies and used the transient co-expression system in *N. benthamiana* to identify the repeats in the LRR domain necessary for PiAVR2 recognition. Domain swaps between R genes interacting or not with PiAVR2 permitted the identification of a region spanning three LRR repeats of the cloned *Rpi* genes that determine the *R2* resistance specificity. Site directed mutagenesis pinpointed one solvent exposed amino acid to be crucial for PiAVR2 recognition.

Chapter 2

Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV

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ABSTRACT

Additionally to *Rpi-blb1* and *Rpi-blb2*, *S. bulbocastanum* appears to harbor *Rpi-blb3* located at a major late blight resistance locus on LG IV, which also harbors *Rpi-abpt*, *R2*, *R2-like* and *Rpi-mcd1* in other *Solanum* spp. Here we report the cloning and functional analyses of 4 *Rpi* genes, using a map-based cloning approach, allele mining strategy, Gateway™ technology and transient complementation assays in *N. benthamiana. Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* contain all signature sequences characteristic of LZ-NBS-LRR proteins, and share amino-acid sequences 34.9% similar to RPP13 from *Arabidopsis thaliana*. The LRR domains of all 4 Rpi proteins are highly homologous whilst LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and those encoded by additional *Rpi-blb3* gene homologs, suggest exchange of LZ, NBS and LRR domains, underlining the modular nature of these proteins. All four Rpi genes recognize the recently identified RXLR effector PiAVR2.

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INTRODUCTION

The potato (*Solanum tuberosum)* ranks as the world's third most important food crop, after wheat and rice. Compared to these other staple crops a greater proportion of the potato crop is edible and a higher yield per hectare is achieved, which, combined with its high nutritional value, underlines its importance and explains ongoing increases in global potato production. The most significant threat to potato production worldwide is the late blight disease, caused by the oomycete pathogen *Phytophthora infestans*, which destroys leaves, stems and tubers. Current disease control relies on multiple (up to 20) fungicide applications per season (Hanson et al. 2007) and costs associated with chemical control amount to €5 billion globally per year (Whisson et al. 2001). Such pesticide inputs are both environmentally and economically undesirable and have a negative impact on the sustainability of potato production. Host resistance is a more environmentally benign means of restricting late blight infection, but the success of this management practices hinge on effective and durable host resistance and an understanding of the pathogen population.

Early in the 20th century the potential of the wild Mexican hexaploid species *Solanum demissum* was recognized for conferring extreme levels of late blight resistance, and hybrids with *S. tuberosum* were generated. Subsequently,11 major dominant resistance (*R*) genes were identified, and introgressed into potato cultivars (Müller and Black 1952; Malcolmson and Black 1966; Malcolmson 1969) However, deployment of these single genes did not provide durable resistance due to the rapidly changing populations of *P. infestans*. As an alternative approach, breeders started to select for quantitative 'field' resistance using races of *P. infestans* that were compatible with the *R* genes present in their breeding material (Toxopeus 1964; Black 1970). Despite considerable progress in genetic analysis of quantitative resistance to late blight using molecular markers (Gebhardt and Valkonen 2001; Simko 2002) under long day conditions breeders have achieved little progress, the major draw backs being tetrasomic inheritance of potato and the strong linkage between foliage resistance and late foliage maturity (Oberhagemann et al. 1999; Visker et al. 2003; Bradshaw et al. 2004). It is therefore anticipated that breeding for late blight resistance in potato, aimed at substantially contributing to disease management, requires the deployment of major resistance to *P. infestans* (*Rpi*) genes. Recent germplasm screens are revealing a wealth of *Rpi* genes in wild *Solanum* species and the challenge now is how to select, judiciously combine, and deploy sets of *Rpi* genes that can confer durable late blight resistance in modern potato varieties.

Key to durable exploitation of *Rpi* genes is also a better understanding of their interactions with effectors from *P. infestans.* All microbes trigger immune responses in plants *via* host receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs). Such PAMP-triggered

immunity (PTI) can be suppressed by secretion of effectors which act either outside or inside host cells and re-program host metabolism to promote 'effector-triggered susceptibility' (ETS). When a successful pathogen has suppressed PTI, the products of plant, *R* genes, as a second 'layer' of defense, can directly or indirectly detect effectors (termed avirulence proteins; AVRs). R proteins subsequently activate resistance responses (effector-triggered immunity; ETI), often including programmed cell death during the hypersensitive response (Jones and Dangl 2006). The ability of a pathogen to evade recognition in the *R*-gene-for-*Avr*-gene concept (Flor 1971), can be explained by an evolutionary 'arms race' (Bergelson et al. 2001; Holub 2001). The generation, through mutation, and selection of *AVR* alleles that evade recognition is matched by strong selection pressure for *R* gene alleles that retain recognition. This is particularly evident in the case ATR1-RPP1 and ATR13-RPP13 interaction in the *Hyaloperonospora arabidopsidis* (formerly *H. parasitica*)-Arabidopsis pathosystem, where considerable allelic diversity has been revealed by studying natural host and pathogen populations (Rehmany et al. 2005; Allen et al.2008). In naturally co-evolving *P. infestans*-potato populations, similar high levels of allelic diversity are expected to be observed. So far, studies of the PiAVR3a-R3a interaction reveal diversifying selection for both genes (Amstrong et al. 2005; Huang et al, 2005). Recently, several oomycete *AVR* genes were identified (Allen et al. 2004; Shan et al. 2004; Armstrong et al. 2005; Rehmany et al. 2005; Vleeshouwers et al. 2008). Although each of the AVR proteins is distinct at the level of primary sequence similarity, they all share a secretion signal peptide followed by the motif RXLR and an acidic region, often ending in the sequence EER (Whisson et al. 2007; Birch et al. 2008a; Birch et al. 2008b).The RXLR-EER region is required for translocation of PiAVR3a, following its secretion from *P. infestans* haustoria, to the inside of host plant cells (Whisson et al. 2007). Translocation of RXLR-containing proteins inside the plant cell is consistent with their recognition by host NBS-LRR resistance proteins (Birch et al. 2006). As all oomycete AVR proteins identified to date have the RXLR motif, it provides a signature to identify AVR candidates from the *P. infestans* genome. Future knowledge of RXLR effectors diversity and their survey in *Phytophtora infestans* population could predict the span life of Rpi-genes in specific areas.

Conventional introgression of resistance often involves inter-specific crosses and repeated backcrosses with cultivated potato, which is a very slow and inefficient process. Currently, *Rpi* genes of natural origin, so called cisgenes (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008), can be introduced using marker-free transformation systems leading to cisgenic plants with only the gene(s) of interest and without linkage drag of other genes from the wild species or from antibiotic resistance genes normally present at the T-DNA (de Vetten et al. 2003; Schaart et al. 2004; Yu et al. 2006). To date six *Rpi* genes have been reported: *R1* and *R3a* from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005) *RB/Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Song et al. 2003; van der Vossen

et al. 2003; van der Vossen et al. 2005),and *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* and *S. papita*, respectively (Vleeshouwers et al. 2008). All belong to the NBS-LRR class of plant R proteins, which are thought to be cytoplasmic (van der Biezen and Jones 1998). Various loci are reported to contain *Rpi* genes on linkage group (LG) VII (Kuhl and Hanneman 2001), LG IX (Smilde and Brigneti 2005; Sliwka et al. 2006), LG X (Ewing and Simko 2000; Rauscher et al. 2006) and LG XI (Huang et al. 2004; Huang 2005; Bradshaw et al. 2006).

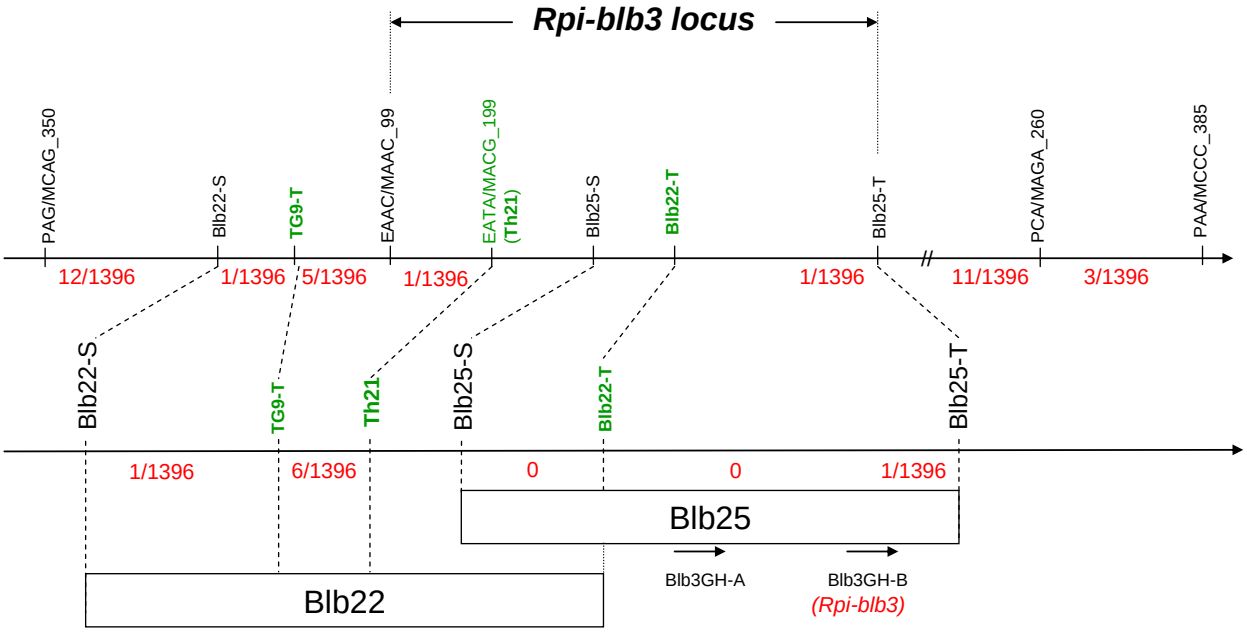
Here we describe the cloning and functional characterization of *Rpi-blb3, Rpi-abpt, R2* and *R2-like* from the major late blight resistance locus on LG IV of potato (Park et al. 2005a, 2005b, 2005c; Li et al. 1998). Upon map based cloning of *Rpi-blb3*, an efficient candidate gene allele mining approach in combination with transient complementation in the heterologous *Nicotiana benthamiana* 'model' system lead to the rapid cloning of the other three genes. All 4 *Rpi* genes code for proteins of the NBS-LRR class of R proteins. Interestingly, with respect to known functional R proteins, they share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to the oomycete *H. arabidopsidis*. Despite apparent differences between the four functional R proteins, all four induce a hypersensitive response upon co-expression with the *P. infestans* derived RxLR effector PiAVR2 (personal communication, E. Gilroy) in *N. benthamiana* leaves, indicating that they recognize the same effector protein.

RESULTS

Cloning of Rpi-blb3 and Rpi-abpt

In order to clone *Rpi-blb3* and *Rpi-abpt*, two BAC libraries were constructed using DNA derived from the resistant clones Blb99-256-3 and 707TG11-1, respectively. Approximately 74000 clones with an average insert size of 85 kb, corresponding to 8 genome equivalents, were obtained for each library. These libraries were screened initially with SCAR marker Th21, which co-segregated with resistance in mapping populations of 1396 and 1383 F1 progeny plants, respectively (Park et al. 2005a). In this way BAC clones Blb22 and TG9 were identified, respectively (Fig. 1A and 1B). By sequencing the ends of these two BACs, new markers were developed which were used to define the genetic intervals of the *R* loci more precisely and to re-screen the BAC libraries to identify clones that overlapped with the initial BAC clones. In this way the *Rpi-blb3* locus was delimited to a 0.1 cM interval (EAAC/MAAC_99- Blb25-T; 2/1396 recombinants) that is physically spanned by the two partially overlapping BAC clones Blb22 and Blb25 (Fig. 1A). In the case of the *Rpi-abpt* locus, the partially

A.



B.

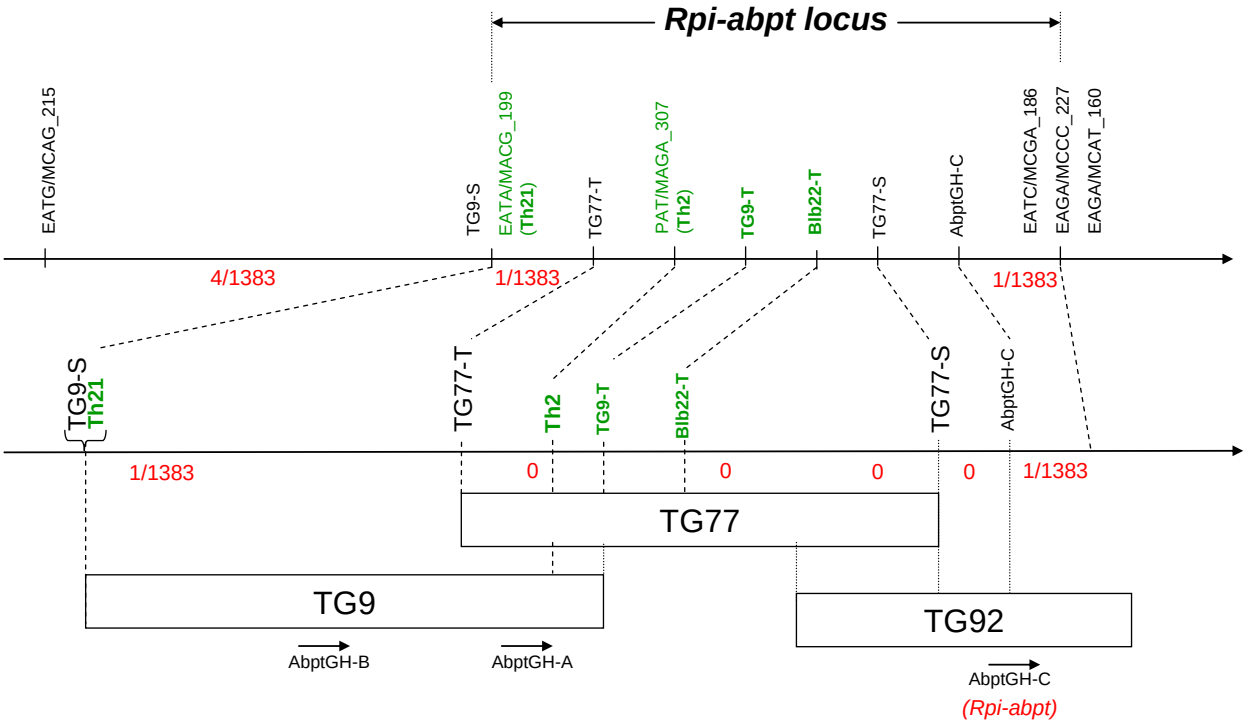


Figure 1: Genetic and physical map of the *Rpi-blb3* (A) and the *Rpi-abpt* (B) loci. Indicated are the relative positions of markers (in green are the one used in the mapping of both genes), the number of recombinants identified between markers, the overlapping BAC clones defining the two loci, and the relative positions of the *R* gene candidates that have been targeted for complementation analysis

overlapping BAC clones TG9 and TG77 were identified. One end of the contig co-segregated with resistance (TG77-S) whilst the other mapped 0.1 cM proximal to *Rpi-abpt* (TG9-S) (Fig. 1B).

Table 1. Overview of markers and primers used for mapping and cloning of *Rpi-blb3*

Name	Type	Polymerase chain reaction primer (5′ to 3′)	Tm[a]	Enzyme[b]
Th21	SCAR	F: ATTCAAAATTCTAGTTCCGCC		a.s.
		R: AACGGCAAAAAAGCACCAC		
Blb22-S	CAPS	F: GTTTGATGTATGTTTGTTCTTGC	56	Msp1
		R: TAATGCACTAATAACTAACTAGG		
Blb22-T	SCAR	F: CTTTATTAGTTCCAAGAGCTAC	56	
		R: ACCCATCCCTTTTTCCTTATC		
Blb25-S	CAPS	F: ACAGATGCTACGTCCATCAC	56	Alu1
		R: CTCCACATGCGATGCAAAAAG		
Blb25-T	CAPS	F: TTTCGATTATGGTGAGCCTTC	56	Hpy 188
		R: TAGAAAAAGGGTGGTTGTGAC		
RGH1	CAPS	F: GGSAAGACCACTCTTGCAAG	50	HpyCH4IV
		R: GGTTTTTAAGCTGCTAATGTTG		
RGH2	SCAR	F: GGSAAGACCACTCTTGCAAG	50	a.s.
		R: TGGTYATAATYACTCTGCTGC		
RGH3	CAPS	F: ATGRCTGATGCMTTTRTGTC	50	HaeIII
		R: CCYAAGTASAGAAAACACTGC		
4-PLOOP		F: GGiATGGGiGGiYTiGGiARGAC	68	
4-GLPL		R: TACiACAATiGCAAGiGGTAAMCC		
4-GLO2		F: GTGTCTCTCAAGAGTACAACAC	56	
		R: GCTCGAACATCAAGTAGTTTCC		
Blb3-start		F: ATGGCTGATGCCTTTCTRTCATTTG	55	
Blb3-end		R: TCAGGAATCTCCTTTAAATTTGGAC		
Blb3-prom		F: TCTTCCTTAGCATTCGTAGC	55	
		R: CTTTAGGAATACTAGTTTTGATTG		
Blb3-ter		F: AGCTTTTCTGCCAAGCACATTGG	55	
		R: GTACCCTCCGTTTGTCGTTTGATC		
Blb3-LRR-1-8		F: CTCTTTATGTATCAGACATGGC	55	
		R: CAACATCTTTCCACTGATCAC		
Blb3-prom -end		F: CCCCAAGTTGTATAATGGTTG	55	
Blb3-orf-bg		R: TGCTTGAGTGATTGAATCTCC		
Sto-orf-bg		R: GGCCATATTCAGACTGGGAG		
Blb3-spe		F: AGCTTTTTGAGTGTGTAATTGG	55	
		R: GTAACTACGGACTCGAGGG		

[a] Annealing temperature. In AF411807R, annealing temperature was 48°C for the first 7 cycles and 54°C for the last 30 cycles.

[b] Restriction enzymes that reveal polymorphism between resistant and susceptible linked alleles of the marker. a.s: allele-specific marker showing polymorphism without digestion

To gain insight into the molecular structure of the *R* loci under study, BAC clones Blb22 and TG9 were sequenced to 6x coverage. This revealed that clone Blb22 did not contain any *R* gene

homologues (RGH) whereas clone TG9 contained two RGH, which shared significant similarity to RGHs present in the sequenced tomato BAC clone AF411807L (Van der Hoeven et al. 2002) (Fig. 1). BAC clones Blb25 and TG77 were subsequently screened for the presence of RGH-specific sequences through PCR analysis using the primers 4-PLOOP-F and 4-GLPL-R (Table 1) which were designed by aligning the RGH sequences of clone AF411807L with those present on BAC clone TG9. Southern analysis of BAC clones Blb22, Blb25, TG9 and TG77 using an RGH-specific PCR fragment amplified from BAC clone Blb25 as a probe identified a minimum of two RGHs on BAC Blb25 and TG9 and one RGH on TG77 located on the overlapping part with TG9 (Fig. 1).Libraries harboring random overlapping binary subclones of 8-10 kb were therefore generated from BAC clones Blb25 and TG9. A total of 1152 clones per library were screened for the presence of RGHs using primers 4-GLO2-F and 4-GLO2-R (Table 1). Based on restriction analyses of the PCR fragments, RGH positive subclones were divided into separate classes: *Blb3GH-A* and *Blb3GH-B* for *Rpi-blb3*, *AbptGH-A* and *AbptGH-B* for *Rpi-abpt*. After determining the relative positions of the RGHs within the 8-13 kb subclones, candidates from each class were targeted for transformation to the susceptible potato cultivar Desiree. Transformation experiments carried out with subclones harboring candidates *Blb3GH-A*, *Blb3GH-B*, *AbptGH-A* and *AbptGH-B*, respectively, resulted in numerous primary transformants. Detached leaf assays using isolates IPO-0 and 90128 revealed that all of the plants transformed with subclones harboring candidates *Blb3GH-A*, *AbptGH-A* and *AbptGH-B* were susceptible to both isolates but that the majority (7/8) of the tested transgenic plants harboring *Blb3GH-B* reacted to both isolates with a hypersensitive response (HR) (Fig. 2). In view of the differential response between the primary transformants harboring *Blb3GH-A* and *Blb3GH-B*, we designated *Blb3GH-B* as the *Rpi-blb3* gene.

Figure 2: Genetic complementation for late blight resistance. Eight days after inoculation with *Phytophtora infestans* isolate 90128, large necrotic lesions and massive sporulation were noted for potato cultivar Desiree, and derived transformant Blb25A-2-4 and Blb25A-2-5, both expressing RGH-Blb25A. Typical resistance phenotype, i.e hypersensitive response (HR) was observed for Blb25B-2-1 and Blb25B-2-2 expressing RGH-Blb25B (*Rpi-blb3*)

In order to identify additional candidate genes for *Rpi-abpt*, the *Rpi-abpt*-specific BAC library was screened with TG77-S, leading to the identification of the TG77 overlapping BAC clone TG92 (Fig. 1B). Screening of this BAC clone with different sets of primers designed to amplify *AbptGH-A*, *AbptGH-B*, *Blb3GH-A* or *Rpi-blb3*, resulted in the identification of a third *Rpi-abpt* candidate gene, *AbptGH-C* (Fig. 1B). *AbptGH-C* LRR domain was amplified, cloned and sequenced using the primers Blb3-LRR-1-8 (Table 1). Sequence comparison of the three *abpt-GHs* enabled to design *AbptGH-C* specific marker (Fig. 1B) which co-segregated with resistance. Southern blot analysis using the AbptGH-C amplicon as a probe revealed that clone TG92 contained one resistance gene encompassing an LRR domain highly similar to the one of *AbptGH-C.* Primers designed on the start and stop codon of *Rpi-blb3* were subsequently used to amplify a full-length AbptGH-C amplicon from clone TG92, which was cloned into the Gateway® entry vector pDONR221. Using Multisite Gateway® technology, the *AbptGH-C* amplicon was subsequently cloned into the binary pKGW-MG destination vector between *Rpi-blb3* derived promoter and terminator sequences of 2723 nt and 883 nt, which were cloned into pDONR^{TM} P4-P1R and pDONR^{TM} P2R-P3 (Suppl. Fig. S1).

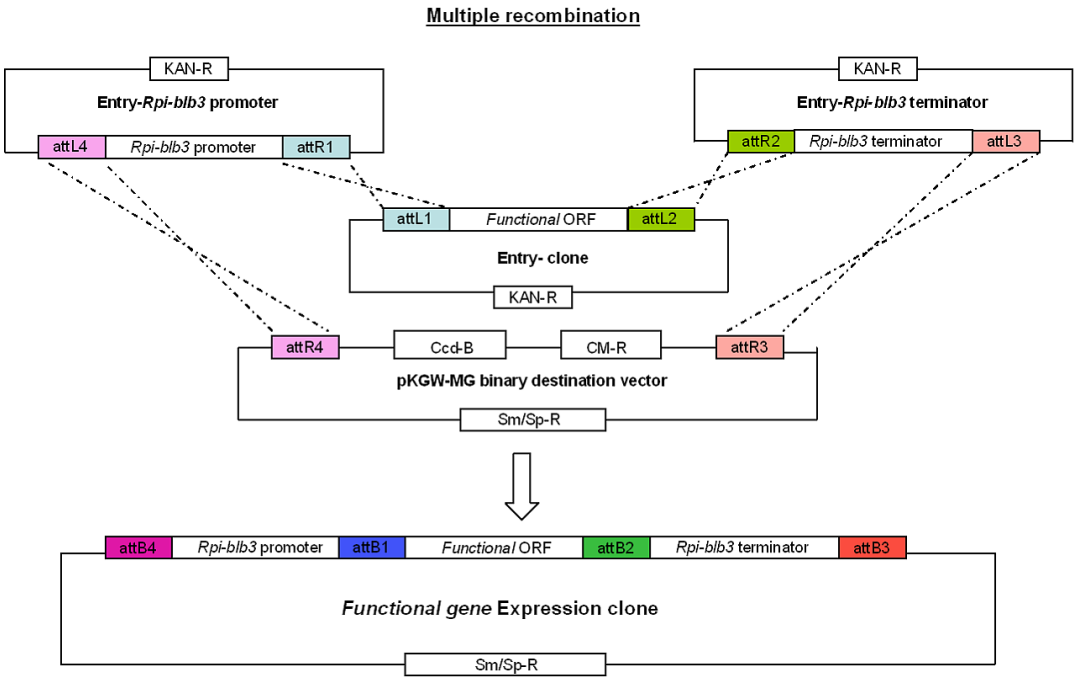


Figure S1: Gateway strategy used to clone *Rpi-abpt*, *R2* and *R2-like***.** LR recombination of the three entry clones bearing the DNA fragments *Rpi-blb3* promoter, *Functional* ORF and *Rpi-blb3* terminator with the Multisite Gateway destination binary vector pKGW-MG, leading to the functional gene expression clone.

Complementation analysis was carried out in *N. benthamiana* using the *Agrobacterium tumefaciens* transient assay (ATTA) whereby leaves from 4-week-old wild type *N. benthamiana* plants were infiltrated with the *Agrobacterium* strain COR308 containing pKGW-AbptGH-C. The binary

clones pBP-Rpi-blb3 and pKGW-Rpi-blb3, comprising the genomic *Rpi-blb3* gene and a Multisite Gateway® reconstituted *Rpi-blb3* gene construct, respectively, were used as positive controls, and pBP-AbptGH-A as a negative control. After two days, infiltrated leaves were inoculated with *P. infestans* strain PY23, IPO-0 and IPO-C in detached leaf assays. Leaves infiltrated with pKGW-AbptGH-C, pBP-Rpi-blb3 and pKGW-Rpi-blb3 developed HRs at the inoculation sites whereas non infiltrated leaves and those infiltrated with pBP-AbptGH-A were susceptible to isolate PY23 and IPO-0. As expected, all leaves inoculated with IPO-C were susceptible (Fig. 3 and Table 2). In view of these results, *AbptGH-C* was designated *Rpi-abpt*.

Allele mining for R2 and R2-like

Rpi-blb3 and *Rpi-abpt* belong to the major late blight resistance locus on LG IV that also harbors *R2* and *R2-like* (Li et al. 1998; Park et al. 2005c). In view of the conserved marker order and observed allelic conservation between the genetic maps of *Rpi-blb3, Rpi-abpt*, *R2*, and *R2-like* (Park et al. 2005c), and the high sequence conservation between *Rpi-blb3* and *Rpi-abpt*, we hypothesised that *R2* and *R2-like* were orthologues of *Rpi-blb3* and thus attempted to clone them through an allele mining strategy. The same primers used to amplify the *Rpi-abpt* candidate gene from BAC clone TG92 were used to amplify full-length Blb3GH from the resistant parental genotypes CEBECO44158-4 (MaR2) and AM3778-16, harboring *R2* and *R2-like*, respectively. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, eight unique sequences were obtained from AM3778-16 (R2-likeGH) and nineteen from CEBECO44158-4 (R2GH), with amino acid identities between Rpi-blb3 and the novel Blb3GH ranging from 86.4% to 97.3% for R2-likeGH and 83.8% to 94.2% for R2GH (Supplementary Table S1 and S2). Phylogenetic analysis of all the available amino acid sequences clustered one R2-like-GH and five R2GH in a clade together with the functional proteins Rpi-blb3 and Rpi-abpt (Fig. 4). The amino acid sequence of R2-likeGH-8 shares 97.3 % amino acid identity with Rpi-blb3. R2GH-2, R2GH-8, R2GH-G3, R2GH-D3 and R2GH-65 share 94.2, 91, 92.6, 89.7, and 92.8% amino acid identity with Rpi-blb3, respectively. This set of candidate genes was targeted for functional analysis and therefore cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences, as described above for the *Rpi-abpt* gene.

Transient complementation assays in *N. benthamiana* indicated that R2GH-G3 and R2-likeGH-8 conferred resistance to Pi isolate IPO-0, whereas R2GH-2, R2GH-8, R2GH-D3 and R2GH-65 were non-functional (Fig. 3). R2GH-G3 and R2-likeGH-8 were therefore designated *R2* and *R2-like*, respective.

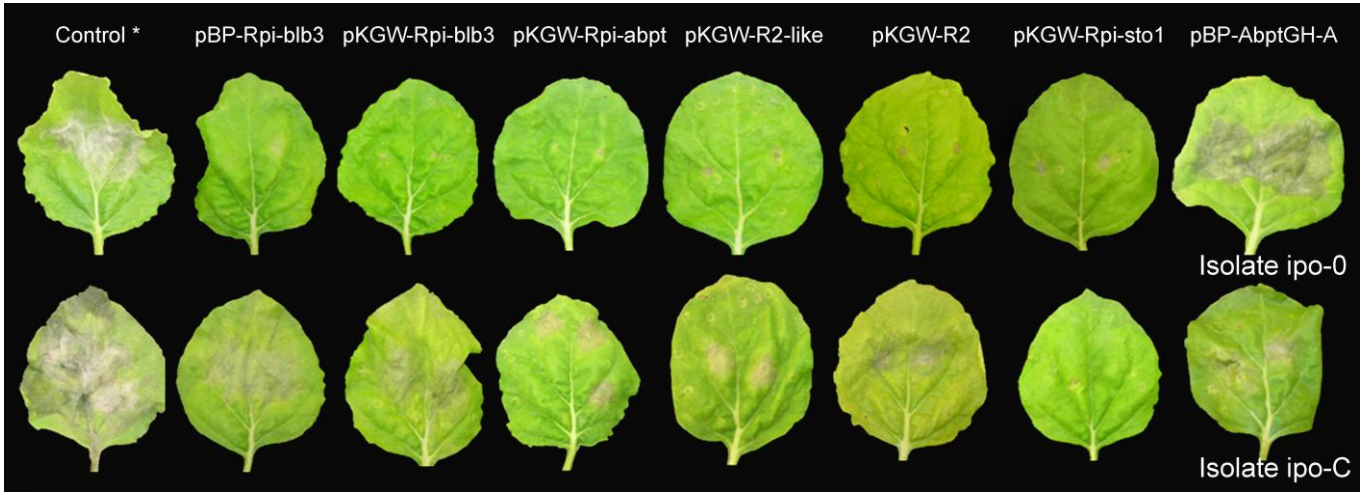


Figure 3. Complementation of *Rpi-blb3, Rpi-abpt , R2, R2-like, Rpi-sto1* by transient expression in *Nicotiana benthamiana*, followed by inoculation by *Phytophtora infestans* isolate Ipo0 (avirulent) and Ipo-C (virulent on all except *Rpi-sto1*). The prefix pBP stand for pBINplus, and pKGW stand for the gateway destination vector containing *Rpi-blb3* regulatory elements. In leaves expressing pB-Rpi-blb3, pKGW-Rpi-blb3, pKGW-Rpi-abpt, pKGW-R2-like, pKGW-R2, a HR was observed at seven days post inoculation with Pi isolate Ipo-0, whereas large necrotic lesions and massive sporulation were evident for Pi isolate Ipo-C. Wild type *N. benthamiana* (Control *) and the non functional AbptGH-A (pBP-AbptGH-A) were included as susceptible controls, and Rpi-sto1 (pKGW-Rpi-sto1) was included as a resistant control **.**

Table 2 Overview of resistance screening with a set of 16 *P. infestans* isolates[a].

spp	Genotype	Gene	Chr.	py 23	IPO-0	90128	H30P04	VK98014	IPO428-2	Dintel	Katshaar	F95573	EC1	89148-09	99183	99189[b]	IPO-C	USA618	99177
BLB	Blb99-256-3	*Rpi-blb3*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
BLB	707TG11-1	*Rpi-abpt*	4	R	R	R	R	R	R	R	R	R	R	R	R	**R**	S	S	S
BLB	AM3778-16	*R2-like*	4	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S
DMS	CEBECO44158-4	*R2*	4	R	R	R	R	Rq	R	Rq	R	R	R	R	R	S	S	S	S
	cv. Desiree	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	cv. Bintje	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

[a] Differential specificity are in bold. Chr.=chromosome, S= susceptible; R= resistant and Rq= quantitatively resistant.

[b] Isolate differentially recognized

All four R genes interact with the RXLR-EER effector PiAVR2

Using a map-based cloning approach, markers flanking the *PiAVR2* locus (van der Lee et al., 1997) were used to delimit a physical region of 742,635 bp of the *P. infestans* genome sequence spanning the genetic interval containing *PiAVR2*. Within this region, two candidate *PiAVR2* genes on supercontig 16 of the *P. infestans* genome (16_7987 and 16_8044), each encoding a secreted RXLR-EER effector, were identified. The cloning and analyses of these gene candidates will be reported in detail in a separate publication (personal communication, E. Gilroy).

To functionally analyze the two RxLR-EER candidate genes identified at the *PiAVR2* locus in this study, *N. benthamiana* leaves were co-infiltrated with *A. tumefaciens* strains carrying a binary construct expressing one of the four *Rpi* genes under study together with strains carrying a binary construct expressing each of the two candidate RxLR-EER genes. Co-infiltration of *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* with *16_7987* resulted in a hypersensitive cell death response (Fig. 5), whereas co-expression of these *Rpi* genes with the *16_8044* candidate or *PiAVR3a*, failed to elicit visible cell death (Fig. 5; personal communication, E. Gilroy). Based on these results *16_7987* was designated *PiAVR2*, and clearly all four *Rpi* genes under study interact with *PiAVR2*.

Gene structure and functionality

Rpi-blb3, *Rpi-abpt*, *R2* and *R2-like* are intronless genes which encode ORFs of 2538-2544 nucleotides (nt) that code for proteins of 845-847 amino acids harboring all the signature sequences characteristic of LZ-NBS-LRR R-proteins (Fig. 6). Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% aa) with RPP13 from *Arabidopsis thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis* isolates containing *ATR13*. The highest similarity with RPP13 resides in the NBS domain with 49.3% sequence identity, and the lowest within the LRR domain (34.3%). The LRR domains of Rpi-blb3, Rpi-abpt, R2, and R2-like are highly homologous and comprise 14 imperfect repeats (Fig. 6). The LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Rpi-abpt and R2-like are identical except for the sequence between LRR2 and LRR3, where Rpi-abpt contains a stretch of amino acids that is identical to that of R2. The LRR domain of R2 is identical to that of Rpi-abpt (Fig. 6).

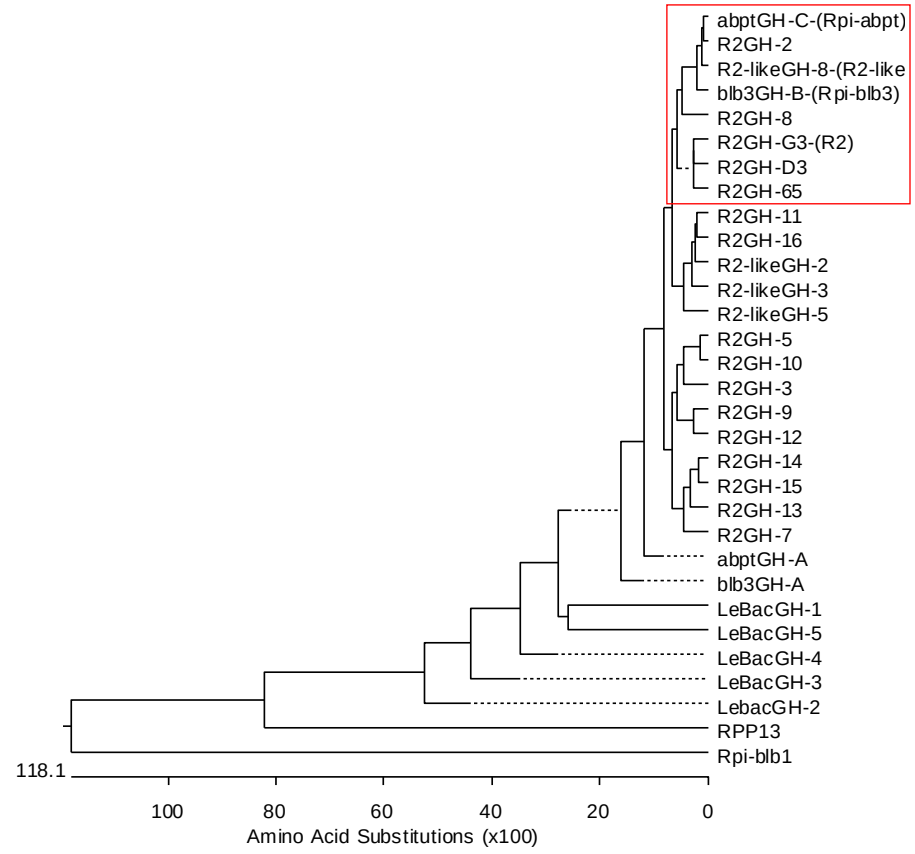


Figure 4: Phylogenetic tree at the amino acid level of Rpi-blb3, RGHs amplified from late blight resistant potato clones harboring *Rpi-abpt*, *R2-like or R2*, RGHs present on the tomato Bac clone AF411807, *RPP13-Nd* and *Rpi-blb1*. Boxed is the group containing Rpi-blb3, Rpi-abpt, R2 and R2-like.

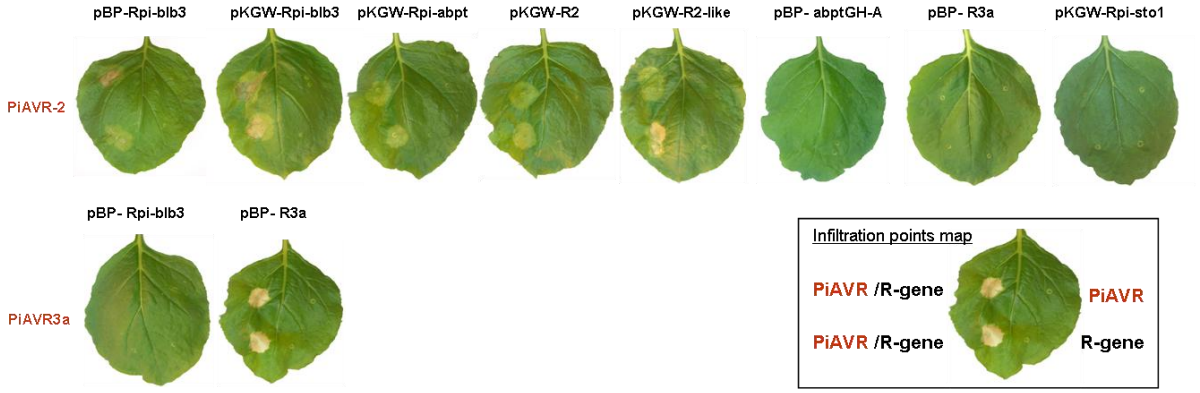


Figure 5. Agrobacterium-mediated co-expression of *PiAvr2* or *PiAvr3a* together with *Rpi-blb3*, *Rpi-abpt* , *R2, R2-like, Rpi-sto1* , the non functional *abptGH-A* or *R3a*. The different infiltration points are indicated on the map. On each leaf, four infiltration spots were performed, duplicated co-infiltration of the *Avr* and the *R* gene on the left , the *Avr* on the top right and the *R* gene on the bottom right. *Rpi-blb3, Rpi-abpt , R2 and R2-like* in combination with *PiAvr2* as well as *R3a* and *PiAvr3a* induced the hypersensitive response (HR). Pictures were taken 6 days post-infiltration.

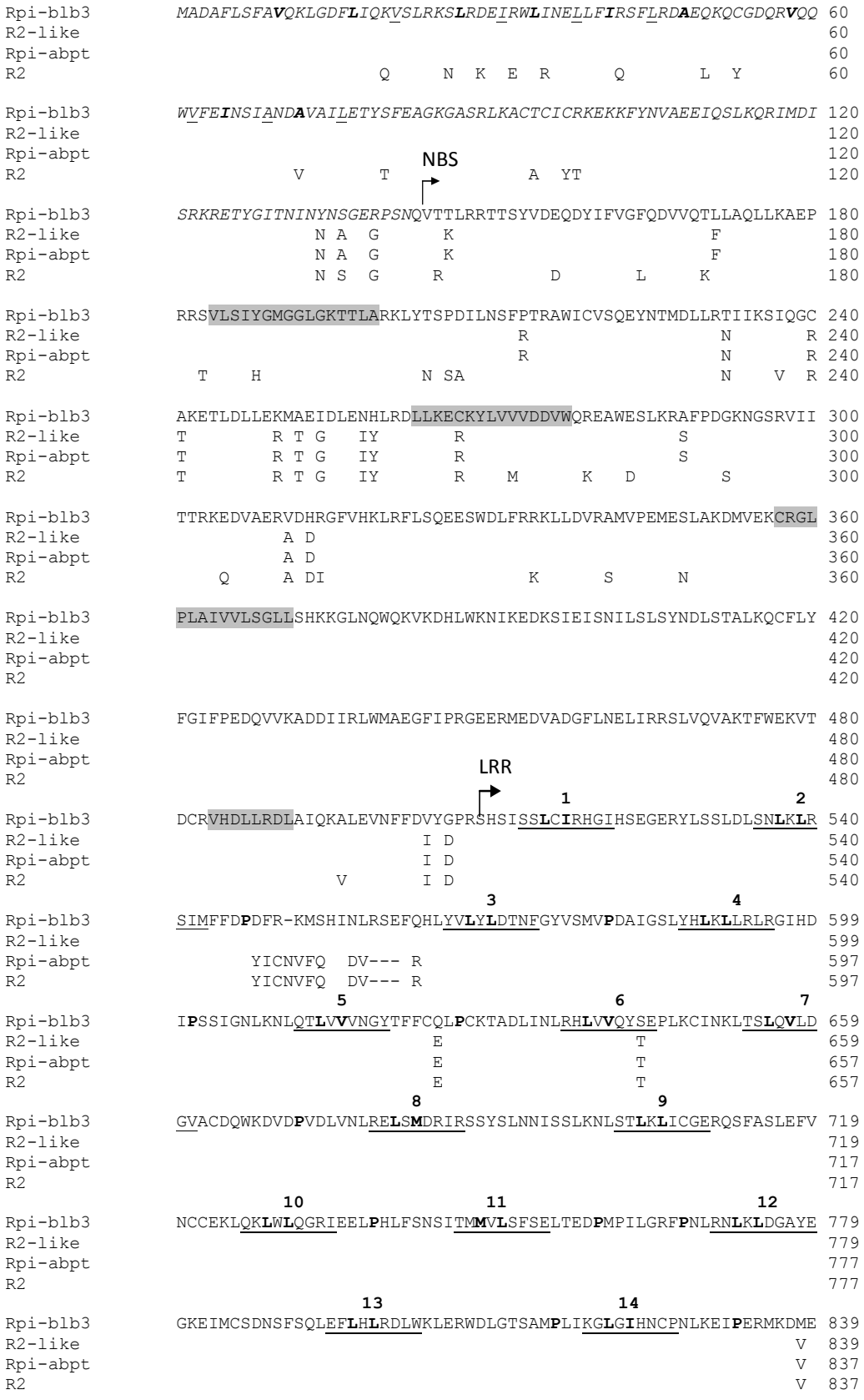

NBS
LRR
1
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 6: Alignment of the deduced protein product encoded by the functional genes *Rpi-blb3, Rpi-abpt, R2-like,* and *R2*. The full amino acid sequence of *Rpi-blb3* and in comparison, differing amino acids of *Rpi-abpt, R2-like,* and *R2* are shown. In italics is the leucine zipper domain with the first and the fifth hydrophobic residues from the heptads in bold and underlined respectively. Positions of the nucleotide binding (NB)-ARC and the leucine reach repeat (LRR) are indicated by arrows. The NB-ARC domain has its characteristic conserved domains highlighted in grey (kinase 1a, kinase 2 and kinase 3). The LRR domain domain is composed of 14 repeats with the motif xx**L**x**L**xxx .

