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Resistance and susceptibility to late blight in *Solanum*:

gene mapping, cloning and stacking

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Resistance and susceptibility to late blight in *Solanum*:
gene mapping, cloning and stacking

Estelle Verzaux

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Contents

Chapter 1

General introduction

General introduction

The *Phytophthora infestans* and potato pathosystem

Potato

Potato, the common name for *Solanum tuberosum*, originated from the Andean region of South America and is produced almost everywhere in the world. The word potato comes form the Spanish word patata, which according to the Real Academia Española, is derived from the two words: batata and papa. Batata is a Taino word for sweet potato, while papa is a word from Quechua that means potato. Like tomato, eggplant, pepper and tobacco, potato belongs to the botanical Solanaceae family. *Solanum* species carry 12 chromosomes as basic chromosome number and their ploidy level can vary from diploid (2n=2x=24) to hexaploid (2n=6x=72). Potato is considered to be the crop that has the highest number of related wild species in its genus; over 200 *Solanum* species are recognized.

Potato is the third most important food crop in the world after wheat and rice. Its production in developing countries has increased since the 1990's, in Asia, Africa and Latin America. Potato has a high production level per hectare and is very nutritious. It is a good staple crop on which a significant number of resource-poor farmers are relying. 2008 was the United Nations International Year of the Potato, which had the objective to raise awareness on the importance of the "humble tuber" as a staple food of humanity (http://www.potato2008.org/). They predicted potato to be food of the future based on four reasons: potatoes are a truly global food, they feed the hungry, they are nutritious and demand for potatoes is growing.

Phytophthora infestans

Phytophthora infestans (Mont.) de Bary is the cause of potato late blight and is responsible for world wide losses in potato production. This pathogen leads to losses of billions of dollars annually to modern agriculture and also impacts subsistence farming in developing countries (Fry 2008; Kamoun and Smart 2005). It is a specialized pathogen affecting potato and tomato that is able to grow on all above-ground parts of the plants and on the tubers. The best condition for

disease development is cool (15 to 25 °C) and humid (relative humidity close to 100%) weather. The blight symptoms are brown and black rapidly expanding lesions. It can also attack the tomato fruit or potato tubers, causing rot in the soil or during storage. The life cycle of *P. infestans* is relatively short, which means that within few days after infection, large lesions causing major damages are appearing and within two to three weeks, the plants can be completely destroyed.

P. infestans is a hemibiotrophic pathogen that first develops on living tissue and then causes necrosis and finally sporulates (Kamoun and Smart 2005). During the biotrophic phase, the pathogen establishes intimate associations with host cells through haustorium production. Haustoria are structures that function in translocation of effector proteins and probably also in nutrient uptake (Birch et al. 2006; Whisson et al. 2007). *P. infestans* is thought to secrete hundreds of effector proteins that are able to target two distinct sites in the host plant: the apoplast and the cytoplast (Haas et al. 2009; Kamoun 2006; Whisson et al. 2007). Among the cytoplasmic effectors, the RXLR protein family is the most studied type of effector, which can also have an avirulence function when recognized by a plant R gene. The conserved RXLR motif enables the delivery of the effector proteins inside plant cells (Bhattacharjee et al. 2006; Dou et al. 2008; Grouffaud et al. 2008; Whisson et al. 2007).

P. infestans can follow a sexual life cycle, as it is a heterothallic organism with two mating types: A1 and A2. Sexual reproduction only occurs when the two mating types are present on the same plant. The oospores resulting from the sexual cycle can survive in the soil for many years. The sexual reproduction also allows a higher genetic variation and quicker adaptation of the pathogen to new resistance which results in the development of new strains able to overcome resistance genes.

The genome of *P. infestans* has been sequenced (Haas et al. 2009). Analyses of the genome revealed large intergenic regions composed of repetitive sequences flanking the RXLR effectors. This is probably the way for *P. infestans* to quickly adapt to new resistances by evolving new effectors. The available *P. infestans* genome sequence will allow a better understanding of the pathogen and the way it evolves in response to plant resistance. This knowledge will be very valuable in the search for resistance.

Natural resistance to late blight

Disease resistance to *P. infestans* in most *Solanum* species is race specific, and almost always based on gene for gene interaction. It relies on the HR response to stop pathogen growth (Kamoun et al. 1999b; Vleeshouwers et al. 2000). The diversity of wild *Solanum* species provides a rich source of genes against stresses. Resistance to late blight occurs frequently in wild *Solanum* species and in the two centers of diversity of the *P. infestans* pathogen, namely Mexico and South America. Since the occurrence of the disease in Western Europe around 1850, search for natural resistance to *P. infestans* has been going on, and many *Solanum* species were found to be resistant, especially the ones collected from regions with high disease pressure. *S. demissum* is the best known and the most exploited source of resistance to late blight in breeding programs. Recently, more efforts were carried out in mapping and cloning of *R* genes to *P. infestans* (*Rpi*) from many different wild species (Hein et al. 2009a). *Rpi* genes are known to be single dominant resistance genes, but they can be located in many different places, frequently in clusters, in the genome. *Rpi* genes from six different clusters have been cloned and they all encode coiled coil nucleotide binding leucine reach repeat (CC-NB-LRR) proteins: *R1*, *R2*, *R3a*, *Rpi-blb1*, *Rpi-blb2* and *Rpi-vnt1.1* (Ballvora et al. 2002; Huang et al. 2004; Lokossou et al. 2009; Pel et al. 2009; van der Vossen et al. 2005; van der Vossen et al. 2000)). The two major late blight (MLB) resistance gene clusters presently found are the *R2* and *R3* clusters. The *R2* cluster lies on the short arm of chromosome 4 and nine functional homologues in that cluster have been cloned by allele mining from several other wild species (Champouret 2010; Lokossou et al. 2009). The *R3* cluster is located on the long arm of chromosome 11 and contains many *R* genes from *S. demissum* of which *R3a* has been cloned (Huang et al. 2005). Within all the cloned *Rpi* genes, the *Avr* genes have been identified for five of them: *ipiO1* for *Rpi-blb1*, *Avrblb2* for *Rpi-blb2*, *Piavr2* for *R2*, *Avr3a* for *R3a*, and *Avr-vnt1* for *Rpi-vnt*1.1 (Armstrong et al. 2005; Champouret et al. 2009; Lokossou et al. 2009; Oh et al. 2009; Pel 2010). The nature of the interaction between the R and the *Avr* protein was never described. It could be a direct or an indirect interaction, but indirect perception by *R* proteins prevails in several gene for gene interactions (Dangl and Jones 2001; Martin et al. 2003). *Rpi* gene cloning provides tools to answer biological questions and material for breeding programs.

Map-based cloning

Principles and requirements

Map-based cloning, also referred to as positional cloning, was described for plants in the early 1990s (Paterson and Wing 1993; Young 1990). It was made possible with the development of molecular biology and the construction of genetic maps. The principles of map-based cloning are simple: it is based on the identification of the mapping position of the trait of interest followed by the assembly of pieces of the genome to form a contig, and the analysis of DNA sequence to identify the gene responsible for the observed phenotype. When gene cloning techniques were introduced and improved, the door was opened to new possibilities for the understanding of molecular mechanisms and plant biology. And until today it has remained a very useful approach, which allows answering important biological questions and solving agricultural problems.

Map-based cloning has only two requirements: a phenotype that segregates in the population and the possibility to construct a genetic map. For the rest the concept is simple, but it is generally difficult in practice. Complicating factors during the map-based cloning process are large genome size, low level of polymorphism, low recombination frequency and repetitive DNA (Young 1990). Technological progress might contribute to more efficiency in the map-based cloning process but it has no influence on the basic requirements that will always have to be met to follow that approach.

Steps of map-based cloning

Here we describe the different steps of map-based cloning, which are also summarized in **Figure 1** and describe the experiments specific for potato R genes conferring resistance to late blight.

Identification of the genotype with the appropriate trait of interest

The first step is to find the genotype with a phenotype of interest. For our purpose, we are looking for a genotype with a good level of resistance. Seeds of several accessions of a particular species are ordered from seed banks. Around 10 seeds per accession are tested. Several screening methods are available to test the resistance level of the different genotypes: in vitro assay, detached leaf assay and field experiment. They are normally performed in that order from the more high-throughput to the more informative and closer to reality. The choice of the *P. infestans* isolates is important: the best is to use an isolate present in the field and against which presently deployed *R* genes do not confer resistance.