Alignment of the nucleotide sequences of the four functional genes and those of four additional *Blb3GHs* and subsequent analysis of informative polymorphic sites (IPS), i.e. sites where two or more genes carry the same nucleotide (Parniske et al. 1997), reveals clear blocks of sequence affiliation between different members of the gene family (Fig. S2), indicating that sequence exchange events between *Blb3GHs* have been involved in the evolution of the gene family (Fig. S2). Interestingly, the observed sequence affiliations in the 5'-terminal half of the genes extend throughout the LZ and NBS domains whereas sequence affiliations in the LRR domain suggest exchange of specific combinations of LRRs, underlining the modular nature of R proteins.

In an attempt to assess the biological relevance of the observed sequence exchange events in relation to resistance spectrum, detached leaves of the parental clones harboring *Rpi-blb3* (Blb99-256-3), *Rpi-abpt* (707TG11), *R2* (CEBECO44158-4(MaR2)) and *R2-like* (AM3778-16) were inoculated with 16 different *P. infestans* isolates (Table 2). For 15 isolates all four clones displayed the same specificity. However, one isolate, PIC99189, displayed a differential interaction; Blb99-256-3, AM3778-16 and CEBECO44158-4(MaR2) were susceptible to this isolate whereas 707TG11-1 was resistant (Table 2). Previously it was reported that 707TG11-1 contained tuber specific *R1-like* (Park et al. 2005) and foliage specific *R3-haplotype* (Huang . 2005). To dissect the resistance specificity of 707TG11-1 we inoculated 48 F1 progeny plants of the *Rpi-abpt* mapping population RH4x103 with isolates 90128 and PIC99189. For both isolates a clear 1:1 segregation of resistance was observed but there was no correlation between resistance to both isolates, confirming that the resistance to isolate PIC99189 is indeed the result of a second gene other than *Rpi-abpt*, and not located on LG IV.

In order to confirm the functionality of *Rpi-abpt*, *R2-like*, and *R2*, transgenic Desiree were generated. Detached leaves of 6, 5, and 2 independent transgenic lines expressing *R2-like*, *Rpi-abpt*, and *R2*, respectively, were inoculated with *P. infestans* isolates IPO-0 and IPO-C. All transformants reacted with a hypersensitive response (HR)to isolate IPO-0 and were susceptible to isolate IPO-C (Fig S3). This result confirms that *Rpi-abpt*, *R2-like*, and *R2* are functional *R* genes with expected specificity to *P. infestans* isolates. Moreover, the use of the *N. benthamiana* transient complementation assay as an efficient tool for rapid functional screen of putative R-genes is verified."

Supplementary Table S2: Amino acid sequence similarity between the LZ, NBS or LRR domains of the different homologues depicted in the phylogenetic tree (Fig. 4) (Rpi-abpt, R2-like, R2, blb3GH-A, abptGH-A, 3 R2likeGHs, 15 R2GHs, 5 LeBacGHs, RPP13, Rpi-blb1) and Rpi-blb3. Highlighted in grey are the genes depicted in the crustal W alignment (Fig. 6).

blb3GH-B -(Rpi-blb3)	blb3GH-B-(Rpi-blb3)	abptGH-C -(Rpi-abpt)	R2-likeGH-8 -(R2-like)	R2GH-G3 -(R2)	blb3GH-A	abptGH-A	R2-likeGH-2	R2-likeGH-3	R2-likeGH-5	R2GH-2	R2GH-3	R2GH-5	R2GH-7	R2GH-8	R2GH-9	R2GH-10
LZ-NBS-LRR	100	95.9	97.3	92.6	81.7	86.3	86.4	89.9	91.4	94.2	84.7	87.9	88.2	91	86.6	86.8
LZ	100	98	98	90.1	87.5	90.8	84.2	84.2	84.2	98	90.1	90.1	97.4	89.5	90.1	89.5
NBS	100	95.5	95.5	91.2	91.8	93.6	89.9	89.7	91.8	94.7	89.4	93.4	92.6	91	89.7	93.4
LRR	100	95.9	99	95.9	81.9	80.4	85.3	92.8	94	93	81.4	83.8	82.6	93.7	84.5	81.9

blb3GH-B-(Rpi-blb3)	blb3GH-B-(Rpi-blb3)	R2GH-11	R2GH-12	R2GH-13	R2GH-14	R2GH-15	R2GH-16	R2GH-D3	R2GH-65	LeBacGH-1	LebacGH-2	LeBacGH-3	LeBacGH-4	LeBacGH-5	RPP13	Rpi-blb1
LZ-NBS-LRR	100	87	85	86.9	84	83.8	86.7	89.7	92.8	75.6	55.7	70.9	78.3	47.6	34.9	24.4
LZ	100	90.8	90.1	98	88.8	90.8	85.5	89.5	90.1	80.9	79.6	76.3	78.3	50	34.9	25
NBS	100	89.4	89.1	88.9	89.1	88.1	89.4	90.2	92.3	80.6	59.7	81.7	86.7	54.9	49.3	31.8
LRR	100	85.7	81.9	83.1	80.4	80.4	85.7	90.8	95.4	73.1	58.6	64.6	74.6	63	34.3	14.5

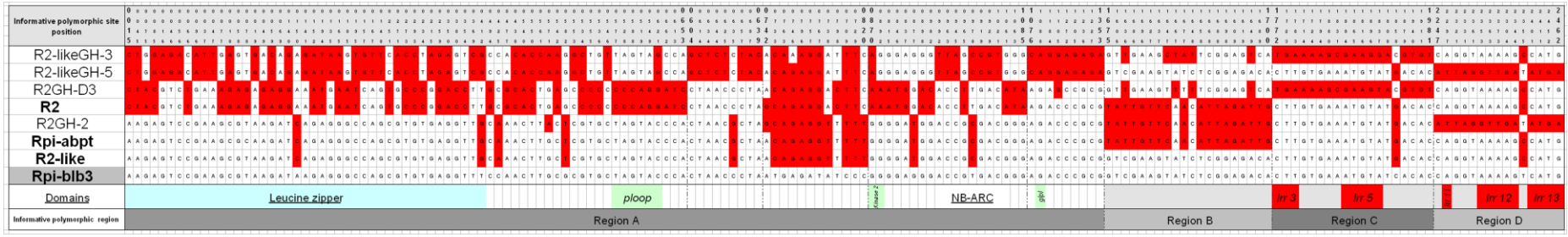


Figure S2: Nucleotide polymorphism analysis of *Rpi-blb3* and 7 homologues sharing informative polymorphic or homologous sequences. In bold are the functional genes *R2*, *Rpi-abpt*, *R2-like*, *Rpi-blb3*. Only informative polymorphic sites are shown, which reduces the sequence size from 2444 bp to 170 bp. Highlighted in red are the positions polymorphic to *Rpi-blb3* sequence. The vertical number at the top of each row indicates the corresponding nucleotide position in the full consensus sequence, the limits of the recombination regions positioned on the bottom of the figure are indicated in bold. The positions of the different domains characterizing LZ-NBS-LRR genes and the informative polymorphic regions A to D are indicated down to the sequences.

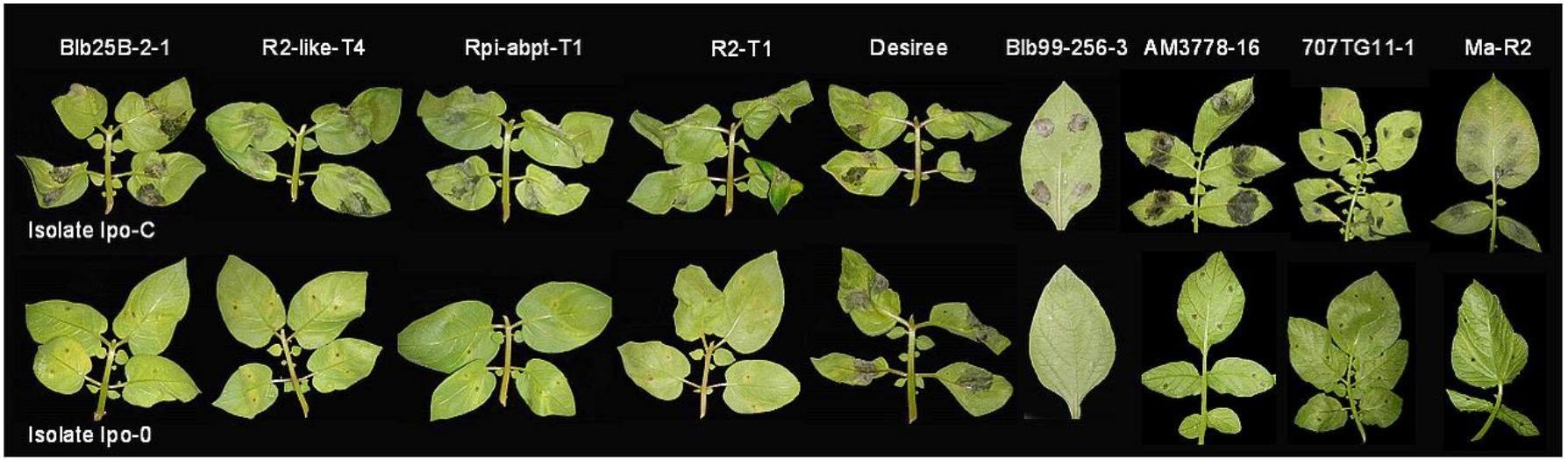


Figure S3: Genetic complementation for late blight resistance. Six days after inoculation with *Phytophtora infestans* isolate IPO-0, the hypersensitive response (HR) was observed for the positive control Blb25B-2-1 carrying *Rpi-blb3*, the transformants expressing *R2*-like (R2-like-T4), *Rpi-abpt* (Rpi-abpt-T1), *R2* (R2-T1) and the parental clones harboring *Rpi-blb3* (Blb99-256-3), *R2-like* (AM3778-16), *Rpi-abpt* (707TG11) and *R2* (MaR2); large necrotic lesions and massive sporulation were noted for potato cultivar Desiree. All the tested leaves were susceptible to the virulent isolate IPO-C.

DISCUSSION

In this study we describe the cloning and functional characterization of four homologous *R* genes from the major late blight resistance locus on LG IV of potato. Previously, marker order and allelic conservation suggested that *Rpi-blb3, Rpi-abpt, R2* and *R2-like* reside at the same locus on LG IV (Park et al. 2005a, 2005b, 2005c). *Rpi-blb3* and *Rpi-abpt* were cloned using a map-based cloning approach whereby physical maps of both loci were generated by screening appropriate BAC-libraries with a highly linked molecular marker, followed by subsequent rescreening of the libraries with novel BAC-end markers until the outer ends of the BAC-contigs exceeded the genetic boundaries of the *R* loci. In the case of *Rpi-blb3,* two candidate NBS-LRR genes were identified and subcloned into a binary vector and targeted for complementation analysis through stable transformation of a susceptible potato variety.

For *Rpi-abpt,* a NBS-LRR candidate was cloned from the BAC using a homology-based PCR cloning strategy using primers designed on the functional *Rpi-blb3* gene. In addition complementation was carried out using an *Agrobacterium*-mediated transient expression assay in *N. benthamiana* followed by detached leaf assays with appropriate *P. infestans* isolates (Rietman et al. in preparation).

Development of the transient complementation assay together with the successful *in planta* expression of *Rpi-abpt* and other *R* genes (Rietman et al. in preparation) using the DNA regulatory elements of *Rpi-blb3,* facilitated for the rapid cloning of *R2* and *R2-like* through an efficient functional allele mining strategy. A prerequisite for such an allele mining strategy is prior knowledge of candidate gene sequences at the locus of interest. Observations within the *Solanaceae* indicate that *R* gene clusters are conserved across species, thus enabling comparative genomics (Grube et al. 2000; Huang. 2005; van der Vossen et al. 2005). As more and more *R* genes are mapped and cloned, the greater the chance that novel *R* genes reside at known and well-characterized loci. Moreover, ongoing potato and tomato genome sequencing projects by international consortia promise to provide a complete survey of the distribution of *R* gene clusters in the *Solanaceae,* enabling even faster cloning of *R* genes through allele mining strategies.

Interestingly, with respect to known functional R proteins, Rpi-blb3, Rpi-abpt, R2, and R2-like share the highest similarity (34.9% aa) with RPP13 from *A. thaliana* (Bittner-Eddy et al. 2000), which confers resistance to *H. arabidopsidis*. Mining of *RPP13* alleles in a diverse set of *A. thaliana* accessions revealed both extreme sequence diversity and functional diversity in pathogen recognition (Rose et al. 2004). In line with this observation a significant excess of amino acid polymorphism was

localized within the LRR domain of *RPP13*. In the case of *Rpi-blb3, Rpi-abpt, R2,* and *R2-like*, the LRR domains are nearly identical despite significant polymorphism at the CC and NBS domains, suggesting conservation of function, and indeed we have shown that all four proteins interact with the same effector protein, PiAVR2, which was recently cloned through a map-based cloning approach (personal communication, E. Gilroy). Although the origin of *Rpi-abpt* and *R2-like* remain unclear, this finding is a second example of how *R* gene recognition specificities can be conserved across *Solanum* species originated from different potatoe superseries, in this case *S. bulbocastanum* (*Rpi-blb3*) belongs to the *Potatoe Stellata Bulbocastana* and *S. demissium* (*R2*) to the *Potatoe Rotata demissa*. Recently we demonstrated both gene conservation and specificity conservation between the *S. bulbocastanum*-derived gene *Rpi-blb1* and the *S. stoloniferum* and *S. papita*-derived orthologues *Rpi-sto1* and *Rpi-pta1,* respectively (Vleeshouwers et al. 2008). Despite apparent positive selection in the LRR domain, the LZ and NBS domains are more polymorphic, those of R2 being the most divergent. Clear blocks of sequence affiliation between the four functional R proteins and additional *Rpi-blb3* gene homologs, suggest exchange of LZ, NBS and LRR domains, and also of specific LRRs, underlining the modular nature of these proteins. *R2* has a particular gene structure likely to be the product of a relatively recent recombination event. The LZ-NBS domain (nucleotides 1-1149) is highly affiliated to that of the non-functional *R2-GH-D3,* whereas the LRR domain (nucleotides 1059-2544) is highly affiliated to that of *Rpi-abpt* and the non-functional *R2-GH-65*, revealing a sequence exchange track between nucleotides 1059 and 1149. Sequence exchange between RGHs has been proposed as a major mechanism shaping *R* gene diversity in plants (Michelmore and Meyers 1998; Meyers et al. 1999; Kuang et al. 2004). Interestingly, the approximate position of this recombination site corresponds to the one described by Rose et al. (2004) for the *RPP13* allele *ksk*-2, a region between the conserved GLPL and VHD motifs of the NB-ARC domain. This is in line with the expectation that in multi-domain proteins, sequence exchange tracks will be concentrated at specific sites that separate domains with independent functions.

Preliminary results from ongoing *Rpi-blb3* allele mining studies in *Solanum* germplasm indicate that *Rpi-blb3* is present in several other species, e.g. *S. brachistotrichum* and *S. pinnatisectum*. In addition several *Rpi* genes recently identified in our laboratory (data not shown) also map to the *Rpi-blb3* interval. The QTLs for late blight resistance (Pi-QTL) (Sandbrink et al 2000) explaining 40% of the phenotypic variation, was mapped to the *Rpi-blb3* locus, and renamed *Rpi-mcd1* (Tan et al. 2008). Similarly, high resolution mapping data of the major Pi-QTL identified in cv. Stirling strongly suggest that this Pi-QTL is also an *Rpi-blb3* homologue (Hein et al. 2007; Ingo Hein, personal communication). And various other examples of Pi-QTLs on the short arm of LG IV have been described (Leonards-Schippers et al. 1994; Sandbrink et al. 2000; Bradshaw et al. 2004; Bradshaw et al. 2006).

Rpi-blb3, Rpi-abpt, R2 and R2-like specifically detect PiAVR2. Do the Rpi-blb3 homologues putatively underlying the Pi-QTL described above also recognize PiAVR2 homologues? If so do they weekly recognize an allele, which is conserved throughout *P. infestans* or do they recognize multiple alleles, thus resulting in non-race-specific resistance?

Current models for NB-LRR R proteins suggest a dual role for the LRR domain, not only as recognition specificity determinant but also as a repressor of inappropriate NB activation (Belkhadir et al. 2004). Furthermore, evolutionary analyses of R proteins and their structure has shown selection pressure on the LRR region but also on other domains within R proteins, which, in light of the dual function, may point towards co evolution in R proteins to establish and maintain recognition of effectors while maintaining the inhibitory function (McDowell and Simon. 2006). Our findings are slightly different, contradictory, indicating conservation in the LRR domain but more variation in the NBS domain, that may point towards different signaling pathways or additional effectors being recognized by these R proteins.

MATERIALS AND METHODS

Plant material and *Phytophthora infestans isolates*

In this study we used the four resistant parental clones Blb99-256-3 harbouring *Rpi-blb3* (Park et al., 2005a), 707TG11-1 *Rpi-abpt* (Park et al., 2005c), CEBECO44158-4 (MaR2) *R2* and AM3778-16 *R2-like*, respectively (Park et al.2005b). F1 progeny plants of population RH4x103 were derived from a cross between the late blight resistant clone 707TG11-1 and the diploid potato clone RH89-039-16 (Park et al., 2005c).The susceptible potato cultivar Desiree was used for *Agrobacterium*-mediated transformation. The characteristics and origin of *P. infestans* isolates used in this study are indicated in supplementary Table S3.

Supplementary Table S3 Characteristics of *P. infestans* isolates used in this study. Resistance genes which didn't have a clear phenotype from one repeat to the other are in brackets in the virulence profile.

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile
90128	1990	Geldrop, Netherlands	A2	Govers, Phytopathology WUR	1.3.4.7.8.10.11
H30P04	unknown	Artificial hybrid	unknown	Govers, Phytopathology WUR	3a, 7, 10
USA618	n.d	Toluca Valley, Mexico	A2	Fry, Cornell, USA	1.2.3.6.7.10.11
89148-09	1989	Netherlands	A1	Govers, Phytopathology WUR	0
IPO-0	n.d	Netherlands	n.d	Kessel, PRI, WUR	0
IPO-C	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11
VK98014	1998	Veenkolonién, Netherlands	A1	Kessel, PRI, WUR	1.(2).4.11
Dinteloord	n.d	Dinteloord, Netherlands	n.d	Kessel, PRI, WUR	1.(2).4
EC1	n.d	Ecuador	n.d	Birch, SCRI, Scotland	1.3.4.7.10.11
F95573	1995	Flevoland, Netherlands	A1	Govers, Phytopathology, WUR	1.3.4.7.10.11
IPO-428-2	1992	Ede, Netherlands	A2	Kessel, PRI, WUR	1.3.4.7.8.10.11
Katshaar	n.d	Katshaar, Netherlands	n.d	Kessel, PRI, WUR	1.3.4.7.10.11
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR)	1.2.3.4.7.11
PIC 99183	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.3.4.5.7.8.10.11
PIC 99189	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.5.7.10.11
88069	1988	Netherlands	A1	Govers, Phytopathology, WUR	1.3.4.7
PY23*	1999	GGO (modified 88069)	A1	Govers, Phytopathology, WUR	1.3.4.7

* References: Kamoun et al. 1998; West et al. 199

BAC library construction

Clones Blb99-256-3 and 707TG11-1 were used as DNA sources for the construction of BAC libraries. High-molecular weight DNA preparation and BAC library construction were carried out as described previously (Rouppe van der Voort et al. 1999). Approximately 74000 clones with an average insert size of 85 kb, corresponding to 8 genome equivalents, were obtained per library. The BAC clones were stored as bacterial pools containing approximately 700 to 1000 white colonies. These were generated by scraping the colonies from the agar plates into LB medium containing 18% glycerol and 12.5 µg ml^{-1} chloramphenicol using a sterile glass spreader. These so-called super pools were stored at -80ºC. Marker screening of the BAC libraries was done, first by isolating plasmid DNA from each pool using the standard alkaline lysis protocol and PCR was carried out to identify positive pools. Bacteria corresponding to positive pools were diluted and plated on LB agar plate containing chloramphenicol (12.5 µg ml^{-1}). Individual white colonies were picked into 384-well microtitre plates and single positive BAC clones were subsequently identified by marker screening as described by Rouppe van der Voort (1999). Names of BAC clones isolated from the super pools carry the prefix Blb (e.g. Blb25) or TG (e.g. TG9).

Subcloning of candidate genes

Candidate RGAs were subcloned from BAC clone BLB25 and TG9 as follows. Aliquots of approximately 1 µg BAC DNA were digested with 0.03U of *Sau3A*l restriction enzyme for 10 min. The partially digested BAC DNA was subjected to electrophoresis at room temperature in 0.5X TAE using a linear increasing pulse time of 1-10 sec and a field strength of 90 V cm^{-1} for 6 h. After electrophoresis, the agarose gel was stained with ethidium bromide to locate the region of the gel containing DNA fragments of approximately 10 Kbp in size. This region was excised from the gel and treated with GELASE (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's instructions. The size-selected DNA was ligated to the *BamH*I-digested and dephosphorylated binary vector pBINPLUS (van Engelen et al. 1995) followed by transformation to ElectroMAX E.coli DH10B competent cells (Life technologies, Paisley, UK).

DNA sequencing and computer analysis

BAC clone sequencing was carried out using a shotgun cloning strategy. Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991).

The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp). Gene structures were predicted using FGENESH++ (Softberry) and protein sequences were deduced by translation of ORF using the standard genetic code. Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Conserved domains were identified using Swiss-Prot (InterProScan, EMBL-EBI, ExPASy, SAPS).

Resistance assay

Detached leaf assays were used to determine the resistance phenotypes of primary transformants of potato and transiently transformed *N. benthamiana* leaves. For complementation analyses, primary transformants were tested with isolates IPO-0 and 90128 (Fig. 2 and Table S3). Inoculum preparations and inoculations were performed as described by Vleeshouwers et al. (1999). Six days after inoculation, plant phenotypes were determined. Leaves showing no symptoms or a localized necrosis at

the point of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Transient complementation in N. benthamiana

Infiltrations of *Agrobacterium* strains carrying the candidate RGH were performed in *N. benthamiana.* Recombinant COR308 *A. tumefaciens* cultures were grown in LB medium (10 gram bacteriological peptone, 10 gram NaCl and 5 gram yeast extract in 1 liter milli-Q water) supplemented with 5 mg/L tetracycline and 50 mg/L kanamycin. After one or two days a calculated amount of culture (according to OD 0.5 at 600 nm) was transferred to YEB medium (5 gram beef extract, 5 gram bacteriological peptone, 5 gram sucrose, 1 gram yeast extract, 2ml 1 M MgSO4 in 1 liter MQ water) supplemented with kanamycin for all strains. After 1 day overnight cells were centrifuged at 3500 rpm and re-suspended in MMA medium (20 gram sucrose, 5 gram MS salts and 1.95 gram MES) supplemented with 1 ml 200 mM acetosyringone to a final OD of 0.2 and infiltrated into leaves of 4 weeks old plants with a 3ml syringe. After two days, infiltrated leaves were detached and inoculated with *P. infestans* strains IPO-C (Table S3) and PY23 in detached leaf assays. Hypersensitive response (HR) or *P. infestans* sporulation was observed between 4 to 8 days post inoculation.

Agrobacterium-mediated coexpression in N. benthamiana

pBP-Rpiblb3, pKGW-Rpi-blb3, pKGW-Rpi-abpt, pKGW-R2, pKGW-R2-like, pKGW-Rpi-sto1, pBP-abptGH-A and *pBP-R3a* were transferred to the *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 5 mg/L tetracycline , 100 mg/L carbeniciline, 75 mg/L choramphenicol and 50 mg/L Kanamycin. The avirulence effectors were cloned into the pGRAB vector (kindly supplied by Sean Chapman, SCRI. Scotland. UK) and transferred to the same *A. tumefaciens* strain AGL1. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 30 mg/L choramphenicol and 50 mg/L kanamycin. The same protocol as the transient complementation was used for the sub-cultures. *PiAVR Agrobacterium* culture and *Rpi-gene Agrobacterium* culture were mixed at a final OD6oo of 0.1 for each and infiltrated into leaves from 5 to 7 week old plants. Specific recognition of the *AVR* gene by the *R* gene induces hypersensitive cell death response (HR) from 5 days post-infiltration.

Transformation of susceptible potato variety

Binary plasmids harboring the candidate genes *Blb3GH-A, Blb3GH-B, AbptGH-A* and *AbptGH-B* were transferred to *Agrobacterium tumefaciens* strain COR308 (Hamilton et al. 1996) and the constructs pKGW-Rpi-abpt, pKGW-R2-like, pKGW-R2 in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* these clones were transformed to the susceptible potato variety Desiree. Overnight cultures of the transformed *A.tumefaciens* strain were used to transform internodal cuttings from *in vitro* grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation. Three to eight plants per transformant were transferred to the greenhouse.

Allele mining

Primers of 32 nucleotides were designed on Rpi-blb3 sequence, with the forward primer beginning at the start codon (Blb3-start) and the reverse primer beginning at the stop codon (Blb3-end; Table 1). The BAC clone TG92 containing *Rpi-abpt* and the genomic DNA of the parental resistant genotype AM-(3778-16) containing R2-like, were used as template in a long range PCR reaction (95 C: 2'40'', 30X [94 C: 20'', 56.8 C: 25'', 64.3 C:7'], 64.3 C: 25') using the high fidelity DNA polymerase *PfuTurbo*® (Stratagene, la Jolla, CA, U.S.A). PCR products were separated on agarose gel and purified using the QIAquick Gel Extraction Kit from Qiagen (Hilden, Germany). The purified pool of R2-likeGH ORFs and the purified Rpi-abpt ORF were used in a BP reaction together with the donor plasmid pDONR 221 according to the protocol described by (Untergasser 2006b). Subsequently, the BP reaction products were transferred to DH10B *E. coli* competent cells and plated on LB-agar plates containing the antibiotic kanamycin at the concentration of 100 µg/ml. Colonies were cultured in LB liquid medium supplemented with kanamycin. Plasmid DNA was subsequently extracted by mini-prep protocol adapted from Sambrook et al (2^{nd} edition, 1989) using the P1, P2, P3 solutions from Qiagen. The first and last 700 bp of plasmids containing R2-likeGH ORFs were sequenced and classified. Then one clone of each group was entirely sequenced and compared to the *Rpi-blb3* sequence. The clones sharing high similarity with *Rpi-blb3* were cloned into the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination, using the protocol of (Untergasser 2006a). The final expression constructs were then transferred to *A. tumefaciens* strain COR308 after the configuration of the constructs was confirmed by PCR.

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Supplementary Table S1: Genebank accessions numbers

Gene	accession number
Rpi-blb3	FJ536346
R2-like	FJ536323
Rpi-abpt	FJ536324
R2	FJ536325
blb3GH-A	FJ536326
R2-likeGH-2	FJ536327
R2-likeGH-3	FJ536328
R2-likeGH-5	FJ536329
abptGH-A	FJ536330
R2GH-2	FJ536331
R2GH-3	FJ536332
R2GH-5	FJ536333
R2GH-7	FJ536334
R2GH-8	FJ536335
R2GH-9	FJ536336
R2GH-10	FJ536337
R2GH-11	FJ536338
R2GH-12	FJ536339
R2GH-13	FJ536340
R2GH-14	FJ536341
R2GH-15	FJ536342
R2GH-16	FJ536343
R2GH-D3	FJ536344
R2GH-65	FJ536345

Chapter 3

Diversity, distribution and evolution of *Solanum bulbocastanum* Late Blight resistance genes

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ABSTRACT

Knowledge on the evolution and distribution of Late Blight resistance genes is important for a better understanding of the dynamics of these genes in nature. We analyzed the presence and allelic diversity of the Late Blight resistance genes, *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*, originating from *Solanum bulbocastanum* in a set of tuber-bearing *Solanum* species. These genes were only present in some Mexican diploid as well as polyploid species closely related to *S. bulbocastanum.* Sequence analysis of the fragments obtained from the *Rpi-blb1* and *Rpi-blb3* gene suggests an evolution through recombination and point mutations. For *Rpi-blb2* only sequences identical to the cloned gene were present in *S. bulbocastanum* accessions, suggesting that it has emerged recently. The three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their spread is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with Rpi-blb1, Rpi-blb2 and Rpi-blb3, respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, Late Blight resistance factors that in addition to the *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes.

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INTRODUCTION

Solanum bulbocastanum is well known for its resistance to Late Blight (Niederhauser and Mills 1953), a disease caused by the oomycete *Phytophthora infestans*. The species belongs to the section *Petota* of the genus *Solanum*. It is a diploid, self-incompatible species native to Central America. It inhabits mountainous areas at an altitude between 1200-2300 m (Spooner et al. 2004) from northwestern Mexico (Durango and Nayarit regions) all the way south to Honduras..

Although characterized by Late Blight resistance, *S. bulbocastanum* resistance sources have not been widely exploited yet, which is mainly due to crossing barriers with cultivated potato (Helgeson et al. 1998; Hermsen and Ramanna 1973). Until now, 4 different NBS-LRR resistance genes have been identified in and cloned from *S. bulbocastanum*, namely *Rpi-Blb1* (van der Vossen et al. 2003) also known as *RB* (Song et al. 2003), *Rpi-Blb2* (van der Vossen et al. 2005) *Rpi-Blb3* (Lokossou et al. 2009) and *Rpibt1* (Oosumi et al. 2009), which opens the possibility to study the distribution and diversity of these genes in the section *Petota.*

The distribution of specific resistance genes in natural populations has been studied in a few cases only, *Rps2* and *Rpp13* in *Arabidopsis thaliana* (Mauricio et al. 2003; Rose et al. 2004), and *Cf-4* and *Cf-9* in tomato (Kruijt et al. 2005; Caicedo 2008) . Mauricio et al. (2003) studied the diversity of *Rps2*, a resistance gene against the bacteria *Pseudomonas syringae*, in a world wide sample of 27 *A. thaliana* accessions. They favored balancing selection as the explanation for the *Rps2* variation. This maintenance of variance by natural selection might be a general feature for the dynamics of *R*-gene evolution (Ellis et al. 1999; Luck et al. 2000). From the study on diversity for *Rpp13*, a resistance gene in *A. thaliana* against *Hyaloperonospora arabidopsis*, which is characterized by an extreme sequence diversity and resistance to various pathogen isolates; the suggestion was made that the co-evolutionary interactions between host and pathogen must be very active (Rose et al. 2004). Confirmation was provided with the analysis of the recently identified *Avr*-gene of *Rpp13*, *Atr13*, which reveals high sequence diversity for this effector as well (Allen et al. 2008) and moreover, one *Rpp13* allele seems to recognize another *H. arabidopsis* effector (Hall et al. 2009). Kruijt et al. (2005) showed that a high proportion of wild tomato plants specifically recognize the *C. fulvum* Avr4 and Avr9 elicitors, and functional homologs of the *C. fulvum* resistance genes *Cf-4* and *Cf-9* are conserved in diverged tomato species. The *Cf-4* and *Cf-9* genes appear to have been maintained by selection pressure imposed by the pathogen *Cladosporium fulvum*. Caicedo (2008) studied the distribution of *Cf-2* homologs in natural populations of *S. pimpinellifolium* and deduced that primarily the geographic distribution of *Cf-2* homolog diversity has been shaped by demographic factors or by selective pressure with a clinal geographic distribution. Levels of homologue diversity varied greatly between populations.

As population genetic processes and dynamics might affect the evolution of *R*-genes, a better understanding of variation within and between populations is crucial. Spooner (2009) evaluated several factors that might be predictive for identifying pest and disease resistant plants in wild relatives of the cultivated potato. For late blight resistance they identified taxonomic, climatic and geographic (latitude and altitude) variables. From these analyses it was clear that species level was the best predictor for resistance against *P. infestans*. These results stressed the need to use more focused evaluations of germplasm and to have a clear picture of geographical patterns of diversity in Late Blight resistance genes in wild *Solanum* species. To analyze these, here, use has been made of well studied *R*-genes like *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* which are terrific candidates since both *Avr* and *R*-genes have been identified and cloned.

The *Rpi-blb1* is located on linkage group VIII, near marker CT64 in a cluster of 4 Resistance Gene Analogues (RGA's) (van der Vossen et al. 2003). Its susceptible haplotype, rb, is characterized by an 18 bp deletion (Song et al. 2003). In an evaluation by Wang et al. (2008) using 44 species of *Solanum* section *Petota* for *Rpi-blb1* (*RGA2*) and its flanking *RGA's* (*RGA1* and *RGA3*) it was shown that the genes had a different distribution and diversity. The flanking *RGA1* appeared to be highly conserved within the tuber bearing *Petota* and was also found in the non tuber bearing species of the section, while *RGA3* was only present in some tuber bearing species. The *Rpi-blb1* gene was found in Mexican diploid species (*S. bulbocastanum* and *S. cardiophyllum*) and the Mexican polyploid species *S. stoloniferum* (including *S. papita* and *S. polytrichon*) (Wang et al. 2008). Functional analysis of the genes found in *S. stoloniferum* (*Rpi-sto1* and *Rpi-pta1*) indeed confirmed the functional homology with *Rpi-blb1* (Vleeshouwers et al. 2008). The occurrence of *Rpi-blb1* homologues in *S. stoloniferum* was suggested to be the result from common ancestry, e.g. *S. bulbocastanum* was suggested as one of the parents of the allopolyploid *S. stoloniferum* (Wang et al. 2008).

Rpi-blb2 is located on linkage group VI near marker CT119, in a locus harboring at least 15 *Mi-1* gene homologues (van der Vossen et al. 2005). The *Mi-1* gene of tomato confers resistance to the root knot nematode *Meloidogyne incognita* (Milligan et al. 1998) and shares 82% overall protein sequence identity with *Rpi-blb2* (van der Vossen et al. 2005). *Rpi-ver1*, a Late Blight *R*-gene from *S. verrucosum* also maps in this region (Jacobs et al. 2010). Other *R*-genes found at this locus in tomato include *Ol-1* and *Ol-3* (Bai et al. 2005), *Cf2* and *Cf5* (Dickinson et al. 1993), conferring resistance to *Oidium neolycopersici* and *Cladosporium fulvum*, respectively. Apart from *S. bulbocastanum*, no other species were found to contain *Rpi-blb2* (Wang et al. 2008).

Rpi-blb3 is located on linkage group IV, near marker TG339 in a major Late Blight resistance gene cluster containing *R2*, *Rpi-abpt* and *R2-like* (Park et al. 2005). Also Late Blight resistances with a

more QTL-like appearance like those of *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008) and those found in tomato (Brouwer et al. 2004) map into the same region. Tan et al. (2008) proposed that the QTL from *S. microdontum,* indentified as *Rpi-mcd1,* is a major *R*-gene. Other pathogen *R*-genes, mapping to the same region, include Ny_{tbr} conferring resistance to *Potato virus Y* (Celebi-Toprak et al. 2002) and the *Hero* resistance gene which confers resistance to all known pathotypes of *Globodera rostochiensis* and partial resistance to *Globodera pallida* (Ernst et al. 2002). Allele mining studies for *Rpi-blb3* are not yet reported.

Distribution of *R*-genes in natural populations is not easy to study phenotypically, as resistance to a particular pathogen might result from the presence of one or more different *R*- genes, which is the case for populations of *S. bulbocastanum*. One approach could be to use *Avr*-genes to determine whether a corresponding *R*-gene is present, in our case Avrblb1, which interacts with Rpi-blb1 (Vleeshouwers et al. 2008), Avrblb2 interacting with Rpi-blb2 (Oh et al. 2009) and Pi-Avr2 interacting with Rpi-blb3 (Lokossou et al. 2009). However the *Rpi-blb3* gene has several close relatives, all recognizing the same set of *Avr*-genes (Lokossou et al. 2009). Also carrying out such analysis in a large number of different species is not straight forward. Studying the distribution of *R*-genes with highly specific molecular markers and deduction of the phenotype for positive genotypes is an alternative. Still, one has to keep in mind that the functionality of *R*-genes might be changed by one single nucleotide polymorphism (SNP) or by insertions/deletions. A combination of the two approaches and screening with key *P. infestans* isolates seems a beneficial strategy as that will also allow the identification of other resistance genes towards this devastating disease.

In this paper, we studied the allelic frequencies of the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes in several wild tuber-bearing *Solanum* accessions by performing allele mining with *R*-gene-specific primers. Additionally, Avr expression assays as well as detached leaf assays with key *P. infestans* isolates were utilized to strengthen the evidence for the presence of functional alleles. We relate the occurrence of the genes to the geographical origin of the accessions.

RESULTS

Screening of Solanum section Petota for the presence of Rpi-blb resistance genes

A total of 196 different taxa from *Solanum* section *Petota* were screened for the presence of the *Rpi-blb1* and *Rpi-blb3* genes. The presence of *Rpi-blb2* was not assessed in this wide set of taxa as a

previous study had already shown that the distribution of this gene was confined to *S. bulbocastanum* (Wang et al. 2008). We used the plant material described by Jacobs et al (2008) and as far as available at least 5 accessions from each species and 5 individuals per accession (in total 4929 genotypes). One screen was carried out with *Rpi-blb1* recognizing 1/1' primer pair, which is specific for the functional allele of *Rpi-blb1* (Colton *et al*. 2006). PCR fragments were detected in *S. bulbocastanum*, *S. cardiophyllum* ssp. *cardiophyllum* and *S. stoloniferum* (sensu Spooner et al. 2004) only.

The collection was also screened with primers specific for *Rpi-blb3* and 6 species were found to contain putative *Rpi-blb3* homologues: *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii*, *S. nayaritense*, *S. brachistotrichum* and *S. stoloniferum*.

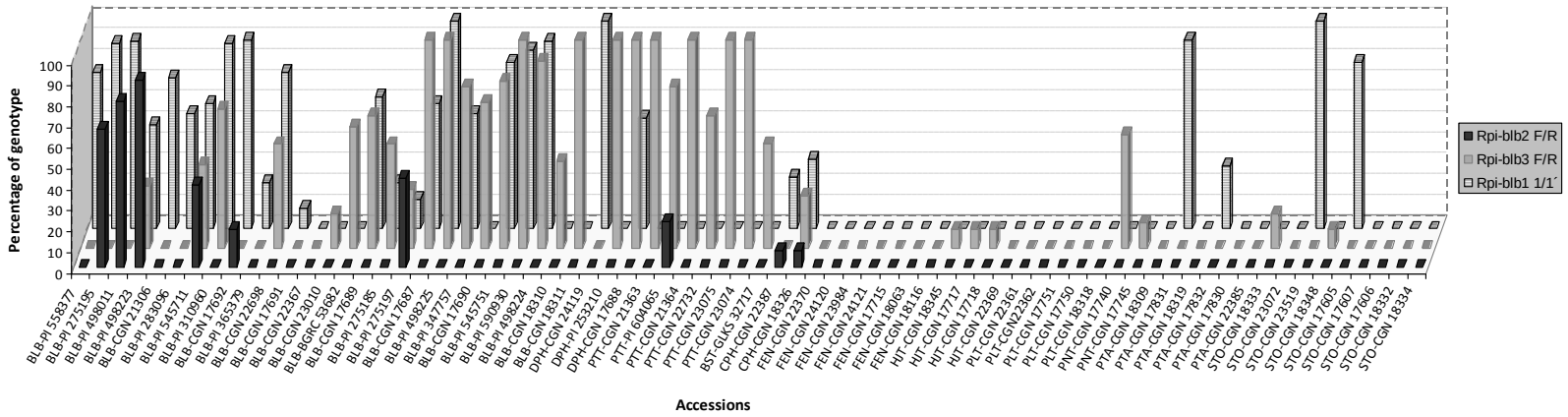
Detailed investigation of S. bulbocastanum and related species

An additional 72 accessions originating from Mexico and Guatemala of the series *Bulbocastana*, *Pinnatisecta* and *Longipedicellata* was evaluated for the presence of *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* genes. For 63 accessions precise coordinates (longitude, latitude data) were available, pointing to 52 different locations (Table S1).

The presence or absence of *Rpi-blb1* was analyzed using the primer combinations Blb1 F/R, detecting the *Rpi-blb1* locus (Wang et al. 2008) and Blb 1/1' (Colton et al. 2006). The RGA1 F/R primer pair served as a positive control for DNA quality as this gene is present in all tuber-bearing *Solanaceae* (Wang et al. 2008).

The primer pair Blb1 F/R amplified fragments of the expected size in 27 *S. bulbocastanum*, 1 *S. brachistotrichum*, 2 *S. cardiophyllum*, and 4 *S. stoloniferum* accessions. The primer pair Blb 1/1' amplified fragments in a total of 29 accessions from 23 different locations and composed of 23 accessions of *S. bulbocastanum*, 2 of *S. cardiophyllum*, and 4 of *S. stoloniferum* (Table S1 and Figure 1A).

A.



B.

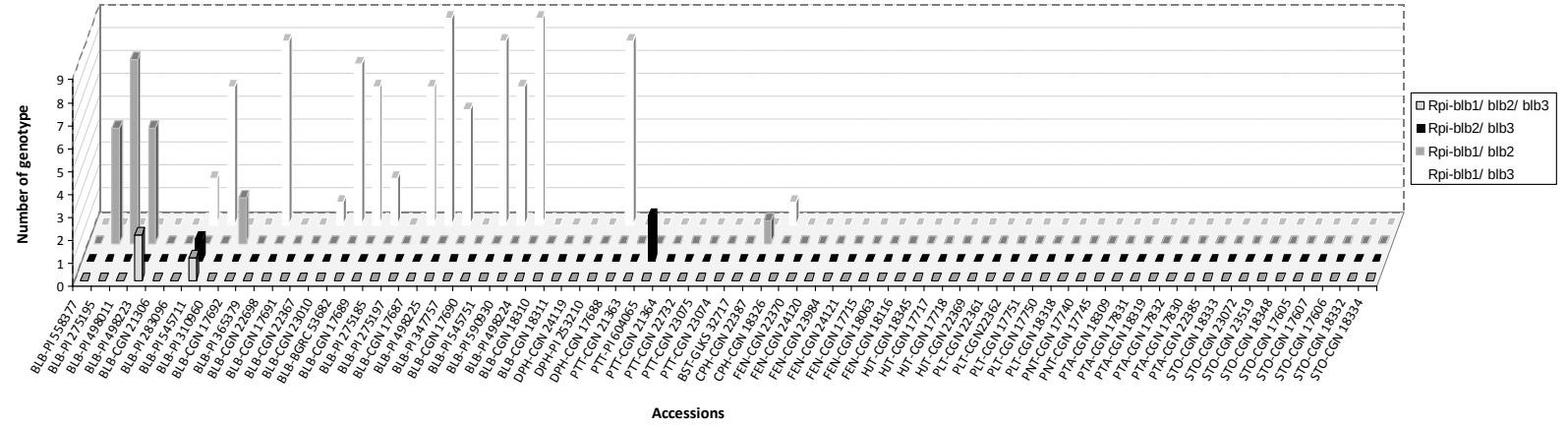


Figure 1:*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* presence in *S.bulbocastanum* and related species. (A) Percentage of genotype per accessions containing *Rpi-blb* genes fragments. (B) Number of genotypes per accessions containing combinations of *Rpi-blb* genes fragments. Abbreviations: BLB: *S. bulbocastanum*, BST*: S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum sbsp dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, PLT: *S. polytrichon*, PNT: *S. pinnatissectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum.*

Supplementary Table S1: List of the accessions used to identify the frequency of the three *S. bulbocastanum* genes *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*. From the left to the right the columns report the plant taxon, the accession number, the country of origin, the location coordinates (latitude-longitude in degrees and minutes); the total number of genotype tested positive by PCR with specific primers to *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, the haplotypes identified after sequencing; the number of genotype per accession which contain the different combination of genes *Rpi-blb1+Rpi-blb2*, *Rpi-blb1+Rpi-blb3*, *Rpi-blb2+Rpi-blb3*, *Rpi-blb1+Rpi-blb2+ Rpi-blb3*.

S1a. *S. bulbocastanum* accessions

Taxon	Accession number	Country	Coordinates		Number of genotypes	Rpi-blb genes identification								Rpi-blb genes Combination			
			lat	lon		RGA1	Blb1 F/R	Blb1 1/1´	Haplotype	Blb2 F/R	Haplotype	Blb3 F/R	Haplotype	blb1/ blb2	blb1/ blb3	blb2/ blb3	blb1/ blb2/ blb3
BLB	PI 558377	MEX	16.16N	96.41W	8	8	7	6	A6, A7	-		-		-	-	-	-
BLB	PI 275195	MEX	17.02N	96.46W	9	9	8	8	A1, A3	6	A	-		**5**	-	-	-
BLB	PI 498011	MEX	17.02N	96.46W	10	10	9	9	A3, C7	8	A	-		**8**	-	-	-
BLB	PI 498223	MEX	17.02N	96.46W	10	10	6	5	A9, A10, C2	9	A	3	A1	**5**	-	-	**2**
BLB	CGN 21306	MEX	17.30N	96.27W	11	11	8	8	A1, A2, A3	-		-		-	-	-	-
BLB	PI 283096	MEX	17.30N	96.52W	9	9	7	5	A4	-		-		-	-	-	-
BLB	PI 545711	MEX	17.33N	99.30W	10	10	6	6	D2, D3	4	A	4	A2		**2**	**1**	**1**
BLB	PI 310960	MEX	17.49N (b)	98.08W	9	9	8	8	H1, I1	-		6	A2	-	**6**	-	-
BLB	CGN 17692	MEX	17.53N	96.33W	11	11	10	10	A2, B1	2	A	-		**2**	-	-	-
BLB	PI 365379	MEX	18.43N	97.19W	9	9	2	2	A8	-		-		-	-	-	-
BLB	CGN 22698	MEX	19.11N	98.13W	16	12	12	12	A1, C2	-		8	A2	-	**8**	-	-
BLB	CGN 17691	MEX	19.13N	98.48W	10	9	5	1	A1, M1	-		-		-	-		-
BLB	CGN 22367	MEX	19.13N	98.48W	10	10	10	-	M1	-		-		-	-	-	-
BLB	CGN 23010	MEX	ND	ND	12	10	8	-	E2, M1	-		2	A2	-	**1**	-	-
BLB	BGRC 53682	MEX	19.15N (a)	98.53W	24	18	11	-	A5, M1	-		14	A2	-	**7**	-	-
BLB	CGN 17689	MEX	19.21N	99.12W	11	11	11	7	A3	-		7	A5	-	**6**	-	-
BLB	PI 275185	MEX	19.21N	99.12W	18	18	10	4	A1, M1	-		9	A2	-	**2**	-	-
BLB	PI 275197	MEX	19.21N	99.12W	7	7	1	1	A3	3	ND	2		-	-	-	-
BLB	CGN 17687	MEX	19.21N (b)	99.12W	10	10	9	6	L1,G1	-		10	A1	-	**6**	-	-
BLB	PI 498225	MEX	19.25N	100.20W	9	9	9	9	A1, M1	-		9	A2		**9**		
BLB	PI 347757	MEX	19.31N	100.15W	9	9	7	5	C3, F1	-		7	A2	-	**5**	-	-
BLB	CGN 17690	MEX	19.50N	101.43W	10	10	3	-	M1	-		7	A1	-	-	-	-
BLB	PI 545751	MEX	20.59N	103.10W	10	10	9	8	B3	-		8	A2		**8**		
BLB	PI 590930	MEX	21.01N	102.59W	7	7	7	6	J1, K1	-		7	A3		**6**		
BLB	PI 498224	MEX	19.24N	100.21W	10	10	10	9	B2	-		9	A2	-	**9**	-	-
BLB	CGN 18310	MEX	ND	ND	12	11	-	-	M1	-		5	B3	-	-	-	-
BLB	CGN 18311		ND	ND	7	7	-	-		-		7	B6	-	-	-	-
DPH	CGN 24119	MEX	18.55N	99.43W	10	10	10	10	C6	-		-		-	-	-	-
DPH	PI 253210	MEX	19.00N (b)	99.06W	5	-	-	-		-		5	A2	-	-	-	-
DPH	CGN 17688	MEX	19.42N	103.31W	15	15	8	8	A6	-		15	A2, A4, A6	-	**8**	-	-
PTT	CGN 21363	GTM	15.09N	90.18W	11	11	-	-	M1	-		11	B2, B8	-	-	-	-
PTT	PI 604065	GTM	15.10N	90.18W	9	9	-	-		2		7	B2	-	-	**2**	-
PTT	CGN 21364	GTM	15.13N	90.18W	11	11	-	-		-		11	B2	-	-	-	-
PTT	CGN 22732	GTM	15.19N	91.31W	11	10	-	-		-		7	B4, B5	-	-	-	-
PTT	CGN 23075	GTM	15.19N	91.33W	11	11	-	-	M1	-		11	B6	-	-	-	-
PTT	CGN 23074	GTM	15.46N	91.30W	12	10	-	-		-		12	A2, B1, B7	-	-	-	-

S1b. *S. brachistotrichum*, *S. cardiophyllum*, *S. nayaritense* and polyploid *Solanum* accessions

Taxon	Accession number	Country	Coordinates		Number of genotypes	Rpi-blb genes identification								Rpi-blb genes Combination			
			lat	lon		RGA1	Blb1 F/R	Blb1 1/1´	Haplotype	Blb2 F/R	Haplotype	Blb3 F/R	Haplotype	blb1/ blb2	blb1/ blb3	blb2/ blb3	blb1/ blb2/ blb3
BST	GLKS 2717	MEX	ND	ND	6	1	1	-		-		3	A2	-	-	-	-
CPH	CGN 22387	MEX	ND	ND	12	10	5	3	D1	1	B	-		**1**	-	-	-
CPH	CGN 18326	MEX	ND	ND	12	10	4	4	D1	1	B	3	A1	-	**1**	-	-
FEN	CGN 22370	MEX	25.25N	101.00W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 24120	MEX	28.08N	107.30W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 23984	MEX	28.19N	107.21W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 24121	MEX	29.08N	108.08W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 17715	USA	31.26N	110.19W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 18063	USA	31.54N	109.16W	10	9	-	-		-		-		-	-	-	-
FEN	CGN 18116	USA	33.23N	108.36W	11	9	-	-		-		-		-	-	-	-
HJT	CGN 18345	MEX	24.52N	100.13W	11	10	-	-		-		1	A1	-	-	-	-
HJT	CGN 17717	MEX	25.25N	100.51W	11	10	-	-		-		1	A1	-	-	-	-
HJT	CGN 17718	MEX	25.25N	101.00W	11	9	-	-		-		1	A1	-	-	-	-
HJT	CGN 22369	MEX	25.25N	100.51W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 22361	MEX	20.20N	100.00W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 22362	MEX	20.20N	100.00W	10	10	-	-		-		-		-	-	-	-
PLT	CGN 17751	MEX	22.47N	102.35W	5	5	-	-		-		-		-	-	-	-
PLT	CGN 17750	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
PLT	CGN 18318	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
PNT	CGN 17740	MEX	20.36N	100.23W	11	-	-	-		-		6	A1	-	-	-	-
PNT	CGN 17745	MEX	20.40N	103.20W	8	-	-	-		-		1	A1	-	-	-	-
PTA	CGN 18309	MEX	23.20N	105.07W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 17831	MEX	23.30N	103.35W	11	10	10	10	C4, C5	-		-		-	-	-	-
PTA	CGN 18319	MEX	24.11N	105.02W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 17832	MEX	ND	ND	10	10	3	3	C4, C6	-		-		-	-	-	-
PTA	CGN 17830	MEX	24.57N	103.54W	10	10	-	-		-		-		-	-	-	-
PTA	CGN 22385	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18333	MEX	19.05N	98.41W	12	11	-	-		-		2	A1	-	-	-	-
STO	CGN 23072	MEX	19.05N	98.41W	10	10	-	-		-		-		-	-	-	-
STO	CGN 23519	MEX	19.07N	98.46W	10	10	10	10	D1	-		-		-	-	-	-
STO	CGN 18348	MEX	19.35N	99.04W	11	10	-	-		-		1	A1	-	-	-	-
STO	CGN 17605	MEX	19.42N	101.07W	10	10	8	8	B3	-		-		-	-	-	-
STO	CGN 17607	MEX	19.42N	101.07W	10	10	-	-		-		-		-	-	-	-
STO	CGN 17606	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18332	MEX	ND	ND	10	10	-	-		-		-		-	-	-	-
STO	CGN 18334	MEX	19.15N (a)	98.53W	10	10	-	-		-		-		-	-	-	-

(a): Coordinates estimated with Google Earth using the CGN route description. (b): Data derived from Heijmans (2007). Abbreviations:ND: not determined BLB: *S. bulbocastanum*, BST*: S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum sbsp dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, PLT: *S. polytrichon*, PNT: *S. pinnatissectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum*.

The primer pair Blb2F/R (Wang et al. 2008) amplified *Rpi-blb2* in 7 accessions of *S. bulbocastanum* and in 2 *S. cardiophyllum* accessions from 6 different locations (Table S1 and Figure 1A).

The primers specific for *Rpi-blb3* amplified distinct fragments in 35 accessions from 28 different locations. Accessions showing amplification originated from 26 *S. bulbocastanum,* 2 *S. pinnatissectum,* 1 *S. brachistotrichum,* 1 *S. cardiophyllum,* 3 *S. hjertingii* and 2 *S. stoloniferum* accessions (Table S1 and Figure 1A).

The *Rpi-blb1* and *Rpi-blb3* gene combinations were found in genotypes from 14 *S. bulbocastanum* accessions originating from 12 different locations. The *Rpi-blb1* and *Rpi-blb2* gene were found together in genotypes from 5 *S. bulbocastanum* accessions from 2 different locations. The *Rpi-blb2* and *Rpi-blb3* fragments were found together in 2 *S. bulbocastanum* accessions from 2 distinct locations (Table S1 and Figure 1B).

The percentages of the individual genotypes of *S. bulbocastanum* containing *Rpi-blb1, Rpi-blb2* or *Rpi-blb3* fragments are 36%, 9% and 56%, respectively.

Three genotypes, belonging to PI498223 and PI545711 accessions, harbored all three genes. Only in a small number of accessions all genotypes harbored the same gene; 2 accessions were fixed for *Rpi-blb1* and 10 for *Rpi-blb3*. Interestingly 91% of the genotypes from the subspecies *S. bulbocastanum* subsp. *partitum* contained *Rpi-blb3*.

Allelic variation in the Rpi-blb1, Rpi-blb2 and Rpi-blb3 gene fragments

To verify the nature of the amplified fragments and to explore the R-gene diversity at the nucleotide level, part of the gene was sequenced for all the positives accessions. The results are presented in Table 1.

The *Rpi-blb1* amplified sequence spans 1734 base pairs in total, composed of two separate parts: the first part is from the start codon to position 545 obtained with primer pair 517/ 1519. The second part is from position 2404 to the stop codon obtained with primer pairs Blb1F/R, 1531/519 and 1/1'. Sequence alignment revealed a total of 56 SNPs compared to the cloned *Rpi-blb1* gene with 34 SNPs in the coding region, 4 SNPs in an intron part and the deletion of 18 nucleotides which is characteristic for the *Rpi-blb1* non-functional allele.

Table 1a: Allelic variation for *Rpi-blb1* in *S. bulbocastanum* accessions and related species.

Haplotype	13	28	42	64	65	78	123	129	155	210	315	387	*455*	*488*	*525*	*538*	2407	2607	2637	2664	2667 - 2684	2745	2809	2818	2897	2908	2981	3021	3134	3180	3255	3314	3376	3390	3442	3481	3517	3574	3588	Accessions
	T	C	T	G	T	T	T	C	A	T	C	C	*A*	*C*	*C*	*T*	T	A	T	A	AAAATTCAATTGTGTTGC	T	A	T	T	C	G	T	A	G	C	T	C	T	A	C	C	T	T	
A 1																																								*Rpi-blb1* , BLB-PI275194, BLB- PI275195, BLB-CGN17689, BLB-CGN21306, BLB-CGN17692
A 2																*a*																								BLB-CGN17682, BLB-CGN21306
A 3														*g*		*a*																								BLB-CGN21306, BLB-PI498011, BLB-CGN17689, BLB-PI498223, BLB- PI275195
A 4														*g*																										BLB-CGN283096
A 5		t			**c**																																			BLB-BGRC53682, BLB-CGN22698
A 6		t			**c**											*a*																								BLB-CGN17688, BLB-PI558377
A 7		t			**c**											*a*																				t	t			BLB-PI558377
A 8		t			**c**						t	t				*a*																								BLB-PI365379
A 9							c				t					*a*																								BLB-PI498223, STO-PI205522
A 10		t			**c**	c	c									*a*																								BLB-PI498223
A 11		Y	Y				c																									c								BLB-CGN17693
B 1																																							**a**	BLB-CGN17692
B 2		t			**c**											*a*																							**a**	BLB-PI498224
B 3		t		**a**	**c**		c			c						*a*																							**a**	STO-CGN17605, BLB-PI545751
B 4		t		**a**	**c**		c			c																													**a**	*Rpi-sto1*
C 1														*g*		*a*													**c**										**a**	BLB-PI498223
C 2		t			**c**																								**M**										**W**	BLB-CGN22698
C 3		t			**c**											*a*													**c**				t						**a**	BLB-PI347757
C 4		t		**a**	**c**		c			c						*a*													**c**										**a**	PTA-CGN17831, PTA-CGN17832
C 5		t		**a**	**c**		c			c						*a*				**g**									**c**										**a**	*Rpi-pta1, Rpi-plt1*
C 6		t			**c**																			c					**M**										**a**	DPH-CGN24119
C 7		t			**c**	c	c									*a*													**c**										**a**	BLB-PI498011
D 1					**c**						t					*a*															**a**									CPH-CGN18326, CPH-CGN22387, STO-CGN23519
D 2		t			**c**										*t*	*a*															**a**				g					BLB-PI545711
D 3		t			**c**											*a*															**a**				g					BLB-PI545711
E 1		t	g				c									*a*						**g**										c								CPH-BGRC55227
E 2					**c**		Y									*a*						**K**		Y																BLB-CGN23010
F 1		t			**c**											*a*						**g**						**c**	**c**				t						**a**	BLB-PI347757
G 1					**c**											*a*						**g**		c		t			**c**				t							BLB-CGN17687
H 1					**c**			g					*t*			*a*							g				**c**		**c**	**c**									**a**	BLB-PI310960
I 1					**c**			S					*t*			*a*							R				**S**			**S**									**a**	BLB-PI310960
J 1	**g**	t			**c**				**t**							*a*		**g**							**c**													c		BLB-PI590930
K 1	**g**	t							**t**							*a*	c	**g**																				c		BLB-PI590930
L 1		t			**c**										*t*	*a*								c					**M**					**c**	g					BLB-CGN17687
M 1		t			**c**										*t*	*a*			**c**		_ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _											c			g				**a**	BLB-CGN17689, BLB-CGN17690, BLB-CGN17691, BLB-CGN22367, BLB-CGN23010, BLB-PI275190, BLB-PI498225, BLB-BGRC53682, BLB-PI275185, BLB-CGN18310, PTT-CGN21363, PTT-CGN23075, BLB-PI275199
Amino acid change	**F/N**	L/L	T/T	**V/I**	**V/A**	G/G	I/I	A/A	**Q/L**	Y/Y	V/V	H/H					T/T	**N/S**	**S/P**	**K/R**		**I/R**	L/L	Y/Y	**F/L**	C/C	**E/Q**	**F/S**	**M/L**	**R/T**	**A/E**	L/L	L/L	**L/P**	L/L	L/L	I/I	N/N	**I/N**	

The *Rpi-blb1* sequence spans 1709 base pairs (bp) in total, composed of two separate parts: (1) from the start codon to position 520 derived from the primer 517/1519. In italic are the located in the intron sequence. (2) from position 2404 to stop codon derived from the primer BLB1F/R and 1521/519.

Table 1b: Allelic variation for *Rpi-blb2* in *S. bulbocastanum* accessions and related species.

Haplotype	SNP position in comparison with *Rpi-blb2* genomic sequence																										Accessions
	2226-2297	2298	2299	2300	2302	2303	2304	2305	2306	2308	2309	2310	2311	2312	2313	2315	2316	2317	2318	2319	2320	2321	2322	2323	2324	2325	2326	
A	TCTGGAAGAAGTGGTGAAAATTTATTTGGATGATTTAATTTCCAGTAGCTTGGTAATTTGTTTCAATGAGAT	A	G	G	G	A	T	T	A	C	C	T	A	C	T	G	C	C	A	A	C	T	T	C	A	T	G	*Rpi-blb2,* BLB-CGN17692, BLB-960631, BLB-PI275195, BLB-PI498011, BLB-PI498223, BLB-
B	"	C	A	C	T	T	G	G	G	T	T	A	A	T	C	A	G	A	T	T	T	T	G	T	A	G	T	CPH-CGN18326, CPH-CGN22387, CPH-BGRC55227

The *Rpi-blb2* sequence spans 715 bp derived from the primers Blb2F/R

Haplotypes code are indicated in the left column. Nucleotide positions are numbered according to the genomic sequences of the genes, beginning at the START codon and including introns in the case of *Rpi-blb1* and *Rpi-blb2*. Amino acid changes are indicated on the bottom line. SNP's part of the intron region are in italic. In light grey are highlighted the position where SNP's did not change the amino-acid. SNP's resulting in non-synonymous amino-acid change are indicated in bold. *Solanum* accessions in which the haplotypes where identified are indicated on the right column.

Nucleotides abbreviations according to the IUB code: K, GT; M,AC; R, AG; S, GC; W,AT; Y, CT.

Table 1c: Allelic variation for *Rpi-blb3* in *S. bulbocastanum* accessions and related species.

Haplotype	SNP position in comparison with *Rpi-blb3* genomic sequence																																			Accessions
	941	972	1001	1016	1021	1027	1026	1036	1043	1045	1065	1103	1107	1116	1131	1133	1196	1235	1239	1255	1258	1272	1293	1309	1326	1338	1352	1371	1396	1439	1487	1501	1510	
	G	T	G	T	G	G	G	A	G	T	A	G	A	G	A	A	T	C	G	C	T	T	G	C	G	C	G	C	C	C	C	T	G	
A 1																																		PNT-CGN17745, PNT-CGN17740, *Rpiblb3,* DPH-CGN17688, PTT-CGN23074, BLB-PI545711, BLB-CGN17690, BLB-CGN17687, HJT-CGN22370, HJT-CGN18345, PNT-CGN17742, STO-CGN18348, STO-CGN18333
A 2																				a														BLB-CGN22698, BLB-CGN23010, BLB-PI 275185, BLB-BGRC53682, BLB-PI275190, BLB-PI310960, BLB-PI347757, BLB-PI498224, BLB-PI498225, BLB-PI545711, BLB-PI545751, BLB-CGN176888, DPH-PI253210, BST-GLKS32717, PTT-CGN23074, BLB-GLKS31741
A 3																																c		BLB-PI590930
A 4																					c													DPH-CGN17688
A 5																						g												PTT-CGN17689
A 6													g		-																			DPH-CGN17688
A 7																-				a														BLB-BGRC53682
A 8																											t							PTT-PI275200
B 1												a		a									a			t		a						PTT-CGN23074
B 2												a		a									a			t		a						PTT-CGN21364, BLB-PI604065, PTT-CGN21363
B 3												a		a									a			t		a		g				BLB-CGN18310
B 4														a									a			t		a						PTT-CGN22732
B 5														a			a						a			t		a						PTT-CGN22732
B 6										c				a									a			t		a	t					BLB-CGN18311, PTT-CGN23075
B 7										c				a									a			t		a						PTT-CGN23074
B 8				Y			K					R						S					R			Y		M						PTT-CGN21363
C 1			a			a													a					t			t							STO-PI205522
C 2			a																					t	a		t							BST-CPC3822, BST-PI320265
C 3			a								c													t			t							BST-PI320265
C 4			a					c																t			t							NYR-PI545825
D 1		c								c																							a	*R2-like, Rpi-abpt*
E 1	t		a		t				a	c																					t		a	*R2*
Amino acid change	R/ I	S/ S	R/ K	V/ A	A/ S	V/ I	M/ I	M/ L	S/ N	L/ L	E/ D	K/ K	G/ G	S/ N	fs	fs	I/ N	T/ S	A/ A	I/ L	Y/ H	F/ L	K/ K	R/ W	E/ E	P/ P	R/ I	D/ E	R/stop	T/ S	A/ V	F/ L	V/ I	

The *Rpi-blb3* sequence spans 618 bp derived from the primers Blb3F/R

The 34 haplotypes could be classified into 13 groups (A-M) based on the identity of the non-synonymous SNP changes (Table 1a). Each haplotype group contained 1 to 18 accessions. Group A, which is the dominant group, contains haplotypes without non-synonymous SNPs and is only composed of *S. bulbocastanum* accessions and the cloned *Rpi-blb1* gene. Group B additionally contains *S. stoloniferum (Rpi-sto1* like) haplotypes while *S. papita* and *S. polytrichon* (*Rpi-pta1* and *Rpi-plt1* like) are part of group C. Haplotype C2, A11, C6, E2, I1 and L1 display double SNPs which are probably the result of heterozygous genotypes. Haplotype M1 represents the known non functional allele, identified by the 18 bp deletion (Song et al. 2003).

The *Rpi-blb2* amplified sequence spans 715 base pairs from position 2157 to 2872 derived from the primers Blb2F/R (Wang et al. 2008). From the 2 sequences obtained, one is 100% identical to the *Rpi-blb2* sequence and the second one displayed a variable region composed of 72 nucleotide deletion at the beginning of the sequence followed by 26 SNPs in the remaining part (Table 1b). The *Rpi-blb3* amplified sequence spans 618 base pairs from position 918 to 1536. Sequence alignment reveals 33 SNPs displayed in 22 haplotypes containing 1 to 16 accessions (Table 1c). Our classification differentiates 5 groups containing 1 to 34 accessions, according to the type of polymorphism present in the sequence. Group A displays one SNP per haplotype, A1 and A2 being the most commonly present in 12 and 16 accessions, respectively. Haplotype A6 and A7 show one nucleotide deletion leading to a frame shift. Group B haplotypes have 4 SNPs in common clearly defining a polymorphic sequence block. Haplotypes B are exclusively identified in accessions originating from Guatemala (Table S1 and Appendix 1). Haplotype B6 contains a SNP in position 1396 resulting in a stop codon. Group C comprises haplotypes with 4 SNPs in common defining as well a polymorphic sequence block, present in *S. brachistotrichum, S. nayaritense* (Table 2) and *S. stoloniferum*. The *R2-like*, *Rpi-abpt* and *R2* alleles are classified in haplotypes D1 and E1.

Validation of Late Blight resistance by effector recognition and detached leaf assay

In an attempt to link the presence of *Rpi-blb* haplotypes to Late Blight resistance, a set of 50 plants from our in vitro collection was screened. It included a molecular characterization by PCR-amplification with *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* gene specific primers followed by sequencing the PCR fragment. An additional combination of Rpi-blb3 primer, blb3 F/Ra was used, with Ra being specific to *Rpi-blb3* and its homolog *R2-like* (Lokossou et al. 2009), in order to pick up intermediates of *Rpi-blb3* and *R2-like*.

Phenotypic characterization was carried out through detached leaf assays with six *P. infestans* isolates (Table S2) and agro-infiltration with Pi-effectors interacting with *Rpi-blb1* and *Rpi-blb3*. The chosen *P. infestans* isolates were genetically very different from each other, as it has been shown by simple sequence repeat (SSR) fingerprinting (Champouret et al. 2009), and display different virulence spectra with respect to the *Rpi-blb* genes tested. The *Rpi-blb2* gene confers resistance to the six isolates used and the *Rpi-blb1* and *Rpi-blb3* gene confer the resistance spectrum shown in Table 2. None of the tested plants contained *Rpi-blb2*, as no fragment was amplified with the primers Blb2F/R. Unfortunately, we observed background responses caused by the agro-infiltration of Avrblb2 in all the plants tested (not shown), thus *Rpi-blb2* presence or absence could not be verified by effector recognition in wild *Solanum* species. Results obtained for *Rpi-blb1* and *Rpi-blb3* are presented in Table 2 and show the diversity of possible interactions, classified in 5 categories.

Table 2: Evaluation of *Solanum* genotypes by molecular characterization, effector agro-infection and detached leaf assay. Form the left to the right are indicated in the column the *Solanum* accession taxon; identification number (genotype number in bracket); geographic coordinates (latitude-longitude); results of the specific amplification of *Rpi-blb1* by the primers combination 1/1' and *Rpi-blb3* by blb3F/R and blb3 F/Ra with Ra being specific to *Rpi-blb3* and *R2-like* (Lokossou et al. 2009); the haplotype identified after sequencing of the specific fragment referring to table 1; results of the effector agro-infection with Avr-blb1, Pi-Avr2 and the controls R3a and Pi-Avr3a/R3a together, are indicated by degree of response intensity from (-) meaning no response to (+++) meaning clear hypersentive reaction; results of the detached leaf assay conducted with the isolates PIC99177, PIC 99189, PIC 99183, IPO-C, 90128 and UK7824 scored R (resistant), S (susceptible), Rq (quantitatively resistant), Q (quantitative).

Abreviations: ND: not determined sequences, BLB: *S. bulbocastanum*, BST: *S. brachistotrichum*, CPH: *S. cardiophyllum*, DPH: *S. bulbocastanum* subsp. *dolichophyllum*, FEN: *S. fendleri*, HJT: *S. hjertingii*, NYR: *S. nayaritense*, PLT: *S. polytrichon*, PNT: *S. pinnatisectum*, PTA: *S. papita*, PTT: *S. bulbocastanum* subsp. *partitum*, STO: *S. stoloniferum*. CPH-BGRC55227* contains *Rpi-blb2* haplotype B.

Taxon	Accession number	Coordinates		Specific amplification			Sequence		Effector Agro-infiltration				*Phytophtora infestans Detached leaf assay*						Comments
		lat	lon	Rpi-blb1 1/1'	Rpiblb3 F/R	Rpiblb3 F/Ra	Rpiblb1 Haplotype	Rpiblb3	Avr-blb1	Pi-Avr2	Controls R3a	Controls R3a/Avr3a	PIC99177	PIC99189	PIC99183	IPO-C	90128	UK7824	
BLB	CGN17692 (Blb8005-8)	17.53N	96.33W	x		x	A1		+ + +	-	-	+++	S	S	R	R	R	R	Genotype containing Rpi-blb1
DPH	CGN17688 (Blb99-256-3)	19.42N	103.31W		x	x		A1	-	+ + +	-	+++	Q	S	R	S	R	S	Genotype containing Rpi-blb3
HJT	CGN17718	25.25N	100.00W			x		A1	-	+ + +	-	+++	S	S	Rq	S	R	Q	
HJT	CGN22370	25.25N	101.00W		x			A1	-	+ + +	-	+++	S	S	R	S	R	S	
HJT	CGN18345	24.52N	100.13W		x			A1	-	+ + +	-	+++	Q	S	R	S	R	S	
HJT	BGRC32671	ND	ND		x			A1	-	+ + +	-	+++	S	R	Rq	S	R	S	
HJT	CGN17717	25.25N	100.51W		x	x		A1	-	+ + +	-	+++	R	S	R	S	R	S	
STO	CGN18333	19.05N	98.41W		x			A1	-	+ + +	-	+++	S	R	R	S	R	S	
PNT	CGN18331	ND	ND		x	x		A1	+	+	-	+++	R	R	R	R	R	R	Plants with Avr2 recognition and Rpi-blb3 fragment.
BST	CGN17681	ND	ND		x	x		A2	-	+ + +	-	+++	S	S	R	S	Rq	S	
BLB	CGN18310	ND	ND		x			B2	-	+ +	-	+++	R	R	R	Q	R	R	
PTT	CGN21364	15.13N	90.18W		x			B2	-	+	-	+++	R	Q	R	R	R	S	
BST	PI320265	29.08N	106.05W			x		C2	-	+ + +	-	++	S	R	R	S	R	Q	
BST	PI320265	29.08N	106.05W			x		C3	-	+ + +	-	+	S	S	R	S	R	S	
NYR	PI545825	ND	ND		x			C4	-	+ + +	-	+++	Q	R	R	S	R	R	
STO	PI205522	19.07N	98.46W	x	x		A9	C1	+ + +	+ + +	-	+++	Q	R	R	R	R	R	Plant with Avr2 and AvrBlb1 recognition and Rpi-blb1 and Rpi-blb3 fragment.
BLB	CGN17689	19.21N	99.12W	x	x		A 1	A5	+ + +	-	-	+++	R	R	R	R	R	R	
BLB	CGN21306	17.30N	96.27W	x	x		A1	nd	+ + +	-	-	+++	R	R	R	R	R	R	Plants with AvrBlb1 recognition and Rpi-blb1 fragment.
BLB	CGN17692	17.53N	96.33W	x		x	A1		+ + +	-	-	++	R	S	R	R	R	R	
STO	CGN18348	19.35N	99.04W		x			A1	+ + +	-	-	+	R	S	R	R	R	R	Plants with AvrBlb1 recognition and no Rpi-blb1 fragment.
BLB	CGN17693	19.43N	99.47W						+	-	-	++	R	R	R	R	R	R	
STO	CGN18333	19.05N	98.41W		x			A1	-	-	-	++	Q	R	S	S	R	R	Plants with Rpi-blb1 and/or Rpi-blb3 fragment
BLB	BGRC53682	19.15N	98.53W	x	x	x	A5	A2	-	-	-	+++	R	S	R	R	R	R	
PTT	PI275200	15.10N	91.31W		x			A8	-	-	-	+++	Q	Q	Q	S	Q	R	
PTT	CGN23075	15.19N	91.33W		x			B6	-	-	-	+++	R	R	R	R	R	R	
BLB	CGN22698	19.11N	98.13W	x		x	A5		-	-	-	+++	R	R	R	R	R	R	
PNT	GLKS1586	ND	ND			x			-	-	-	+++	R	R	R	R	R	R	
CPH	GLKS99	ND	ND			x				-	-	+++	Q	S	S	S	S	R	
BLB	CGN18310	ND	ND			x			-	-	-	+++	Q	Q	S	S	S	Q	
PTA	CGN17830	24.57N	103.54W			x			-	-	-	+++	R	Q	S	S	S	S	
BLB	CGN23010	ND	ND						-	-	-	+++	R	R	R	R	R	R	
BLB	PI275199	19.29N	98.54W			x			-	-	-	+	R	R	R	R	R	R	
CPH	CGN22387	ND	ND						-	-	-	+	R	R	R	R	R	R	
CPH	CGN18325	ND	ND						+	-	-	+++	R	R	R	R	R	R	Plants containing different late blight resistance genes other than the tested Rpi-blb genes.
CPH	CGN18326	ND	ND						-	-	-	+++	Q	Q	S	R	Q	R	
CPH	CGN18326	ND	ND						-	-	-	+++	Q	R	Q	R	S	R	
CPH	BGRC55227	ND	ND						-	-	-	+++	Q	Q	S	R	S	R	
PTT	CGN23074	15.46N	91.30W		x			B1	-	-	-	++	S	S	S	S	S	S	
PTT	CGN21363	15.09N	90.18W		x			B2	-	-	-	+++	S	S	S	S	S	S	
PTT	CGN23074	15.46N	91.30W		x			B7	-	-	-	+	S	S	S	S	S	S	
BST	PI320265	29.08N	106.05W			x		C2	-	-	-	++	S	S	S	S	S	S	Late Blight suceptible plants
BST	CGN17681	ND	ND						+	-	-	+++	S	S	S	S	S	Q	
FEN	CGN18116	33.23N	108.36W						-	-	-	+++	S	S	S	S	S	S	
BST	CPC3822	ND	ND		x	x		nd	-	+ + +	-	+++	S	S	S	S	S	S	
STO	GLKS0620	ND	ND		x			nd	-	+	-	++	S	S	S	S	S	S	
PNT	CGN17743	ND	ND			x			+	++	++	++	R	R	R	R	R	R	
BLB	CGN22367	19.13N	98.48W			x			-	-	-	-	R	Q	R	R	R	R	
PNT	CGN17740	20.36N	100.23W		x			A1	+	+	++	++	R	R	R	R	R	R	Plants not suitable for agro-infiltration
PNT	CGN17742	21.07N	101.40W		x			A1	-	-	-	-	R	R	R	R	R	R	
BST	GLKS2717	ND	ND						-	+	+	+++	S	S	S	S	Q	S	

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The first category comprises plants harboring the *Rpi-blb1* and/or *Rpi-blb3* gene, as judged from the marker analysis. They also interacted with the Avrblb1 and/or Pi-Avr2 elicitor and displayed a resistance spectrum identical or broader than the *S. bulbocastanum* derived *R*-genes. Based on the assay with Avrblb1, 3 *S. bulbocastanum* and 1 *S. stoloniferum* plants contained *Rpi-blb1*. PCR amplification and sequencing strengthened the presence of functional *Rpi-blb1* haplotypes, identified as A1 and A9.Their resistance spectrum matches with that of *S. bulbocastanum* plant (CGN17692), which was confirmed to harbor *Rpi-blb1* (van der Vossen et al. 2003). However, they were also resistant to isolates PIC99189 and PIC99177, known to break *Rpi-blb1* (Champouret et al. 2009), which suggests the presence of additional resistance factors in these genotypes. The *Rpi-blb3* haplotypes A1, B2, C1, C2, C3 and C4 were found in 14 plants from *S. bulbocastanum*, *S. brachistotrichum*, *S. hjertingii*, *S. nayaritense* and *S. stoloniferum* accessions. They all interact with Pi-Avr2 and display an identical or broader resistance spectrum in comparison to the genitor used to isolate *Rpi-blb3*, thus suggesting the presence of a functional *Rpi-blb3* haplotype and additional Late Blight resistance factors.

From plants classified in the second category fragments were amplified with primers specific for the *Rpi-blb1* and/or *Rpi-blb3* gene, but no interaction with Avrblb1 or Pi-Avr2 was observed. The *Rpi-blb* genes amplified might contain mutations preventing the interaction with the tested effectors. Moreover, these plants putatively contain Late Blight resistance genes other than the one tested, as judged from the responses to the isolates (Table 2).

The third category comprises resistant plants lacking *Rpi-blb1* or *Rpi-blb3* as judged from the marker assays and effector interaction. Interestingly, 2 *S. bulbocastanum* and 2 *S. cardiophylum* plants featured complete resistance to the full set of isolates. These plants probably contain late blight resistance genes other than the tested *Rpi-blb* genes. One *S. cardiophyllum* plant (CPH-BGRC55227*) was tested positive for *Rpi-blb2* haplotype B and displays a resistance spectrum not identical to the one of *Rpi-Blb2* containing plant.

Late Blight susceptible plants not containing any functional *R*-gene as judged from the response with the tested isolates, but showing amplification with *Rpi-blb3* primers, are found in category 4. One *S. brachistotrichum* clone contains *Rpi-blb3* haplotype C2 and interacts strongly with Pi-Avr2 but is completely susceptible to the set of *P. infestans* isolates tested. Three accessions from *S. bulbocastanum* subsp. *partitum*, 2 of *S. brachistotrichum* and 1 of *S. stoloniferum* contain *Rpi-blb3* haplotypes B1, B2, B7 and C2 but do not interact with Pi-Avr2 and display a susceptible phenotype when infected with the set of isolates.

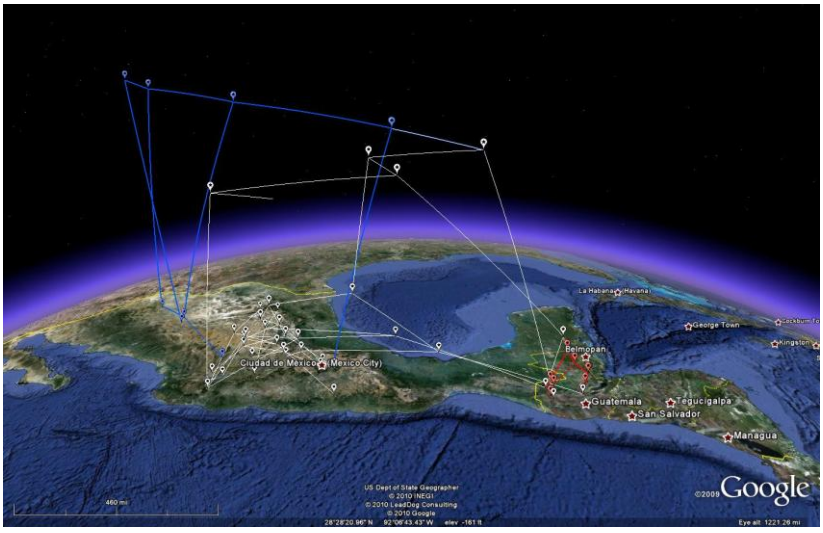
The last category of plants was not suitable for the agro-infiltration. The reactions with the effectors were non specific and resulted in a hypersensitive response to all the tested effectors, or gave no reaction to control *Pi-Avr*/*R*-gene co-infiltration (Table 2). However plants showing resistance to all tested isolates are likely to contain new late blight resistance gene.

Geographical distribution of the Rpi-blb genes

The 3 *Rpi-blb* genes are mainly present in the *S. bulbocastanum* accessions from Central Mexico (Figure 2) and plants not containing the 3 studied *Rpi-blb* genes originate from the northern part of Mexico and the southern part of the USA. Also the allotetraploid species *S. fendleri*, which is found in the north of Mexico, lacks the *Rpi-blb* genes tested.

Solanum stoloniferum from the Durango region contains *Rpi-blb1* while *Rpi-blb3* is present in *S. hjertingii* originating from Monterray, *S. brachistotrichum* from Chihuahua and *S.bulbocastanum* subsp. *partitum* from Guatemala.

In order to see if haplotypes diverge when they originate from more geographically distant areas, the haplotypes were phylogenetically mapped in Google Earth using Supramap (Janies et al. 2007). From the results, it can be seen that the M1 haplotype of *Rpi-blb1* (Appendix 1) is present in Central Mexico as well as in Guatemala and that the less supported clade containing the C and B haplotypes from *S. stoloniferum* is confined to the upper part of Mexico. For *Rpi-blb3*, the haplotypes of group C originate from Central towards Northern Mexico, while haplotypes recognized as group B all are from Guatemala.



Appendix 1 (KMZ file): *Rpi-blb* genes diversity and geographical origin. Phylogenetic and geographical localization of genotypes containing *Rpi-blb* haplotypes. *Rpi-blb3* groups B and C haplotypes and *Rpi-blb1* haplotype M1 are indicated in red, blue and white, respectively. The original kmz file can be provided as and be opened in a Google Earth desktop application. Additionally to the phylogenetic tree, the location of the collection sites of the accessions can be visualized.

A.