Genetic mapping

The second step is the genetic mapping which is composed of two phases: the generation of a mapping population and the identification of the chromosomal position. The generation of the mapping population starts with the identification of susceptible genotypes, that can be easily crossed with the resistant one and produce a large number of F1 seeds. Both intra- and inter-specific F1 populations can be produced and tested, to increase the chance of finding a suitable population. The efficiency of the crosses and the seed set can be very variable and is not predictable. Around 50 individuals of each population are phenotyped for resistance to *P. infestans*. The population is suitable for further use when: the resistance segregates in the population, can be clearly scored and follows a 1:1 segregation ratio. Resistance to the pathogen can be incomplete or quantitative which renders the scoring difficult. A clear phenotype following a 1:1 segregation ratio indicates the presence of a single dominant R gene, which can be cloned by map-based cloning approach.

In this study, for the identification of the chromosomal position, we use two marker technologies: simple sequence repeats (SSR) and NBS profiling (van der Linden et al. 2004) combined with a bulk segregant analysis (BSA, Michelmore et al. 1991). After the identification of a putative chromosomal arm position, cleaved amplified polymorphism sequence (CAPS) markers were used to confirm the mapping position and to construct a genetic map. The SGN or the Gaby primary databases are valuable sources of CAPS markers mapped all over the genome of plants from the Solanaceae family (http://www.sgn.cornell.edu/; http://www.gabipd.org/projects/Pomamo/). The best is to develop as many markers as possible to saturate the region, but in theory, two flanking markers are sufficient to go to the next step: high resolution mapping.

High resolution mapping

To achieve high resolution mapping two elements are necessary: recombination events around the *R* gene locus, and markers closely linked to the gene. Recombination events or meiotic crossovers are recombination between two non-sister chromatids of the homologous chromosome during meiosis. The recombination frequency depends on the chromosomal position. Recombination may be suppressed in the telomeric or centromeric regions. The screen for recombination events between flanking markers is performed in a high-throughput manner on a large number of individuals. A screen of around 3000

individuals should be sufficient for high resolution mapping and successful further map-based cloning.

The development of cosegregating markers can be performed through comparative genomics and the use of physical maps, like the RH89-039-16 (RH) physical map (Borm 2008). Relevant new markers can also be obtained by targeted gene family marker approaches when sequence information of the locus containing the targeted gene is available. The genotyping of the recombinant with closely linked markers will allow the construction of a high resolution genetic map of the DNA region flanking the *R* gene, and will be an important milestone for physical mapping.

Physical mapping

Physical mapping requires a BAC library of the resistant genotype and one marker very closely linked to the R-gene, to screen the library. The objective is the construction of a contig covering the R gene region delimited by flanking markers.

Bacterial artificial chromosome (BAC) libraries are the best tool for *R* gene map-based cloning. It consists of a DNA sequence from the genome of the resistant genotype inserted into a vector and transformed into *E. coli*. The average insert size that can be obtained goes up to 120kb. To ensure complete genome coverage, a library with the equivalent of 10 times the genome is constructed, and stored at -80°C. The library can be picked or pooled depending on the distance between the closest marker and the targeted gene. BAC picking is necessary when that distance cannot be reduced, and chromosome walking is expected to require several BAC clones to close the contig. It takes a lot of effort to pick but the identification of BAC clones is then much easier. BAC pooling requires much less effort at the beginning but the identification of a single BAC clone is more tedious. BAC pooling is suitable for chromosome landing (Tanksley et al. 1995). Chromosome landing is the direct identification of the BAC clone containing the targeted gene with the cosegregating marker(s). The availability of a cosegregating marker in a large population gives good promises for the success of a BAC landing approach. It implies that the physical distance between the gene and the marker should be smaller than the average insert size of the BAC library. If only a small number of BAC clones are necessary to close the contig, BAC pooling can also be advantageous compared to BAC picking.

Physical mapping is the assembly of BAC clones for the construction of the contig. The BAC clones are assembled by logical deduction from the marker data, the

orientation of the BAC clones, the BAC end sequence and the BAC end markers. The BAC clones covering the contig are fully sequenced and subsequently annotated by looking at gene prediction and blastX results (NCBI database).

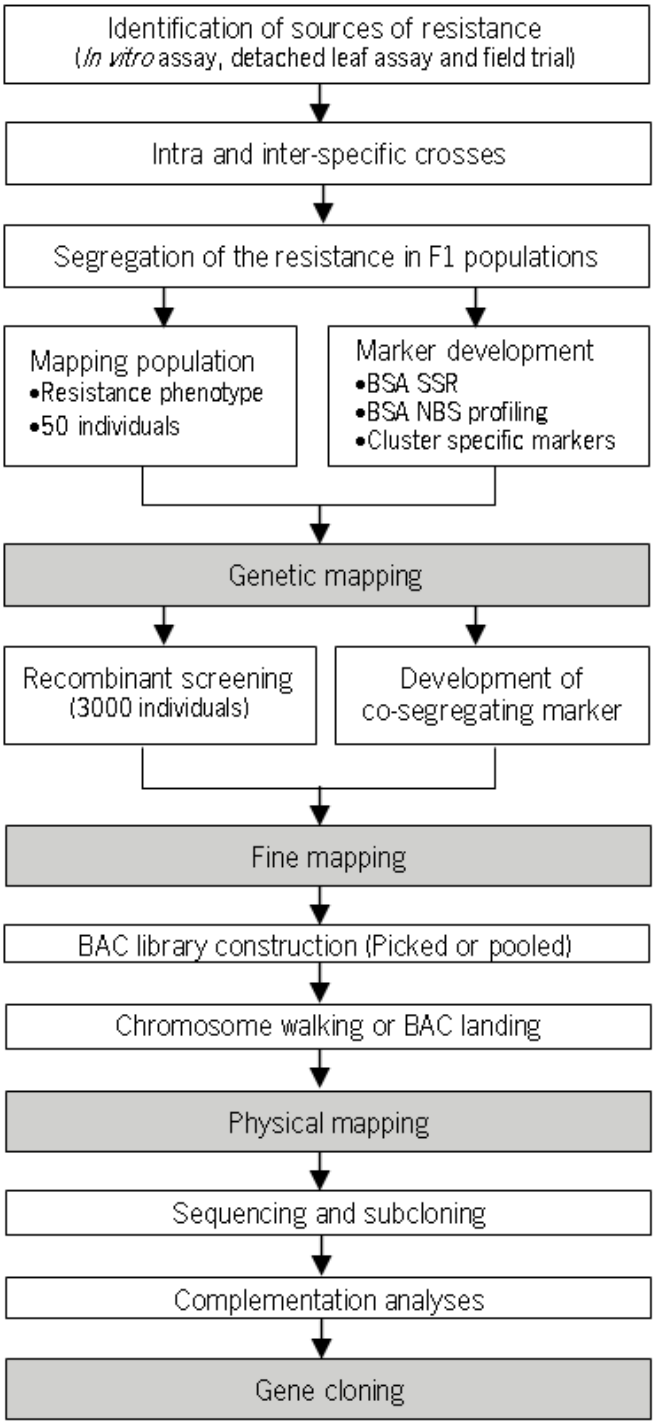

Figure 1. Steps of the map-based cloning strategy. In grey are the mile stone steps, with first the identification of the mapping position and the development of flanking and cosegregating markers, which lead to the genetic mapping. The next step is the fine mapping with the recombinant screen and the precise localization of the markers. Finally the physical mapping followed by complementation analyses which results to gene cloning.

Complementation

The complementation assay aims to confirm that one candidate gene present in the contig, confers resistance to *P. infestans* when transferred to a susceptible genotype. The candidate genes are first inserted in a binary vector and transformed in *Agrobacterium tumefaciens*. Then the plants are transformed with the different constructs and phenotyped for their resistance to *P. infestans*. This will normally allow the identification of the functional gene.

The BAC clone or contig that should contain the functional R gene may contain several candidate genes. This is especially true for *R* genes of the NB-LRR type. The individual candidate genes need to be sub-cloned. For the sub-cloning of the candidate genes several approaches are available: the classical BAC subcloning, LR-PCR followed by ligation into a binary vector or LR-PCR followed by cloning with the gateway technology. The first approach only has the advantage to avoid PCR mistakes and thus the sequencing step. The last two approaches require primers generally designed 3kb in front and 1 kb after the open reading frame (ORF).