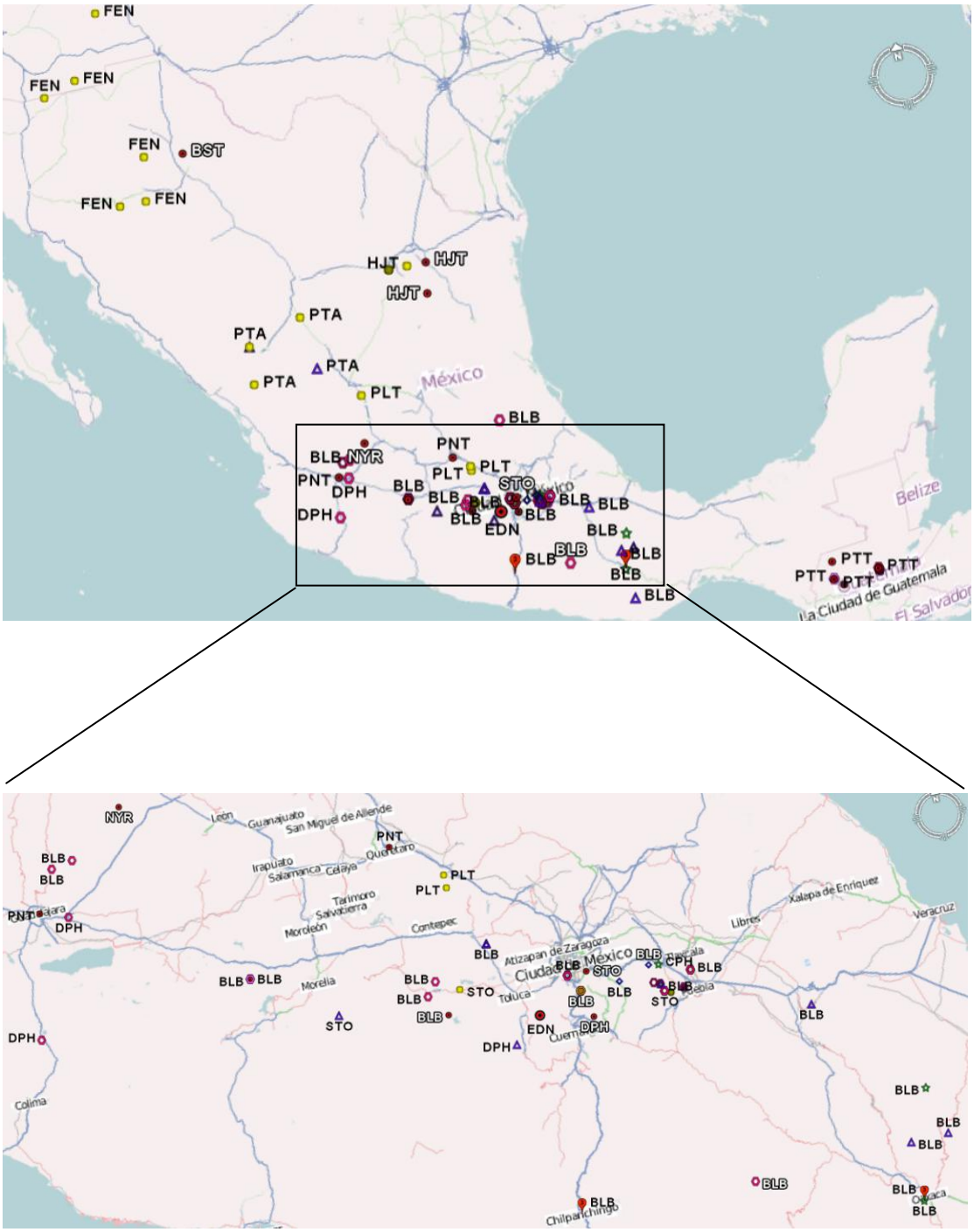


Figure 2: Geographical location of the *Solanum* accessions under study containing only *Rpi-blb1* (blue triangular), only *Rpi-blb3* (red dots), *Rpi-blb1* and *Rpi-blb3* (purple lozenges), *Rpi-blb1* and *Rpi-blb2* (green stars), the three *Rpi-blb* genes (red drops) and the accessions not containing any Rpi-blb gene (yellow dots)

DISCUSSION

The use of gene specific primers and gene functionality

Gene specific primers are powerful tools for the evaluation of presence or absence of resistance genes (Arens et al. 2010). Although *R*-genes often have numerous close homologues, in most cases it is possible to develop markers in such a way that a fragment specific to the gene of interest is amplified. Subsequent sequencing of the fragment makes it possible to verify its identity, to differentiate the haplotypes and to discard recognized pseudo-genes thus indicating the presence or absence of most likely functional genes. We validated this approach by phenotyping 50 individual plants using effectors and detached leaf assays. The plants were first characterized for the presence of *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* with markers. In the 50 cases we only observed two cases for *Rpi-blb1* in which the markers predicted the presence of the gene where there was no effector response. This indicates that there is a good but not 100% correlation between markers and phenotype for *Rpi-blb1* (table 2). All plants tested negative when using the gene specific markers for *Rpi-blb2*. Unfortunately, this could not be validated by an effector response due to background that was observed in the agro-infiltration experiment with Avrblb2. For *Rpi-blb3* we found 13 cases in which there was amplification with the gene specific primers but no response to the effector. Also there were 3 cases in which there was a response to the effector but no amplification of the gene specific fragment and one case in which we observed an effector response and no resistance in the DLA assay. An additional primer pair (Rpiblb3 F/Ra) did amplify in some of the cases where there was no amplification with the *Rpi-Blb3* specific primers was found (Table 2), suggesting that similar but slightly different genes are present. This was also clear from the sequence analysis of some of the fragments obtained with the *Rpi-blb3* specific primers (e.g. the B and C haplotypes of *Rpi-blb3*). These results reflect the complexity of *Rpi-blb3* gene family and underline the usefulness of the combined techniques to identify *Rpi-blb3* from the other alleles of the cluster. However, it should be stressed that in most cases where there was a discrepancy observed between marker assays and phenotypic assays this was outside *S. bulbocastanum*.

Discrepancies between molecular and functional assays can have several causes. This can be the case when plants containing an *R*-allele specific fragment are for some reason unable to interact with the Avr-protein. In such cases the *R*-gene fragment found is probably not translated into a protein interacting with the Avr-protein. Hall et al. (2009) studied natural variation of *Atr13* in a set of *Hyaloperonospora arabidopsidis* isolates and showed differential interaction of *Atr13* with *Rpp13* alleles. The *Atr13* alleles are recognized by *Rpp13* alleles and by *R* genes or genes at other loci in *A.*

thaliana. Interestingly, the *Rpp13-Rld* allele is unable to recognize *Atr13* alleles, and most probably detects an alternative effector protein in *H. arabidopsidis* isolates. Recognition specificity of different effectors by different alleles has previously also been demonstrated at the *RPM1* locus of *A. thaliana* (Bisgrove et al. 1994; Grant et al. 1995), at the *L* locus in flax (Dodds et al. 2004) and at the *Pto* locus in tomato (Kim et al. 2002; Ronald et al. 1992).

Also, plants may produce an *R*-allele fragment and show recognition of the Avr-protein (in our case for example in CPC3822), but still be susceptible to some of the key isolates. This might occur because of the high concentrations of the AVR-protein in our transient expression assay. A similar case was demonstrated by Schornack et al. (2004) for the Bs4/AvrBs3 interaction. Another explanation could be that the *R*-gene is probably active but unable to initiate a hypersensitive response in this specific phenotypic context. Finally, also plants not containing an *R*-allele specific fragment, but able to cause necrosis in presence of the Avr- protein will give discrepancies. Genotypes without any of these discrepancies and no indication for known *R*-gene presence, or plants with indication for known *R*-genes but with enhanced resistance spectra are good candidates to contain new *R*-genes.

Distribution of Rpi-blb genes in Solanum

The presence of *Rpi-blb1* and *Rpi-blb3* has been evaluated in approximately 1000 *Solanum* accessions. In addition, 72 accessions from *S. bulbocastanum* and related species were tested for *Rpi-blb1, Rpi-blb2* and *Rpi-blb3.* In total 35 *Rpi-blb1* haplotypes were found in the Mexican diploid species *S. bulbocastanum* and *S. cardiophyllum* as well as in the polyploid Longipedicellata *S. stoloniferum* (including *S. papita* and *S. polytrichon*), all geographically located in Central and South Mexico. Although the data have to be treated with caution, similar observations were made for *Rpi-blb3* were 22 haplotypes were found in the Mexican diploids *S. bulbocastanum, S. cardiophyllum S. pinnatissectum, S. nayaritense*, and *S. brachistotrichum* , as well as in the polyploid Longipedicellata *S. stoloniferum* and *S. hjertingii*. Interestingly, one *Rpi-blb3* haplotype was exclusively found in *S. bulbocastanum* originating from Guatemala thus showing a clear geographical/ *R*-gene haplotype association. The *Rpi-blb2* gene was only identified in a small number of *S. bulbocastanum* accessions from the Mexican provinces Oaxaca, Guerrero and Toluca. Since no haplotype variation within *Rpi-blb2* was found, this is a strong indication that this *R*-gene is from recent origin, as was suggested by Wang et al. (2008)

The results for the 3 genes show that their spread is limited to the diploid Mexican and Longipedicellata group and confined to Central America. The occurrence of similar haplotypes in different diploid species may be explained by the fact that these species are able to form interspecific hybrids, especially *S. bulbocastanum* and *S. cardiophyllum* (Graham 1965). The presence of both *Rpi-blb1* and *Rpi-blb3* in the polyploid Longipedicellata may be explained by the fact that this group of *Solanum* section *Petota* is of allotetraploid origin. It has been shown that *S. hjertingii*, for example, consists of an *S. verrucosum* like and an *S. cardiophyllum* like genome (Pendinen et al. 2008). Wang et al. (2008) suggested that *S. bulbocastanum* may be one of the parental species of the allotetraploid *S. stoloniferum*. The observation that the Late Blight resistance genes evaluated are confined to Central America might be true for the distribution of other *R*-genes as well.

Rpi-blb genes are found in different frequencies in S. bulbocastanum populations

Genebank accessions used in this study consist of plant material that has been generatively multiplied by the genebank prior to usage. In the Netherlands, at CGN, this is done without any *P. infestans* pressure, since protective spraying is applied every week. This might have resulted in slight frequency changes in the presence of the *Rpi-blb* genes compared to the actual situation in Mexico.

We assessed the frequency in which the different genes are present in the *S. bulbocastanum* accessions under study. Individual plants containing all 3 *Rpi-blb* genes have been found at very low frequency (less than 1 %) and in two *S. bulbocastanum* accessions only, probably because of the rarity of *Rpi-blb2*. The *Rpi-blb1* and *Rpi-blb3* genes have been detected together in 27% of the *S. bulbocastanum* plants analyzed (52% of the accessions). Only *S. bulbocastanum* PI498225 is fixed for *Rpi-blb1* and *Rpi-blb3*, suggesting that accumulation of these genes in *S. bulbocastanum* is a rare event.

Pyramiding of resistance genes is suggested to enhance durable resistance under agricultural conditions (Park et al. 2009; Haverkort et al. 2009; Pink 2002) and is also observed in polyploid *Petota* species, like *S. demissum*. Although pyramiding of resistance genes appears to be a common mechanism in polyploids, in diploid species it seems to be rare with respect to *Rpi-blb1, Rpi-blb2* and *Rpi-blb3*. On the other hand, our functional test with the 50 plants showed frequently a broader resistance spectrum than expected with the tested *Rpi-blb* genes, indicating the presence of other, unknown, *Rpi*-genes.

Almost all the accessions tested contain putative resistant and susceptible individuals. This might reflect a strongly fluctuating *P. infestans* pressure. The coexistence of resistant and susceptible individuals might also have an ecological meaning; in susceptible genotypes, newly recombined *R*-genes are under full pathogen pressure and functional *R*-genes are allowed to enter the population level.

The susceptible haplotype of *Rpi-blb1,* denoted as M1, is abundantly present and not confined to one specific geographical region, but scattered around in Central America (Appendix 1). The wide distribution of such a non-functional allele could imply that carrying a functional copy of *Rpi-blb1* affects the plants fitness in the absence of *P. infestans*. It is known that *R*-genes can result in serious fitness costs (Tian et al. 2003; McDowell and Simon 2006) and Kramer et al. (2009) showed that the *Rpi-blb1* gene is highly upregulated (37 times) once inoculated with *P. infestans*. Such observations suggest that we deal with a 'net cost of fitness under attack' (Korves and Bergelson 2004). In such cases null alleles might counterbalance potent but costly R-genes (McDowell and Simon 2006). Long-lived co-existence of resistant and susceptible alleles across the range of *Arabidopsis thaliana* ecotypes might be an example, as both alleles frequently occurred together within natural populations (Stahl et al. 1999). Another example would be RPS5 from *A. thaliana* (Henk et al. 1999).

Evolution of Rpi-blb genes

Sequence analysis of the fragments obtained for the *Rpi-blb1* and *Rpi-blb3* genes suggests an evolution through recombination and point mutations. In this respect there are clear differences between the 2 genes. The *Rpi-blb1* is thought to have resulted from a recombination event involving *RGA1-blb* and *RGA3-blb* (van der Vossen et al. 2003) and sequence analysis (Table 1) suggests a further evolution through point mutations, an evolutionary pattern typical for a Type II resistance gene as described for *R*-genes in natural lettuce populations (Kuang et al. 2004).

The situation with *Rpi-blb3* is more complex than with *Rpi-blb1* and most likely related to the complex nature of the cluster in which the gene is present. In this cluster several genes have almost identical sequences (Lokossou et al. 2009). Although all sequence information available has been taken into account for the design of the gene specific primers for *Rpi-blb3* it can not be excluded that some variants may not be discriminated by these primers. In *Solanum* as a whole even more *Rpi* genes have been mapped to the *Rpi-blb3* cluster. Examples are *Rpi-demf1* from *S. demissum* (Hein et al. 2007), *Rpi-Skn1* (Jacobs et al. 2010) and QTL's from *S. microdontum* (Colon et al. 1995; Micheletto et al.

2000; Sandbrink et al. 2000). Such resistances might represent *R* genes which are homologues or recombinants sharing ancestry with *Rpi-blb3* as well as *R*-genes that have evolved independently and interact with other effectors.

The presence of conserved SNP's patterns among the haplotypes suggests recombinations between ancestors of *Rpi-blb3*. Such recombinations might have resulted in the *Rpi-blb3* group of B and C haplotypes which are similar to the *R2* gene as suggested by Lokossou et al. (2009).

Recent studies show that the recombination level at *R*-clusters is increased following pathogen infection, suggesting a mechanism that induces temporary genome instability in response to extreme stress, as recently reviewed by Friedman (2007). This instability might well be connected with the high intensity of transcription of some *R*-genes seen just after infection (e.g. Kramer et al. 2009) in which the genome temporary must be accessible for efficient transcription. It is conceivable that, depending on the disease pressure, we have a fast pattern of evolution of *R*-gene clusters dominated by sequence exchange or a slow pattern of evolution dominated by point mutations. Both evolutionary mechanisms appear to have shaped the *Rpi-blb1* and *Rpi-blb3* clusters. Additionally, the distinction between type I and II seems rather artificial; in real life a continuum is seen with type I and II as extreme borders.

The absence of mutation or recombination in *Rpi-blb2* fragment amplified from *S. bulbocastanum* and the low percentage of *Rpi-blb2* containing plants show that this gene emerged recently and didn't evolve yet.

New P. infestans R-genes identified

In our analysis, 76% of Late Blight resistant genotypes can not be explained by only the presence of the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes. Clear examples are *S. bulbocastanum* accession CGN23010 and P1275199, but also genotypes from CGN17689 and CGN21306 contain additional resistance gene(s), because of their resistance spectrum different from *Rpi-blb1*, *Rpi-blb2* or *Rpi-blb3* and thus providing new source(s) for future Late Blight resistance breeding. The *S. stoloniferum* accession PI205522, containing *Rpi-blb1* and *Rpi-blb3* based on the marker assays, contains an additional gene because of the resistance spectrum observed (Table 2). The gene involved is probably a paralog of $R4^{MA}$ (Van Poppel et al. 2009) as it confers resistance to isolate PIC99189 (avirulent on $R4^{MA}$, see supplementary Table 2) and a hypersensitive response with Avr4 effector (van Poppel et al. 2008) was found (unpublished results). It can not be excluded that the recently described gene *Rpi-bt1* (Oosumi et al. 2009), is responsible for some of the resistant phenotypes found in *S. bulbocastanum.*

Since we found *Rpi-blb1* and *Rpi-blb3* in species related to *S. bulbocastanum* it would make sense that some of newly identified resistance genes in *S. bulbocastanum* might already have been identified in related species, like the resistances found in *S. cardiophyllum* and *S. pinnatisectum* (Kuhl et al. 2001).

CONCLUSION

Our study provides a clear picture of the distribution of Late Blight resistance genes so far isolated from *S. bulbocastanum.* It shows that *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* have a very limited distribution among species. Few other species outside *S. bulbocastanum* contain the genes underlining the restricted interaction between *S. bulbocastanum* and other species. Several different haplotypes were detected for *Rpi-blb1* and *Rpi-blb3* and for 27 % of them functionality was shown. Our functional screen reveals the presence of more Late Blight resistance genes in *S. bulbocastanum*, confirming that this species is a rich source of Late Blight resistance genes as indicated by Budin (2002).

Allele mining in *Petota* germplasm with primers identifying known *R*-genes, allows the breeders to identify more amenable species for breeding. For example, *S. bulbocastanum* hardly crosses with cultivated potato (Helgeson et al. 1998), and the identification of *Rpi-blb1* alleles in *S. stoloniferum* largely facilitates the introgression breeding of *Rpi-blb1* for commercial usage (Vleeshouwers et al. 2008; Wang et al. 2008).

As observed by us and others (e.g. Budin. (2002)) a hotspot for resistance genes is found were host and pathogen co-evolve. This is also reflected by the putative new *R*-genes identified in this study. Given the high diversity of genes found, some promising *R*-genes might even not be present in the germplasm collections. New extensive collections in hot-spots of co-evolution and subsequent evaluations will undoubtedly provide new sources of resistance that can be used in breeding.

MATERIAL & METHODS

Plant material

The *Solanum* species used are listed in Table S1. They were obtained from the Centre for Genetic Resources of the Netherlands (CGN). DNA samples of 14 *S. bulbocastanum* accessions originating from the United States potato Genebank, in Sturgeon Bay, WI, USA, were kindly supplied

by Dr. A. del Rio, Univ. Wisconsin, USA. (referred to as PI accessions in Table 1). When available up to 10 genotypes per accession were used. Individual clones listed in Table 2 were retrieved from the plant material described in Jacobs et al. (2008) for *in vivo* experiments consisting of detached leaf assays and effector agro-infiltrations.

PCR amplification, DNA sequencing and computer analyses

Specific primers used to amplify fragments of the 3 *R* genes studied are listed in Table S3 and Fig.S1. PCR products of randomly selected positive genotypes within an accession were sequenced directly to confirm their identity and to identify single nucleotide polymorphism (SNPs). Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK) and analyzed on an ABI3700 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Multiple sequence alignments were conducted using DNASTAR Lasergen MegAlign version 8.0.2, permitting the distinction of the SNP's in comparison to the cloned *Rpi-blb* gene sequence (*Rpi-blb1* GenBank accession number AY426259.1; *Rpi-blb2* GenBank: DQ122125.1; *Rpi-blb3* GenBank: FJ536326.1). Unique sequences are reported in Table 1a and 1b. Nucleotides are numbered from the first nucleotide of the coding sequence of the cloned gene. The synonymous or silent SNP's not leading to a change in amino acid were differentiated from the non synonymous SNP's.

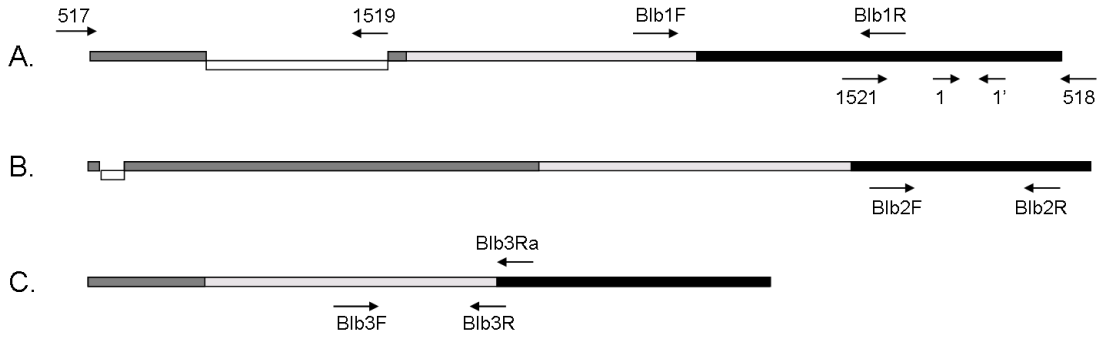


Figure S1: Location of *Rpi-blb* genes specific primers. *Rpi-blb1* (A), *Rpi-blb2* (B) and *Rpi-blb3* (C) nucleotide sequences are from the start to the stop codon and drawn to scale. The regions corresponding to the leucine zipper or the coil coiled, the intron, the NBS domain, and the LRR domain are highlighted in grey, white, light grey and black, respectively. Primer pairs used in this study are 517/1519, Blb1 F/Blb1 R, 1521/518, 1/1', Blb2F/Blb2R, Blb3F/Blb3R and Blb3F/Blb3Ra.

Resistance assay

Detached leaf assays were used to determine the resistance phenotypes of the tested genotypes with 8 *Phytophthora infestans* isolates, PIC99177, PIC99189, PIC99183, IPO-C, 90128 and UK7824. Inoculum preparations and inoculations were performed as described by Vleeshouwers et al. (1999). Six days after inoculation, infection symptoms were scored on a scale from 1-8, to discriminate between very susceptible until very resistant; score 1-3 means clear infection with 1 heavily sporulating lesion and 3 ample sporulating lesion thus characterized as susceptible (S). Scores 4 and 5 are not clearly susceptible or resistant, therefore called quantitative (Q). Resistant (R) phenotypes are characterized by the score 6 with hypersensitive lesions larger than 10 mm, score 7 in between 4 and 10 mm and score 8 implicates hypersensitive lesions smaller than 4 mm in diameter.

Effector agro-infiltration on wild species

The Avr-effectors *Avrblb1* (Vleeshouwers et al. 2008) and *Avrblb2* were cloned into vector pK7WG2 and *Pi-Avr2* was cloned into vector pGRAB (Lokossou et al. 2009). The resistance genes *R3a* (Huang et al. 2004) and its cognate effector *Pi-Avr3a* (Bos et al. 2006) were cloned into the vector pGRAB (kindly provided by S.N. Chapman) and used as control for the agro-transformation efficiency. The constructs were transferred into the same *A. tumefaciens* strain AGL1 carrying pVirG. The different constructs cultures were grown in LB medium supplemented with 75 mg/L choramphenicol and 50 mg/L kanamycin and 4 mg/L tetracycline for *Pi-Avr2, Avr3a and R3a,* with 100 mg/L spectinomycin, 75 mg/L chloramphenicol and 4 mg/L tetracycline for *Avrblb1*and *Avrblb2*. Inoculum of the first experiment was prepared using the growth of bacteria in LB media (15 gr/L Agar, 10 gr/L Bacterial Peptone, 10 gr/L NaCl and 5 gr/L Yeast Extract) until stationary phase and subsequently spinned down at 3600 RPM. Bacteria were resuspended in MMA media (10mM Acetosyringone, 10mM MES and 5 gr/L MS salts) In the second experiment the bacteria were precultured in 3 ml LB, where after they were transferred to YEB medium(5 gr/L Beef Extract, 1 gr/L Yeast Extract, 5 gr/L sucrose, 5 gr/L Bacterial Peptone and 2mM MgSO4) and allowed to grow to OD_{600} 0.4-2.0, afterwards they were spinned down at 3600 RPM for 10 min. and subsequently resuspended in MMA medium. Individual constructs were prepared at an OD_{600} of 0.2 and the combination *Pi-Avr3a* /*R3a* at 0.3 were used to infiltrate 3 to 5 weeks old wild species plants, reaching their 9^{th} leaf stage (Vleeshouwers and Rietman 2009). Specific recognition of the *Avr*-gene by a putative *R* gene present induces hypersensitive cell death response (HR) from 5 days post-infiltration. Response intensity was scored in percentage of

infiltrated area showing cell death. Zero to 20% of dark lesion were characterized by a (-), 21 to 35% by (+), 36 to 65% by (++) and 66 to 100% by (+++).

Geographical localization of the accessions

Geographical origin (latitude, longitude) of the accessions was retrieved from the CGN website (www.cgn.wur.nl) and from literature (Rodriguez and Spooner 1997; Hijmans et al. 2007). The desktop application Google Earth (GE) was used to visualize the location of the genotypes. Different tags were associated to genotypes containing *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3* or their combinations.

We used the internet application SUPRAmap (http://supramap.osu.edu; Janies. 2007) in order to associate the different haplotypes with their geographic distribution. The out-group for the *Rpi-blb1* and *Rpi-blb3* was assigned by performing a phylogenetic analysis in Mega Software (version 4), using the 'minimal evolution' option with standard parameters. The classification described in Table 1a and 1c was used to illustrate the geographical localization of groups B and C (for Table 1c) and the M1 clade from Table 1a is highlighted to illustrate the spread of the M1 allele (see supplementary KMZ file).

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Supplementary Table S2: Characteristics of the set of *P.infestans* isolates used in this study

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile	Reference
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.7.9*.11	Flier et al. 2002
PIC 99189	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.5.7.10.11	Flier et al. 2002
PIC 99183	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.5.7.8.10.11	Flier et al. 2002
IPO-C	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11	
90128	1990	Geldrop, Netherlands	A2	Govers, Phytopathology WUR	1.3.4.7.8.10.11	Vleeshouwers et al. 1999
UK7824	1978	United Kingdom	A1	Govers, Phytopathology, WUR	1.2.3.6.7	

Supplementary Table S3. Overview of primers used for the screening of the wild potatoes species

Marker	PCR primer (5′ to 3′)	Tma	Fragment lenght	Reference
Blb1 F/R	F: AACCTGTATGGCAGTGGCATG	58 ºC	820 bp	Wang et al. 2008
	R: GTCAGAAAAGGGCACTCGTG			
1/1’	F: CACGAGTGCCCTTTTCTGAC	50 ºC	213 bp	Colton et al. 2006
	R: ACAATTGAATTTTTAGACTT			
1521/518	F: GAAAGTCTAGAGTTACACTGG	58 ºC	704 bp	Wang et al. 2008
	R: CAATCACAATGGCAGGAACC			
517/1519	F: CATTCCAACTAGCCATCTTGG	58 ºC	750 bp	Wang et al. 2008
	R: TATTCAGATCGAAAGTACAACG			
RGA1F/R	F: CAGTCACTTTCTTGTTTGCCG	55 ºC	845 bp	Wang et al. 2008
	R: CAGTAGTGAAGTCACTGTGTG			
Blb2F/R	F: GGACTGGGTAACGACAATCC	58 ºC	773 bp	Wang et al. 2008
	R: AGCACGAGTTCCCCTAATGC			
Blb3F/R	F: TGTCGCTGAAAGAGTAGGCC	50 ºC	618 bp	
	R: TATGGAGTGGCTTCTTGAAC			
	Ra: TCCTGAGGTTTATATGACTCATC		757 bp	

Chapter 4

Phytohthora infestans resistance gene *Rpi-mcd1.1* from the Argentinean *Solanum microdontum* is functionally distinct from other *R2* homologues of Mexican origin

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ABSTRACT

Wild *Solanum* species are an important reservoir of disease resistance (*R*) genes, which are under constant evolutionary pressure amongst others via recombination events between alleles. The major Late Blight (MLB) resistance locus on linkage group IV harbors *R2* homologues isolated from *Solanum bulbocastanum*, *S. demissum*, *S. edinense*, *S. hjertingii* and *S. schenckii* that all originate from Mexico. Here we report about the cloning of the NBS-LRR gene *Rpi-mcd1.1* which belongs to the same MLB locus in *S. microdontum* originating from Argentina. *R2* allele mining in the resistant parent RH90-038-21 resulted in 27 homologues among which *Rpi-mcd1.1* was identified. *Rpi-mcd1.1* shares 91% nucleotide identity with *R2* and polymorphic nucleotides are mainly located in the LRR region. *Rpi-mcd1.1* is a unique member of the *R2* family since it provides a resistance that is functionally different from the resistance conferred by the Mexican *R2* gene family. The analysis of recombinant genotypes and the amino acid sequence both reveal multiple recombination events which recently occurred inside the *Rpi-mcd1.1* resistance locus. Phenotypically, *Rpi-mcd1.1* was found to confer qualitative resistance to some *Phytophthora infestans* strains, but quantitative resistance to some other, more aggressive strains. Further characterizations of parents and RH94-076 progeny permitted the identification of two additional *R* genes segregating in the population, namely *Rpi-mcd1.2* also originating from *S. microdontum*, and *Rpi-phu2* originating from *S. phureja.*

INTRODUCTION

Potato is an important staple food originating from South America and is grown in more than 100 countries, under temperate, subtropical and tropical conditions. About 200 wild potato species, partly domesticated by generations of local farmers, have been collected in South America by different expeditions in the last decades (Hawkes and Hjerting 1969). In the rest of the world, all potato cultivars are derived from the wild species *Solanum tuberosum*. Potato monoculture and the lack of genetic diversity helped the emergence and dominance of late blight disease caused by the oomycete *Phytophthora infestans* (*Pi*).

In general the plant defense system is triggered upon pathogen effector recognition by the plant's resistance genes resulting in cell death of the cells attacked (McHale et al. 2006) and consequently decreasing or even blocking the pathogen's growth. So far, the cloned late blight resistance (*R*) genes belong to the NBS-LRR gene family and *P.infestans* avirulence (*PiAVR*) effectors to the RXLR family. Key domains necessary for R/AVR interactions have been identified in the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006).

The attempt to combat the disease by introgression of *Solanum demissum* resistance genes (R-genes) into susceptible cultivars failed because of the rapid emergence of virulent *P. infestans* isolates, thus resulting in the so-called "broken R-genes". To control the disease, the potato polyculture as applied in the Toluca valley of Mexico and in the Andes inspired modern potato breeders to R-gene pyramiding in cultivated potatoes. Pyramiding is pictured in mixing individual *R-gene* containing cultivars or creating a cultivar containing stacked R-genes. In the attempt to assemble this molecular weapon, additionally to the *Solanum demissum* R-genes, many R-genes were identified and isolated from wild *Solanum* species comprising *S. bulbocastanum* hosting *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* (Lokossou et al. 2009; Oosumi et al. 2009; van der Vossen et al. 2003; van der Vossen et al. 2005), *S. stoloniferum* harboring *Rpi-blb1* allelic versions *Rpi-sto1* and *Rpi-pta1* (Wang et al. 2008) *; S. venturii* containing *Rpi-vnt1.1*, *Rp-vnti1.2* and *Rpi-vnt1.3* (Foster et al. 2009; Pel et al. 2009).

R2 allele mining using homologous primers successfully permitted the cloning of *Rpi-abpt*, *R2like*(Lokossou et al. 2009; Park et al. 2005a; Park et al. 2005b; Park et al. 2005c) and *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010; Jacobs et al. 2010), originating from *S. demissum, S. schenckii* , *S. edinense* and *S. hjertingii*. A focused allele mining study using *Rpi-blb3* specific primers in *Solanum* germplasm identified *S. pinnatisectum, S. bulbocastanum* (including some subspecies), *S. hjertingii, S. nayaritense, S. brachistotrichum, S. cardiophyllum* and *S.*

stoloniferum as containing putative functional *Rpi-blb3* homologues (Chapter 3). All *Rpi-blb3*-like containing *Solanum* species are geographically located in Mexico and Guatemala. The functional *R* genes that confer resistance to *P. infestans* among them, all interact with the cognate *P. infestans* avirulence gene *PiAvr2* (RXLRfam7) (Champouret 2010; Lokossou et al. 2009). Another strategy to control late blight is to identify and introgress chromosome loci harboring genes responsible for quantitative resistance traits. Quantitative resistance loci (QRL) have been largely studied and are believed to provide a more stable durable resistance (Gebhardt 1994; Leonards-Schippers et al. 1994; Oberhagemann et al. 1999), so called horizontal resistance in contrast to the vertical resistance provided by *R* genes. Late blight QRL have been identified in many wild *Solanum* species including the Argentinean wild diploid species *S. microdontum* (Colon et al. 1995; Micheletto et al. 2000; Sandbrink et al. 2000). Interestingly, several of the identified PiQRL mapped to the short arm of linkage group IV (Bradshaw et al. 2004; Bradshaw et al. 2006; Leonards-Schippers et al. 1994; Sandbrink et al. 2000), on the *R2* locus (Hein et al. 2009; Hein et al. 2007). Recent studies determined the quantitative resistance of *S. microdontum* accession BGRC 18302-34 (CGN 21342) to be conferred by *Rpi-mcd1,* which was mapped to the *Rpi-blb3* locus at linkage group IV. *Rpi-mcd1* was characterized as a weak-effect resistance gene and expected to be a member of the NBS-LRR gene family (Tan et al. 2008). Since *Rpi-mcd1* was mapped with a complex race IPO-C that is virulent on known members of *R2* cluster, we were intrigued by the possibility to identify a functionally distinct R gene at the *R2* locus.

In our study, we investigated the late blight QRL *Rpi-mcd1* identified in *S. microdontum.* We characterized the resistance spectrum of the population that segregates for *Rpi-mcd1* (Tan et al. 2008) with genetically diverse *P. infestans* isolates permitting the identification of at least three segregating genes. Allelic mining strategies resulted in the identification of *Rpi-mcd1.1*which was functional in a transient complementation assay with *Nicotiana benthamiana*.

RESULTS

Characterization of the parental and progeny resistance spectra

To functionally characterize the resistance of *Rpi-mcd1*, detached leaves of the parental clones RH90-038-21 and RH88-025-50 and their RH94-076 progeny were inoculated with *P. infestans* isolates IPO-C, IPO-0 and PIC99177. In addition, Blb99-256-3, 707TG11-1, AM3778-16 and

Cebeco44158-4 from which *Rpi-blb3*, *Rpi-abpt*, *R2-like* or *R2* were isolated, respectively, were included as controls (Table 1 and Fig 1). As expected for typical *R2*or *Rpi-blb3* activity, Blb99-256-3, 707TG11-1, AM3778-16 and Cebeco44158-4 were resistant to IPO-0 and susceptible to IPO-C and PIC99177 (Fig 1). Both clones RH90-038-21 and RH88-025-50 were resistant to PIC99177, with clear cell death lesions at the inoculation spot. However the population segregated into 41 resistant and 7 susceptible genotypes, which suggests that the resistance to PIC99177 is based on 2 (or more) *R* genes originating from both parents. When inoculated with IPO-0, RH90-038-21 was moderately resistant displaying small to large lesions with no or weak sporulation, starting at day 7, whereas RH88-025-50 was susceptible. The resistance segregated in a 1:1 manner in the RH94-76 population, with 27 resistant and 21 susceptible genotypes, suggesting monogenic inheritance of a single dominant *R* gene. In the first experiment, the highly aggressive isolate IPO-C generally caused susceptible phenotypes with large sporulating lesions (Table 1), although previous studies detected *Rpi-mcd1* with this isolate under field conditions. In order to have a better comparison of the resistance phenotypes, we challenged the RH94-076 population plants with the isolates IPO-C and PIC99177, and applied both isolates to the same leaf (Fig.2). In this second experiment, three resistance patterns were observed; tested plants were resistant to PIC99177 and IPO-C, or only to PIC99177, or susceptible to both isolates. None of the tested plants was simultaneously resistant to IPO-C and susceptible to PIC99177. Half of the RH94-76 population displayed clear hypersensitive response (HR)-like cell death lesions at 5 days post inoculation with isolate IPO-C, followed by a low degree of sporulation at 7 dpi, and this was interpreted as quantitative resistance. More than three quarters of the progeny displayed clear hypersensitive response lesions as the parental clones upon PIC99177 inoculation (Table 1). Additionally, there appeared to be a correlation between the resistance to PIC99177 and IPO-C. Champouret et al (2009) described PIC99177 as relatively weak isolate, which can detect a "QRL" or weak R genes in detached leaf assays (DLA). In contrast, for the much more aggressive IPO-C isolate, DLA is considered less appropriate to detect QRL or weak R genes . In line with this, the resistance to IPO-C was mapped under field conditions (Tan et al. 2008). We decided that for our mapping studies using DLA, PIC99177 is an appropriate isolate to use. In conclusion, the genotypes from RH94-76 population which are resistant to the isolates PIC99177, IPO-0 and quantitatively resistant to IPO-C contain the targeted resistance gene *Rpi-mcd1*.

Markers bordering the late blight resistant locus on linkage group IV, TG 339, T0703 and CT 229 (Tan et al. 2008, Park et al. 2005b) fully co-segregate with resistance to IPO-0 PIC99177 and IPO-C with the exception of 1/3 of the PIC99177 resistant offspring in which the markers were not detected (Table 1). This is in accordance with the resistance phenotype of the parental clones RH90-038-21 and RH88-025-50, confirming the presence of two different genes conferring resistance to PIC99177.

Table 1: Overview of the resistance screening of the parental clones RH88-025-50 and RH90-038-21 and their progeny, the cross population RH94-076. Plants were challenged with three *P. infestans* isolates, IPO-C, IPO-0 and PIC99177. The plants genomic DNA was tested for the presence of the markers TG339, T0703, CT229 and Mf. Abbreviations: S: susceptible; R: resistant.

		RH88-025-50	RH90-038-21	RH94-076 progeny																																												rc1	rc2	rc3
Isolates	IPO-C	S	S	R	R	R	R	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	Q	S	S	S	Q	Q	Q	Q	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	Q	S	S	S	S	S	S	S	S	S
	PIC99177	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	R	R
	IPO-0	S	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R
Markers	TG339	aa	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	ab	ab	ab
	T0703	aa	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	ab	ab	ab
	CT229	aa	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa
	M-f	aa	ab	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	aa	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	ab	aa	aa

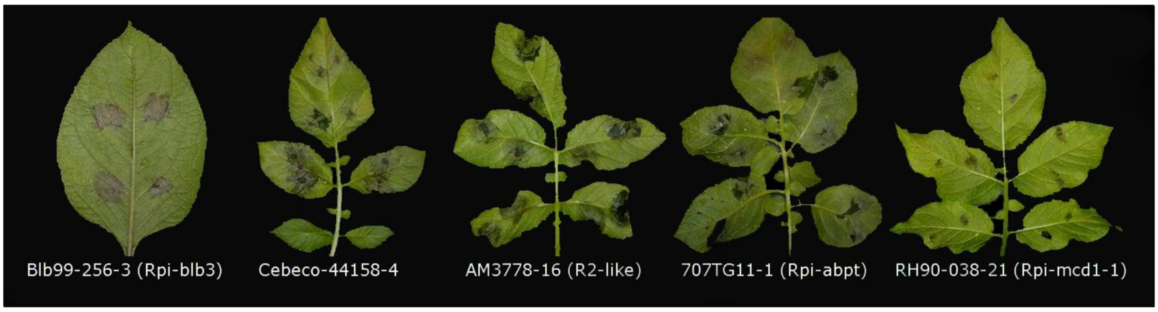


Figure 1: Typical disease phenotypes 8 days after inoculation with a sporangiospore suspension of Phytophtora infestans isolate PIC99177. The genotypes carrying the resistance genes *Rpi-blb3* (Blb99-256-3), *R2* (R2-differential), *R2-like* (AM3778-16), *Rpi-abpt* (707TG11-1) couldn't stop the pathogen proliferation. While the genotype carrying *Rpi-mcd 1-1*(RH90-038-21) showed clear hypersensitive response (HR) thus stopping *Phytophtora* growth.

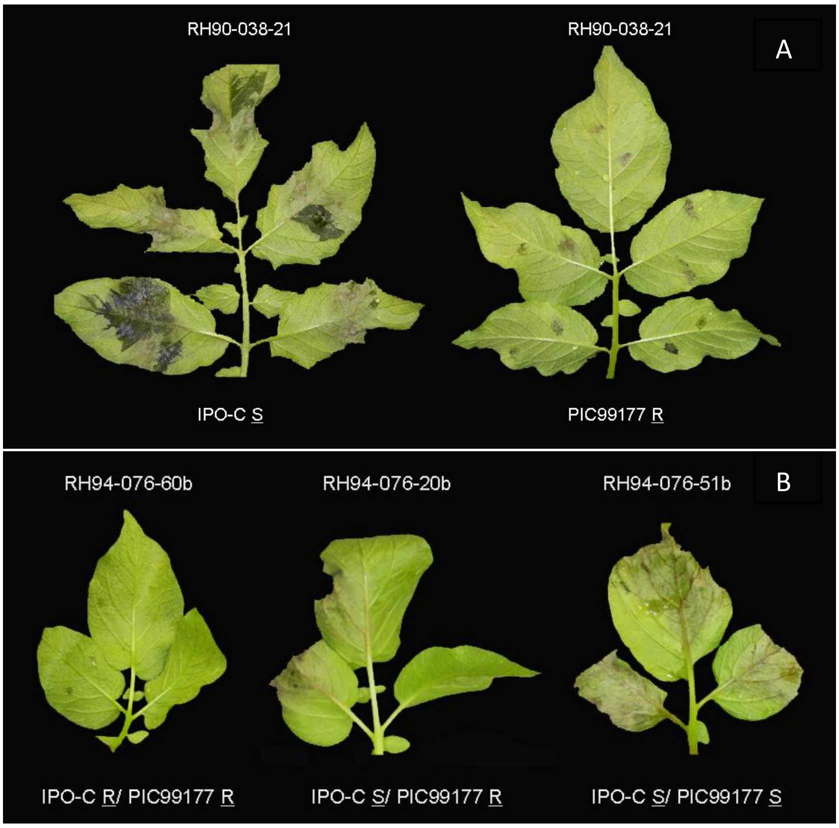


Figure 2: (A) Phenotype of the resistant parent RH90-038-21 with clear susceptibility to IPO-C and resistance to PIC99177 and (B) different possible phenotypes of RH94-076 individuals at seven days post inoculation with *P. infestans* isolates IPO-C and Pi99177. The isolate IPO-C has been inoculated on the left part of individual leaves, and PIC99177 on the right part.

The parental clones are resistant to the isolate PIC99177 and are derived from *S. microdontum* for RH90-038-21 and from *S. phureja* for RH88-025-50. We deduced that these genotypes carry different independently inheriting genes which confer resistance to PIC99177 that we named *Rpi-mcd1.1* and *Rpi-phu2*, respectively.

In addition, three recombinant genotypes were identified (Table 1). Two recombinant genotypes have resulted from one recombination event which occurred between the marker CT229 and the resistance locus. Remarkably, the existence of the third recombinant genotype can only be explained by the existence of 2 R genes in the locus, *Rpi-mcd1.1* and *Rpi-mcd1.2* conferring resistance to PIC99177 and IPO-0, respectively. The recombination event occurred between the two resistance genes, with *Rpi-mcd1.1* located next to the marker CT229 and *Rpi-mcd1.2* next to the markers TG339 and T0703.