The most reliable way to confirm the functionality of the gene is by complementation of the resistance phenotype after transformation with the gene. Transient and stable transformations both are valid for that purpose. The transient transformation can be done in two ways: by co-agroinfiltration of the *R* gene and the *Avr* gene into *N. benthamiana* (Bos et al. 2006). This is only applicable in cases where the cognate *Avr* gene is known and available. It can also be carried out by agroinfiltration of the *R* gene in *N. benthamiana*, followed by *P. infestans* inoculation (Lokossou et al. 2009; Pel et al. 2009). The stable transformation into potato still provides the most clear and definitive evidence.

Map-based cloning of *Rpi* genes

The map-based cloning approach as described above is well adapted for the cloning of *Rpi* genes. Almost all new *Rpi* gene cloned from potato were cloned through this approach. One, *Rpi-mcd1*, was cloned by allele mining with homologous genes in the same resistance cluster (Lokossou 2010). In potato, there are at least 19 *R* gene clusters known containing R genes conferring resistance to all pathogens (Gebhardt and Valkonen 2001; Grube et al. 2000; Pan et al. 2000) and the NB-LRR gene family is large and diverse. So map-based cloning is the best strategy to clone *Rpi* genes from wild *Solanum* species.

Map-based cloning approaches in crop species like potato or tomato, can now be carried out within the same amount of time as in *Arabidopsis thaliana* 10 years ago, which was inconceivable 30 years ago (Peters et al. 2003). Nowadays, gene cloning in the model plant Arabidopsis can be done in less than a year due to the availability of the genome sequence, the availability of thousands of markers and advances in the methods used to detect DNA polymorphism (Jander et al. 2002). Such progress has also been taking place for potato: an ultra-high-density (UHD) map (van Os et al. 2006) and a physical map (Borm 2008) are already available, and in the very near future the potato genome sequence will be released. These developments are promising for speeding up the process of map-based cloning of important genes, such as genes involved in pathogen resistance.

Breeding for resistance to *P. infestans* in potato

Classical potato breeding is a long process and requires tremendous efforts. It normally requires the screen of 100 000 seedlings, and more than 10 years will be needed to obtain a new cultivar after the cross between breeding parents. This is mainly due to the need to get rid of all the unwanted genes in back cross generations and to limit linkage drag (Jacobsen and Schouten 2007). Two alternatives are available to improve the efficiency of potato breeding: marker assisted selection (MAS) and genetic modification (GM). These two approaches will also help to avoid the problems caused by the earlier mentioned linkage drag.

Potato breeding first essentially relied on *Rpi* genes from *S. demissum*. The hope to control late blight with *Rpi* genes was lost when the deployed *demissum Rpi* genes got quickly overcome by virulent isolates from the pathogen. Recently, the hope to use *R* genes was revived with the idea that a large part of the natural *R* gene resources had not been exploited. It started with the discovery that, although *S. demissum* counts more than 11 *R* genes, except *R1, R2* and *R4*, they are all located in the same cluster (Huang 2005). The cloning of *Rpi-blb1* and later *Rpi-blb2* from *S. bulbocastanum* (van der Vossen et al. 2003; van der Vossen et al. 2005) also brought more hope due to the fact that these *Rpi* genes conferred resistance to (almost) all *P. infestans* isolates tested. In addition, the mapping of many *Rpi* genes from wild species (Hein et al. 2009a) also revealed the diversity of *Rpi* genes available in nature. The large potato gene pool with its potential source of resistance has not been fully exploited and offers possibilities for resistance breeding against potato late blight.

A lesson from the past is that using single *Rpi* genes in one variety is not durable. It is better to start with the proposition that all *R* genes are or can be overcome by the pathogen. Therefore, for more durable resistance, it is necessary to pyramid or stack two or more *Rpi* genes together in a single variety, which can be achieved through MAS and GM. However, stacking of two or more *R* genes implies a lot of linkage drag. To be able to stack R genes in a potato variety to achieve more durable resistance to late blight, *R* genes from resistant wild *Solanum* species first have to be detected, mapped and/or cloned. The mapping results will provide the diagnostic markers that may serve as a tool for the pyramiding approach using MAS and the cloning will provide genes to make *Rpi* gene combinations to directly transform potato varieties.

Scope of the thesis

The isolation of *R* genes against *P. infestans* from wild *Solanum* species and subsequent introduction of a combination of these genes as cisgenes (genes from the potato plant itself or crossable species with their own regulatory elements) into existing potato varieties is currently the fastest means of exploiting potentially durable resistance present in the *Solanum* gene pool (Schouten et al. 2006). In this study, we are pursuing map-based cloning to isolate novel late blight R genes from a diverse set of wild *Solanum* species. We are also looking at other alternatives for durable resistance through map-based cloning of a gene involved in the cell death response mediated by a *P. infestans* elicitin: INF1.

In **Chapter 2**, we studied the genetic basis of late blight resistance from a wild Bolivian *Solanum* species: *S. avilesii*. We identified *Rpi-avl1*, a single dominant R gene, located in a new cluster for resistance to late blight, and constructed a high resolution genetic map of the R gene locus.

In **Chapter 3**, the same strategy as in **Chapter 2** was applied on two other Bolivian species: *S. capsicibaccatum* and *S. circaeifolium* ssp. *quimense* with R genes (*Rpi-cap1* and *Rpi-qum1*) located in the same cluster as *Rpi-avl1*. Their broad resistance spectrum and strong level of resistance make these R genes very attractive to be included in breeding programs.

In **Chapter 4**, we studied the resistance occurring in the pentaploid hybrid *S. x edinense.* We identified the presence of at least three *R* genes located on different chromosomes, a natural example of *R* gene stacking. The resistance conferred by

each *R* gene has been overcome by some *P. infestans* isolates, but the three R genes combined provide resistance to all the isolates tested, except for one.

In **Chapter 5**, another trait was the object of investigation: the INF1 elicitin mediated cell death response in *S. microdontum.* Map-based cloning identified a functional candidate gene, *Rinf1-mcd,* which encodes a receptor like protein (RLP). This gene maps in a novel RLP cluster present in *Solanum*, located on the long arm of chromosome 12.

The general discussion (**Chapter 6**) summarizes the findings from the map-based cloning of the different *Rpi* genes from different wild *Solanum* species. The importance of the cluster in which four new *Rpi* genes were mapped is presented. The requirements for successful R gene cloning are emphasized. Furthermore, future work for *Rpi* gene cloning and the implications of our results for breeding durable late blight resistant potato varieties are discussed.

Chapter 2

High resolution mapping of *Rpi-avl1*, an *R* gene from the wild Bolivian species *Solanum avilesii* conferring resistance to late blight

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Abstract

The large diversity of the potato gene pool and the potential source of resistance to *Phytophthora infestans* available within wild *Solanum* species revived the hope of potato breeders to rely on *R* genes in the fight against potato late blight. Both, Mexico and South America are areas that are rich in *Solanum* species showing late blight resistance, from which the presence of resistance (*R*) genes should be investigated. Here, we focus on an *R* gene present in the diploid Bolivian species *S. avilesii*. The identification of a resistant genotype and the generation of a segregating population allowed the mapping of a single dominant *R* gene, *Rpi-avl1*, which is located in an *R* gene cluster on chromosome 11. This *R* gene cluster is considered as "hot spot" containing *R* genes to at least five different pathogens. High resolution mapping of the *Rpi-avl1* gene revealed a marker cosegregating in 3890 F1 individuals, which may be used for marker assisted selection in breeding programs or for completing the cloning of *Rpi-avl1*.

Introduction

Late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating disease for potato and tomato cultivation. It is responsible for important losses, and chemical control drastically increases the production costs and is not sustainable (Haverkort et al. 2008). *P. infestans* is a specialized pathogen that primarily causes lesions on the foliage and that can also infect the tubers. *P. infestans* is a problem since the Irish potato famine in the mid-19th century. Breeders included late blight resistance in the potato breeding programs, and first focused on the introgression of dominant resistance (*R*) genes from *Solanum demissum,* a hexaploid wild species originating from Mexico

(Malcolmson et al. 1966;Wastie 1991). It is important to realize that introgression breeding is connected with linkage drag, especially when stacking of *R* genes originating from different wild species is needed (Jacobsen and Schouten 2007). Later, the focus was on quantitative resistance, but it is more complex to phenotype and the effects of partial resistance factors are often difficult to determine (Leonards-Schippers et al. 1994). In addition, it has been indicated by Vleeshouwers et al. (2000) that inoculated potato plants with partial resistance do show a hypersensitivity reaction, which is an indication that classical *R* genes can also be involved in quantitative resistance. Recently, breeder's interest went back to major broad spectrum *R* genes, especially since the cloning of *Rpi-blb1* (van der Vossen et al. 2003). The large diversity of potato species and their resistance to late blight offer good possibilities for breeding for high levels of resistance that remain durably effective. The isolation of major *R* genes and their stacking into potato varieties appears today as the objective aimed for by breeders. For such stacking, breeders have two alternative approaches. 1: *R* genes can be transferred to the existing potato varieties by genetic transformation, but European laws do not allow easy release of GM organisms. However, the approach of cisgenesis has been introduced with the potential to simplify release of cisgenic varieties with one or more *R* genes by exemption of the GM-directive 2001/18/EC (Schouten and Jacobsen 2007). Cisgenes are natural genes from the crop plant itself or from crossable species with own regulatory elements and introns. 2: Breeders can also follow the traditional breeding approach, accelerated with marker assisted selection (Barone 2004; Collard et al. 2005) but still hampered by the problem of linkage drag, especially when stacking of different *R* genes is needed.

A good place to search for resistance is the center of origin of late blight where plant hosts co-evolved with the pathogen. Many wild *Solanum* species that evolved in Mexico or South America have been identified as source of resistance to late blight (Hawkes 1990). The center of origin of *P. infestans* is subject of debate, and contradictory evidence points to Mexico or South America (Gomez-Alpizar et al. 2007; Goodwin 1994; Grunwald and Flier 2005). Their observations led to the suggestion that both areas are centers of diversification. So South American and Mexican *Solanum* species are expected to carry a diversity of *R* genes that might be valuable for resistance breeding. In practice, the Mexican *S. demissum* and *S. bulbocastanum* are the best known examples as sources of late blight resistance, and thus far, mainly *R* genes originating from Mexico have been used. Perhaps South American species evolved other *R* genes with distinct

recognition specificities, either in a known *R* gene cluster with different specificity or in new *R* gene clusters. The identification of a new *R* gene specificity with resistance to *P. infestans* would provide additional and very valuable tools for cisgenic or marker assisted resistance breeding. The higher the number and diversity of available *R* genes, the bigger the chance to finally win the battle against late blight, for example, by stacking strong *R* genes from different *R* gene clusters. This could be compared with stacking of genes from a single cluster.

In this study we focus on the diploid species *S. avilesii* (avl) Hawkes & Hjerting, which originates from Bolivia (Gabriel et al. 2001; Ruiz de Galarreta et al. 1998). We aimed at identifying the gene responsible for resistance to *P. infestans* in *S. avilesii*. A resistant genotype was selected from a screen on *S. avilesii* accessions, to be crossed with a susceptible parent and to generate a segregating F1 mapping population. Several marker techniques were applied to construct a high resolution genetic map of the *Rpi-avl1* locus, a single dominant *R* gene located on the northern arm of chromosome 11.

Figure 1. Field experiment in 2007 with the resistant genotype avl478-2 next to susceptible potato cultivar Bintje.

Materials and methods

Plant material and mapping population

Accessions of *S. avilesii* were provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. A screen for resistance to *P. infestans* was performed on three *S. avilesii* accessions (477, 478 and 479, representing CGN

18255, CGN 18256 and CGN 18257, respectively), with 5 genotypes per accession. The genotype avl478-2 (**Figure 1**) with the highest level of resistance was crossed with the susceptible genotype avl477-1 to generate an intraspecific F1 mapping population.

Phytophthora isolates and disease tests

The *P. infestans* isolates 90128, IPO-C, H30P04, EC1, USA618, VK98014, IPO428-2, NL01096, F95573, and 89148-09 were kindly provided by Geert Kessel, Francine Govers and Paul Birch. Resistance levels were assessed by three different assays, i.e. the *in vitro* assay, detached leaf assay (DLA), or field experiment. The *in vitro* assay was performed once as a high-throughput screening on five plantlets per genotype using *P. infestans* isolate 90128 (Huang 2005). Plantlets were visually scored after 1 and 2 weeks, and a 5-scale score ranging from 9 (fully resistant) to 1 (susceptible) was applied.

The detached leaf assay was performed as described by Vleeshouwers et al. (1999). Briefly, one leaf was harvested between the third and the fifth fully developed leaves from five week old plants, grown in the greenhouse, and inoculated with a zoospore suspension of the isolate of interest. The leaves were scored six days after inoculation. For initial germplasm screenings (**Table 1**), lesions sizes were measured. Lesion size was converted to a scale from 9 (resistant) to 1 (susceptible), with the resistance level of the susceptible control cultivar Bintje set at 2 (**Table 1**). For phenotyping of the parental genotypes with multiple isolates and the F1 progeny with 90128 and USA618 (**Table 2**), macroscopic scoring was applied and leaves were scored as resistant (R) when hypersensitive responses or no disease symptoms were observed, susceptible (S) when expanding lesions with sporulation were observed, or quantitative (Q) for a response not clearly resistant or susceptible.

Two field trials were performed in the summer of 2005 and 2007 (**Figure 1**), in Wageningen, the Netherlands, as described by Colon and Budding (1988). Each field trial consisted of two randomized blocks, and within the blocks, genotypes were represented as four-plant subplots which were treated as a single experimental unit as described by Colon and Budding (1988). For comparisons between years, standard cultivars Ostara, Bildtstar, Eersteling, Pimpernel, Robijn and Biogold were included. Spreader rows consisted of potato cultivar Bintje, the border rows of potato cultivar Nicola. For the inoculation, a large number of potato cultivar Bintje leaves were inoculated with the isolate IPO-C. After 6 days, the spore suspension was collected in large containers and zoospore release was induced by incubating the containers at 10°C. At nightfall, the zoospore

suspension was sprayed on the potato field using a tractor with two spraying arms. Disease assessments were made at weekly intervals. The percentage of leaf area covered with late blight lesions was estimated for each plot (Colon and Budding 1988). From these readings the area under the disease progress curve (AUDPC) was calculated (Fry 1978), and subsequently, the AUDPC values were transformed to a 9 (resistant) to 1 (susceptible) scale.

Table 1. Resistance of 15 *S. avilesii* genotypes from three different accessions to two *Phytophthora infestans* isolates 90128 and IPO-C, using three different assays: in vitro assay, detached leaf assay (DLA) and field experiment in two years. Resistance phenotypes were rated on the scale from 1 (fully susceptible) to 9 (fully resistant). avl for *S. avilesii* and dms for *S. demissum*. In grey, the genotypes used in DLA and field experiments, in bold the genotypes used in this study.

Genotypes		In vitro	DLA		Field 2005	Field 2007
		90128	90128	IPO-C	IPO-C	IPO-C
avl	**477-1**	**1**	**3**	**3**	**6**	**6**
avl	477-2	9	nd	nd	nd	nd
avl	477-3	1	nd	nd	nd	nd
avl	477-4	9	4	4	9	nd
avl	477-5	9	nd	nd	nd	nd
avl	478-1	1	nd	nd	nd	nd
avl	**478-2**	**9**	**6**	**6**	**9**	**8**
avl	478-3	3	nd	nd	nd	nd
avl	478-4	3	nd	nd	nd	nd
avl	478-5	1	nd	nd	nd	nd
avl	479-1	1	nd	nd	nd	nd
avl	479-2	1	nd	nd	nd	nd
avl	479-3	3	nd	nd	nd	nd
avl	479-4	1	nd	nd	nd	nd
avl	479-5	1	nd	nd	nd	nd
cv. Bintje		2	2	2	2	2
dms	344-14	9	9	9	nd	nd
dms	344-18	nd	9	9	9	9

DNA isolation

Genomic DNA was isolated following two different protocols: CTAB and NaOH. The CTAB protocol was performed on material that needed long storage like the mapping population and the interesting recombinants. Young leaf tissue was collected for DNA isolation according to the CTAB protocol with the Retsch machine (RETSCH INC., Hannover, Germany). The NaOH protocol was used for screening for recombination events in the population. Leaf samples were collected on three weeks old seedlings and DNA was isolated by following the NaOH protocol (Wang et al. 1993). The PCR amplifications were directly performed on these samples.