Cloning of Rpi-mcd1.1

Rpi-mcd1.1 belongs to the major late blight resistance locus on LG IV that also harbors *Rpi-blb3* (Tan et al. 2008). In view of the conserved markers CT229 and TG339 we hypothesized that *Rpi-mcd1.1* and *Rpi-blb3* were homologues and thus attempted to clone the gene through an allele mining strategy. The same primers used to amplify the *Rpi-abpt*, *R2* and *R2-like* (Lokossou et al 2009) candidates were used to amplify full-length *R2GH* from the potato clone RH90-038-21. Amplicons of the expected size were cloned into pDONR221 and fully sequenced. In total, 27 unique sequences were obtained from RH90-038-21 featuring 88 % to 92% nucleotide (nt) identity with *Rpi-blb3* and 89% to 94% nt identity with *R2*.

Phylogenetic analyses using UPGMA algorithm method resulted in the classification of the *R2GH* sequences in three clades (Fig.3). Specific primers differentiating between and within the clades of homologues (Table S2 and Fig 3) were tested on RH94-076 population to search for a correlation with the resistant phenotype observed. The primer pairs M-a and M-d could differentiate the clades I and IV, respectively, but there was no correlation with the resistance phenotype. M-f primer pair is specific to clade II and interestingly, M-f and M-h primer pairs amplified specific fragments which segregate in the progeny of RH94-076 in repulsion phase when comparing to the resistant phenotype (Table 1). This result means that the candidate *R2GH1-18*, *R2GH1-19*, *R2GH1-23*, *pseudoR2GH1-3*, *pseudoR2GH1-5* and *-pseudoR2GH1-9* originate from the *S. tuberosum* genotype SH 76-128-1857. (Tan et al.2008). Since these candidates are not from *S. microdontum* descent they were discarded from further analyses.

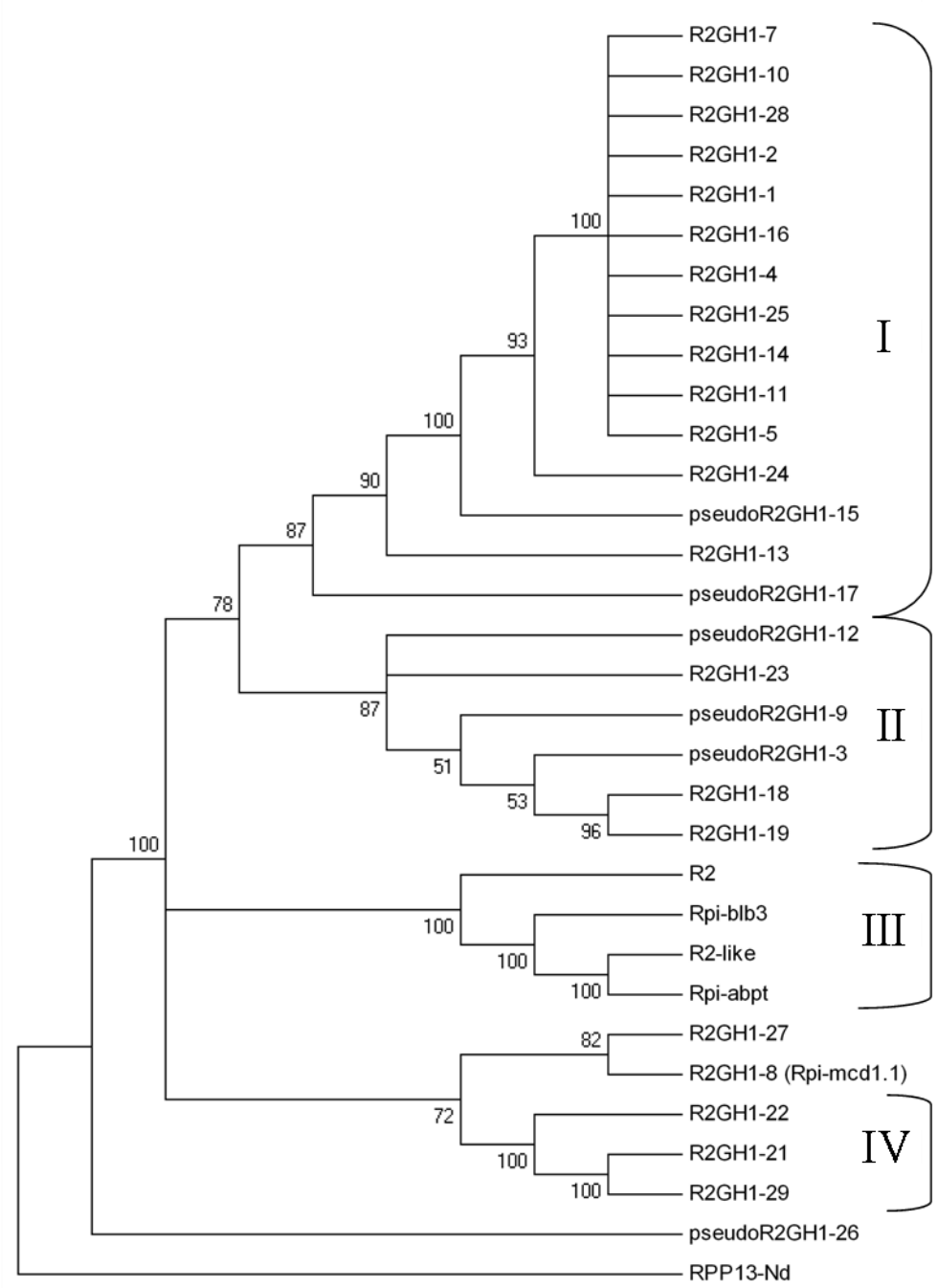


Figure 3: Phylogenetic tree at the nucleotide level of 27 *R2GH* candidates including *Rpi-mcd1-1*and four resistance genes *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* belonging to the same locus on LG IV. RPP13 had been used as an out-group. The clade's numbers are indicated on the right side. UPGMA algorithm with bootstrap analysis of 500 replicates. Numbers above the branches are bootstrap values in percentages.

Additionally, nucleotide and amino acid sequence analyses identified six pseudo-genes harboring early stop codons in the open reading frame which were also discarded from further studies Finally, the remaining eleven *R2GHs* which are coding for unique amino acid sequences were considered candidates for *Rpi-mcd1.1*.

To study which of the eleven *R2GHs* confers late blight resistance, they were targeted for functional analysis. Candidates were transiently expressed in *N. benthamiana* leaves and further inoculated in DLA with the avirulent *P. infestans* isolates PIC99177 and IPO-C. Phenotypes were observed at seven days post inoculation (Fig 4). No traces of sporulation were observed for both isolates on *N. benthamiana* leaves transformed with *R2GH1-8*, whereas the leaves expressing the other constructs showed large sporulating lesions (Fig S1). *P. infestans* isolate PIC99177 displayed clearly smaller lesion at the inoculation point in comparison to IPO-C, which may be explained by the higher aggression level of the latter strain (Champouret et al. 2009). In conclusion, scoring of the tested leaves indicated that *R2GH1-8* conferred strong resistance to PIC99177 and was therefore designated *Rpi-mcd1.1*.

To confirm complementation by *Rpi-mcd1.1* in potato, stable transformants of potato Desiree were generated. Detached leaves of mature transgenic potato plants were challenged with *P. infestans* isolate PIC99177 and IPO-0. As expected, stable transformed plants containing *Rpi-mcd1.1* were resistant to PIC99177, but susceptible to IPO-0 (data not shown), indirectly confirming the existence of the second R-gene in the locus of the parental plant RH90-038-21, which was named *Rpi-mcd1.2.*

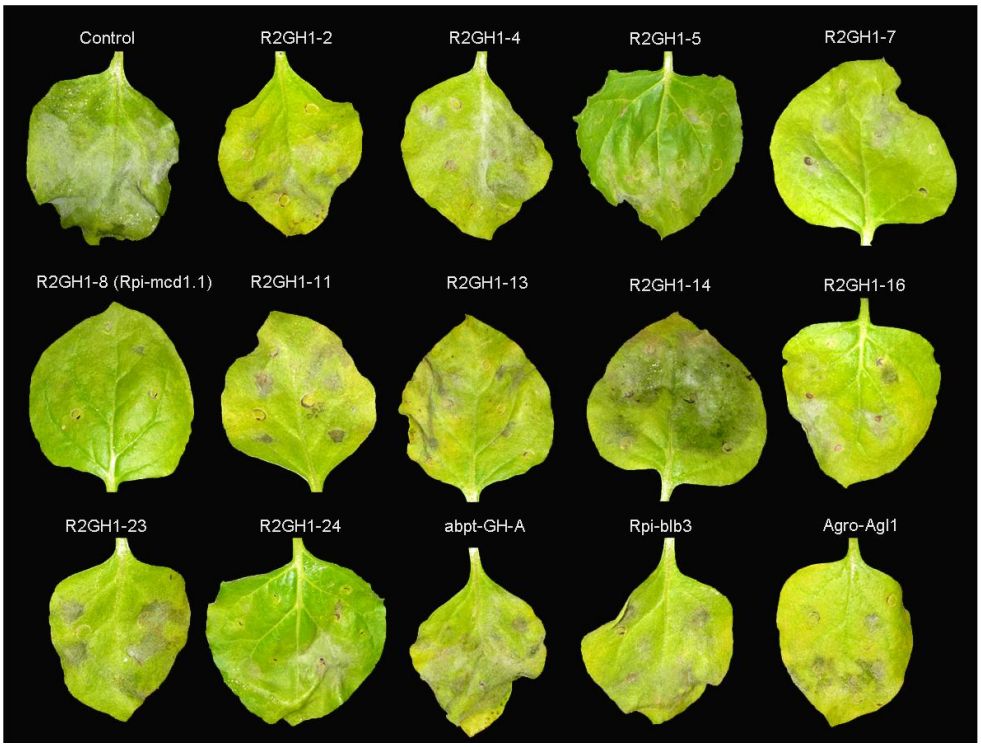


Figure 4: *N. benthamiana* infiltrated leaves with *R2GH*-candidates, the resistance gene *Rpi-blb3* or an empty *Agrobacterium* (Agl1) displaying different resistance phenotypes when inoculated with *P. infestans* isolates IPO-C (left part of the leaf) and PIC99177 (right part of the leaf).

Molecular structure and recognition specificity

Alignment of the nucleotide sequences coding for complete ORFs, and subsequent analysis of informative polymorphic sites (IPS), i.e. sites where two or more genes carry the same nucleotide (Parniske et al. 1997), reveals a region of 1200 nucleotides residing between kinase 2 and LRR6 (Fig 5) where recombination is increased. Three constant nucleotide structures are present in the *R2GH* pool represented in the clustal W alignment (Fig.5) by *R2GH1-1*, *R2GH1-18* and *R2GH1-21* belonging to clades I, II and IV, respectively (Fig 3). Recombination between these constant structures gave rise to seven different homologues, i.e. *R2GH1-8, R2GH1-22, R2GH1-13, R2GH1-19, R2GH1-23, R2GH1-24* and *R2GH1-27*. Interestingly, *R2GH1-19* and *R2GH1-24* appear to be complementary recombinant genes which arose from a double recombination event in the region of 379 nucleotides between nt positions 1259 and 1638 (Fig 5). *Rpi-mcd1.1* (*R2GH1-8)* appears to be a combination of sequences of the three constant structures plus one unknown gene between the nt positions 1043 and 1259 (Fig 5 and Fig 6). These clear blocks of sequences from different members of *R2GH*s pool indicate that sequence exchange events have been involved in the rise of resistance gene *Rpi-mcd1.1*.

To study the nature of specificity conferred by *Rpi-mcd1.1*, the nucleotide sequences of R2GHs were first aligned with *R2*/ *Rpiblb3* homologues. The *R2GHs* contain ORFs of 2538-2541 nucleotides that encode proteins of 845-846 amino acids harboring the same signature sequences as R2 homologues. Clustal W amino acid sequence alignment of *Rpi-mcd1.1* together with the three constant structures described above *R2GH1-1, R2GH1-18* and *R2GH1-21*, and with *R2* reveals identical LZ-NBS region with *R2* delimited by the same break point described in the structural analyses of *R2* homologues by Lokossou et al (2009) (Fig.6). *Rpi-mcd1.1* LRR domain differs from *R2* LRR in the polymorphic amino acids which are mainly localized in the xxLxLxxx repeats indicating a putative Pi-Avr differential recognition.

To study *Rpi-mcd1.1* specificity at the functional level, the R-AVR interaction was reconstructed in *N. benthamiana*. The avirulence protein PiAvr2 that is known to evoke cell death responses when co-expressed with R protein of the R2 family was tested for response to Rpi-mcd1.1. *Agrobacterium* strains expressing PiAvr2 were co-infiltrated together with *Agrobacterium* strains expressing Rpi-mcd1.1 in *N. benthamiana* leaves. Co-infiltration of Pivr2 together with R2 or Rpi-blb3 constructs were used as a positive control and single R-gene or PiAvr2 gene infiltration were used as negative controls.

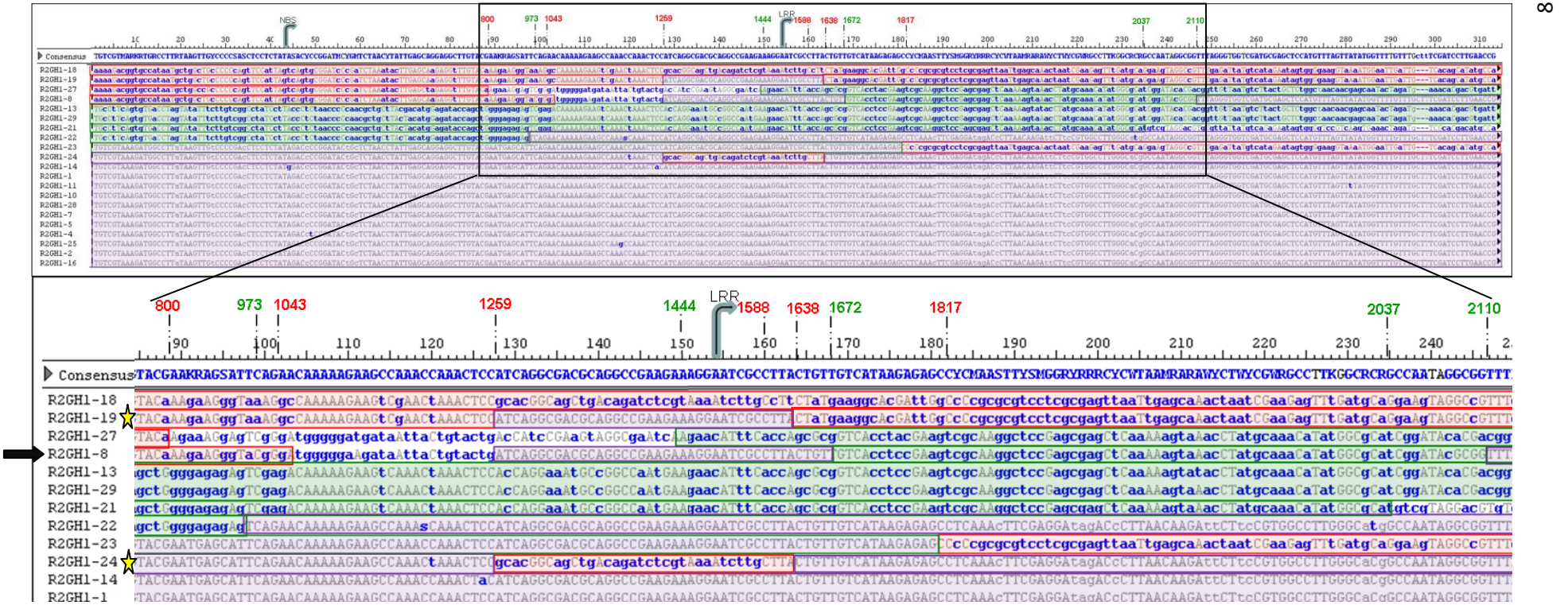
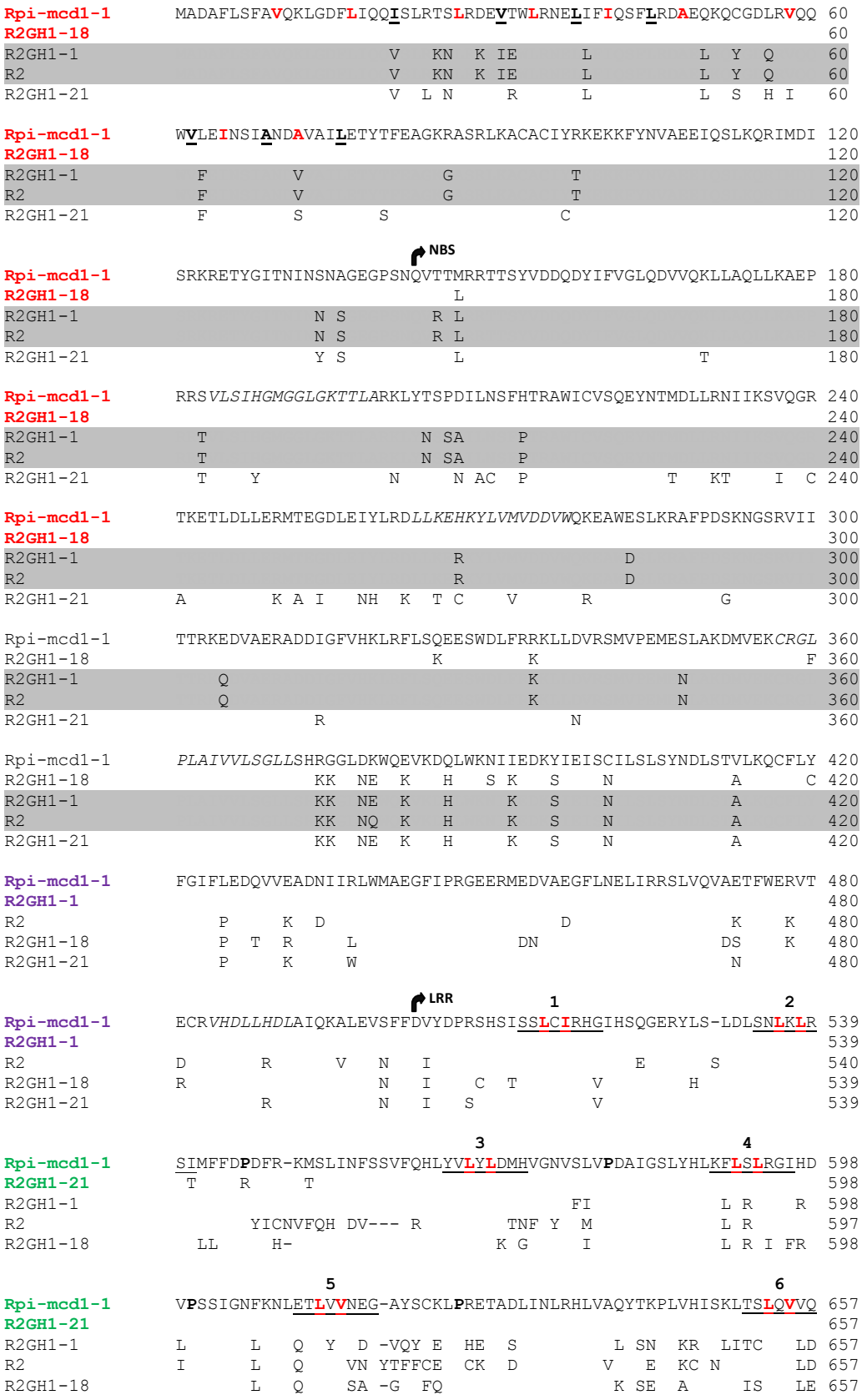


Figure 5: Modular structure analyses of *R2GH* candidates. Sequence of the 314 IPS (Informative Polymorphic Sites) which appear in blue, from the alignment of 21 *R2GH* candidates spanning complete ORFs. The arrow indicates the functional candidate *R2GH1-8* (*Rpi-mcd1-1*). Highlighted in red, green or purple are the sequence blocks referring to R2GH1-18, R2GH1-21 and R2GH1-1, respectively. On the top are indicated the breaking points between the different blocks referring to the original sequence numbering. The figure lights up 3 recombination regions: between [800-1043 bp], [1444-1917 bp], [2037- 2110], and one recombination point at [1259 bp]. *Rpi-mcd-1-1* modular structure is depicted in detail in figure 7. The stars indicate the complementary recombinant *R2GH1-19* and *R2GH1-24*. The beginning of the NBS and LRR regions are indicated by arrows.