Table 2. List of isolates of *Phytophthora infestans* used in the detached leaf assays with information on their origin and virulence spectrum. The phenotypes on the parental genotypes avl478-2 and 477-1 are characterized as resistant (R), susceptible (S) or unclear (Q). *(Champouret et al. 2009)

P.i. isolate	Country of origin	Race*	avl478-2	avl477-1
90128	The Netherlands	1,3,4,7,8,10,11	R	S
H30P04	The Netherlands	3[a], 7,10,11	R	Q
EC1	Ecuador	1,3,4,7,10,11	R	S
IPO-C	Belgium	1,2,3,4,5,6,7,10,11	R	S
USA618	Mexico	1,2,3,6,7,10,11	R	S
VK98014	The Netherlands	1,2,4,11	R	S
IPO428-2	The Netherlands	1,3,4,7,8,10,11	R	S
NL01096	The Netherlands	1,3,4,7,8,10,11	S	S
F95573	The Netherlands	1,3,4,7,10,11	S	S
89148-09	The Netherlands	0	R	Q

Marker development and map construction

A set of approximately 80 SSR markers covering all chromosomes of the potato genome (Collins et al. 1999; Feingold et al. 2005; Bakker et al., manuscript in preparation; Ghislain et al. 2004) was applied to determine the chromosomal position of segregating *R* genes. Parental genomic DNA and 12 resistant and 12 susceptible F1 individuals from the mapping population were used for the screen. PCR reactions for the SSR markers were performed using a single PCR profile: an initial cycle at 95°C for 2 min; then 30 cycles of 95°C for 30s sec, 56°C for 30 sec, using a ramp of 1°C/min, and 72°C for 45 sec, using a ramp of 1°C/min; and a final step at 72°C for 3 min. Subsequently the PCR products were visualized by electrophoresis on polyacrylamide gels on the LI-COR DNA sequencer (Lincoln, Nebraska, USA).

PCR markers were developed on the northern arm of chromosome 11 (**Table 3**). Primer pairs from markers mapped on the northern arm of chromosome 11 were tested. The degenerate primer pair N2527 was designed on the conserved region of the two mRNA clones Nl25 and Nl27 (Hehl et al. 1999). The primer pairs were first tested for amplification on the parents and screened with a selection of enzymes for the identification of polymorphism. The polymorphism was only of interest if a fragment or a restriction site was present in the resistant parent and absent in the susceptible parent. The marker was later tested on the F1 individuals to determine the segregation pattern of the fragment and its possible association with the resistance phenotype. In case the polymorphic fragment occurred in all progeny of the mapping population, the resistant parent was homozygous for that polymorphism and therefore could not be used. Genetic maps were constructed using the number of recombination events between the markers and between the markers and the resistant/susceptible phenotypes.

Table 3. PCR markers used in the *Rpi-avl1* population, with primer sequences based on BAC sequences located on the northern arm of chromosome 11 from the reference genotype RH or references for primers already published, annealing temperature (TM), and restriction enzyme to identify a polymorphism. .*SSR marker ramp for TM

Marker	Sequence or reference	Annealing temperature (°C)	Enzyme[1]
RH008P12	F gggtcgatgatccatttattg R ccctttttgttccatatcagttg	56	a.s.
N2527	F gaaacacaggggaatattcacc R ccatrtcttgwattaagtcatgc	60	a.s.
Ct182	Brigneti et al., 1997	50	HpyCH4IV
M33	Brigneti et al., 1997	55	AluI
ADG2	Hämäläinen et al., 1998	55	Cfr13I
Gp163	Brigneti et al., 1997	55	Cfr13I
Nst9256	atggcatcttcttcttcttttgcg	55	BspLI

[1] a.s., allele specific.

Table 4. List of primers used for the N profiling.

Primer name	Position on the gene	Sequence
Nbs13-R	NBS	AAGAARCATGCDATATCTARAAATAT
Nbs12-R	NBS	YTTSARSGCTAAAGGRAGRCC
Nbs-12-F	NBS	CTTTAGCBYTSAARKTGTKKGG
Nbs15-F	NBS	ATGCATGAYTTRATWVAAGABATGGG
Tir270-F	TIR	TATGCTACRTCDAGNTGGTGC
Tir300-F	TIR	NTAGTRAAGAYATGGAATGC
Lrr3050-R	LRR	YGATGGTGGAACCAHCTTGGG
Lrr3150-R	LRR	CAGAGTAACATACARCAAATCCC
Nst9256-F	Start codon	ATGGCATCTTCTTCTTCTTTTGCG
Nst3467-F	Start codon	ATGGCATCATCATCTTCTCCTTCTGAG
Nst381-F	Start codon	CTTCTTCTGCTAAATCGTCACAG
Nst516-F	Start codon	AGTAATTCACAATATTGTCCTCC
Nst911-F	Start codon	TGCAAGTAACTCACTCTACTGG
Nst307-F	Start codon	TCACAGTACTGTCCTCCATGG
Nst055-F	Start codon	TAGATCGTCGCAGTTGACTCC

N profiling was developed by following the NBS profiling protocol (van der Linden et al. 2004) and using *N*-like specific primers. We designed a total of 15 primers (**Table 4**) on N-like sequences, viz. eight degenerated primers were based on conserved regions from the TIR, NBS and LRR domains and seven non degenerated were based on the start codon region of several *N*-like sequences from the RH89-039-16 (RH) physical map (Borm 2008). These primers were used in combination with five enzymes generating blunt ends: AluI, HaeIII, RsaI, DpnI and HincII. The analysis was performed on the LI-COR DNA sequencer using fluorescent-labeled primers. An adapted bulk segregant analysis (BSA, Michelmore et al. 1991) was carried out as follows: bulks with non recombinant plants (10 *R* and 10 S), and bulks of plants with a recombination event on either side of the gene (10 *R* and 10 S). The identified linked markers were first tested

on the F1 individuals of the bulks to confirm the linkage and then applied on the complete population for final validation.

Recombinant screening

The F1 seeds were placed on 96 well format trays for germination in order to facilitate the screen. Two weeks after germination, the second true leaf was harvested and DNA isolated by following the NaOH protocol. The markers Ct182 and M33 and Gp163, flanking *Rpi-avl1*, were applied on the freshly isolated DNA. In total 3840 individuals were screened for recombination events with these two markers. The plant individuals recombinant between the flanking markers were transplanted into pots. CTAB DNA was isolated to confirm the recombination and test the markers co-segregating in the original population of 50 individuals. Recombinant plants were tested for resistance, using late blight isolate 90128 in detached leaf assay.

Results

Resistance in *S. avilesii* accessions

To identify a resistant genotype for *R* gene mapping and cloning, in total 15 genotypes from three *S. avilesii* accessions were tested for their resistance to *P. infestans*. The 15 individuals were first tested by an *in vitro* assay with isolate 90128. Four genotypes from two accessions (avl477-2; avl477-4; avl477-5 and avl478-2) gave a strong level of resistance and all the other 11 genotypes showed clear susceptibility (**Table 1**). In accession number 479 with five tested plants no resistance was observed. Two resistant genotypes, plant 4 from the accession 477, and plant 2 from accession 478, and one susceptible genotype, plant 1 from accession 477, were subsequently tested in a detached leaf assay again with isolate 90128 and with the additional isolate IPO-C. In this particular experiment, the plants were relatively old and almost flowering. The two resistant genotypes showed a medium level of resistance to both isolates which was not highly different from the susceptible control (**Table 1**). However, in various subsequent DLA with younger plants, the genotype avl478-2 showed clearly higher levels of resistance than avl477-4 (see below). The field experiments with the same genotypes revealed much higher levels of resistance to IPO-C than the detached leaf assay. Avl478-2 (**Figure 1**) and avl477-1 were therefore selected for further investigation. The controls cv Bintje and genotypes 14 and 18 from accession 344 of *S. demissum* showed in all 3 resistance assays the expected high level of susceptibility and resistance, respectively. Also the level of

resistance of the standard cultivars present in the field experiment was according to expectation (data not shown).

***P. infestans* resistance spectrum in avl478-2 and avl477-1**

The spectrum of resistance of avl478-2 and avl477-1 was investigated in DLA with 11 *P. infestans* isolates from different origin and with varying virulence spectrums. Macroscopic observation revealed that avl478-2 clearly showed resistance to nine of the 11 isolates tested, and susceptibility to the remaining two isolates (**Table 2**). The susceptible parent showed susceptibility to all the isolates tested, except two isolates that showed intermediate phenotypes. Considering the genetically diverse set of isolates used in this experiment (Champouret et al. 2009), we conclude that *S. avilesii* plant avl478-2 is resistant to several isolates of diverse origins.

Population development

Resistant genotype avl478-2 and susceptible genotype avl477-1 were crossed to generate a mapping population. Fifty F1 individuals were tested for their resistance to *P. infestans* in a detached leaf assay with the isolates 90128 and USA618. However, the resistance phenotype could not be clearly defined for all the individuals. From the 50 individuals, 17 were scored as resistant, 23 as susceptible and 10 individuals could not be characterized unambiguously. It appeared that the age of the plants influenced the resistance phenotype. It turned out that the plants have to be tested when they are young, i.e., before flowering to give a reliable resistant or susceptible phenotype. The segregation of the resistance in the population suggested a 1:1 ratio ($p<0.05$) which is consistent with the presence of a single dominant *R* gene in the resistant parental plant avl478-2, that we designated *Rpi-avl1*. This intra-specific F1 population was suitable to map the *R* gene found in the resistant genotype avl478-2.

Mapping of *Rpi-avl1*

The screening of a global set of approximately 80 SSRs applied on the parents and 20 F1 individuals (10 resistant and 10 susceptible) resulted in one linked marker, marker RH008P12, located on the northern side of chromosome 11 (**Figure 2**, Bakker et al., manuscript in preparation). Linkage with resistance was confirmed on the 40 individuals with 5 recombinants (12 cM). This indicated that *Rpi-avl1* is located on the northern side of chromosome 11. More markers were needed to determine more precisely the position of *Rpi-avl1.* Firstly, known CAPS markers located in the cluster were tested in *S. avilesii*. The four markers Ct182,

M33, ADG2 and Gp163 were polymorphic between the two parents and segregated in the population. The design of primers on the cDNA clones Nl25 and Nl27 (Hehl et al. 1999) resulted in the development of the SCAR marker N2527. The N2527 primers amplified many homologous sequences of the same size (around 2 kb) with an extra smaller fragment of around 1kb on the resistant parent, linked in repulsion phase to the resistant phenotype. The three markers M33, ADG2 and N2527 cosegregated with the resistance in the population of 40 individuals. Ct182 was proximal and Gp163 was distal to *Rpi-avl1* (**Figure 2**). The markers available for this population are summarized in **Table 3**.

Fine mapping of *Rpi-avl1*

To map *Rpi-avl1* at higher resolution, a larger population was required in order to obtain more recombination events. A recombinant screen was performed on around 3840 individuals with the flanking markers Ct182 and Gp163. The 70 recombinant individuals, identified from the screening of the first seven 96 well plates (672 F1 individuals), were characterized for their resistance phenotype. This allowed the separation between the previously cosegregating markers M33 and *Rpi-avl1*. M33 was proximal to the gene and therefore was used instead of Ct182. In total 66 recombinant individuals were found between the markers M33 and Gp163 from the total screen of 3840 F1 individuals (40 x 96 well plates). The resistance phenotype of these recombinants was determined, and the genetic map was constructed (**Figure 2**). The closest flanking markers to *Rpi-avl1* were the marker N2527 located 17 recombinants proximal to *Rpi-avl1* (0.5 cM), and ADG2 with 49 recombinants distal to the gene (1.3 cM). The screen for recombinant F1 individuals revealed that some markers are closely linked to the gene of interest and resulted in a good set of recombinants for further marker development and potential map based cloning of *Rpi-avl1*.

Development of cosegregating N-like profiling marker

The *N* cluster contains a large number of *N*-like sequences which represent a good potential for marker development (**Chapter 3**). The PCR products obtained with the N2527 primers on the DNA of parental and F1 individuals was digested with several restriction enzymes in an attempt to obtain additional markers that mapped closer to *Rpi-avl1*. Unfortunately, the number of homologues amplified with this primer pair was so high that the digestion resulted only in a smear of fragments and no individual fragment could be identified and isolated. This precluded the CAPS marker approach, so we decided to follow a more sensitive approach by adapting the NBS profiling to the specific *N*-like gene family.

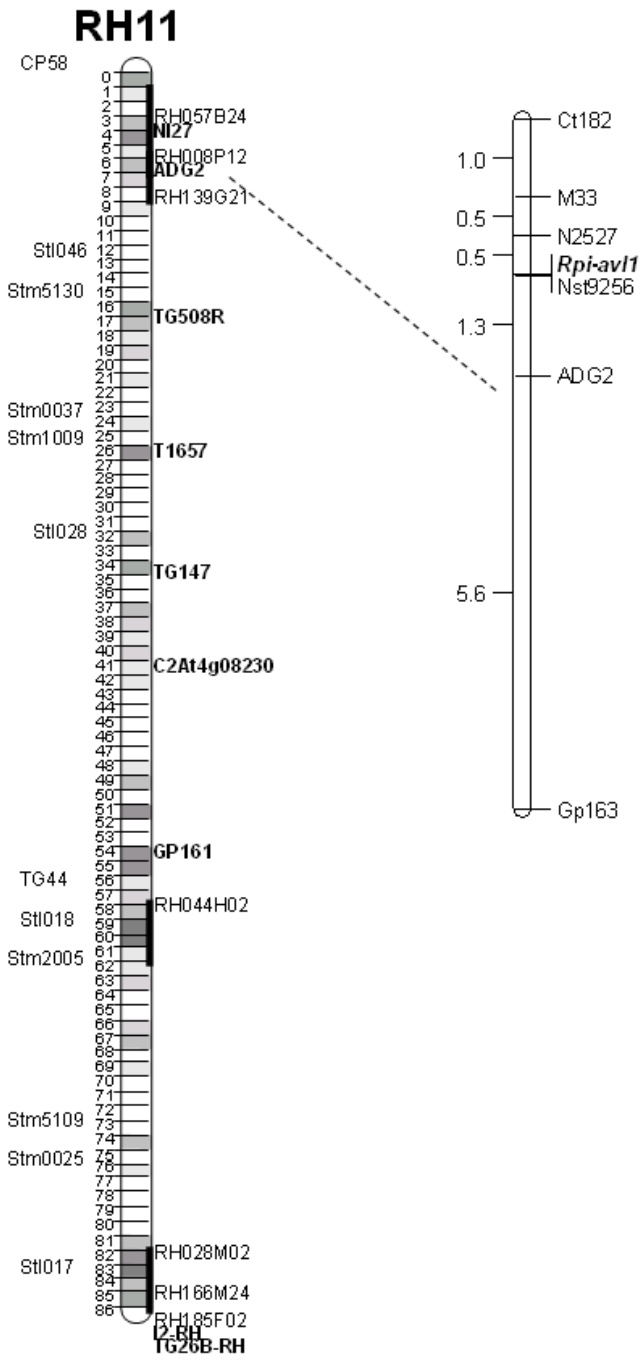

Figure 2. The genetic map of *Rpi-avl1* locus on chromosome 11 compared to the genetic map of the complete RH chromosome 11. On the RH map, the numbers on the left indicate the bin numbers and available markers are located. The mapping position of the markers on the right are accurate whereas the one of the markers on the left are less accurate. The vertical black bars indicate *R* gene clusters. On the *Rpi-avl1* map, the numbers on the left indicate the distance in cM (n=3890). All the markers are PCR markers except the N profiling markers.