NBS
LRR
1
2
3
4
5
6

```
                                    7                                   8
Rpi-mcd1-1      NICCDQWKDVDPVDLVNLRELIMDDINKSYSLNNISSLKNLSTLRLFCRGGQSFPDLEFV 717
R2GH1-21                                                      R YVNE   S     717
R2GH1-1         GLG         I        G HN K                              717
R2              GVA                  S  R RS               K I GER   AS     717
R2GH1-18        RV                   R NS K                T   EDYE   S     717

                          9                   10                      11
Rpi-mcd1-1      NCCEKLQKLWFEGRIEKLPHPFPNSITMIVLSLSVLIEDPMPILGILPNLRNLVLFRASY 777
R2GH1-1                                                                       777
R2                    LQ    E   L S     M   F E T        RF      K DG -  776
R2GH1-18             ML  K     DL       M  RK   K                E I  -  776
R2GH1-21             LLL        L       ML EN K T        M       I ED -  776

                               12                  13
Rpi-mcd1-1      EGKEIMCSDNSFSQLEFLILRDLEKLERWHLGTSVMPLIKGLGINNCPNLKEIPERMKDV 837
R2GH1-1                                                                       837
R2                          H    W     D    A         H                    836
R2GH1-18             N      H      V                D YD                   836
R2GH1-21            KN      H          D    A         YE                     836

Rpi-mcd1-1      ERLKRNYML 846
R2GH1-1                 846
R2               L      845
R2GH1-18         Q      845
R2GH1-21         L      845
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Figure 6: Structure and functional analyzes. ClustalW comparing Rpi-mcd1-1 to R2GH1-1, R2GH1-18, R2GH1-21 and R2.The sequence names appear in red, green or purple color when Rpi-mcd1-1 sequence is identical to R2GH1-18, R2GH1-21 or R2GH1-1, respectively. Highlighted in grey are the identical block sequences between R2GH1-1 and R2. The NBS and LRR regions are indicated by an arrow. Conserved domains present in the NBS region are in italic. LRR repeats are underlined and numbered. The amino acid L, I, F, A, M and V from the leucine zipper heptads and the LRR repeats appear in bold red

Specific recognition of *AVR* gene by the *R* gene induces hypersensitive cell death response (HR) from 5 days post-infiltration. In contrast to R2, the Rpi-mcd1.1coinfiltrated with PiAvr2 did not show any cell death (data not shown). This is in line with the disease tests with *P. infestans* isolates that are virulent on plants expressing R2 but avirulent on plants expressing Rpi-mcd1.1, and indicates that *Rpi-mcd1.1* is interacting with other effectors than previously described *R2* homologues from Mexican origin.

DISCUSSION

In this study, we cloned and elucidated the function of one resistance gene, *Rpi-mcd1.1*, originating from *Solanum microdontum* accession BGRC 18302-34 (CGN 21342) collected in northern Argentina. This *Rpi*-gene provides qualitative resistance to some *P. infestans* isolates and a typical hypersensitive reaction was observed after infection with the late blight isolate PIC99177 and IPO-0. To other isolates e.g. IPO-C, a quantitative resistance was observed, in line with previous studies (Sandbrink et al. 2000; Tan et al. 2008). Resistance assays and screening of the closest known markers of linkage group IV in

the population RH94-076 indicated the presence of at least three *R*-genes, *Rpi-mcd1.1* and *Rpi-mcd1.2* conferring resistance to the *Pi* isolates PIC99177 and IPO-0, respectively, and *Rpi-phu2* conferring resistance to PIC99177. *Rpi-mcd1.1* and *Rpi-mcd1.2* originate from *S. microdontum* and are located on linkage group IV. *Rpi-mcd1.1* was cloned by *R2*/ *Rpi-blb3* adapted allele mining and conferred resistance to PIC99177 when transiently expressed in *N. benthamiana* leaves and in stable transformants of potato cultivar. Stable transformants confirmed the functionality of *Rpi-mcd1.1* by complementing the susceptibility of the cultivar Desiree when challenged with the isolate PIC99177. No complementation was evaluated against the isolate IPO-0, confirming that the resistance against this isolate comes from a different gene.

Correlation between quantitative and qualitative resistance

Rpi-mcd1.1 is characterized as a "weak" resistance gene by Tan et al (2008) which is in line with the description of PIC99177 by Champouret et al (2009) as a "weaker" isolate than IPO-C or IPO-0. The differences in degree of aggressiveness of these isolates permitted the mapping of *Rpi-mcd1.1* in the field and via detached leaf assay. Additionally, we hypothesize that the quantitative resistance to IPO-C evaluated in the population RH94-076 is also due to different resistance factors including *Rpi-mcd1.1, Rpi-mcd1.2 and Rpi-phu2.* The last one being also characterised as a QRL when the population RH94-076 was challenged to the isolate IPO-C (Dr. C. Celis Gamboa, Wageningen UR Plant Breeding, unpublished results).

Transfer of genes or common ancestor?

The presence of *Rpi-blb3* homologues in *S. microdontum* emphasises the existence of a common ancestor with *S. bulbocastanum* from Mexico. According to Hawkes classification (2004), both belong to the *Petota* section, series *Bulbocastana* for *S. bulbocastanum* and series *Tuberosa* for *S. microdontum*. Using AFLP markers, (Kardolus et al. 1998) unexpectedly found that *S. microdontum* was associated with ser. *Megistacroloba* rather than with ser. *Tuberosa.* Recent phylogenetic studies using AFLP molecular markers classified *S. microdontum* as a distinct genetic unit or species (Jacobs et al. 2008). These results highlight the difficulty to properly classify *S. microdontum* and find phylogenetic links with other *Solanum* species. Our findings shed light on a clear link between the Mexican polyploid *S. demissum* and the Argentinean diploid *S. microdontum*. *S. bulbocastanum* (*Rpi-blb3*), *S. demissum* (*R2*) and *S. microdontum* (*Rpi-mcd1.1*) appear to have a common ancestor but

different evolutionary forces dependant on their geographical location and the exposure to local *P. infestans* isolates may have shaped functionally distinct R genes for specificity to Avr-genes. *S. bulbocastanum* (*Rpi-blb3*) and *S. demissum* (*R2*) evolved in Mexico and are specifically resistant to P.infestans isolates expressing the effectors *PiAvr2*. In contrast, *S. microdontum* (*Rpi-mcd1.1*) originates from Argentina and probably specifically recognizes different effectors than PiAvr2. Perhaps, an R-AVR pair other than R2-PiAvr2 has driven a co-evolutionary arms race of the Argentinean *Solanum* with local *P. infestans* isolates in the Andes. We hypothesize that the *P. infestans* populations in Mexico and Argentina may have evolved independently, and co-evolutionary forces might have been targeted at different R-AVR pairs. The presence of the effector Pi-Avr2 in isolates might have maintain the LRR structures of *Rpi-blb3* or functional *R2* homologues in Mexico. A survey of the Argentinean late blight population is not yet available in order to relate the presence of Avr-mcd1 with the maintenance of *Rpi-mcd1.1*. in *S. microdontum*.

Intragenic recombination resulted in new recognition specificity of the MLB resistant cluster located on linkage group IV

Rpi-mcd1.1 is so far the only and thus a unique member of the *R2*/ *Rpi-blb3* cluster providing resistance to the late blight isolate PIC99177. Significant excess of amino acid polymorphism resides in the LRR domain when compared to the one of *Rpi-blb3*, indicating that *Rpi-mcd1.1* probably interacts with another PIC99177-specific effector(s). Similar results were obtained in *RPP13* (*Hyaloperonospora arabidopsidis* resistance gene) allele mining in a diverse set of *Arabidopsis thaliana* accessions (Rose et al. 2004). *RPP13* exhibits the highest reported level of sequence diversity among known R-genes. Hall et al (2009) demonstrated the recognition of the effector ATR13 was mediated by alleles belonging to just a single clade, as it had been reported for *Rpi-blb3*, *R2*, *R2-like* and *Rpi-abpt* (Lokossou et al 2009). Additionally RPP13 alleles in other clades have evolved the ability to detect other *H. arabidopsidis* ATR protein(s) as it might be the case for *Rpi-mcd1.1*. Although certain resistance gene clusters like the one of *Rpi-vnt1* (Foster et al. 2009; Pel 2010; Pel et al. 2009), *Rpi-blb1* (van der Vossen et al. 2003; Vleeshouwers et al. 2008) and *Rpi-blb3* (Champouret 2010; Lokossou et al. 2009), tend to yield only similar genes, it is still worthwhile to further study them in geographically and phylogenetically diverse *Solanum* to find new R genes with distinctive specificity.

Molecular structure studies showed that *Rpi-mcd1.1* has in common five segments with at least three genes present in the gene pool, underlining the role of genetic recombination inside the *R-gene* cluster, resulting in the generation of novel resistance specificities (Hammond-Kosack and Kanyuka 2007;

Kuang et al. 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Despite the absence of data about the genetic position of each *R2GH* gene, the presence of recombinants in the gene pool implies the close proximity of these genes from each other.*R2*/ *Rpi-blb3* homologues originating from *S. tuberosum* were identified among the candidate genes isolated from the hybrid *S. microdontum*/ *S. tuberosum* clone RH90-038-21. Additionally, the presence of a *R2GH* fragment identified as being from the *S. tuberosum* parent, inside *Rpi-mcd1.1* and the identification of complementary recombinant genes demonstrate the occurrence of multiple recent recombination events inside the *Rpi-mcd1.1* resistance locus. The profusion of alleles identified in different studies, 27 alleles in this study, 24 alleles described in Lokossou et al (2009) and 17 alleles isolated by N. Champouret (2010) represent a natural powerful reservoir available for new recombinations and the birth of new resistance alleles in the MLB located on linkage group IV.

MATERIAL AND METHODS

Plant material and late blight isolates

In this study we used the parental clones RH90-038-21, RH88-025-50, and fifty-one new RH94-076 plants from the cross earlier described in Tan et al (2008). The clone RH90-038-21 and the RH94-76 population contain essentially a susceptible *S. tuberosum* genetic background resulting in a germplasm with about 25% *S. microdontum* introgression for the parental clone and less than 12.5% for the progeny (Tan et al. 2008). The clone RH88-025-50 is a hybrid of the *S. phureja* genotype DB-207 and the *S. tuberosum* genotype SH76-128-1865. The characteristics and origin of *P. infestans* isolates IPO-0, IPO-C and PIC99177 are indicated in supplementary Table S1.

Table S1: Race specificity of the different isolates used in this study

Isolate	Year	Geographic origin	Mating type	Obtained from	Virulence profile	Reference
IPO-0	n.d	Unknown	n.d	Kessel, PRI, WUR	0.3b. 4. 7. 10. 11	
IPO-C*	1982	Belgium	A2	Kessel, PRI, WUR	1.2.3.4.5.6.7.10.11	
PIC 99177	1999	Metepec, Mexico	A2	Kessel, PRI, WUR	1.2.3.4.7.9**.11	Flier et al. 2002

* : also known as IPO-82001

** : not conclusive

Resistance assay

Detached leaf assays were performed as described by (Vleeshouwers et al. 1999). Six days after inoculation, phenotype responses were determined. Leaves showing no symptoms or a localized necrosis at the site of inoculation were scored as resistant and those with clear sporulating lesions (visible by naked eye) as susceptible.

Allele mining

The *Rpi-blb3* allele mining primers Blb3-start and Blb3-end described in Lokossou et al (2009) were used to amplify *Rpi-blb3* homologous ORFs present in the parental resistant genotype RH90-038-21. The *R2GHs* were cloned into the binary vector pKGW-MG between the *Rpi-blb3* promoter and terminator sequences of 2,723 and 883 nucleotides, which were cloned into pDONR P4-P1R and pDONR P2R-P3. The binary clones were subsequently transferred to *Agrobacterium* strain AGL1 and tested in a transient complementation assay in *N. benthamiana,* (Lokossou et al 2009).

Transient complementation in N. benthamiana

Infiltration with *Agrobacterium* strains carrying R2GH were performed in *N. benthamiana.* After the configuration of the constructs were confirmed by PCR, the final expression constructs were then transferred to *A. tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Recombinant AGL1 *A. tumefaciens* cultures were grown in LB medium (10 g/L bacteriological peptone, 10 g/L NaCl and 5 g/L yeast extract in MQ water) supplemented with 5 mg/L tetracycline , 100 mg/L carbeniciline, 75 mg/L choramphenicol and 100 mg/L spectinomycin. Recombinant *A. tumefaciens* cultures were grown in LB medium supplemented with 100 mg/L spectinomycin. After one or two days a calculated amount of culture (according to OD 0.5 at 600 nm) was transferred to YEB medium (5 g/L beef extract, 5 g/L bacteriological peptone, 5 g/L sucrose, 1 g/L yeast extract, 2 ml/L 1 M $MgSO_4$ in MQ water) supplemented with spectinomycin. After 1 day overnight cells were centrifuged at 3500 rpm and re-suspended in MMA medium (20 g/L sucrose, 5 g/L MS salts and 1.95 g/L MES) supplemented with 1 ml 200 mM acetosyringone to a final OD of 0.2 and infiltrated into leaves of 4 weeks old plants with a 3ml syringe. Three leaves from four plants were infiltrated. After two days, leaves were detached, and spot-inoculated with *P. infestans* strains IPO-C and PIC99177. Seven phenotypes were distinguishable under the binocular and

were scored from 0 to 6 reflecting the resistance level provided by the tested R2GH to the leaf (Fig S1). Scores of the 24 leaves per construct were computed and the average was calculated.

Agrobacterium-mediated co-expression in *N. benthamiana*

The selected clone *R2GH1-8* was co-expressed with the avirulence effector *Pi-Avr2* cloned in pGRAB vector (kindly supplied by Dr. P. Birch, SCRI. Scotland. UK). Pi-Avr2 clone was transferred to *A. tumefaciens* AGL1 cultures and grown in LB medium supplemented with 30 mg/L choramphenicol and 50 mg/L kanamycin. *Rpi-blb3* was used as a control. The same protocol as the transient complementation was used for the sub-cultures. PiAVR *A. tumefaciens* cultures and R2GH1-8 *A. tumefaciens* cultures were mixed at a final OD600 of 0.1 for each and infiltrated into leaves from 5 to 7 week old plants.

Wild type plant Pi-Avr infiltration was done as described in Vleeshouwers and Rietman (2009). In order to verify that the resistance pathway works, co-infiltration of *Rpi-blb3* and *Pi-Avr2* was used as control.

Transformation of susceptible potato variety

The constructs pKGW-R2GH were transferred in AGL1 (Lazo et al. 1991). After verifying their stability in *Agrobacterium* these clones were transformed to the susceptible potato variety Desiree. Overnight cultures of the transformed *A. tumefaciens* strain were used to transform internodal cuttings from *in vitro* grown plants (Heilersig et al. 2006). A total of 100 to 200 explants were used for each transformation

DNA sequencing, primers design and computer analysis

Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK), M13 universal forward and reverse primers, and an ABI377 automated sequencer (Applied Biosystem, La Jolla, CA, USA). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991). The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp). Multiple sequence alignments were conducted using ClustalW2 program accessible from EMBL-EBI web page. Alignments were

produced using DNASTAR Lasergen Seqman version 8.0.2. The "CLC main workbench 5" program, version 5.0.2 was used to design the specific primers and MEGA4 to produce the phylogenetic trees.

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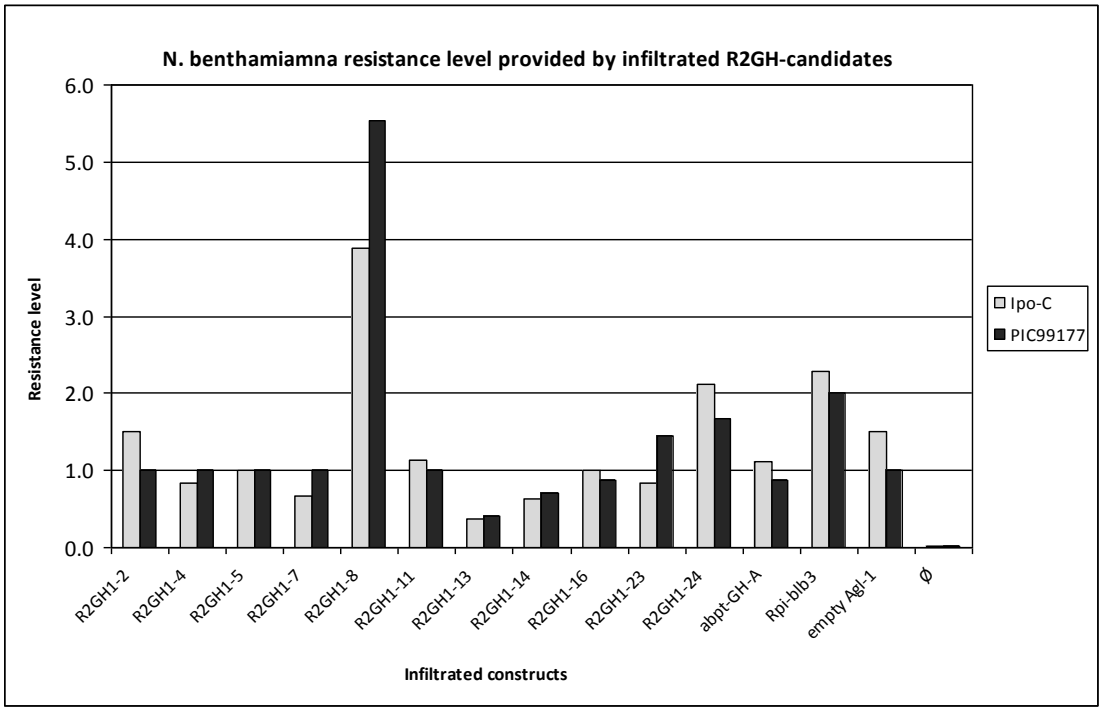
Table S2: List of primers used in this study

Marker	Primers	sequence (5'----> 3')	Region	Tm C°	Fragment Length (bp)	Specific to
M-a	mcd-Fa	GAGTTCCTTATTCTTSGTGA	2389-2408	61	146	Clade I
	mcd-Ra	TTCAACCGCTCCACGTC	2518-2534			
M-d	mcd-Fd	GAGGATGTCGCTGAAAGAG	922-940	61	1520	Clage III
	mcd-Rd	GTGCCTAAATCCCATCTTTCT	2421-2441			
M-f	mcd-Ff	AAAAACCTTAGCACTCTCAC	2098-2117	61	336	Clade II
	mcd-Rf	ATGCCATCTTTCTACCTTC	2415-2433			
M-h	mcd-Fh	CCAAGAAAGCACTCCAAA	1528-1545	50	469	pseudoR2GH-5b
	mcd-Rh	CACAACCAACCCAATTAAGA	1977-1996			

Primers	sequence (5'----> 3')	Enzyme	Tm C°	Reference
TG339-F	GCTGAACGCTATGAGGAGATG	*Mnl*I	58	Tan et al .2008
TG339-R	TGAGGTTATCACGCAGAAGTTG			
CT229-F	TTGTGAGTGGTGAACTACGGGC	*Hpy*CH4IV	58	Park et al . 2005b
CT229-R	CGGCAATGGTTATGGGAACG			
T0703-F	CCAGTAAGAACAAGCCGATT	*Bme*1390I	54	Tan et al .2008
T0703-R	ATCACCAATTACGCGATCTA			



0	Heavy sporulation
1	Lesion + heavy sporulation on dead tissue
2	Lesion + moderate sporulation on dead tissue
3	Large lesion and weak sporulation
4	Lesion + weak sporulation on dead tissue
5	Large lesion but no sporulation
6	hypersensitive response

Figure S1: *N. benthamiana* resistance level to Pi isolates IPO-C and PIC99177, provided by infiltrated *R2GH*-candidates and the controls *abpt-GH-A* and *Rpi-blb3* known not to complement susceptibility of *N. benthamiana* to both isolates. Score from 1 to 6 characterize the different phenotypes observed; 1 being susceptible and 6 highly resistant.

Chapter 5

Exploration of sequence polymorphism in *R2* gene homologues permitted the identification of a single amino acid which triggers the potato/ *P. infestans* interaction R2/PiAVR2

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ABSTRACT

The major late blight resistance cluster on linkage group IV has been extensively investigated in various *Solanum* species leading to the identification and/ or isolation of an abundance of late blight (LB) resistance allelic variants. These genes share high percentage identity and define a new *R* gene family characterized by the already known functional *Rpi-blb3, Rpi-abpt, R2 and R2-like* alleles (Lokossou et al. 2009). Functional LB resistance homologues were identified by susceptible phenotype complementation and/ or effector recognition using the transient co-expression system in *N. benthamiana*. Resistance against a set of *Phytophthora infestans* isolates was coupled with the recognition of the RXLR effector PiAVR2. In our study, we used the sequence information provided by the alleles to identify and functionally characterize the repeats in the LRR domain which are necessary for PiAVR2 recognition. Domain swaps between *Rpi-abpt* or *R2* which interact with PiAVR2 and R2GH-2, which is non functional, permitted the identification of the critical LRR repeats 11, 12 and 13 involved in recognition. Site directed mutagenesis on *Rpi-abpt* pointed to one solvent exposed amino acid in LRR13 crucial for PiAVR2 recognition. These results are an indication of evolutionary differentiation between both partners because of recognition failure by the pathogen.

INTRODUCTION

The pathosystem formed by the oomycete *Phytophthora infestans* and its hosts among *Solanaceae* is under intensive investigation since the Irish potato famine in 1845. Classical breeding strategies first succeeded in combating Late Blight (LB) by introgression of dominant resistance genes from the wild specie *Solanum demissum* into cultivated potato (Malcolmson 1969; Malcolmson and Black 1966; Müller and Black 1952). The set of eleven *R* genes (*R1* to *R11*) were exploited in monoculture, which besides providing an efficient crop protection, was exerting a strong selection pressure on the pathogen resulting in its rapid adaptation and consequently the defeat of those *R* genes (Fry 2008; McDonald and Linde 2002). A need for new resistance genes appeared and more wild *Solanum* species were investigated for their LB resistance potential. In the last decade, many *R*-genes were identified and isolated from wild *Solanum* species comprising *S. demissum* harboring *R1* (Ballvora et al.), *S. bulbocastanum* hosting *Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* (Lokossou et al. 2009; Oosumi et al. 2009; van der Vossen et al. 2005; van der Vossen et al. 2003), *S.stoloniferum* and *S. papita* harbouring *Rpi-blb1* allelic versions *Rpi-sto1* and *Rpi-pta1* (Wang et al. 2008)*; S. venturii* containing *Rpi-vnt1-1* and *Rpi-vnt1-3* (Foster et al. 2009; Pel et al. 2009).

An explanation of the rapid adaptability of *P.infestans* was found in the recent sequencing of its genome which appears to be large and complex with about 74% of repetitive DNA. Annotation of the genome identifies many gene families encoding for effector proteins that are involved in causing pathogenicity. Additionally, comparison with the sequenced *Phytophthora sojae* and *Phytophthora ramorum* showed rapid turnover and extensive expansion of these effector genes (Haas et al. 2009). *P.infestans* effector proteins are classified in two groups, the effectors secreted in the cytoplasm or the apoplasm. The RXLR effectors have been identified among the cytoplasmic secreted proteins and are characterized by an arginine-X-leucine-arginine motif at the N-terminus of the protein that defines a domain required for delivery inside plant cells followed by an acidic region (Jiang et al. 2008; Morgan and Kamoun 2007; Whisson et al. 2007; Win et al. 2007).

Avirulent RXLR effectors (AVR) are detected by the potato plant upon *P.infestans* infection resulting in a hypersensitive reaction stopping the growth of the pathogen (McHale et al. 2006). AVR protein detection is possible in a direct way or indirectly by the resistance genes harboring key domains for pathogen recognition, the nucleotide binding site (NBS) and the leucine rich repeat (LRR) domains (Catanzariti et al. 2010; Collier and Moffett 2009; Ellis et al. 2007; Jones and Dangl 2006; Rairdan and Moffett 2006). Although, the N terminal domain of NBS-LRR protein has been demonstrated to

play a crucial role in AVR protein recognition (Moffett et al. 2009), multiple studies showed that the LRR domain is the region of the protein that confers recognition specificity (Hulbert et al. 2001; Meyers et al. 1998; Mondragon Palomino and Meyers 2002; Noel et al. 1999). In silico secondary structure of the NBS-LRR protein shows that the LRR repeats with the motif xxLxLxx form a β-strand/β-turn structure in which the solvent x residues are exposed and available for interactions with potential ligands (Kobe and Deisenhofer 1995). These solvent exposed residues are hyper variable and subject to diversifying selection, suggesting that these residues have evolved to detect variation in pathogen-derived ligands (Michelmore and Meyers 1998).

So far six matching pairs of *PiAVR* and potato *R*-gene have been identified, *PiAVR3a/R3a* (Armstrong et al. 2005), *AVR4/ R4* (van Poppel et al. 2008), *AVR-blb1/Rpi-blb1* (Vleeshouwers et al. 2008), *AVR-blb2/ Rpi-blb2* (Oh et al. 2009), *PiAVR2 /R2* (Lokossou et al.) and *AVRvent1 /Rpi-vent1-1* (Pel 2010). Detailed investigation of PiAVR/R interaction was so far orientated to the effector side to identify the crucial domain necessary for the activation of the hypersensitive reaction. The C terminal domains were found indispensable for PiAVR3a and AVR4 recognition (Bos et al. 2006); (van Poppel et al. 2009). Remarkably, PiAVR2 is so far recognized by 10 R genes, *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* (Lokossou et al.) *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010).

In this study, we explore the information provided by the alleles generated in different *Rpi-blb3* allele mining strategies and use the transient co-expression system in *N. benthamiana* to identify and functionally characterize the repeats in the LRR domain which are necessary for PiAVR2 recognition. Domain swaps between R genes interacting or not with PiAVR2 permitted the identification of a region spanning three LRR repeats of the cloned *Rpi* genes that determine the *R2* resistance specificity. Site directed mutagenesis pinpointed one solvent exposed amino acid to be crucial for PiAVR2 recognition.

RESULTS

Allelic variation

A total of 75 full length amino acid sequences identified on linkage group IV were retrieved from the public nucleotide sequence database and this thesis. These alleles originated from *Rpi-blb3* or *R2* allele mining in *S. bulbocastanum, S. microdontum, S. demissum, S. schenckii, S. edinense,* and *S.*

hjertingii. A blast research on the public nucleotide sequence database resulted in the identification of three BAC clones from the *Solanum tuberosum* diploid genotype RH89-039-16 and two BAC clones from tomato. All BAC clones are mapped on linkage group IV. Annotation of the BAC sequences for *Rpi-blb3* like genes resulted in 5 genes present on the *S. tuberosum* BACs and 4 genes in the tomato BACs, spanning full length ORF. The corresponding protein sequences are classified in a phylogenetic tree into 12 clades (Fig 1). Functional resistance genes interacting with PiAVR2 share 95 to 100% identity with each other, and are bulked in clade X. The functional gene *Rpi-mcd1-1* which doesn't interact with PiAVR2, belongs to clade VI. The genes isolated from wild *Solanum* species which are non functional and do not interact with PiAVR2 share 88 to 99% identity with the functional genes belonging to clade X. The genes identified in *S. tuberosum* and tomato BAC clones share 78 to 89% identity with *Rpi-blb3* and are bulked in clade XII.

Interestingly, three non functional genes, *R2GH 2*, *R2GH-D3* and *R2GH-65*, share 95 to 99% amino acid identity with *Rpi-abpt* or *R2*. In an attempt to identify the causes of non functionality, nucleotide sequences were aligned by clustal W and informative polymorphic sites were identified in comparison to *R2* (Fig 2). R2GH-65 is almost identical to R2 in the exception of a region between the nucleotide positions 693 and 1338, comprising the kinase 2 and glpl domain, which confirm that disruption of the NB-ARC conserved motifs alters the functionality of the gene. The same explanation for non functionality can be given for the polymorphism found in the leucine zipper and NB-ARC domains of the R2GH-6 sequence.

Identification of the LRRs of R2 required for PiAVR2 specific interaction

Remarkably, the R2-GH-2 nucleotide sequence is almost identical to *Rpi-abpt* except in a region comprising LRR11 to LRR13 (Fig 2). Comparison of the amino acid sequences confirms that the majority of the R2GH-2-specific amino acids are concentrated in the putative solvent exposed residues of LRR11-LRR13 (Fig. 3), suggesting that the corresponding LRRs in the functional genes play an important role in determining *R2* specificity.

To analyse the role of LRR11-LRR13 in the interaction with PiAVR2, chimeras were constructed between *R2* and the non-functional *R2GH-2* allele. The unique but conserved restriction sites *Spe*I (located between LRR6 and LRR7), *Bsu*316 (located between LRR11 and LRR12) and *Bsg*I (located between LRR12 and LRR13) were used to swap 5' and 3' ends of the two homologues (Figs. 3 and 4). The constructs were cloned under *Rpi-blb3* regulatory elements and tested by agro-infiltration together with PiAVR2.

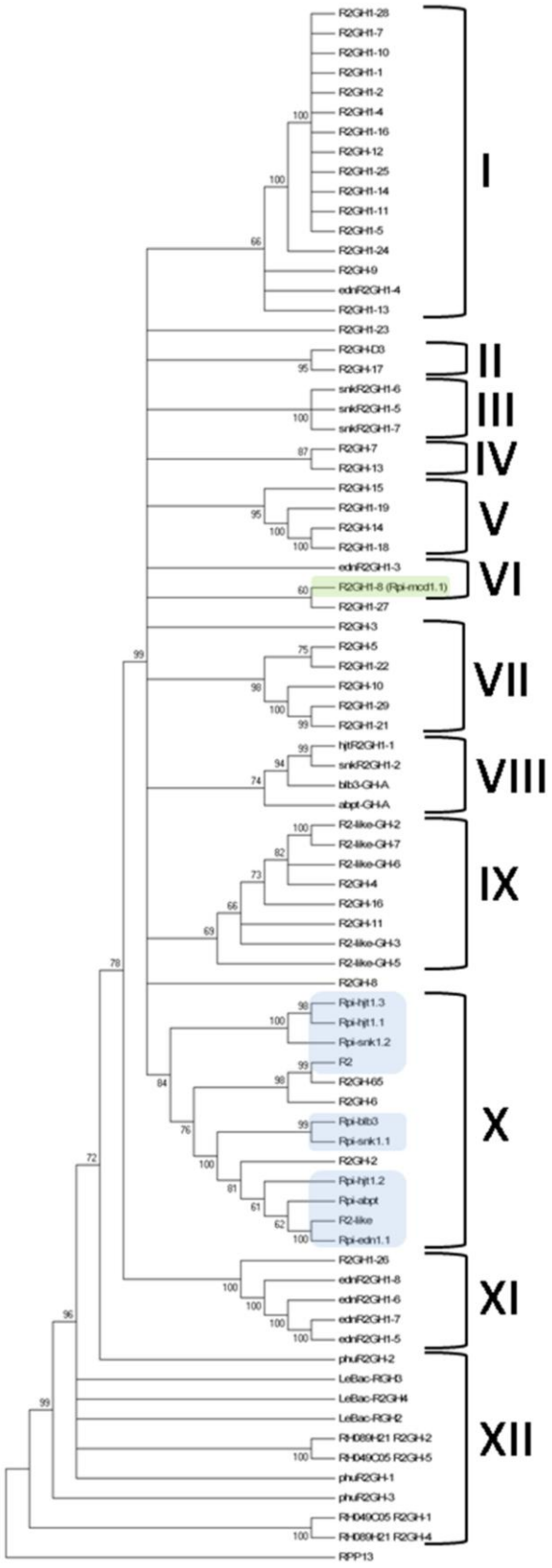


Figure 1: Phylogenetic tree of 75 amino acid sequences of Rpi-blb3 like genes. Functional genes interacting with PiAVR2 are highlighted in blue; the one not interacting with PiAVR2 is highlighted in green. The clade numbers are indicated on the right side. RPP13 had been used as an out-group. UPGMA algorithm with bootstrap analysis of 500 replicates. Numbers above the branches are bootstrap values percentages.

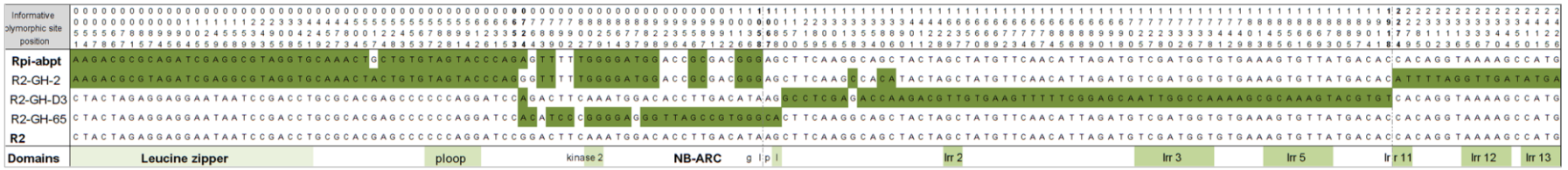


Figure 2: Nucleotide polymorphism analysis of *R2* and 4 homologues sharing informative polymorphic or homologous sequences. In bold are the genes *R2* and *Rpi-abpt* which interact with *PiAVR2*. Only informative polymorphic sites are shown, which reduces the sequence size from 2444 bp to 156 bp. Highlighted in green are the positions polymorphic to *R2* sequence. The vertical number at the top of each row indicates the corresponding nucleotide position in the full consensus sequence, the limits of the recombination regions positioned on the bottom of the figure are indicated in bold. The positions of the different domains characterizing LZ-NBS-LRR genes are indicated down to the sequences.

Table 1: List of primers used for the site-directed mutagenesis

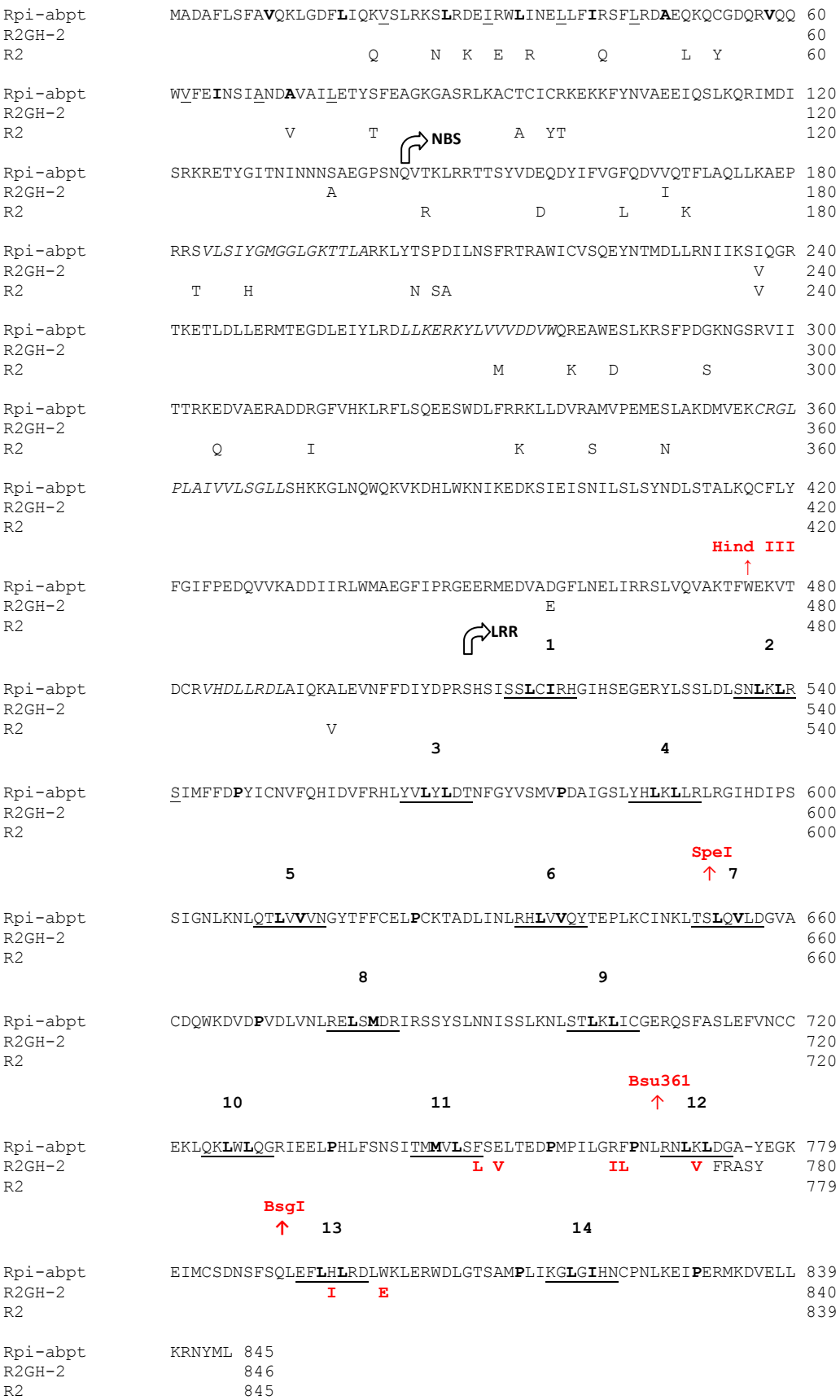
Primer	Sequence (in 5'----> 3' order)	Mismatches	% GC	Tm
lrr11-misLV-F	GATGGTTCTGAGTTT**A**TCAG**T**ACTGACAGAAGATCCGATGC	2	44	78
lrr11-misLV-R	GCATCGGATCTTCTGTCAGTACTGATAAACTCAGAACCATC			
inter-lrr11-12-misIL-F	GAAGATCCGATGCCTATTTTGGGAA**TA**TT**G**CCAAACCTAAGGAATCTC	3	42	78
inter-lrr11-12-missIL-R	GAGATTCCTTAGGTTTGGCAATATTCCCAAAATAGGCATCGGATCTTC			
lrr12-misV-F	GGTTTCCAAACCTAAGGAATCTC**GTT**TTAGATGGAGCTTACGAAGG	3	43	78
lrr12-misV-R	CCTTCGTAAGCTCCATCTAAAACGAGATTCCTTAGGTTTGGAAACC			
lrr12-misK-F	GAAGGTTTCCAAACCTAAGGAATCTC**AAA**TTAGATGGAGCTTACGAAGG	3	41	78
lrr12-misK-R	CCTTCGTAAGCTCCATCTAATTTGAGATTCCTTAGGTTTGGAAACCTTC			
lrr13-misI-F	CTTCAGTCAACTAGAGTTCCTT**AT**TCTTCGTGATCTTTGGAAGC	2	41	78
lrr13-misI-R	GCTTCCAAAGATCACGAAGAATAAGGAACTCTAGTTGACTGAAG			
lrr13-misE-F	GTTCCTTCATCTTCGTGATCTT**GA**GAAGCTAGAAAGATGGG	2	44	78
lrr13-misE-R	CCCATCTTTCTAGCTTCTCAAGATCACGAAGATGAAGGAAC			

The chimeric constructs R2GH2-SpeI-R2 and R2GH2-Bsu316-R2 were the only constructs able to induce an HR when co-infiltrated with PiAVR2. However, we observed a weaker response with R2GH2-Bsu316-R2 compared to R2GH2-SpeI-R2, suggesting that LRR11, LRR12 and LRR13 are all three involved in the interaction or recognition of PiAVR2. We can also conclude that the two amino acid differences in the NBS domain do not influence the interaction with PiAVR2 (Figs. 3 and 4) while the four amino acids polymorphic between R2 and R2GH-2 in LRR11 influence the recognition but do not suppress the response. However, presence of the R2-specific LRR11, LRR12 and LRR13 alone is not sufficient for R2 specificity, as illustrated by the non-functional homolog R2GH-65, which contains the R2 specific LRR11-LRR13 sequence but differs from the functional genes in the region harbouring NB-ARC conserved motifs. Additionally, R2GH-D3 contains the R2 specific LRR11-LRR13 sequence and differs from R2 in the region harbouring LRR3-LRR10 (Fig 2). The latter observation illustrates the crucial role that putative intra-molecular interactions within the LRR domain and possibly between the LRR domain and the LZ or NBS domain play in determining recognition by the functional Blb3GHs.

R2GH2-Bsu-R2 recognized PiAVR-2 but induced a weaker response than R2GH2-Spe-R2. Our observation indicates that the 4 polymorphic amino-acids in R2GH-2-LRR11 negatively influence but don't completely suppress PiAVR2 recognition. R2GH2-Spe-R2 recognizes PiAVR2 indicating that the 2 SNPs present in the NBS domain of R2GH-2 are not loss of function SNPs. R2-Spe-R2GH2 and Abpt-Spe-R2GH2 are non-functional, indicating that the repeats 11, 12 and 13 are crucial in PiAVR2 recognition. These results are supported by the protein structure prediction of Rpi-abpt and R2GH-2 which show a clear difference of helix, strand and coil structures in the LRR11-LRR13 region (Fig 5).

On the opposite page:

Figure 3: Identification of regions and amino acids crucial for PiAVR2 recognition. Clustal W comparing the amino acid sequences of the functional genes Rpi-abpt and R2 to the non functional gene R2GH-2. The NBS and LRR regions are indicated by an arrow. The unique restriction sites used in domain swapping experiment are indicated in red. Conserved domains present in the NBS region are in italic. LRR repeats are underlined and numbered. The amino acid L, I, F, A, M and V from the leucine zipper heptads and the LRR repeats appear in bold red.


Rpi-abpt MADAFLSFAVQKLGDFLIQKVSLRKSLRDEIRWLINELLFIRSFLRDAEQKQCGDQRVQQ 60
R2GH-2 60
R2 Q N K E R Q L Y 60
Rpi-abpt WVFEINSIANDAVAILETYSFEAGKGASRLKACTCICRKEKKFYNVAEEIQSLKQRIMDI 120
R2GH-2 120
R2 V T NBS A YT 120
Rpi-abpt SRKRETYGITNINNNSAEGPSNQVTKLRRTTSYVDEQDYIFVGFQDVVQTFLAQLLKAEP 180
R2GH-2 A I 180
R2 R D L K 180
Rpi-abpt RRSVLSIYGMGGLGKTTLARKLYTSPDILNSFRTRAWICVSQEYNTMDLLRNIIKSIQGR 240
R2GH-2 V 240
R2 T H N SA V 240
Rpi-abpt TKETLDLLERMTEGDLEIYLRDLLKERKYLVVVDDVWQREAWESLKRSFPDGKNGSRVII 300
R2GH-2 300
R2 M K D S 300
Rpi-abpt TTRKEDVAERADDRGFVHKLRFLSQEESWDLFRRKLLDVRAMVPEMESLAKDMVEKCRGL 360
R2GH-2 360
R2 Q I K S N 360
Rpi-abpt PLAIVVLSGLLSHKKGLNQWQKVKDHLWKNIKEDKSIEISNILSLSYNDLSTALKQCFLY 420
R2GH-2 420
R2 420
Hind III
Rpi-abpt FGIFPEDQVVKADDIIRLWMAEGFIPRGEERMEDVADGFLNELIRRSLVQVAKTFWEKVT 480
R2GH-2 E 480
R2 LRR 480
1 2
Rpi-abpt DCRVHDLLRDLAIQKALEVNFFDIYDPRSHSISSLCIRHGIHSEGERYLSSLDLSNLKLR 540
R2GH-2 540
R2 V 540
3 4
Rpi-abpt SIMFFDPYICNVFQHIDVFRHLYVLYLDTNFGYVSMVPDAIGSLYHLKLLRLRGIHDIPS 600
R2GH-2 600
R2 600
SpeI
5 6 7
Rpi-abpt SIGNLKNLQTLVVVNGYTFFCELPCKTADLINLRHLVVQYTEPLKCINKLTSLQVLDGVA 660
R2GH-2 660
R2 660
8 9
Rpi-abpt CDQWKDVDPVDLVNLRELSMDRIRSSYSLNNISSLKNLSTLKLICGERQSFASLEFVNCC 720
R2GH-2 720
R2 720
Bsu361
10 11 12
Rpi-abpt EKLQKLWLQGRIEELPHLFSNSITMMVLSFSELTEDPMPILGRFPNLRNLKLDGA-YEGK 779
R2GH-2 L V IL V FRASY 780
R2 779
BsgI
13 14
Rpi-abpt EIMCSDNSFSQLEFLHLRDLWKLERWDLGTSAMPLIKGLGIHNCPNLKEIPERMKDVELL 839
R2GH-2 I E 840
R2 839
Rpi-abpt KRNYML 845
R2GH-2 846
R2 845

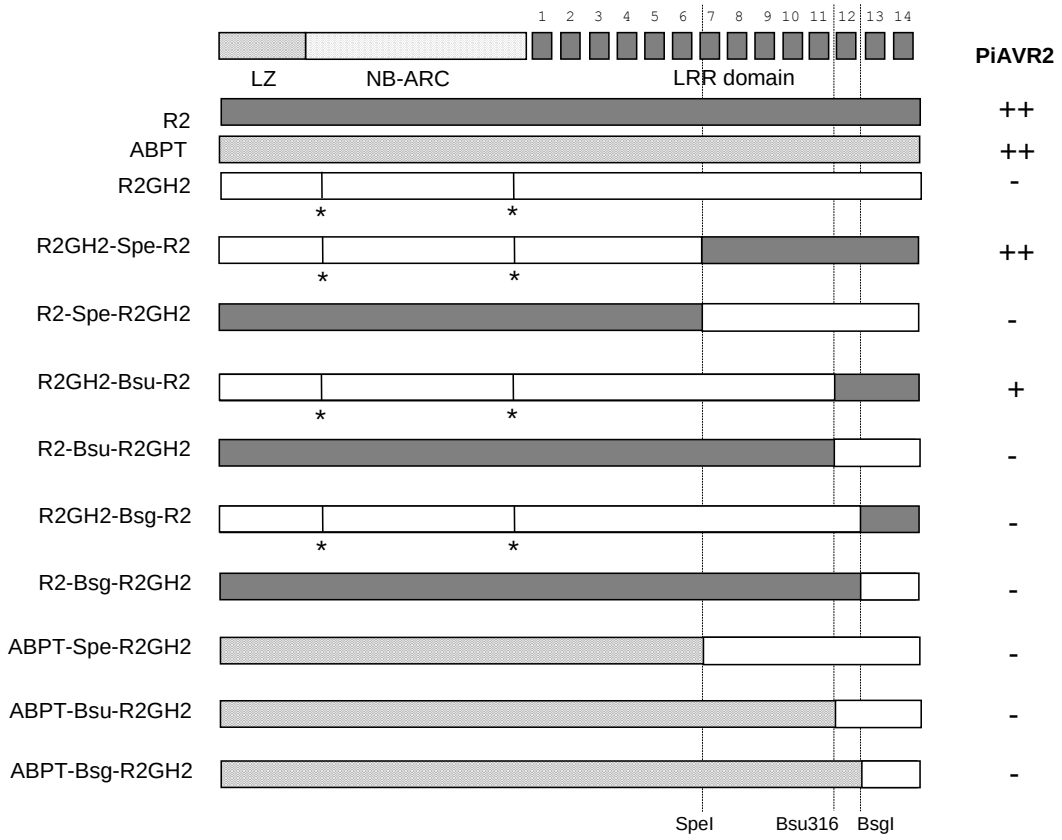


Figure 4: Domain swaps between the functional gene *R2* or *Rpi-abpt* and the non-functional gene R2GH-2. On the top are indicated the different domains (LZ: leucine zipper, NB-ARC and LRR domain with its 14 repeats). Below are indicated the position of the restriction sites used to swap the domains. (*) indicates single nucleotide polymorphism present in R2GH- sequence .in comparison with *Rpi-abpt*. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+)and no visible cell death region were scored as (-).

Pinpointing of a single amino acid important for PiAvr2 recognition

R2GH-2 is almost identical to Rpi-abpt with the exception of 11 amino acids residing in LRR11, 12 and 13. These amino acids differ from R2GH-2 and Rpi-abpt sequences by their chemical propriety (Fig 3). In an attempt to determine which amino acid(s) in the non functional R2GH2 are critical for the avirulence protein recognition Pi-AVR2, a PCR-based site-directed mutagenesis strategy was used to convert the Rpi-abpt residue individually into the corresponding R2GH-2 residue (Fig 6). Five constructs were made which target seven amino acids individually or in combinations. The construct mut-abpt-1 displays L and V amino acid instead of F and E in the LRR11, mut-abpt-2 has the amino acids I and L instead of R and F also in LRR11, mut-abpt-3 contains the amino acid V instead of K in LRR repeat 12, mut-abpt-4 spans the amino acid I instead of H in LRR13, mut-abpt-5 has the amino acid E instead of W also in LRR13.

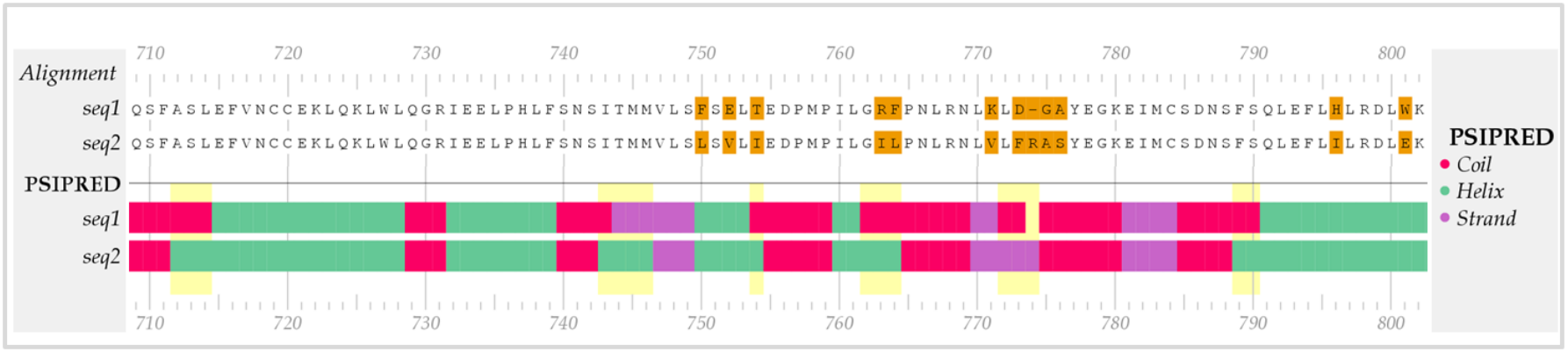


Figure 5: Comparison of the secondary structure prediction of Rpi-abpt (seq1) and R2GH-2 (seq2) in the LRR9 to LRR13 region. Polymorphic amino acids and secondary structure are highlighted in yellow. The figure is a combination of the positional features and differential prediction outputs obtained after analyses of the protein sequences by EPipe 0.922 Server from the center for biological sequences analysis (CBS).

The four constructs mut-abpt- 1, 2, 3 and 5 showed variable intensity of cell death regions. The construct mut-abpt-1 and mut-abpt-2 interact in a less intense reaction with PiAVR2 than the positive control Rpi-abpt. This observation is in correlation with the weak reaction obtained when the chimeric construct R2GH2-Bsu-R2 is co-infiltrated with PiAVR2. Mut-abpt-1 and mut-abpt-2 harbour a double mutation while R2GH2-Bsu-R2 harbours the combination of those double mutations. The constructs mut-abpt-3 and mut-abpt-5 showed a weaker response than mut-abpt-1 and mut-abpt-2. The construct mut-abpt-4 co-infiltrated with PiAVR2 did not induce a hypersensitive response in the *N. benthamiana* leaves. These observations reflect the impact which could have the mutation of strategic amino acids in the structural conformation of Rpi-abpt protein. The mutated amino acids in the constructs mut-abpt-1,2,3 and 5 are identified as residues under strong positive selection pressure (Champouret 2010) with a high posterior probability (p>99%) when the model M8 of the PAML method as applied. The amino acid H in LRR13 displays a posterior probability of 75%. These observations indicate the malleability degree of certain residues like the amino acids K and W belonging to LRR12 and 13, and the crucial importance of others like the amino acid H in LRR13 for *Rpi-abpt* recognition of PiAVR2.

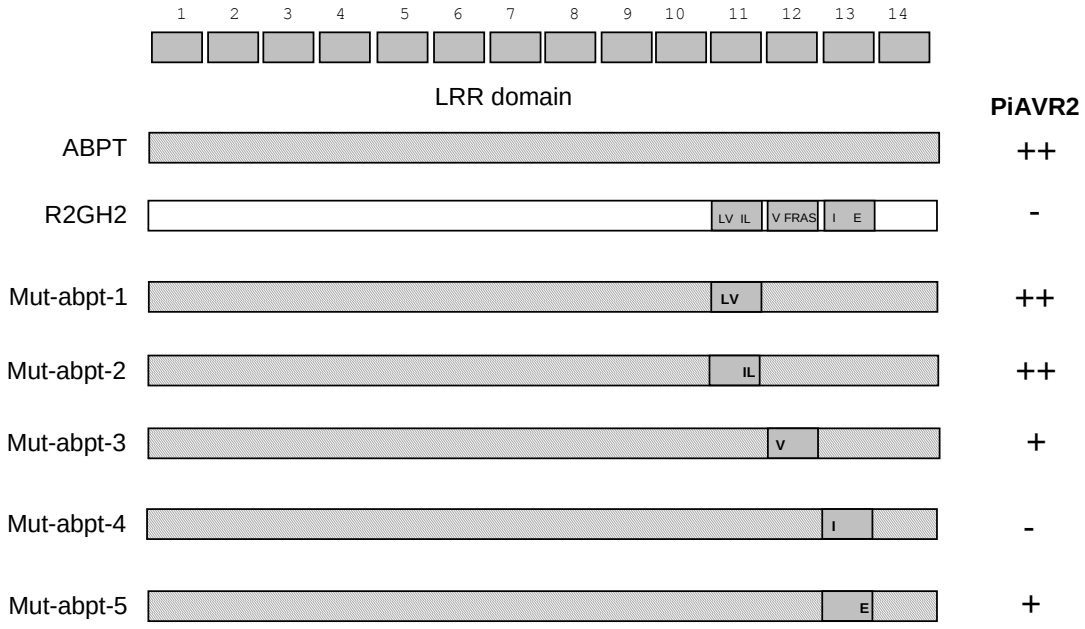


Figure 6: Site-directed mutated constructs tested by co-infiltration with PiAVR2. On the top are indicated the LRR domain with its 14 repeats. Polymorphic amino acids are indicated in comparison with the Rpi-abpt amino acid sequence. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+) and no visible cell death region were scored as (-).

DISCUSSION

The major late blight resistance cluster on linkage group IV harbors a profusion of alleles sharing high percentage identity. These alleles define a new R gene family already identified by the cloning of *Rpi-blb3, Rpi-abpt, and R2-like* (Lokossou et al. 2009). Additionally, N. Champouret (2010) cloned *Rpi-edn*, *Rpi-hjt* and *Rpi-snk* alleles by R2 allele mining. These genes provide resistance against late blight by recognizing the *Phytophthora infestans* RXLR effector PiAVR2. Sequence analyses showed identical C terminal regions starting at the LRR repeat 3 (Lokossou et al. 2009; Champouret 2010). Three non functional R2GHs share high sequence identity with R2 and Rpi-abpt, giving us the opportunity to investigate PiAVR2 recognition specific region(s).

PiAVR2 recognition specificity resides in LRR11, LRR12 and LRR13.

Domain swap experiments have provided direct evidence that LRRs play a major role in determining resistance specificity (Thomas et al. 1997). In depth studies of the *P* and *P2* genes at the *P* locus in flax (Dodds et al. 2001), the *Mi* locus in tomato (Hwang et al. 2000; Hwang and Williamson 2003), and the *Pi2* and *Piz-t* at the *Pi* locus in rice (Zhou et al. 2006) identified essential amino acids in the LRR domain involved in the recognition of the AVR(s) or the functions of the genes. In depth investigation of the tomato Cf-9 disease resistance protein demonstrated that the major specificity-determining amino acids reside at hypervariable solvent exposed positions (Wulff et al. 2009). Here we demonstrate that LRR11, LRR12 and LRR13 are important for the interaction of R2 with PiAVR2. Further more seven amino acids were pinpointed in site directed mutagenesis experiments resulting in the identification of one of the crucial amino acids determining Rpi-abpt and its homologues specificity. The identified Histidine-795 is located in a solvent exposed position of LRR13 (xxLHLxx). Mutation of this amino acid into an Isoleucine as in R2GH-2 resulted in non recognition of PiAVR2. Our results support the positive selection identified in LRR 11, 12 and 13 by N. Champouret (2010) when analyzing 17 *R2* variants using the PAML method. The Histitine-795 had a posterior probability value lower than the other mutated residues reflecting its reduced potential to mutate and maintain the recognition of PiAVR2. This indicates the R protein potential of adaptation to PiAVR conformational change. Modification in the R protein structure might occur in a slow process of searching for the perfect match by trial and error, thus resulting in the profusion of alleles indentified in our allele mining strategy

Type I and II evolution at the MLB locus on linkage group IV

Sequence analyzes of the 24 *Rpi-blb3*/*R2* and 27 *Rpi-mcd1-1* homologues (Chapter 2 and 4) reveal a modular structure of the genes which evolution was dominated by sequence exchange, an evolutionary pattern typical for a Type I resistance gene as described for *R*-genes in natural lettuce populations (Kuang et al.). Investigation of the 17 *R2* variants isolated by N. Champouret et al (2010) shows residues in the LRR domain under positive selection thus submitted to single mutation like Type II resistance genes (Kuang et al.), with the aim to counter act to *P. infestans* escape. In our study, we demonstrated that a single mutation on a solvent exposed residue can lead to the lack of recognition of the cognate effector. This could also be interpreted as an adaptation of the gene to recognize an evolved version of the effector, or by the presence in the germplasm of R genes ready to protect the plant against a sudden change in the effector structure. This simultaneous evolution in host and pathogen for survival is also reflected in *Rpi-blb3* allele mining in *S .bulbocastanum* and related species (Chapter 3).

Non functionality or differential P. infestans resistance spectrum or differential pathogen recognition

Fifteen % of the full length homologous proteins reproduce the expected LB resistance spectrum. Thus, the other homologues remain with undetermined functionality. The resistance spectrum covered by *Rpi-mcd1-1* is different from the R2 spectrum and proves the existence of R2 homologues with different recognition specificity (Chapter 4). The demonstrated synteny between potato and tomato (Bonierbale et al. 1988; Gebhardt et al. 1991), and the identification of multiple pathogen resistance genes at one locus open the debate of the existence of homologues genes conferring resistance to other pathogens than *P. infestans* among the *Rpi-blb3*/*R2* homologue gene collection. Such cluster diversity has been found at the Mi-1/ Rpi-blb2 locus on LG VI which shares 82% protein sequence identity (van der Vossen et al.). *Mi-1* originates from tomato and confers resistance to three species of root knot nematodes (*Meloidogyne* spp.) as well as to the potato aphid *Macrosiphum euphorbiae* (Milligan et al. 1998; Rossi et al. 1998; Vos et al. 1998), and to both B and Q biotypes of whitefly *Bemisia tabaci* (Nombela et al. 2003). *Rpi-blb2* originated from potato and conferred resistance to late blight. *Tm2*, which provides resistance to tomato against the *Tomato mosaic virus,* shares 75% amino acid identity with the late blight resistance gene *Rpi-vent*, both are located on LG IX (Lanfermeijer et al. 2003; Pel et al. 2009). The tomato gene *I2* and potato gene *R3a* which are on LG XI share 88% identity and provide resistance against the fungi *Fusarium oxysporum* and *P. infestans*, respectively (Huang et al. 2005; Ori et al. 1997; Simons et al. 1998). Resistance against the

potato virus X and the potato cyst nematode *Globodera pallida* are provided by *Rx1* and *Gpa2*, respectively. Both are located on LG V and share an overall homology of over 88% amino-acid identity (Bendahmane et al. 1999; Rouppe van der Voort et al. 1999; van der Vossen et al. 2000). A way to reveal the putative functionality of the *R2/Rpi-blb3* homologues would be to create a library of potato transformants harboring the different alleles and test them with a differential set of *P. infestans* and other pathogens.

MATERIEL ANS METHODS

Domain swaps

pDONR221 constructs harboring *R2*, *Rpi-abpt* and *R2GH-2* were digested according to the manufacturers instructions with the restriction enzymes *Spe*I (Fermentas, Germany), *Bsu*316 (Fermentas, Germany) or *Bsg*I (New England Biolabs, The Netherlands). Digested products were separated by agarose electrophoresis and isolated using the QIAQUICK Gel Extraction Kit from Qiagen (The Netherlands). Different combinations of digested fragments were used in ligation reactions with the T4 DNA Ligase (Fermentas, Germany). Chimeric constructs were verified by comparing restriction digestion patterns obtained after digestion with *Bsu*316 and *Nhe*I (Fermentas, Germany). Additional verification was done through sequencing of the 5'-end, 3'-end and chimeric region of the inserts.

Site direct mutagenesis

Site directed mutations on *Rpi-abpt* in pDONR221 vector were generated by PCR with PfuUltra ® High-Fidelity DNA polymerase (Stratagene) and specific primers containing the desired mutations (Table S1).The PCR product were digested with the enzyme Dpn I according to the manufacturer's instruction. The resulted digestion mix was used to transform XL1-Blue super competent cells (Stratagene QuikChange ® II Site-Directed Mutagenesis kit).

The occurrence of the mutation was verified by sequencing each construct.

Cloning and testing

Confirmed chimeric and site directed mutated constructs were transferred to the destination vector pKGW-MG (kindly provided by Andreas Untergasser) in between *Rpi-blb3* regulatory elements via a multiple LR reaction with the *Rpi-blb3* promotor in pDONR-P4P1R, the *Rpi-blb3* terminator in pDONR-P2RP3, the ORF of interest in pDONR221, and the binary destination, using the protocol of (Untergasser 2006). The final expression constructs were then transferred to *A. tumefaciens* AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG and pSoup (van der Fits et al. 2000). Each construct was tested by co-infiltration with the *P.infestans* avirulence protein Pi-Avr2 in *N. benthamiana* (as described by Lokossou et al.2009). *pKGW-Rpi-abpt* construct co-infiltrated with Pi-Avr2 was used as positive control. Negative controls were the infiltration with the R-gene or the AVR-gene construct alone. The chimeras R2GH2-Spe-R2, R2-Spe-R2GH2, R2GH2-Bsu-R2, R2-Bsu-R2gh2, R2GH2-Bsg-R2, R2-Bsg-R2gh2 and Abpt-Spe-R2GH2, and the site directed mutants mut-abpt-1 to 5 were functionally tested by co-infiltration with PiAVR2. Infiltrated leaves were scored 5 days post infiltration, white or black infiltration spots were scored as (++), yellow pale spots were scored as (+) and no visible cell death region were scored as (-).

DNA sequencing and computer analyses

Sequencing reactions were performed using a dye terminator cycle sequencing reaction kit (Perkin-Elmer, Pt Biosystem, Warrington, UK). Sequence contigs were assembled using the STADEN sequence and analysis program (Dear and Staden 1991). Alignment and phylogenetic trees were produced using "CLC main workbench 5" program, version 5.0.2. Gene structures were predicted using FGENESH++ (Softberry). Multiple sequence alignments were conducted using CLUSTALX 1.81 (Thompson et al. 1997). The search for genes homologous to *Rpi-blb3* was carried out using the Basic Local Alignment Search Tool (BLAST). Secondary structure domains were identified using the beta version of EPipe 0.922 Server from the center for biological sequences analysis (CBS) (BioSapiens Network of Excellence et al. 2008; (Jones 1999); (Tress et al. 2007).

ACKNOWLEDGEMENTS

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Chapter 6

General discussion

The research described in this thesis comprises the isolation of five late blight resistance genes clustering in the major late blight (MLB) locus positioned on potato linkage group (LG)IV. A combination of plant and pathogen investigation techniques permitted the dissection of the MLB locus in terms of functionality, molecular interaction, allelic variation, diversification and evolution. This study could serve as an example for future investigation of resistance gene clusters.

A model for resistance gene cluster studies

We used different techniques in our research, starting with classical map based cloning to isolate the genomic sequence of *Rpi-blb3*, including its regulatory elements. Allelic diversity investigation was done by employing an allele mining strategy permitting the isolation of *Rpi-abpt*, *R2* and *R2-like* (**Chapter 2)**, and leading to the additional identification of 22 *Rpi-blb3* haplotypes among *S. bulbocastanum* and related species (**Chapter 3)**. Functionality of new R genes was best demonstrated in stable transformation experiments with suitable (susceptible) potato cultivars. Allelic versions of a specific R gene could better be tested in an alternative functional test, like described in **Chapter 2,** which is a transient expression of R genes in *N benthamiana* leaves followed by inoculation with *P. infestans* isolates. This method was successfully deployed in **Chapter 4** for the identification of *Rpi-mcd1.1*. Detection of known R genes in *Solanum* species under study in **Chapter 3** was possible using a socalled effectoromics approach, where a hypersensitive response, obtained upon agro infiltration of the avirulence gene in the leaves, gave an accurate diagnostic reaction in the presence of a cognate functional R gene. Finally, co-infiltration of R and AVR gene pairs in *N. benthamiana* leaves allowed the accurate identification of critical amino acid(s) for the determination of a particular R2/PiAVR2 molecular interaction **(Chapter 5)**.

Tracing back the origin of the R2GHs

The major late blight resistance cluster on LG IV harbors a profusion of alleles sharing high percentages of identity. These alleles define a new R gene family, first identified by cloning of *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2-like* (**Chapter 2**). *Rpi-blb3* and *R2* originate from *S. bulbocastanum* and *S. demissum*, respectively. Other allele mining studies revealed an identical sequence of *R2-like* in *S. edinense* (Champouret 2010), thus shedding light on its origin. However, the indistinguishable

sequence of *Rpi-abpt* was isolated from a *S. tuberosum*/ *S. demissum* clone (Mahdi Mortazavian Wageningen UR Plant Breeding personal communication) adding to the complexity of the origin of this gene that we previously had suspected to be derived from one of the crossing parents used for creation of the quadruple species hybrid ABPT, *S. acaule*, *S. bulbocastanum*, *S. phureja* or *S. tuberosum*.

In Chapter 5 we report about all the *R2GHs* coding for full length protein sequences. The different alleles isolated in this research and in N. Champouret's (2010) study have been identified in *S. bulbocastanum, S. demissum, S. microdontum*, *S. schenckii*, *S. edinense* and *S. hjertingii*. Interestingly, the *R2GHs* recovered from the clone *S. tuberosum*/ *S. demissum* (Cebeco44158-4) described in **Chapter 2** and the *R2GHs* retrieved from the clone *S. tuberosum*/ *S. microdontum* (RH90-038-21) described in **Chapter 4** ended up in the same phylogenetic clades (**Chapter 5;** Fig.1) and shared a high percentage of amino acid identity. The alleles that the two clones possess in common are seemingly originating from *S. tuberosum.* In addition, the R2GHs identified in the *S. tuberosum* clone RH89-039-16 shared the same phylogenetic clade with R2GHs discovered in tomato. Also, a focused allele mining study using *Rpi-blb3* specific primers in *Solanum* germplasm identified *Rpi-blb3* haplotypes in *S. pinnatisectum*, *S. bulbocastanum* (including some subspecies), *S. hjertingii*, *S. nayaritense*, *S. brachistotrichum, S. cardiophyllum* and *S. stoloniferum* **(Chapter 3).**

Using diverse *Solanum* classification systems (Hawkes 1994; Jacobs et al. 2008; Spooner et al. 1991), we failed in our attempt to find phylogenic relationship between the various *Solanum* species harboring *R2GHs* and to deduce the ancestral origin of the MLB residing on linkage group IV.

Intergenic recombination events and mutations, directed by positive selection, shaped the resistance locus

Sequence analyses of the genes isolated by *R2* or *Rpi-blb3* adapted allele mining showed the modular structure of this LZ-NBS LRR R gene family. We identified clear sequence exchanges between the different members of the cluster, nicely exemplified by the structure of *R2* which displayed a different N-terminal sequence when compared to other members of the MLB also interacting with the same avirulence effector PiAVR2. In addition, *R2GHs* identified by N.

Champouret (2010) showed residues in the LRR domain under positive selection. Sequence exchanges and point mutations directed by positive selection appeared to have contributed to the birth of different functional resistance genes identified in this investigation. The same phenomenon is observed in linkage group V, where multiple intergenic recombinations resulted in the unique *RB*/*Rpiblb1* (Song et al. 2003; van der Vossen et al. 2003). Following the same evolution pattern as *R2GHs*, only point mutations were detected in the *Rpi-blb1* haplotypes identified in **Chapter 3**.

A previous study demonstrates the rare occurrence of meiotic recombination per chromosome arm in potato (Park et al. 2007). In addition, the presence of identical *R2GH* sequences in different species reflects the conservation of the functional gene during the evolution of *Solanum*. We presume from this observation that the evolution process which led to the actual functional resistance genes studied here, preceded the divergence of the *Solanum* species from an apparent common ancestor.

Co-evolution between Phytophthora infestans and Solanum is permanent

P. infestans originated from the Toluca valley, in the central highlands of Mexico where it evolved together with wild *Solanum* species. Mating type A1 and A2 were first localized in Mexico, while only mating type A1 was present in Europe . In the 1980s the A2 mating type appeared, followed by a dramatic increase of genetic variation in *P. infestans* suggested to be caused by sexual reproduction (Drenth et al. 1994; Fry et al. 1992; Goodwin and Drenth 1997). This led to the rapid defeat of *Solanum demissum* R genes introgressed into breeding material. *Rpi-blb3*, *Rpi-abpt*, *R2* and *R2 like* which have been identified in **Chapter 2**, together with *Rpi-snk1-1*, *Rpi-snk1-2*, *Rpi-edn1-1*, *Rpi-hjt1-1*, *Rpi-hjt1-2* and *Rpi-hjt1-3* (Champouret 2010), which originated from Mexican species and all interact with the avirulence effector PiAVR2. In **Chapter 3** we discovered a new *Rpi-blb3* haplotype which did not interact with PiAVR2 and geographically is located in Guatemala. In addition in **Chapter 4**, *Rpi-mcd1-1* which originated from Argentina also did not interact with PiAVR2. These examples demonstrate the adaptation of the *R2/Rpi-blb3* cluster to the local population of the pathogen and reflect the co-evolution occurring between *Solanum* and *P.infestans*. Since a rapid evolution of *P. infestans* is witnessed under the presence of the two mating types, it is expected to observe a similar fast evolution of R genes present in wild *Solanum.*

Molecular mechanism underlying quantitative resistance

Among the *Solanum* species known to exhibit quantitative resistance, *Solanum microdontum* is well described for its quantitative resistance gene locus (PiQRL), localized on linkage group IV (Sandbrink et al. 2000; Tan et al. 2008). Cytological characterisation of partial resistance is explained by Vleeshouwers et al. (2000) as a less effective hypersensitive response which is possibly caused by an inadequate or delayed recognition of effectors by weak R genes. Additionally, Champouret et al. (2009) categorized *P.infestans* isolates in use for R-gene characterisation, according to their aggressiveness level. **Chapter 4** depicts the dissection of the quantitative resistance map in a population derived from *S. microdontum.* Leaves of susceptible cultivars transformed with *Rpi-mcd1-1* react with a clear hypersensitive response when inoculated with PIC99177 which displays a very low level of aggression, presuming an underlying "weak" R/ Avr gene pair interaction. Taken together, this information contributes to a better understanding of some, previously called, partial resistance factors and gives an example of a suitable way to characterise these partial resistance factors which now appear to be weak R genes.

Future research and breeding advises

Exploring the synteny existing among Solanaceae

Comparison of genomic positions of phenotypically defined disease resistance genes and R gene homologues in two solanaceous crop genera, *Solanum* (potato and tomato) and *Capsicum* (pepper) highlight syntenous resistance loci. These positions harbor genes or QTL providing resistance to various pathogens. For example, the potato linkage group IV which anchors the major MLB studied in this research is syntenous to the tomato chromosome IV loci hosting the *Hero* gene conferring resistance to *G. rostochiensis*. In pepper, the genomic equivalent position is on chromosome V with *phyt1* QRL conferring resistance to *P. capsici* (Grube et al. 2000a). Only 15% of the R2GHs described in **Chapter 5** reproduce the expected late blight resistance spectrum. The remaining homologues stay with undetermined functionality but the demonstrated synteny between the well studied crops tomato, potato, pepper (Grube et al. 2000b) and the less well studied eggplant (Doganlar et al. 2002) opens speculation to the putative resistance provided by some alleles against more different pathogens than *P. infestans*.

Broad spectrum and durable resistance

The preference of breeders in introgression of resistance loci providing field resistance, and their believe in a different mechanism underlying quantitative resistance is not strongly supported by the findings in this thesis. In **Chapter 3**, we showed that most of the *Solanum* species displaying large resistance spectrums, often host multiple resistance genes. **Chapter 4** revealed that a classical R gene is in fact encoding the previously identified PiQRL on linkage group IV. This new knowledge about the nature and the distribution of R genes in wild *Solanum* species suggests that achievement of durable resistance can be possible by stacking resistance genes with different resistance spectra and which target functionally different Pi avirulence genes.

P. infestans effector forecast

Taking actual efficient decision support systems (Skelsey et al. 2009) as an example and making use of the current knowledge gathered about *P. infestans*, a complementary forecast approach is proposed. Harvest of *P. infestans* spores present in the target potato cultivation region, followed by the characterization of the avirulence effector content would identify the weakness of the pathogen (Zhu et al.2010). Cultivars containing cognate R genes would be recommended for next season planting. Ongoing studies in different laboratories are conducted to understand the natural evolution of the R genes and the Avr genes, in their natural environment. Using this knowledge, better predictions about the level and the duration of resistance will be possible in the future.

LITTERATURE CITED

Allen, R. L., Meitz J. C, Baumber R. E., Hall S. A., Lee S. C., Rose L. E and Beynon J. L. 2008. Natural variation reveals key amino acids in a downy mildew effector that alters recognition specificity by an Arabidopsis resistance gene. Mol. Plant Pathol.9: 511-523

Allen, R. L., Bittner Eddy, P. D., Grenville Briggs, L. J., Meitz, J. C., Rehmany, A. P., Rose, L. E., and Beynon, J. L. 2004. Host-parasite coevolutionary conflict between Arabidopsi*s* and downy mildew. Science 306:1957-1960.

Arens, P., Mansilla, C., Deinum, D., Cavellini, L., Moretti, A., Rolland, S., van der Schoot, H., Calvache, D., Ponz, F., Collonnier, C., Mathis, R., Smilde, D., Caranta, C., and Vosman, B. 2010. Development and evaluation of robust molecular markers linked to disease resistance in tomato for distinctness, uniformity and stability testing. Theor. Appl. Genet. doi 10.1007/s00122-009-1183-2

Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I. B., Venter, E., Avrova, A. O., Rehmany, A. P., Bo¨hme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M. A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J. L., and Birch, P. R. J. 2005. An ancestral oomycete locus contains late blight avirulence gene *Avr3a*, encoding a protein that is recognized in the host cytoplasm. PNAS 102:7766-7771

Bai, Y. L., van der Hulst, R., Bonnema, G., Marcel, T. C., Meijer-Dekens, F., Niks, R. E., and Lindhout, P. 2005. Tomato defense to *Oidium neolycopersici*: Dominant *Ol* genes confer isolate-dependent resistance via a different mechanism than recessive *ol-2*. Mol. Plant-Microbe Interact. 18:354-362.

Ballvora, A., Ercolano, M. R., Weiss, J., Meksem, K., Bormann, C. A., Oberhagemann, P., Salamini, F., and Gebhardt, C. 2002. The R1 gene for potato resistance to late blight (Phytophthora infestans) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. Plant J 30:361-71

Belkhadir, Y., Nimchuk, Z., Hubert, D. A., Mackey, D., and Dangl, J. L. 2004. Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. Plant Cell 16:2822-2835.

Bendahmane, A., Kanyuka, K., and Baulcombe, D. C. 1999. The *Rx* gene from potato controls separate virus resistance and cell death responses. Plant Cell 11:781-791.

Bergelson, J., Dwyer, G., and Emerson, J. J. 2001. Models and data on plant-enemy co evolution. Annu. Rev. Genet. 35:469-499.

Birch, P. R. J., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L., and Whisson, S. C. 2008a. Oomycete RXLR effectors: delivery, functional redundancy and durable disease resistance. Curr Opin in Plant Biol. 11:373-379.

Birch, P. R. J., Whisson, S., Boevink, P., Armstrong, M., Gilroy, E., Pritchard, L., Grouffaud, S., Sandanandom, A., Taylor, R., Kamoun, S., and Bos, J. 2008b. Translocated oomycete effectors

that target the plant immune system. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology 150:S176-S177.

Birch, P. R. J., Rehmany, A. P., Pritchard, L., Kamoun, S., and Beynon, J. L. 2006. Trafficking arms: oomycete effectors enter host plant cells. Trends Microbiol. 14:8-11.

Bisgrove, S. R., Simonich, M. T., Smith, N. M., Sattler, A., and Innes, R. W. 1994. A disease resistance gene in *Arabidopsis* with specificity for 2 different pathogen avirulence genes. Plant Cell 6:927-933.

Bisognin, D. A., Douches, D. S., Buszka, L., Bryan, G., and Wang, D. 2005. Mapping Late Blight Resistance in Solanum microdontum Bitter. Crop Sci 45:340-345.

Bittner-Eddy, P. D., Crute, I. R., Holub, E. B., and Beynon, J. L. 2000. *RPP13* is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. Plant Journal 21:177-188.

Black, L. M. 1970. Potato yellow dwarf virus. Assoc. Appl. Biologists, Wellesbourne, UK No. 35.

Bonierbale, M. W., Plaisted, R. L., and Tanksley, S. D. 1988. RFLP Maps Based on a Common Set of Clones Reveal Modes of Chromosomal Evolution in Potato and Tomato. Genetics 120:1095-1103.

Bos, J. I. B., Kanneganti, T.-D., Young, C., Cakir, C., Huitema, E., Win, J., Armstrong, M., Birch, P. R. J., and Kamoun, S. 2006. The C-terminal half of *Phytophthora infestans* RXLR effector AVR3a is sufficient to trigger R3a-mediated hypersensitivity and suppress INF1-induced cell death in Nicotiana benthamiana. Plant Journal in press.

Bradshaw, J. E., Bryan, G. J., Lees, A. K., McLean, K., and Solomon Blackburn, R. M. 2006. Mapping the *R10* and *R11* genes for resistance to late blight (*Phytophthora infestans*) present in the potato (*Solanum tuberosum*) R-gene differentials of Black. Theoretical and applied genetics 112:744-751.

Bradshaw, J., Bryan, G., Hackett, C., McLean, K., Pande, B., Stewart, H., and Waugh, R. 2004. Dissection and analysis of quantitative disease resistance in tetraploid potato. Euphytica 137:13-18.

Brouwer, D. J., Jones, E. S., and St Clair, D. A. 2004. QTL analysis of quantitative resistance to *Phytophthora infestans* (late blight) in tomato and comparisons with potato. Genome 47:475-492.

Brown, C. R. 1990. Modern Evolution of the Cultivated Potato Gene Pool. In Vayda, M.E. and Park, W.D. The Molecular and Cellular Biology of the Potato Wallingford. CAB international. Wallingford:1-12.

Budin, K. Z. 2002. Genetic foci of *Solanum* species, *Petota* Dumort, resistant to *Phytophthora infestans* (Mont.) De Bary. Genet. Res. Crop Evol. 49:229-235.

Caicedo, A. L. 2008. Geographic diversity cline of *R* gene homologs in wild populations of *Solanum pimpinellifolium* (Solanaceae). Am. J. of Bot. 95:393-398.

Catanzariti, A.-M., Dodds, P. N., Ve, T., Kobe, B., Ellis, J. G., and Staskawicz, B. J. 2010. The AvrM Effector from Flax Rust Has a Structured C-Terminal Domain and Interacts Directly with the M Resistance Protein. Molecular Plant-Microbe Interactions 23:49-57.

Celebi-Toprak, F., Slack, S. A., and Jahn, M. M. 2002. A new gene, Ny_{tbr}, for hypersensitivity to potato virus Y from *Solanum tuberosum* maps to chromosome IV. Theor. Appl. Genet. 104:669-674.

Champouret, N. 2010. Functional Genomics of *Phytophthora infestans* Effectors and *Solanum* Resistance Genes. PhD Thesis, Wageningen UR, Plant Breeding.

Champouret, N., Bouwmeester, K., Rietman, H., van der Lee, T., Maliepaard, C., Heupink, A., van de Vondervoort, P. J. I., Jacobsen, E., Visser, R. G. F., van der vossen, E. A. G., Govers, F., and Vleeshouwers, V. G. A. A. 2009. *Phytophthora infestans* isolates lacking class I IpiO variants are virulent on *Rpi-blb1* potato. Mol. Plant-Microbe interact.22:1535-1545

Collier, S. M., and Moffett, P. 2009. NB-LRRs work a "bait and switch" on pathogens. Trends in Plant Science 14:521-529.

Colon, L. T., Jansen, R. C., and Budding, D. J. 1995. Partial resistance to Late Blight (*Phytophtora infestans*) in hybrid progenies of 4 south-american *Solanum* species crossed with diploid *Solanum tuberosum* Theor. Appl. Genet. 90:691-698.

Colton, L. M., Groza, H. I., Wielgus, S. M., and Jiang, J. M. 2006. Marker-assisted selection for the broad-spectrum potato late blight resistance conferred by gene *RB* derived from a wild potato species. Crop Science 46:589-594.

Damasceno, C. M. B., Bishop, J. G., Ripoll, D. R., Win, J., Kamoun, S., and Rose, J. K. C. 2008. Structure of the Glucanase Inhibitor Protein (GIP) Family from Phytophthora Species Suggests Coevolution with Plant Endo-Î²-1,3-Glucanases. Molecular Plant-Microbe Interactions 21:820-830.

Danan, S. 2009. Diversité structurale des locus de résistance a Phytophthora infestans chez la pomme de terre et syntenie chez les Solanacées. Thèse doctorale (INRA d'Avignon et Centre international d'études supérieures en sciences agronomique SupAgr Momtpellier).

Dickinson, M., Jones, D., and Jones, J. 1993. Close linkage between the *Cf-2/Cf-5* and *Mi* resistance loci in tomato. Mol. Plant-Microbe Interact. 6:341-347.

de Vetten, N., Wolters, A. M., Raemakers, K., van der Meer, I., ter Stege, R., Heeres, E., Heeres, P., and Visser, R. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nature biotechnology 21:439-442.

Dear, S., and Staden, R. 1991. A sequence assembly and editing program for efficient management of large projects. Nucleic Acids Res. 19:3907-3911.

Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Ayliffe, M. A., and Ellis, J. G. 2004. The Melampsora lini *AvrL567* avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell 16:755-768.

Dodds, P. N., Lawrence, G. J., and Ellis, J. G. 2001. Contrasting modes of evolution acting on the complex *N* locus for rust resistance in flax. The Plant Journal 27:439-453.

Doganlar, S., Frary, A., Daunay, M. C., Lester, R. N., and Tanksley, S. D. 2002. Conservation of gene function in the Solanaceae as revealed by comparative mapping of domestication traits in eggplant. Genetics . Aug 161:1713-1726.

Dou, D., Kale, S. D., Wang, X., Chen, Y., Wang, Q., Wang, X., Jiang, R. H. Y., Arredondo, F. D., Anderson, R. G., Thakur, P. B., McDowell, J. M., Wang, Y., and Tyler, B. M. 2008. Conserved

C-Terminal Motifs Required for Avirulence and Suppression of Cell Death by Phytophthora sojae effector Avr1b. Plant Cell 20:1118-1133.

Drenth, A., Tas, I. C. Q., and Govers, F. 1994. DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. European Journal of Plant Pathology 100:97-107.

Dumas, B., Bottin, A., Gaulin, E., and Esquerré-Tugayé, M.-T. 2008. Cellulose-binding domains: cellulose associated-defensive sensing partners? TRENDS in plant science 13:160-164.

Ellis, J. G., Lawrence, G. J., and Dodds, P. N. 2007. Further analysis of gene-for-gene disease resistance specificity in flax. Molecular Plant Pathology 8:103-109.

Ellis, J. G., Lawrence, G. J., Luck, J. E., and Dodds, P. N. 1999. Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell 11:495-506.

EPipe 1.0: a pipeline for discovery of functional differences in proteins originating from alternative splicing, pan-genome variation or protein family evolution. In preparation by several groups from the BioSapiens Network of Excellence, 2008.

Ernst, K., Kumar, A., Kriseleit, D., Kloos, D. U., Phillips, M. S., and Ganal, M. W. 2002. The broad-spectrum potato cyst nematode resistance gene (*Hero*) from tomato is the only member of a large gene family of NBS-LRR genes with an unusual amino acid repeat in the LRR region. Plant J. 31:127-136.

Ewing, E. E., and Simko 2000. Genetic mapping from field tests of qualitative and quantitative resistance to Phytophthora infestans in a population derived from Solanum tuberosum and Solanum berthaultii. Molecular breeding 6:25-36.

Flor, H. H. 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. 78:275-298.

Foster, S. J., Park, T.-H., Pel, M., Brigneti, G., Śliwka, J., Jagger, L., van der Vossen, E., and Jones, J. D. G. 2009. Rpi-vnt1.1, a Tm-22 Homolog from Solanum venturii, Confers Resistance to Potato Late Blight. Molecular Plant-Microbe Interactions 22:589-600.

Friedman, A. R., and Baker, B. J. 2007. The evolution of resistance genes in multi-protein plant resistance systems. Curr. Opin. Genetics Dev. 17:493-499.

Fry, W. 2008. *Phytophthora infestans*: the plant (and *R* gene) destroyer. Molecular plant pathology 9:385-402.

Fry, W. E., Goodwin, S. B., Matuszak, J. M., Spielman, L. J., Milgroom, M. G., and Drenth, A. 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30:107-130.

Gaulin, E., Drame, N., Lafitte, C., Torto Alalibo, T., Martinez, Y., Ameline Torregrosa, C., Khatib, M., Mazarguil, H., Villalba Mateos, F., Kamoun, S., Mazars, C., Dumas, B., Bottin, A., Esquerre Tugaye, M. T., and Rickauer, M. 2006. Cellulose binding domains of a Phytophthora cell wall protein are novel pathogen-associated molecular patterns. Plant cell 18:1766-1777.

Gebhardt, C., and Valkonen, J. P. 2001. Organization of genes controlling disease resistance in the potato genome. Annu. Rev. Phytopathol. 39:79-102.

Gebhardt, C. 1994. RFLP mapping in potato of qualitative and quantitative genetic loci conferring resistance to potato pathogens. American Potato Journal 71:339-345.

Gebhardt, C., Ritter, E., Barone, A., Debener, T., Walkemeier, B., Schachtschnabel, U., Kaufmann, H., Thompson, R. D., Bonierbale, M. W., Ganal, M. W., Tanksley, S. D., and Salamini, F. 1991. RFLP maps of potato and their alignment with the homologous tomato genome. Theoretical and Applied Genetics 83:49-57

Goodwin, S. B., and Drenth, A. 1997. Origin of the A2 mating type of *Phytophthora infestans* outside Mexico. Phytopathology 87:992-999.

Graham, K. M. 1965. Experimental hybridization in certain diploid mexican solanum species. Euphytica 14:113-119.

Grant, M. R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R. W., and Dangl, J. L. 1995. Structure of the *Arabidopsis Rpm1* gene enabling dual-specificity disease resistance. Science 269:843-846.

Grube, R. C., Blauth, J. R., Arnedo, M. S., Caranta, C., and Jahn, M. K. 2000. Identification and comparative mapping of a dominant potyvirus resistance gene cluster in Capsicum. Theor. Appl. Genet. 101:852-859.

Ghislain, M., and Trognitz 2001. Genetic loci associated with field resistance to late blight in offspring of Solanum phureja and S. tuberosum grown under short-day conditions. Theoretical and applied genetics 103:433-442.

Hall, S. A., Allen, R. L., Baumber, R. E., Baxter, L. A., Fisher, K., Bittner-Eddy, P. D., Rose, L. E., Holub, E. B., and Beynon, J. 2009. Maintenance of genetic variation in plants and pathogens involves complex networks of gene-for-gene interactions. Mol. Plant Pathol. 10:449 - 457.

Hammond-Kosack, K. E., and Kanyuka, K. 2007. Resistance genes (R genes) in plants. Encyclopedia of Life Sciences.

Hanson, P., Sitathani, K., Sadashiva, A., Yang, R.., Graham, E., and Ledesma, D. 2007. Performance of Solanum habrochaites LA1777 introgression line hybrids for marketable tomato fruit yield in Asia. Euphytica 158:167-178.

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M. V., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., Bollmann, S. R., Bos, J. I. B., Bulone, V., Cai, G., Cakir, C., Carrington, J. C., Chawner, M., Conti, L., Costanzo, S., Ewan, R., Fahlgren, N., Fischbach, M. A., Fugelstad, J., Gilroy, E. M., Gnerre, S., Green, P. J., Grenville-Briggs, L. J., Griffith, J., Grunwald, N. J., Horn, K., Horner, N. R., Hu, C.-H., Huitema, E., Jeong, D.-H., Jones, A. M. E., Jones, J. D. G., Jones, R. W., Karlsson, E. K., Kunjeti, S. G., Lamour, K., Liu, Z., Ma, L., MacLean, D., Chibucos, M. C., McDonald, H., McWalters, J., Meijer, H. J. G., Morgan, W., Morris, P. F., Munro, C. A., Oâ€™Neill, K., Ospina-Giraldo, M., Pinzon, A., Pritchard, L., Ramsahoye, B., Ren, Q., Restrepo, S., Roy, S., Sadanandom, A., Savidor, A., Schornack, S., Schwartz, D. C., Schumann, U. D., Schwessinger, B., Seyer, L., Sharpe, T., Silvar, C., Song, J., Studholme, D. J., Sykes, S., Thines, M., van de Vondervoort, P. J. I., Phuntumart, V., Wawra, S., Weide, R., Win, J., Young, C., Zhou, S., Fry, W., Meyers, B. C., van West, P., Ristaino, J., Govers, F., Birch, P. R. J., Whisson, S. C., Judelson, H. S., and Nusbaum, C. 2009. Genome sequence and analysis of the Irish potato famine pathogen Phytophthora infestans. Nature 461:393-398.

Haverkort, A. J., Struik, P. C., Visser, R. G. F., and Jacobsen, E. 2009. Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. Potato Res. 52:249-264.

Hawkes, J. G. 1994. Origins of cultivated potatoes and species relationships. Pages 3-42. In: Potato Genetics. Bradshaw and MacKay, eds.

Hawkes, J. G. 1978. History of the potato. In Harris, P.M. The potato crop: the scientific basis for improvement Ed. P. M. harris. Chapman and Hall. London:1-69.

Hawkes, J. G., and Hjerting, J. P. 1969. The Potatoes of Argentina, Brazil, Paraguay and Uruguay Oxford University Press.

Heilersig, H. J. B., Loonen, A., Bergervoet, M., Wolters, A. M. A., and Visser, R. G. F. 2006. Post-transcriptional gene silencing of *GBSSI* in potato: Effects of size and sequence of the inverted repeats. Plant Mol. Biol. 60:647-662.

Hein, I., Birch, P., Danan, S., Lefebvre, V., Achieng Odeny, D., Gebhardt, C., Trognitz, F., and Bryan, G. 2009a. Progress in Mapping and Cloning Qualitative and Quantitative Resistance Against Phytophthora infestans in Potato and Its Wild Relatives. Potato Research 52:215-227.

Hein, I., Gilroy, E. M., Armstrong, M. R., and Birch, P. R. J. 2009b. The zig-zag-zig in oomycete–plant interactions. Molecular and plant pathology 10:547-562.

Hein, I., McLean, K., Chalhoub, B., and Bryan, G. J. 2007. Generation and Screening of a BAC Library from a Diploid Potato Clone to Unravel Durable Late Blight Resistance on Linkage Group IV. *International Journal of Plant Genomics* Vol 2007 Article ID 51421:5 pages doi:10.1155/2007/51421.

Helgeson, J. P., Pohlman, J. D., Austin, S., Haberlach, G. T., Wielgus, S. M., Ronis, D., Zambolim, L., Tooley, P., McGrath, J. M., James, R. V., and Stevenson, W. R. 1998. Somatic hybrids between *Solanum bulbocastanum* and potato: a new source of resistance to late blight. Theor. Appl. Genet. 96:738-742.

Henk, A. D., Warren, R. F., and Innes, R. W. 1999. A New Ac-Like Transposon of Arabidopsis Is Associated With a Deletion of the RPS5 Disease Resistance Gene. Genetics 151:1581-1589.

Hermsen, J. G. T., and Ramanna, M. S. 1973. Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. Euphytica 22:457-466.

Hijmans, R. J., Gavrilenko, T., Stephenson, S., Bamberg, J., Salas, A., and Spooner, D. M. 2007. Geographical and environmental range expansion through polyploidy in wild potatoes (*Solanum* section *Petota*). Global Ecol. Biogeography 16:485-495.

Holub, E. B. 2001. The arms race is ancient history in Arabidopsis, the wildflower. Nat. Rev. Genet. 2:516-527.

Huang, S. 2005. Discovery and characterization of the major late blight resistance complex in potato: genomic structure, functional diversity, and implications. PhD thesis. Wageningen University.

Huang, S., van der Vossen, E. A. G., Kuang, H., Vleeshouwers, V. G. A. A., Zhang, N., Borm, T. J. A., van Eck, H. J., Baker, B., Jacobsen, E., and Visser, R. G. F. 2005a. Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. The plant journal 42:251-261.

Huang, S., Vleeshouwers, V. G. A. A., Visser, R. G. F., and Jacobsen, E. 2005b. An accurate in vitro assay for high-throughput disease testing to *Phytophthora infestans* in potato. Plant Disease 89:1263-1267.

Huang, S., Vleeshouwers, V. G. A. A., Werij, J. S., Hutten, R. C. B., Eck, H. J. v., Visser, R. G. F., and Jacobsen, E. 2004. The R3 resistance to Phytophthora infestans in potato is conferred by two closely linked R genes with distinct specificities. Molecular Plant Microbe Interactions 17:428-435.

Hwang, C. F., and Williamson, V. M. 2003. Leucine-rich repeat-mediated intramolecular interactions in nematode recognition and cell death signaling by the tomato resistance protein Mi. Plant journal 34:585-593.

Hwang, C. F., Bhakta, A. V., Truesdell, G. M., Pudlo, W. M., and Williamson, V. M. 2000. Evidence for a role of the N terminus and leucine-rich repeat region of the Mi gene product in regulation of localized cell death. Plant Cell 12:1319-1329.

Hulbert, S. H., Webb, C. A., Smith, S. M., and Sun, Q. 2001. Resistance gene complexes: Evolution and Utilization. Annual Review of Phytopathology 39:285-312.

Jacobs, M., Vosman, B., Vleeshouwers, V., Visser, R., Henken, B., and van den Berg, R. 2010. A novel approach to locate Phytophthora infestans resistance genes on the potato genetic map. Theo. Appl.Genet. DOI 10.1007/s00122-009-1199-7

Jacobs, M. M. J., van den Berg, R. G., Vleeshouwers, V. G. A. A., Visser, M., Mank, R., Sengers, M., Hoekstra, R., and Vosman, B. 2008. AFLP analysis reveals a lack of phylogenetic structure within *Solanum* section *Petota*. BMC Evol. Biol. 18479504 8:145

Jacobsen, E., and Schouten, H. J. 2007. Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends in Biotechnology 25:219-223.

Janies, D., Hill, A. W., Guralnick, R., Habib, F., Waltari, E., and Wheeler, W. C. 2007. Genomic Analysis and Geographic Visualization of the Spread of Avian Influenza (H5N1). Syst. Biol. 56:321-329.

Jiang, R. H. Y., Tripathy, S., Govers, F., and Tyler, B. M. 2008. RXLR effector reservoir in two Phytophthora species is dominated by a single rapidly evolving superfamily with more than 700 members. Proceedings of the National Academy of Sciences 105:4874-4879.

Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. Nature 444:323-329.

Jones, D. T. 1999. Protein secondary structure prediction based on position-specific scoring matrices. Journal of Molecular Biology 292:195-202.

Kamoun, S. 2006. A Catalogue of the Effector Secretome of Plant Pathogenic Oomycetes. Annu. Rev. Phytopathol. 44:41-60.

Kamoun, S., van West, P., Vleeshouwers, V. G. A. A., de Groot, K. E., and Govers, F. 1998. Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* is mediated by the recognition of the elicitor protein INF1. Plant Cell 10:1413-1425.

Kardolus, J. P., Eck, H. J., and Berg, R. G. 1998. The potential of AFLPs in biosystematics: A first application inSolanum taxonomy(Solanaceae). Plant Systematics and Evolution 210:87-103.

Kim, Y. J., Lin, N. C., and Martin, G. B. 2002. Two distinct *Pseudomonas* effector proteins interact with the Pto kinase and activate plant immunity. Cell 109:589-598.

Kobe, B., and Deisenhofer, J. 1995. Proteins with leucine-rich repeats. Current Opinion in Structural Biology 5:409-416.

Korves, T., and Bergelson, J. 2004. A Novel Cost of R Gene Resistance in the Presence of Disease. Amer. Nat. 163:489-504.

Kramer, L. C., Choudoir, M. J., Wielgus, S. M., Bhaskar, P. B., and Jiang, J. 2009. Correlation Between Transcript Abundance of the RB Gene and the Level of the RB-Mediated Late Blight Resistance in Potato. Mol. Plant-Microbe Interact. 22:447-455.

Kruijt, M., Kip, D. J., Joosten, M. H. A. J., Brandwagt, B. F., and de Wit, P. J. G. M. 2005. The *Cf-4* and *Cf-9* resistance genes against *Cladosporium fulvum* are conserved in wild tomato species. Mol. Plant-Microbe Interact. 18:1011-1021.

Kuang, H., Woo, S-S., Meyers, B. C., Nevo, E. and Michelmore, R. W. 2004. Multiple genetic processes result in heterogeneous rates of evolution within the major cluster disease resistance genes in lettuce. Plant Cell 16:2870-2894.

Kuhl, J. C., and Hanneman 2001. Characterization and mapping of Rpi1, a late-blight resistance locus from diploid (1EBN) Mexican Solanum pinnatisectum. Molecular genetics and genomics 265:977-985.

Lanfermeijer, F. C., Dijkhuis, J., Sturre, M. J. G., de Haan, P., and Hille, J. 2003. Cloning and characterization of the durable tomato mosaic virus resistance gene Tm-22 from Lycopersicon esculentum. Plant Molecular Biology 52:1039-1051.

Lazo, G. R., Stein, P. A., and Ludwig, R. A. 1991. A DNA Transformation-Competent Arabidopsis Genomic Library in Agrobacterium. Nat. Biotech. 9:963-967.

Leonards-Schippers, C., Gieffers, W., Schafer Pregl, R., Ritter, E., Knapp, S. J., Salamini, F., and Gebhardt, C. 1994. Quantitative resistance to *Phytophthora infestans* in potato: a case study for QTL mapping in an allogamous plant species. Genetics 137:67-77.

Leonards-Schippers, C., Gieffers, W., Salamini, F., and Gebhardt, C. 1992. The *R1* gene conferring race-specific resistance to *Phytophthora infestans* in potato is located on potato chromosome V. Molecular and General Genetics 233:278-283.

Li, X., Eck, H. J. v., Rouppe van der Voort, J. N. A. M., Huigen, D. J., Stam, P., and Jacobsen, E. 1998. Autotetraploids and genetic mapping using common AFLP markers: the *R2* allele conferring resistance to *Phytophthora infestans* mapped on potato chromosome 4. Theoretical and applied genetics 96:1121-1128.

Lokossou, A. A., Park, T. H., van Arkel, G., Arens, M., Ruyter-Spira, C., Morales, J., Whisson, S. C., Birch, P. R. J., Visser, R. G. F., Jacobsen, E., and van der Vossen, E. A. G. 2009. Exploiting knowledge of *R/Avr* genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. Mol. Plant-Microbe Interact. 22:630-41.

Luck, J. E., Lawrence, G. J., Dodds, P. N., Shepherd, K. W., and Ellis, J. G. 2000. Regions outside of the leucine-rich repeats of flax rust resistance proteins play a role in specificity determination. Plant Cell 12:1367-1377.

Malcolmson, J. 1969. Factors involved in resistance to blight (*Phytophthora infestans* (Mont.) de Bary) in potatoes and assessment of resistance using detached leaves. Annals of Applied Biology 64:461-468.

Malcolmson, J. F., and Black, W. 1966. New *R* genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. Euphytica 15:199-203.

Mauricio, R., Stahl, E. A., Korves, T., Tian, D. C., Kreitman, M., and Bergelson, J. 2003. Natural selection for polymorphism in the disease resistance gene *Rps2* of *Arabidopsis thaliana*. Genetics 163:735-746.

McDonald, B. A., and Linde, C. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. Annual Review Of Phytopathology 40:349-379.

McDowell, J. M., and Simon, S. A. 2006. Recent insights into R gene evolution. Mol. Plant Pathol. 7:437-448.

McGillivary, J. H., and Clemente, L. J. 1956. Effect of tomato size on solids content. Proc. Am. Soc. Hprt. Sci. 68: 466-469.

McHale, L., Tan, X., Koehl, P., and Michelmore, R. 2006. Plant NBS-LRR proteins: adaptable guards. Genome Biology 7:212.

Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., and Young, N. D. 1999. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J. 20:317-332.

Meyer, R. C., Milbourne, D., Hackett, C. A., Bradshaw, J. E., McNichol, J. W., and Waugh, R. 1998a. Linkage analysis in tetraploid potato and association of markers with quantitative resistance to late blight (Phytophthora infestans). Molecular and General Genetics MGG 259:150-160.

Meyers, B. C., Chin, D. B., Shen, K. A., Sivaramakrishnan, S., Lavelle, D. O., Zhang, Z., and Michelmore, R. W. 1998b. The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10:1817-1832.

Micheletto, S., Boland, R., and Huarte, M. 2000. Argentinian wild diploid Solanum species as sources of quantitative late blight resistance. Theor. Appl.Genet. 101:902-906.

Michelmore, R., and Meyers, B. 1998. Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113 - 1130.

Milligan, S. B., Bodeau, J., Yaghoobi, J., Kaloshian, I., Zabel, P., and Williamson, V. M. 1998. The root knot nematode resistance gene *Mi* from tomato is a member of the leucine zipper, nucleotide binding, leucine-rich repeat family of plant genes. Plant Cell 10:1307-1319.

Moffett, P., Gad, L., and John, P. C. 2009. Mechanisms of Recognition in Dominant R Gene Mediated Resistance. Pages 1-33, 228-229. In: Advances in Virus Research. Academic Press.

Mondragon Palomino, M., and Meyers 2002. Patterns of positive selection in the complete NBS-LRR gene family of Arabidopsis thaliana. Genome research 12:1305-1315.

Morgan, W., and Kamoun, S. 2007. RXLR effectors of plant pathogenic oomycetes. Current opinion in microbiology 10:332-338.

Müller, K. O., and Black, W. 1952. Potato breeding for resistance to blight and virus diseases during the last hundred years. Zeitschrift für Pflanzenzüchtung 31:305-318.

Niederhauser, J., and Mills, W. 1953. Resistance of *Solanum* species to *Phytophthora infestans* in Mexico. Phytopathology 43:57.

Noel, L., Moores, T. L., van der Biezen, E. A., Parniske, M., Daniels, M. J., Parker, J. E., and Jones, J. D. G. 1999. Pronounced intraspecific haplotype divergence at the *RPP5* complex disease resistance locus of *Arabidopsis*. Plant Cell 11:2099-2111.

Nombela, G., Williamson, V. M., and MuÃ±iz, M. 2003. The Root-Knot Nematode Resistance Gene Mi-1.2 of Tomato Is Responsible for Resistance Against the Whitefly Bemisia tabaci. Molecular Plant-Microbe Interactions 16:645-649.

Oberhagemann, P., Chatot-Balandras, C., Schäfer-Pregl, R., Wegener, D., Palomino, C., Salamini, F., Bonnel, E., and Gebhardt, C. 1999. A genetic analysis of quantitative resistance to late blight in potato: towards marker-assisted selection. Molecular Breeding 5:399-415.