RH89-039-16 (RH) BACs containing *R* genes have been sequenced, including several that are located in the vicinity of the *N* cluster on chromosome 11. N-like sequences from RH were aligned and eight degenerate primers were designed on the conserved region of the three domains: TIR, NBS and LRR. Seven primers

were also designed on specific and unique sequences of the start codon (**Table 4**). The 75 primer/enzyme combinations (15 primers x 5 enzymes) were tested on the parents and on the bulks of resistant and susceptible individuals. This screen resulted in 11 putative markers, six of which were confirmed on the individuals. Five markers still mapped a few recombinants away from the gene and were therefore not informative. One marker revealed 100% linkage with the resistance (Nst9256-BspL). The primer Nst9256 is a specific primer designed on the start codon of two similar RH sequences from the BAC clones RH170N15 and RH125M10 and both were mapped in RH Bin 4. This indicates that the *Rpi-avl1* gene indeed lies within an *N* gene like cluster and that it is highly likely that *Rpi-avl1* is an *N* gene homologue.

Figure 3. N profiling marker in coupling phase (Nst9256-BspL) cosegregating with the resistance in 3890 individuals. The first two samples are the parents, followed by the closest F1 recombinant individuals grouped as 14 resistant and 8 susceptible genotypes, indicated by R and S respectively.

Discussion

The search for late blight resistance of South American origin led to the mapping of *Rpi-avl1*, an *R* gene from the Bolivian species *S. avilesii*. *Rpi-avl1* is located on the northern arm of chromosome 11 in the so-called *N* cluster. We call it *N* cluster because sequences with homology to the *N* gene, conferring resistance in *Nicotiana tabacum* to tobacco mosaic virus (TMV, Whitham et al. 1994), have been identified in this cluster in the RH physical map and in other studies (Borm 2008; Hehl et al. 1999; Vidal et al. 2002). The *N* cluster is a hot spot for resistance (Gebhardt and Valkonen 2001) with genes conferring resistance to many pathogens including nematodes, bacteria, fungi and viruses (Brown et al. 1996; Hämäläinen et al. 1998; Hämäläinen et al. 1997; Hehl et al. 1999; Zimnoch-Guzowska et al. 2000). So far *Rpi-avl1* is the first major *R* gene to late blight to be mapped in this cluster, in addition to one QTL conferring resistance to late blight reported to be located there (Leonards-Schippers et al. 1994; Oberhagemann et al. 1999). Interestingly, other *Rpi* genes were subsequently found to be located in the same cluster (**Chapters 3 and 4**).

The gene family specific marker approach made use of the fact that the *N* cluster contains many *N* like sequences. This allowed the development of markers cosegregating with *Rpi-avl1*. The adaptation of NBS profiling to a specific *R* gene family was proven to be successful here and in **Chapters 3 and 4**. The primers mainly target the *N* cluster on chromosome 11 (Dr. Jack Vossen, Wageningen UR Plant Breeding, personal communication), the most important and largest cluster containing genes from the *N* family. The degenerate *N*-like primers and the primers specific for one N-like homologue are both useful and have different advantages. The degenerate primers have a higher chance to lead to the development of a linked marker, whereas the *N*-like sequence specific primers give the possibility to target more loci from the same gene cluster. In this study, the only primer leading to a cosegregating marker was an N-like sequence specific primer, whereas the degenerate primers resulted in the development of markers a few centiMorgans away from the gene of interest. The cosegregating marker could be a good diagnostic single marker for marker assisted selection during introgression breeding. Flanking markers from degenerate primers could be used to select to select for recombinations to reduce the size of the introgression, and hence minimize undesired linkage drag. It must be realized that during the process of introgression breeding with *S. tuberosum* as recurrent parent, the frequency of cross-over events in the introgressed "hybrid" region of the chromosome is often reduced , especially if the donor accession is a distant wild relative. The presence of N-like sequences at the *Rpi-avl1* locus and the identification of the N-like marker that cosegregates with the resistance against *P. infestans* leads to the hypothesis that *Rpi-avl1* might be an N-like *R* gene.

Rpi-avl1 conferred resistance to a relatively large number of different *P. infestans* isolates. However, additional isolates should be tested to draw solid conclusions about the resistance spectrum. The resistance reaction in the detached leaf assay was not in all cases sufficient to fully arrest lesion development, but did reduce pathogen growth, resulting in lesions that were slightly larger than the original inoculation spot. This is different from the observation in the field where a stronger resistance reaction occurred. Clearly, the age of the plant influenced the results in the detached leaf assay. This plant age effect is probably inherent to the particular *R* gene or to the cognate avirulence gene. The *N* gene of *N. tabacum* consists of five introns and six exons and belongs to the TIR-NBS-LRR class of *R* genes, which allows the splicing of different variants from the same pre-mRNA (Kazan 2003). Dinesh-Kumar and Baker (2000) indeed found the

presence of two splicing variants of the *N* gene with a ratio evolving upon pathogen challenge and necessary for the resistance. Other intron-containing genes are predicted to encode multiple variants (Jordan et al. 2002). In these cases, the ratio between the two splicing variants might be easily disturbed, which could affect the level of resistance. If a similar situation applies to *Rpi-avl1*, this could explain the variation of the resistance reaction with aging of the plant. A low level of expression of the effector recognized by *Rpi-avl1* might also be the reason for the relatively weak resistance in aged plants. Future cloning of the gene should give an answer to these questions and offer the possibility to get insight into the mechanism of this *Rpi* gene. Transformation could be helpful to answer the question whether the resistance level of this gene is dependent on the insertion site of the genome. The combination of several *Rpi* genes with different mechanisms and belonging to different clusters could result in more durable resistance. Such a combination may be achieved by introgression breeding or more easily by genetic modification of existing varieties with cisgenic *Rpi* genes.

This study constitutes a good basis to continue with the cloning of *Rpi-avl1*. Depending on the recombination frequency, the cosegregating marker might allow BAC landing (Tanksley et al. 1995). The availability of the potato genome sequence will facilitate eventual chromosome walking and marker development required to finalize the cloning (http://www.potatogenome.net). Knowing the avirulence gene of *Rpi-avl1* could allow quick complementation studies by co-infiltration of the candidate *R* and *Avr* genes in the model plant *N. benthamiana* and monitoring for occurrence of specific cell death (Armstrong et al. 2005;Goodin et al. 2008;Vleeshouwers et al. 2008). An alternative, but less reliable way, is co-infiltration of the candidate *Rpi-avl1* gene in *N. benthamiana* followed by *P. infestans* inoculation of the plant, and observation of the site specific reaction of the plant for resistance or susceptibility (Lokossou et al. 2009; Pel et al. 2009). At this moment, *Rpi-avl1* could already be introduced in a conventional potato breeding program and be selected for with the cosegregating N-like marker.

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Chapter 3

High resolution mapping of genes conferring resistance to late blight from two wild species of the *Solanum circaeifolium* group

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Abstract

Resistance to *Phytophthora infestans* in *Solanum* is based on the gene for gene interaction. Identification of hitherto unknown *R* genes could help fighting against late blight, caused by this devastating oomycete, by pyramiding or *R* gene stacking through cisgenesis. *Rpi-cap1* and *Rpi-qum1*, the resistance genes to *P. infestans* from the wild species *S. capsicibaccatum* and S. *circaeifolium* spp. *quimense* respectively, are located on the northern part of chromosome 11 in a hot spot for resistance. Despite the difficulties encountered for marker development, a high resolution genetic map with CAPS markers was constructed. A gene specific profiling approach led to the development of a co-segregating marker. The two *R* genes are hypothesized to be homologous to the *N* gene, a TIR-NBS-LRR type of resistance gene to TMV in tobacco. Cloning of the *Rpi-cap1* should be pursued as it is a good *R* gene to be included in a cisgenic breeding approach.

Introduction

The gene-for-gene hypothesis was first proposed by Harold Flor in the 1940's. It is the most studied model explaining plant resistance to pathogens. This model, which was later reviewed by the same author (Flor 1971), states that for each resistance (R) gene of the plant there is a corresponding avirulence gene in the pathogen. Recently, the term avirulence has been replaced by the term effector (Hogenhout et al. 2009). Recognition of the effector protein by the R protein will lead to the activation of plant defenses and stop pathogen growth. Two main R gene domain architectures have been identified: NB-LRR and eLRR (Chisholm et al. 2006). The NB-LRR, which is the largest class of R genes, is characterized by two domains: the nucleotide binding site (NBS) and leucine-rich repeats (LRR)

(Rairdan and Moffett 2007). On the plant chromosomes these genes tend to occur in clusters containing pseudogenes and functional genes (Michelmore and Meyers 1998). In their review, these authors described the theory for the evolution of *R* genes and concluded that the presence of complex *R* gene clusters can explain how plants can generate and maintain large numbers of resistance specificities against ever-changing pathogen populations.