Oh, S.-K., Young, C., Lee, M., Oliva, R., Bozkurt, T. O., Cano, L. M., Win, J., Bos, J. I. B., Liu, H.-Y., van Damme, M., Morgan, W., Choi, D., Van der Vossen, E. A. G., Vleeshouwers, V. G. A. A., and Kamoun, S. 2009. In Planta Expression Screens of Phytophthora infestans RXLR Effectors Reveal Diverse Phenotypes, Including Activation of the Solanum bulbocastanum Disease Resistance Protein Rpi-blb2. Plant Cell 21:2928-2947.

Oosumi, T., Rockhold, D., Maccree, M., Deahl, K., McCue, K., and Belknap, W. 2009. Gene Rpi-bt1 from Solanum bulbocastanum Confers Resistance to Late Blight in Transgenic Potatoes. American journal of potato research 86:456-465.

Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., Zamir, D., and Fluhr, R. 1997. The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. Plant Cell 9:521-32.

Park, T., Gros, J., Sikkema, A., Vleeshouwers, V. G. A. A., Muskens, M., Allefs, S., Jacobsen, E., Visser, R. G. F., and Vossen, E. A. G. v. d. 2005a. The late blight resistance locus *Rpi-blb3* from *Solanum bulbocastanum* belongs to a major late blight R gene cluster on chromosome 4 of potato. Molecular Plant Microbe Interactions 18:722-729.

Park, T. H., Vleeshouwers, V. G. A. A., Huigen, D. J., van der Vossen, E. A. G., van Eck, H. J., and Visser, R. G. F. 2005b. Characterization and high-resolution mapping of a late blight resistance locus similar to *R2* in potato. Theoretical and applied genetics 111:591-597.

Park, T. H., Vleeshouwers, V. G. A. A., Hutten, R. C. B., Eck, H. J. v., Vossen, E. v. d., Jacobsen, E., and Visser, R. G. F. 2005c. High-resolution mapping and analysis of the resistance locus *Rpi-abpt* against *Phytophthora infestans* in potato. Molecular Breeding 16:33-43.

Park, T., Vleeshouwers, V. G. A. A., Kim, J., Hutten, R. C. B., and Visser, R. G. F. 2005. Dissection of foliage and tuber late blight resistance in mapping populations of potato. Euphytica . 2005; 143:75-83

Parniske, M., Hammond-Kosack, K. E., Golstein, C., Thomas, C. M., Jones, D. A., Harrison, K., Wulff, B. B., and Jones, J. D. 1997. Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91:821-32.

Pel, M. 2010. Mapping, Isolation and Characterization of Genes Responsible for Late Blight Resistance .PhD thesis, Wageningen UR, Wageningen.

Pel, M. A., Foster, S. J., Park, T.-H., Rietman, H., van Arkel, G., Jones, J. D. G., Van Eck, H. J., Jacobsen, E., Visser, R. G. F., and Van der Vossen, E. A. G. 2009. Mapping and Cloning of Late Blight Resistance Genes from Solanum venturii Using an Interspecific Candidate Gene Approach. Molecular Plant-Microbe Interactions 22:601-615.

Pendinen, G., Gavrilenko, T., Jiang, J. M., and Spooner, D. M. 2008. Allopolyploid speciation of the Mexican tetraploid potato species *Solanum stoloniferum* and *S. hjertingii* revealed by genomic in situ hybridization. Genome 51:714-720.

Pink, D. 2002. Strategies using genes for non-durable disease resistance. Euphytica 124:227-236.

Rairdan, G. J., and Moffett, P. 2006. Distinct domains in the ARC region of the potato resistance protein Rx mediate LRR binding and inhibition of activation. Plant cell 18:2082-2093.

Rauscher, G. M., Smart, C. D., Simko, I., Bonierbale, M., Mayton, H., Greenland, A., and Fry, W. E. 2006. Characterization and mapping of *RPi-ber*, a novel potato late blight resistance gene from *Solanum berthaultii*. Theoretical and applied genetics 112:674-687.

Rehmany, A. P., Gordon, A., Rose, L. E., Allen, R. L., Armstrong, M. R., Whisson, S. C., Kamoun, S., Tyler, B. M., Birch, P. R. J., and Beynon, J. L. 2005. Differential recognition of highly divergent downy mildew avirulence gene alleles by *RPP1* resistance genes from two Arabidopsis lines. Plant Cell 17:1839-1850.

Rodriguez, A., and Spooner, D. M. 1997. Chloroplast DNA Analysis of Solanum bulbocastanum and S. cardiophyllum, and Evidence for the Distinctiveness of S. cardiophyllum Subsp. ehrenbergii (Sect. Petota). Syst. Botany 22:31-43.

Ronald, P. C., Salmeron, J. M., Carland, F. M., and Staskawicz, B. J. 1992. The cloned avirulence gene *Avrpto* induces disease resistance in tomato cultivars containing the *Pto* resistance gene. J. Bacteriol.174:1604-1611.

Rose, L. E., Bittner-Eddy, P. D., Langley, C. H., Holub, E. B., Michelmore, R. W., and Beynon, J. L. 2004. The maintenance of extreme amino acid diversity at the disease resistance gene, *RPP13*, in *Arabidopsis thaliana*. Genetics 166:1517-1527.

Rossi, M., Goggin, F. L., Milligan, S. B., Kaloshian, I., Ullman, D. E., and Williamson, V. M. 1998. The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proceedings of the National Academy of Sciences of the United States of America 95:9750-9754.

Rouppe van der Voort, J., Kanyuka, K., van der Vossen, E., Bendahmane, A., Mooijman, P., Klein-Lankhorst, R., Stiekema, W., Baulcombe, D., and Bakker, J. 1999. Tight physical linkage of the nematode resistance gene *Gpa2* and the virus resistance gene *Rx* on a single segment introgressed from the wild species *Solanum tuberosum subsp. andigena* CPC 1673 into cultivated potato. Mol. Plant-Microbe Interact. 12:197-206.

Sambrook, J., Fritsch, E. F., Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual (Second Edition).

Sandbrink, J. M., Colon, L. T., Wolters, P. J. C. C., and Stiekema, W. J. 2000. Two related genotypes of *Solanum microdontum* carry different segregating alleles for field resistance to *Phytophthora infestans*. Molecular Breeding 6:215-225.

Savenkov, E. I., Germundsson, A., Zamyatnin, A. A., Sandgren, M., and Valkonen, J. P. T. 2003. Potato mop-top virus: the coat protein-encoding RNA and the gene for cysteine-rich protein are dispensable for systemic virus movement in Nicotiana benthamiana. Journal of general virology 84 Part 4:1001-1005.

Schaart, J. G., Krens, F. A., Pelgrom, K. T. B., Mendes O., and Rouwendal, G. J. A. 2004. Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnology Journal 2:233-240.

Schornack, S., Ballvora, A., Gürlebeck, D., Peart, J., Ganal, M., Baker, B., Bonas, U., and Lahaye, T. 2004. The tomato resistance protein Bs4 is a predicted non-nuclear TIR-NB-LRR protein that mediates defense responses to severely truncated derivatives of AvrBs4 and overexpressed AvrBs3. Plant J. 37:46-60.

Schouten, H. J., and Jacobsen, E. 2008. Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends in Plant Science 13:260-261.

Shan, W. X., Cao, M., Leung, D., and Tyler, B. M. 2004. The *Avr1b* locus of *Phytophthora sojae* encodes an elicitor and a regulator required for avirulence on soybean plants carrying resistance gene *Rps1b*. Molecular Plant Microbe Interactions 17:394-403.

Simko, I. 2002. Comparative analysis of quantitative trait loci for foliage resistance to *Phytophthora infestans* in tuber-bearing Solanum species. Am. J. Potato Res. 79:125-132.

Simons, G., Groenendijk, J., Wijbrandi, J., Reijans, M., Groenen, J., Diergaarde, P., Van der Lee, T., Bleeker, M., Onstenk, J., de Both, M., Haring, M., Mes, J., Cornelissen, B., Zabeau, M., and Vos, P. 1998. Dissection of the *Fusarium I2* gene cluster in tomato reveals six homologs and one active gene copy. Plant Cell 10:1055-68.

Skelsey, P., Kessel, G. J. T., Holtslag, A. A. M., Moene, A. F., and van der Werf, W. 2009. Regional spore dispersal as a factor in disease risk warnings for potato late blight: A proof of concept. Agricultural and Forest Meteorology 149:419-430.

Sliwka, J., Jakuczun, H., Lebecka, R., Marczewski, W., Gebhardt, C., and Zimnoch Guzowska, E. 2006. The novel, major locus Rpi-phu1 for late blight resistance maps to potato chromosome IX and is not correlated with long vegetation period. Theoretical and applied genetics 113:685-695.

Smilde, W. D., and Brigneti 2005. *Solanum mochiquense* chromosome IX carries a novel late blight resistance gene *Rpi-moc1*. Theoretical and applied genetics 110:252-258.

Song, J., Bradeen, J. M., Naess, S. K., Raasch, J. A., Wielgus, S. M., Haberlach, G. T., Liu, J., Kuang, H., Austin-Phillips, S., Buell, C. R., Helgeson, J. P., and Jiang, J. 2003. Gene RB cloned from Solanum bulbocastanum confers broad spectrum resistance to potato late blight. Proc Natl Acad Sci U S A 100:9128-33.

Spooner, D. M., Jansky, S. H., and Simon, R. 2009. Tests of Taxonomic and Biogeographic Predictivity: Resistance to Disease and Insect Pests in Wild Relatives of Cultivated Potato. Crop Sci. 49:1367-1376.

Spooner, D. M., van den Berg, R. G., Rodrigues, A., Bamberg, J. B., Hijmans, R. J., and Lara-Cabrera, S. I. 2004. Wild potatoes (*Solanum* section *Petota*; *Solanaceae*) of North and Central America. Amer. Soc. Plant Tax. Ann Arbor, Michigan.

Spooner, D. M., Sytsma, K. J., and Conti, E. 1991. Chloroplast DNA Evidence for Genome Differentiation in Wild Potatoes (Solanum Sect. Petota: Solanaceae). American Journal of Botany 78:1354-1366.

Stahl, E. A., Dwyer, G., Mauricio, R., Kreitman, M., and Bergelson, J. 1999. Dynamics of disease resistance polymorphism at the *Rpm1* locus of *Arabidopsis*. Nature 400:667-71.

Tan, M. Y. A., Hutten, R. C. B., Celis, C., Park, T. H., Niks, R. E., Visser, R. G. F., and van Eck, H. J. 2008. The *Rpi-mcd1* locus from Solanum microdontum involved in resistance to *Phytophthora infestans*, causing a delay in infection, maps on potato chromosome 4 in a cluster of NBS-LRR genes. Molecular Plant-Microbe Interactions 21:909-918.

Thomas, C. M., Jones, D. A., Parniske, M., Harrison, K., Balint-Kurti, P. J., Hatzixanthis, K., and Jones, J. D. 1997. Characterization of the tomato Cf-4 gene for resistance to Cladosporium fulvum identifies sequences that determine recognitional specificity in Cf-4 and Cf-9. Plant Cell 9:2209-24.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Tian, M., Huitema, E., da Cunha, L., Torto Alalibo, T., and Kamoun, S. 2004. A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B. Journal of Biological Chemistry. 2004; 279:26370-26377.

Toxopeus, H. J. 1964. Treasure-digging for blight resistance in potatoes. Euphytica 13:206-222.

Tress, M. L., Martelli, P. L., Frankish, A., Reeves, G. A., Wesselink, J. J., Yeats, C., Ã“lason, P. l. Ä. s. l., Albrecht, M., Hegyi, H., Giorgetti, A., Raimondo, D., Lagarde, J., Laskowski, R. A., LÃ³pez, G., Sadowski, M. I., Watson, J. D., Fariselli, P., Rossi, I., Nagy, A., Kai, W., StÃ¸rling, Z., Orsini, M., Assenov, Y., Blankenburg, H., Huthmacher, C., RamÃ-rez, F., Schlicker, A., Denoeud, F., Jones, P., Kerrien, S., Orchard, S., Antonarakis, S. E., Reymond, A., Birney, E., Brunak, S. r., Casadio, R., Guigo, R., Harrow, J., Hermjakob, H., Jones, D. T., Lengauer, T., A. Orengo, C., Patthy, L. s., Thornton, J. M., Tramontano, A., and Valencia, A. 2007. The implications of alternative splicing in the ENCODE protein complement. Proceedings of the National Academy of Sciences 104:5495-5500.

Untergasser, A. 2006a. Cloning - Multiple Gateway Reaction II without a Mastermix. Untergasser's Lab. (access to the page: 11/2006) <http://www.untergasser.de/lab/protocols/lr_multiple_gateway_reaction_ii_v1_0.htm>.

Untergasser, A. 2006b. Cloning - Gateway BP-Reaction II. Untergasser's Lab. (access to the page: 11/2006) <http://www.untergasser.de/lab/protocols/bp_gateway_reaction_ii_v1_0.htm>.

van der Biezen, E. A., and Jones, J. D. 1998. The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Current Biology 8:R226-7.

van der Fits, L., Deakin, E. A., Hoge, J. H. C., and Memelink, J. 2000. The ternary transformation system: constitutive virG on a compatible plasmid dramatically increases Agrobacterium-mediated plant transformation. Plant Mol. Biol. 43:495-502.

van der Hoeven, R., Ronning, C., Giovannoni, J., Martin, G., and Tanksley, S. 2002. Deductions about the Number, Organization, and Evolution of Genes in the Tomato Genome Based on Analysis of a Large Expressed Sequence Tag Collection and Selective Genomic Sequencing. Plant Cell 14:1441-1456.

van der Lee, T., De Witte, I., Drenth, A., Alfonso, C., and Govers, F. 1997. AFLP linkage map of the oomycete *Phytophthora infestans*. Fungal Genet. Biol. 21: 278-291.

van der Vossen, E. A. G., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., Pereira, A., and Allefs, S. 2005. The *Rpi-blb2* gene from *Solanum bulbocastanum* is an *Mi-1* gene homolog conferring broad-spectrum late blight resistance in potato. Plant Journal 44:208-222.

van der Vossen, E., Sikkema, A., Hekkert, B. L., Gros, J., Stevens, P., Muskens, M., Wouters, D., Pereira, A., Stiekema, W., and Allefs, S. 2003. An ancient R gene from the wild potato species *Solanum bulbocastanum* confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. Plant Journal 36:867-82.

van der Vossen, E. A. G., Rouppe van der Voort, J. N. A. M., Kanyuka, K., Bendahmane, A., Sandbrink, H., Baulcombe, D. C., Bakker, J., Stiekema, W. J., and Klein Lankhorst, R. M. 2000. Homologues of a single resistance-gene cluster in potato confer resistance to distinct pathogens: a virus and a nematode. Plant Journal 23:567-576.

van Engelen, F. A., Molthoff, J. W., Conner, A. J., Nap, J.-P., Pereira, A., and Stiekema, W. J. 1995. pBINPLUS: An improved plant transformation vector based on pBIN19. Transgenic Res. 4:288-290.

van Poppel, P.M.J.A, Jiang, R.H.J., Sliwka J., and Govers, F. 2009. Recognition of Phytophthora infestans Avr4 by potato R4 is triggered by C-terminal domains comprising W motifs. Molecular Plant Pathology 10:611-620.

van Poppel, P. M. J. A., Guo, J., van de Vondervoort, P. J. I., Jung, M. W. M., Birch, P. R. J., Whisson, S. C., and Govers, F. 2008. The Phytophthora infestans Avirulence Gene Avr4 Encodes an RXLR-dEER Effector. Molecular Plant-Microbe Interactions 21:1460-1470.

van West, P., Kamoun, S., van ' t Klooster, J. W., and Govers, F. 1999. Internuclear gene silencing in *Phytophthora infestans*. Mol. Cell 3:339-348.

Villamon, F. G., Spooner, D. M., Orrillo, M., Mihovilovich, E., Pérez, W., and Bonierbale, M. 2005. Late blight resistance linkages in a novel cross of the wild potato species Solanum paucissectum (series Piurana). TAG Theoretical and Applied Genetics 111:1201-1214.

Visker, M., Keizer, L. C. P., Budding, D. J., Van Loon, L. C., Colon, L. T., and Struik, P. C. 2003. Leaf position prevails over plant age and leaf age in reflecting resistance to late blight in potato. Phytopathology 93:666-674.

Vleeshouwers, V. G. A. A., and Rietman, H. 2009. *In planta* expression systems. In: Oomycete Genetics and Genomics. K. H. Lamour and S. Kamoun, eds. John Wiley & Sons, Hoboken, New Jersey. p455-475.

Vleeshouwers, V. G. A. A., Rietman, H., Krenek, P., Champouret, N., Young, C., Oh, S.-K., Wang, M., Bouwmeester, K., Vosman, B., Visser, R. G. F., Jacobsen, E., Govers, F., Kamoun, S., and Vossen, E. A. G. V. d. 2008. Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora Infestans* avirulence genes. PLoS ONE 3 (8):e2875.

Vleeshouwers, V. G. A. A., van Dooijeweert, W., Govers, F., Kamoun, S., and Colon, L. T. 2000. The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210:853-864.

Vleeshouwers, V. G. A. A., van Dooijeweert, W., Keizer, L. C. P., Sijpkes, L., Govers, F., and Colon, L. T. 1999. A laboratory assay for *Phytophthora infestans* resistance in various Solanum species reflects the field situation. Eur. J. Plant Pathol. 105:241-250.

Vos, P., Simons, G., Jesse, T., Wijbrandi, J., Heinen, L., Hogers, R., Frijters, A., Groenendijk, J., Diergaarde, P., Reijans, M., Fierens, O. J., De, B. M., Peleman, J., Liharska, T., Hontelez, J., and Zabeau, M. 1998. The tomato Mi-1 gene confers resistance to both root-knot nematodes and potato aphids. Nature Biotechnology 16:1365.

Wang, M., Allefs, S., van den Berg, R. G., Vleeshouwers, V. G. A. A., van der Vossen, E. A. G., and Vosman, B. 2008. Allele mining in *Solanum*: conserved homologues of *Rpi-blb1* are identified in *Solanum stoloniferum*. Theor. Appl. Genet. 116:933-943Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., Armstrong, M. R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I. K., Pritchard, L., and Birch, P. R. J. 2007. A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature 450:115-118.

Whisson, S. C., van derLee, T., Bryan, G. J., Waugh, R., Govers, F., and Birch, P. R. J. 2001. Physical mapping across an avirulence locus of *Phytophthora infestans* using a highly representative, large-insert bacterial artificial chromosome library. Molecular Genetics And Genomics. Oct 266:289-295.

Win, J., Morgan, W., Bos, J., Krasileva, K. V., Cano, L. M., Chaparro-Garcia, A., Ammar, R., Staskawicz, B. J., and Kamoun, S. 2007. Adaptive Evolution Has Targeted the C-Terminal Domain of the RXLR Effectors of Plant Pathogenic Oomycetes. Plant Cell 19:2349-2369.

Wulff, B. B. H., Heese, A., Tomlinson-Buhot, L., Jones, D. A., de la Pena, M., and Jones, J. D. G. 2009. The Major Specificity-Determining Amino Acids of the Tomato Cf-9 Disease Resistance Protein Are at Hypervariable Solvent-Exposed Positions in the Central Leucine-Rich Repeats. Molecular Plant-Microbe Interactions 22:1203-1213.

Yu, W., Lamb, J. C., Han, F., and Birchler, J. A. 2006. Telomere-mediated chromosomal truncation in maize. Proceedings of the National Academy of Sciences 103:17331-17336.

Zhou, B., Qu, S., Liu, G., Dolan, M., Sakai, H., Lu, G., Bellizzi, M., and Wang, G.-L. 2006. The Eight Amino-Acid Differences Within Three Leucine-Rich Repeats Between Pi2 and Piz-t Resistance Proteins Determine the Resistance Specificity to Magnaporthe grisea. Molecular Plant-Microbe Interactions 19:1216-1228.

Zhu, S., Zhu, J., LI, Y., Nijenhuis, M., Bergervoet, M., Rietman, H., and Jacobsen, E.. 2010. Broad Spectrum Resistance from *Rpi-blb1* Homologous *R*-genes Has Been Broken by *Phytophthora infestans* Isolates Collected from *Solanum stoloniferum*. Acta Horticulturae Sinica, 37(2): 241–246

SUMMARY

Potato is consumed worldwide and represents the fourth most important staple food crop after rice and wheat. Potato cultivars display a large variety of color, shape, taste, cooking properties and starch content but are all derived from the same species; *Solanum tuberosum*. Potato breeding is an economic important activity for international breeding companies, but also plays an important role in breaking the circle of poverty for small farmers. In the Andean region, most farmers use many different potato genotypes combined with farming practices transmitted orally over thousands of years.

The most prominent menace to potato production is Late Blight caused by the oomycete *Phytophthora infestans* which destroys leaves, stems and tubers. Differences of breeding methods between the potato grown in South America and in the rest of the world are related to differences in the consequences of Late Blight infection. In the 19^{th,} century, entire potato fields in Ireland were devastated while in South America *P. infestans* proliferation was readily inhibited. This difference is found in the biodiversity reserve such as that of the Chiloé archipelago in Chile where local small farmers cultivate about 200 varieties of native potato. Obviously, the genetic diversity of cultivated native potato acts as a shield against this versatile pathogen. Inspired by this model to solve the problems raised by the extensive use of potato monoculture, large scale growers and breeders need to maintain genetic diversity in the European staple food crops.

In exploring the South American native potato collection, *Solanum demissum* and later on *Solanum bulbocastanum* appeared to be a source of resistance genes (*Rpi*) to *P. infestans*. The *S. demissum Rpi* genes were transmitted to potato breeding clones by traditional introgression breeding. However the fading of their ability in providing effective resistance against Late Blight infection was witnessed within a decade. In the pursuit to provide a hopefully more durable protection in existing potato cultivars, breeding scientists proposed to directly introduce South American native potato *Rpi* genes in modern potato varieties by using a so-called cisgenic approach. This is in contrast to transgenic plants which can contain genes which have originated from non related genera or even different kingdoms. Breeding of cisgenic plants is on its way to public acceptance because of its inherent resemblance to natural crossing and because efforts are made by the scientific community to explain the principles of cisgenesis.

Lessons were learned from the flexibility of *P. infestans* to overcome the effect of newly introduced *Rpi* genes and, therefore, efforts are still ongoing to discover and clone new *Rpi* genes from native

potatoes. With this in mind, a new family of *Rpi* genes represented by *Rpi-blb3, Rpi-abpt, R2, R2-like* and *Rpi-mcd1.1* were characterized in clones derived from *S. bulbocastanum, S. demissum, S. edinense* and *S. microdontum.* We accomplished in this research the physical isolation of these genes, the molecular characterization of their functionality and the allelic distribution in the Petota collection.

Rpi-blb3, Rpi-abpt, R2, R2-like and *Rpi-mcd1.1* belong to the potato linkage group IV and all contain signature sequences characteristic of LZ-NBS-LRR proteins. The closest known *R* gene so far is RPP13 from *Arabidopsis thaliana w*hich shares an amino-acid sequence similarity of 35%. The LRR domains of Rpi-blb3, Rpi-abpt, R2 and R2-like proteins are highly homologous, whilst LZ and NBS domains are more polymorphic with those of R2 being the most divergent. All four Rpi genes recognize the recently identified RXLR effector protein PiAVR2 which is secreted by *P.infestans* in the cytoplasm of plant cells during the infection process. Unlike *Rpi-blb3, Rpi-abpt, R2* and *R2-like*, the *S. microdontum* resistance gene *Rpi-mcd1.1* does not interact with PiAVR2 and provides a different resistance spectrum. *Rpi-mcd1.1* shares 90% nucleotide identity with *Rpi-blb3* and polymorphic nucleotides are mainly located in the LRR region.

The *S. bulbocastanum* haplotypes of *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* were discovered in several Mexican diploid as well as polyploid species closely related to *S. bulbocastanum*. These three resistance genes occurred in different combinations and frequencies in *S. bulbocastanum* accessions and their distribution is confined to Central America. A selected set of genotypes was tested for their response to the avirulence effectors IPIO-2, Avr-blb2 and Pi-Avr2, which interact with *Rpi-blb1, Rpi-blb2* and *Rpi-blb3*, respectively, as well as by disease assays with a diverse set of isolates. Using this approach some accessions could be identified that contain novel, yet unknown, Late Blight resistance factors in addition to the *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* genes

Analysis of the sequences obtained in different allele mining strategies suggests an evolution of the major late blight locus on linkage group IV through recombination and point mutations. The identification of the repeats and amino acids in the LRR domain which are specific for PiAVR2 recognition was possible by making use of the sequence information provided by the alleles.

Finally the results, described in this thesis, have been discussed in a potato/ *P. infestans* co-evolution context.

SAMENVATTINGl

Aardappel wordt wereldwijd gegeten en is het op twee na belangrijkst voedingsgewas na rijst en tarwe. Aardappelrassen laten een grootte verscheidenheid aan kleur, vorm, smaak, kookeigenschappen en zetmeelgehalten zien, maar zijn allemaal afkomstig van dezelfde soort: *Solanum tuberosum*. Aardappelveredeling is voor internationale georiënteerde veredelingsbedrijven een economisch belangrijke activiteit, maar het speelt ook een belangrijke rol bij het doorbreken van de armoedecirkel waar kleine boeren zich in bevinden. In de Andes gebruiken boeren verschillende aardappelgenotypen gecombineerd met landbouwtechnieken die al duizenden jaren mondeling worden doorgegeven.

De belangrijkste bedreiging voor de aardappelproductie is de Aardappelziekte die wordt veroorzaakt door de oömyceet *Phytophthora infestans* die bladeren, stengels en knollen aantast en verwoest. Verschillen tussen de manier waarop aardappel wordt gecultiveerd in Zuid-Amerika en de rest van de wereld houdt verband met de verschillen in de consequenties van *Phytophthora* infecties. In de 19de eeuw werden hele aardappelvelden in Ierland verwoest terwijl in Zuid-Amerika de verspreiding van *P. infestans* gemakkelijk werd tegengehouden. Dit verschil wordt verklaard door de grote hoeveelheid biodiversiteit zoals die te vinden is in de Chiloé archipelago in Chili waar de plaatselijke bevolking ongeveer 200 verschillende variëteiten van de inheemse aardappel verbouwen. De genetische diversiteit van de gecultiveerde inheemse aardappel gedraagt zich als een schild tegen dit onberekenbare pathogeen. Geïnspireerd door dit model zouden landbouwers en veredelaars de genetische diversiteit in de Europese aardappel meer moeten gebruiken, om problemen te voorkomen die veroorzaakt worden door het te intensief gebruikt van aardappel als monocultuur.

Tijdens het verkennen van de Zuid-Amerikaanse inheemse aardappelcollectie, *bleken Solanum demissum* en later ook *Solanum bulbocastanum* een bron te zijn van resistentiegenen tegen *P. infestans (Rpi)*. De *S. demissum Rpi* genen werden overgebracht in aardappelveredelingsklonen door traditionele introgressie veredeling. Toch werd doorbreking van deze mogelijkheid, om effectieve resistentie tegen de Aardappelziekte te verschaffen, binnen een decennium waargenomen. Met als doel om een hoopvolle en meer duurzame bescherming in bestaande cultivars te verkrijgen, stelden wetenschappers in de plantenveredeling voor, om inheemse Zuid-Amerikaanse aardappel *Rpi* genen direct in moderne aardappelrassen te introduceren via de zogenaamde cisgenese aanpak. Hierbij worden resistentiegenen van aardappel zelf of van kruisbare wilde soorten gebruikt. Dit in tegenstelling tot de transgene aanpak, waarbij planten genen geïntroduceerd kunnen krijgen die afkomstig zijn van niet gerelateerde geslachten of zelfs andere rijken. Veredelen van cisgenese planten is goed op weg naar publieke

acceptatie door de impliciete gelijkenis met natuurlijke kruisingen en omdat de principes van cisgenese vanuit de wetenschappelijke gemeenschap goed uitgelegd kunnen worden.

Door de flexibele eigenschap van *P. infestans*, om de bescherming van de nieuw geïntroduceerde *Rpi* genen te overwinnen, wordt nog steeds getracht nieuwe meer duurzame *Rpi* genen te ontdekken en te kloneren uit inheemse aardappelen. Hierdoor is er een nieuwe familie van *Rpi* genen gekarakteriseerd die vertegenwoordigd wordt door *Rpi-blb3, Rpi-abpt, R2, R2-like* en *Rpi-mcd1.1* die afkomstig zijn van *S. bulbocastanum, S. demissum, S. edinense* en *S. microdontum.* Tijdens dit onderzoek zijn we erin geslaagd deze genen fysiek te isoleren, de moleculaire karakterisatie van hun functionaliteit te bepalen en hun allelische verdeling in de Petota collectie in kaart te brengen.

Rpi-blb3, Rpi-abpt, R2, R2-like en *Rpi-mcd1.1* behoren tot de aardappel koppelingsgroep IV en hebben allemaal een specifieke sequentie die karakteristiek is voor LZ-NBS-LRR eiwitten. Het meest gelijkende R gen dat tot nu toe bekend is, is RPP13 van *Arabidopsis thaliana* die wat betreft aminozuur sequentie 35% ermee overeenkomt. De LRR domeinen van de Rpi-blb3, Rpi-abpt, R2 en R2-like eiwitten zijn zeer homoloog, terwijl de LZ en NBS domeinen het meest polymorf zijn waarbij die van R2 het meest divergent is. Alle vier de *Rpi* genen herkennen het recent geïdentificeerde RXLR effectoreiwit PiAVR2 welke door *P. infestans* in het cytoplasma van de plantencel wordt uitgescheiden tijdens het infectieproces. In tegenstelling tot *Rpi-blb3, Rpi-abpt, R2* en *R2-like*, gaat het *S. microdontum* resistentiegen *Rpi-mcd1.1* geen interactie aan met PiAVR2 en heeft een ander resistentiespectrum. *Rpi-mcd1.1* komt op DNA nucleotide niveau voor 90% overeen met *Rpi-blb3* en de polymorfe nucleotiden zijn vooral gelokaliseerd in de LRR regio.

De *S. bulbocastanum* haplotypen van *Rpi-blb1, Rpi-blb2* en *Rpi-blb3* werden ontdekt in een aantal Mexicaanse diploïde en polyploïde soorten die verwant aan *S. bulbocastanum* zijn. Deze drie resistentiegenen komen in verschillende combinaties en frequenties voor in *S. bulbocastanum* accessies en hun verspreiding is beperkt tot Centraal Amerika. Een geselecteerde set genotypen was getest op hun reactie op de avirulentie effectoren IPIO-2, Avr-blb2 en Pi-Avr2, welke een interactie aangaan met *Rpi-blb1*, *Rpi-blb2* en *Rpi-blb3*, respectievelijk. Deze set was ook getest na inoculatie met verschillende isolaten. Met deze aanpak konden enkele accessies geïdentificeerd worden die nieuwe, nog onbekende, *Phytopthora* resistentiefactoren bevatten naast de al bekende *Rpi-blb1, Rpi-blb2* en *Rpi-blb3* genen.

Analyse van de sequenties verkregen uit de verschillende “allele mining” strategieën suggereren het optreden van een evolutieproces van dit belangrijke *Phytopthora* resistentielocus, dat op recombinaties en puntmutaties gebaseerd is. Door gebruik te maken van de sequentie informatie van deze allelen

konden repeats en aminozuren in het LRR domein geïdentificeerd worden die specifiek voor de PiAVR2 herkenning zijn.

In het laatste hoofdstuk worden de resultaten die in dit proefschrift beschreven zijn in de context van een co-evolutie van aardappel/ *P. infestans* besproken.

RESUME

La pomme de terre joue un rôle clé dans le système alimentaire mondial. C'est la principale denrée alimentaire non céréalière du monde. Dans les pays développés, la consommation de pommes de terre augmente considérablement et représente plus de la moitié de la récolte mondiale. Comme elle est facile à cultiver et que sa teneur énergétique est élevée, c'est une culture commerciale précieuse pour des millions d'agriculteurs. Elle est vivement recommandée pour atteindre la sécurité alimentaire et elle peut aider les agriculteurs à faibles revenus et les consommateurs vulnérables à surmonter la crise actuelle des disponibilités alimentaires et de la demande mondiales.

La pomme de terre est consommée dans les Andes depuis 8 000 ans environ, les agriculteurs sont parvenus à sélectionner et à améliorer les premiers spécimens de ce qui allait donner, au fil des millénaires, une diversité inouïe de tubercules. Bien qu'offrant à ses consommateur un choix de couleurs, forme, saveur, propriétés de cuisson, de contenu en amidon etc..La pomme de terre que nous connaissons, l'espèce *Solanum tuberosum*, ne contient en réalité qu'une infime partie de la diversité génétique contenue dans les sept espèces reconnues et dans les 5 000 variétés de pommes de terre qui sont encore de nos jours cultivées dans les Andes.

Cette absence de diversité génétique chez les clones de tubercules cultivés en Amérique du Nord et en Europe a pour conséquence de les rendre extrêmement vulnérables: si un ravageur ou une maladie s'attaquait à une plante, il pouvait se propager rapidement aux autres. Un exemple fut le mildiou de la pomme de terre causé par l'oomycète *Phytophthora infestans*, qui dévasta les champs de pommes de terre d'Europe continentale, de la Belgique à la Russie. Mais le pays le plus affecté fut l'Irlande, où la pomme de terre représentait plus de 80 pour cent de la ration énergétique. Entre 1845 et 1848, le mildiou de la pomme de terre ravagea trois récoltes, provoquant une famine qui causa la mort d'un million de personnes.

De toute évidence, la diversité génétique de la pomme de terre ainsi que les méthodes agricoles pratiquée dans la cordières des Andes agissent comme un bouclier contre les différents bio agresseurs. Inspiré par ce modèle pour résoudre les problèmes soulevés par l'utilisation intensive de la monoculture de pommes de terre et des épidémies de mildiou, agriculteurs et ingénieurs agronomes pensent qu'il est nécessaire de maintenir la diversité génétique dans les cultures de pomme de terre, en mettant au point des variétés plus productives et résistantes aux maladies..

En explorant le contenu génétique des pommes de terres indigènes collectionnées a l occasion de différentes expéditions en en Amérique du Sud dans les années 60, il fut découvert de nouvelles espèces contenant une variétés de gènes de résistances au mildiou (Rpi : resistance à *Phytophthora infestans*), qui sont *Solanum demissum* et, plus tard, *Solanum bulbocastanum* . Les gènes de résistance au mildiou présent dans le matériel génétique de *S. demissum*. ont été transmis aux nouvelle variétés de pomme de terre par sélection traditionnelle. L effet souhaité en champs fut de courte durée car la résistance aux mildiou apportée par les nouveaux gènes fut contournée en une décennie par une adaptation rapide de *P. infestans*.

Une autre solution pour rapidement fournir une protection totale, espérons-le, plus durable aux varietes existantes de pommes de terre est d'y introduire directement les gènes de résistances indigènes d'origine sud américaine en utilisant une approche dite cisgenic. Les plantes cisgeniques sont modifiees exclusivement par des gènes provenant du meme genre de plante, à l'inverse des plantes transgéniques qui elles, peuvent contenir des gènes qui provenant de genre non apparentés ou même du monde animal. L'acceptation des plantes cisgenic par le public est en bonne voie grâce aux efforts déployés par la communauté scientifique pour expliquer les principes de cisgenesis ainsi que du fait que ces plantes soient fortement comparable à celle obtenues par sélection traditionnelle.

Des leçons ont été tirées sur la flexibilité de P. infestans pour contourner l'effet de resistance procurer par les gènes nouvellement introduits et, par conséquent, les efforts sont maintenus pour en découvrir et en cloner de nouveaux. Dans cet esprit, une nouvelle famille de gènes de résistance aux mildiou représentée par *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* et *Rpi-mcd1.1* ont été caractérisés dans des clones issus des especes de pommes de terre *S.bulbocastanum* , *S.demissum*, *S.edinense* et *S.microdontum*. Nous avons accompli dans cette recherche l'isolement physique de ces gènes, la caractérisation moléculaire de leurs fonctionnalités ainsi que leur distribution allelic dans la collection de pommes de terre sud américaine dénommée « Petota ».

Les gènes *Rpi-blb3*, *Rpi-abpt*, *R2*, *R2-like* et *Rpi-mcd1.1* sont localisés sur le chromosome numéro IV de la pomme de terre et tous contiennent des séquences caractéristiques des protéines appartenant a la catégorie LZ-NBS-LRR (LZ : leucine zipper, NBS : nucleotide binding site, LRR : leucine rich repeat). Le gène de résistance le plus similaire est *RPP13* provenant de la plante modèle *Arabidopsis thaliana*, avec un taux de similitude des séquence protéiques de 35 %. Les domaines LRR des genes *Rpi-blb3*, *Rpi-abpt*, *R2* et *R2-like* sont très homologues, tandis que les domaines LZ et BNS sont plus polymorphes avec ceux de R2, ce dernier étant le plus divergent. Les protéines issue des quatre gènes reconnaissent la protéine de type RXLR récemment identifiée, PiAVR2, qui est sécrétée par *P.infestans* dans le cytoplasme des cellules végétales au cours du processus d'infection. Contrairement à *Rpi-blb3*,

Rpi-abpt, *R2* et *R2-like*, la proteine issue du gène de résistance provenant de *S.microdontum*, *Rpi-mcd1.1*, n'interagit pas avec PiAVR2 et fournit un spectre de résistance différent. *Rpi-mcd1.1* est similaire à 90 % au niveau nucléotidique à *Rpi-blb3* et les nucléotides polymorphes sont principalement situées dans la région LRR.

Les haplotypes *Rpi-blb1*, *Rpi-blb2* et *Rpi-blb3* à l'origine identifiés dans l'èspèce *S. bulbocastanum*, ont été découverts dans plusieurs espèces mexicaines diploïdes et polyploïdes étroitement apparentées à *S. bulbocastanum*. Différentes combinaisons des trois gènes de résistance ont été trouvées et leur distribution est limitée à l'Amérique centrale. Un jeu de génotypes a été sélectionné et testé pour leur réponse aux protéines secretees par *P. infestans*, IPIO-2, Avr-blb2 et PI-Avr2, qui interagissent avec Rpi-blb1, Rpi-blb2 et Rpi-blb3, respectivement. Leurs niveaux de résistance à différent isolats de *P. infestans* ontaussi été évalués. Cette approche as permis d' identifier de nouvelles especes pouvant contenir des facteurs de résistance au mildiou encore inconnus, en plus de *Rpi-blb1*, *Rpi-blb2* et *Rpi-blb3*.

L'analyse des séquences obtenues à partir des différentes stratégies d'« allele mining » suggère une évolution du locus de résistance au mildiou localisé sur le chromosome IV, par recombinaison et mutations ponctuelles. En explorant les différences existant entre les différent allèles, nous avons identifié quelles parties du domaine LRR, sont importantes dans la reconnaissance de PiAVR2.

Enfin, les résultats décrits dans cette thèse ont été discuté dans un contexte de co-évolution entre la pomme de terre et *P. infestans*.

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When I started my PhD, scientific research was the core of my life, working late in the nights and weekends. And I met **Teun**, my angel on earth, who makes me realize that the sky was blue, birds were singing, flowers were colorful and beautiful. It was in spring 2006. We got married, two times, in Togo and in the Netherlands….nine months later **Maelya** was born. It was the 1st of July 2009. Teun, thank you for this wonderful gift you gave me, your LOVE;

Thank you for your patience, your tolerance, your energy, your support in this life. **Teun** and **Maelya**, you are the core of my life. I love you.

Ria, **Riny**, **Jan**, **Nelly**, **Maarten**, **Melina**, **Wendy**, **Nathalie**, **Iris** en **Mike,** dank jullie wel**.** Jullie hebben jullie armen en hart geopend en me warm en onvoorwaardelijk ontvangen in jullie familie.

This acknowledgment part wouldn't be complete without a special part to **those who left**.

I first would like to thank you **Bud** for your humor and positive attitude in all circumstances, until the end. Rest in peace.

Thank you to the anonymous **donator** who made a legacy gift to Wageningen University fund and permitted to finance my project. I hope that I fulfill his/her wish.

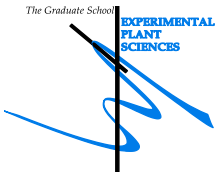
To finish, Thank you to my **ancestors**, **family** and **friends** who kept an eye on me and guide me on the good tracks through this life. M' do Akpe ne mawu. Akpe ne mia togbe o. Agnigban ne fa na mi.

Anoma Lokossou van Herpen

CURRICULUM VITAE

Anoma Akuvi Lokossou was born on September the 7th, 1978 in Nice located on the sunny Mediterranean coast of France. In 1981, she moved with her family to Lomé, the capital of Togo. After she completed her high academic school at the "Lycée français de Lomé" in 1996, she returned to Nice to study biology at the University of Sofia Antipolis. In 2002, she continued with plant biotechnology and bioingeniery study at the University of Paul Sabatier in Toulouse (France). In February 2005, Prof. Dr. Richard Visser gave her the opportunity to do a 6 months internship in Wageningen University at the Laboratory of Plant Breeding (The Netherlands). She worked in the team of Dr. Krit Raemakers on improving the in vitro genetic transformation of Cassava and analyzing the protein content of transgenic pea plants. After she obtained her master degree in September 2005, she started her PhD in Plant breeding under the supervision of Prof. Dr. Richard Visser, Prof. Dr. Ir. Evert Jacobsen and Dr. Edwin van der Vossen. The results of her research are described in this thesis.

Education Statement of the Graduate School
Experimental Plant Sciences



Issued to: **Anoma A. Lokossou**
Date: **7 June 2010**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Late blight resistance genes: Rpi-blb3, Rpi- abpt, R2-like	Mar 20, 2006
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
Genetic Analysis, Tools and Concepts	Oct 31-Nov 19, 2005
► **Laboratory use of isotopes**	
Radiation expert level 5B	Mar 19, 2007
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student day, Wageningen University	Sep 19, 2006
EPS PhD Student day, Wageningen University	Sep 13, 2007
EPS PhD Student day, Leiden University	Feb 26, 2009
► **EPS theme symposia**	
EPS theme 4 symposium 'Genome Plasticity', Wageningen University	Feb 09, 2005
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting, Lunteren	Apr 02-03,2007
NWO-ALW meeting, Lunteren	Apr 07-08, 2008
NWO-ALW meeting, Lunteren	Apr 08-09, 2009
► **Seminars (series), workshops and symposia**	
Seminar Sophien Kamoun	Oct 05, 2005
Seminar Tony Conner at Keygene	Jun 29, 2005
Seminar Dr. Kurt H. Lamour: "Phytophthora capsici: the key to unlocking Oomycete genetics and host specificity".	Nov 06, 2006
Seminar Dr. Roger Hellens: Identification and functional analysis of transcription factor genes from apple and their role in fruit quality-related traits	Feb 14, 2007
Research day (Plant breeding)	2007
Research day (Plant breeding)	Mar 2009
► **Seminar plus**	
► **International symposia and congresses**	
Oomycete Molecular Genetics Network Wageningen	May 04-07, 2006
MPMI (Italy: Sorrento)	Jul 21 -27, 2007
Sol meeting (Koln)	Oct 12-16, 2008
► Bio-exploit meeting	Feb 25-27, 2009
► **Presentations**	
Poster presentation at Oomycete Molecular Genetics Network Wageningen	May 04-07, 2006
Poster presentation at lunteren	Apr 02-03,2007
Poster presentation at Sorento	Jul 21 -27, 2007
Oral presentation at lunteren	Apr 07-08, 2008
Poster presentation at 5th Solenaceae genome workshop	Oct 12-16, 2008
► **IAB interview**	Sep 13, 2007
► **Excursions**	
Avebe excursion	Sep 2006
Subtotal Scientific Exposure	*13.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Signaling in plant development	Jun 19-21, 2006
Gateway to gateway technology	Nov 20-24, 2006
International PhD summerschool "Environmental signaling: Arabidopsis as a model"	Aug 27-29, 2007
► **Journal club**	
► Literature discussion "Plant Breeding"	2005-2009
► **Individual research training**	
Subtotal In-Depth Studies	*6.0 credits**

4) Personal development	*date*
► **Skill training courses**	
6th Frans-Nederlandse Ontmoetingsdagen: Promoveren zonder grenzen (Paris)	May 22-24, 2006
PhD competence assessment	Jun 02, 2006
Teaching and supervising Thesis students	Jun 13-14, 2006
Personal Efficacy	Sep 27-Oct 25, 2006
Toefle test (personal training)	Apr 26, 2008
Course Career perspectives	Oct 20-Nov 24, 2009
► **Organisation of PhD students day, course or conference**	
Lab trip of Plant breeding laboratory	Jun 2006
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*6.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**35.2**

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 ECTS credits

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

Cover Art / Photo CV / Invitation:

Remko Zijlstra 2010

www.RemkoZijlstra.vpweb.nl

Ezel1@live.nl

Remko Zijlstra created this cover inspired by the history of the potato combined with reading this thesis summary.

The cover depicts the journey of the potato across time, presented by a serie of maps, from the front to the back, as a “come back” to the origin.

This project was financed by a legacy to the Wageningen University Funds (WUF)

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