The oomycete *Phytophthora infestans* (Mont. de Bary) causing late blight in potato is the most devastating pathogen in potato (*Solanum tuberosum*) production. Currently the disease is controlled by chemicals which involves high costs and raises environmental concerns. Past experiences indicated that relying on single *R* genes was not suitable because of the quick breakdown of the *R* gene by the rapidly evolving pathogen (McDonald and Linde 2002). So pyramiding several *R* genes in a single genotype might be better and may be a more durable option to fight late blight (McDowell and Woffenden 2003; Pink 2002). A practical way to stack several hitherto unexploited *R* genes in potato cultivars is through cisgenesis (Haverkort et al. 2008; Jacobsen and Schouten 2007; Schouten et al. 2006). This approach requires the availability of a larger number of diverse *R* genes with complementary spectra of resistance. Resistance spectrum and effector recognition are important aspects to take into account in selecting the most promising *R* genes for stacking to reach a durable resistance.

The genomic region on the northern distal end of chromosome 11 in potato has been characterized as a resistance "hotspot" in a review by Gebhardt and Valkonen (2001), harboring two *R* genes giving resistance to viruses, one to a nematode and one to a fungus and two QTL, one giving resistance to potato late blight and one to *Erwinia* soft rot. Currently, one more gene can be added to that list: *Y-1* occurring in *S. tuberosum* subsp. *andigena* and conferring cell death upon infection with potato virus Y (PVY), and homologuous to the *N* gene (Vidal et al. 2002). This *R* gene rich potato genomic region on chromosome 11 is syntenic to the tobacco genome segment containing the *N* gene (Whitham et al. 1994). The *N* gene was one of the first *R* genes to be cloned (Whitham et al. 1994), and confers resistance to tobacco mosaic virus (TMV virus) in tobacco. It is an NBS LRR gene preceded by a TIR (toll interleukin 1 receptor) domain. The syntenic *R* gene cluster in potato contains sequences homologous to the *N* gene, as was demonstrated by cDNA clones (Hehl et al. 1999), N-like specific primers and sequences (Hämäläinen et al. 1998; Leister et al. 1996) and BAC end sequences (Zhang and Gassmann 2007). This *R* gene rich region apparently

contains a large number of N-like sequences, suggesting that the *R* genes present in this region must be homologous to the *N* gene. Therefore we refer to this region as the *N* cluster. To date only one *R* gene is mapped in the syntenic tomato genomic region, the gene *I* conferring resistance to *Fusarium oxysporum* f. sp. *Lycopersici* (Sarfatti et al. 1991).

In a recent study, Jacobs et al. (2010) identified the mapping position of *Rpi-cap1,* the gene giving resistance to *P. infestans* in the diploid wild *Solanum* species *S. capsicibaccatum* Cardenas, on chromosome 11 in the *N* cluster. The *R* gene from the wild *S. capsicibaccatum* species seems very interesting for cloning as the wild potato genotype shows high levels of resistance to late blight in the field and has a broad resistance spectrum. To broaden this *R* gene mapping study, the *S. circaeifolium ssp. quimense* Hawkes & Hjerting diploid wild species, also from the *Circaeifolia* series, was included here. *S. circaeifolium ssp. quimense* is phylogenetically related to *S. capsicibaccatum* and also shows strong resistance to late blight. The gene occurring in this species is referred to as *Rpi-qum1*. *S. capsicibaccatum* and *S. circaeifolium,* which contains two subspecies *quimense* and *circaeifolium,* are both diploid *Solanum* species endemic to Bolivia (Hawkes and Hjerting 1989). The aim of this study was to fine map the genes *Rpi-cap1* and *Rpi-qum1* to facilitate their map based cloning. Large mapping populations were tested for each *R* gene. A gene specific marker approach combined with bulk segregant analysis (BSA, Michelmore et al. 1991), described in **Chapter 2**, was applied to obtain markers cosegregating with *Rpi-cap1*.

Material and methods

Plant material and mapping population

Accessions of *S. capsicibaccatum*, *S. circaeifolium* ssp. *quimense* and *S. circaeifolium* ssp. *circaeifolium* (**Table S1**) were provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. The two resistant genotypes *S. capsicibaccatum* (cap) 536-1 and *S. circaeifolium* ssp. *quimense* (qum) 567-1 were selected and were crossed with susceptible plants of *S. circaeifolium* ssp. *circaeifolium* (crc) 564-3 and crc564-4 to generate intra-specific F1 mapping populations for *Rpi-cap1* and *Rpi-qum1,* respectively (**Table 1**).

Phytophthora and disease test

The *P. infestans* isolates used in this study which were kindly provided by Francine Govers, Geert Kessel, and Paul Birch (**Table S2)**. *P. infestans* isolate 90128 was used to screen for resistant genotypes from the accessions *S.*

capsicibaccatum and *S. circaeifolium* ssp. *quimense.* The F1 mapping populations were inoculated with the same isolate to test for segregation of resistance. The segregants were also tested with four other isolates VK98014, IPO-4282, NL01096 and EC1. The resistance spectrum of the parents of the crosses was characterized by testing 21 isolates (**Table S2)**.

Screening the *Solanum* section Circaeifolia accessions for resistance to *P. infestans* isolate 90128 was performed using an *in vitro* inoculation assay (Huang 2005). Further resistance phenotyping of selected genotypes was performed with a detached leaf assay (**Figure 1**), as described by Vleeshouwers et al., (1999). Depending on their size, the leaves of five weeks old plants were inoculated with 6 to 10 drops of inoculum with 50,000 zoospores per ml. The leaves were scored six days after inoculation as resistant due to a hypersensitive response (HR, small necrotic lesion) or as susceptible if a sporulating lesion appeared (large lesion with sporangiophores).

Table 1. Description of the *Rpi-cap1* and *Rpi-qum1* F1 populations. Segregation of resistance in the detached leaf assay is indicated as: R for resistant and S for susceptible F1 plants.

Gene	R parent	S parent	Population size	R	S	Chi 2
Rpi-cap1	cap536-1	crc564-3	108	64	44	<0.05
Rpi-qum1	qum567-1	crc564-4	50	25	25	<0.05

Figure 1. Detached leaf assay on some F1 individuals of *Rpi-cap1* population, six days after inoculation. The phenotype resistant or susceptible in response to *P. infestans* (isolate 90128) inoculation is easy to determine between the green leaves and the sporulating, brownish and wrinkled leaves, respectively.

DNA isolation

Genomic DNA was isolated following two different protocols: CTAB and NaOH. The CTAB protocol was performed on material of which the DNA needs long storage like the mapping population and the interesting recombinants. Young leaf tissue was collected for DNA isolation according to the CTAB protocol with the Retsch machine (RETSCH INC., Hannover, Germany). The NaOH protocol was used for screening for recombination events in the population. Leaf samples were collected on three weeks old seedlings and DNA was isolated by following the NaOH protocol (Wang et al. 1993). The PCR amplifications were directly performed on these samples.

Marker development and map construction

PCR markers were developed for the northern arm of chromosome 11. Two different approaches were followed to identify additional markers. First, primers derived from known markers mapped on the northern arm of chromosome 11 were tested for polymorphism. Second, primers were designed on the basis of BAC sequences mapped on the northern arm of chromosome 11 from RH89-039-16 (RH) (http://www.potatogenome.net/). These primers were first tested for amplification on the parents. If a fragment was present for the two parents, the PCR product was screened with a selection of restriction enzymes for the identification of polymorphism. A polymorphism was only of interest if a fragment was present in the resistant parent and absent in the susceptible parent. The marker was later tested on the F1 individuals to determine the segregation and possible association with the resistance phenotype. In case the polymorphic fragment occurred in all progeny of the mapping population, the resistant parent was homozygous for that polymorphism and it could not be used. Genetic maps were constructed using the number of recombination events between the markers and between the markers and the resistant/susceptible phenotype.

Recombinant screening

Seeds from the crosses were placed on 96 well format trays for germination in order to facilitate the screen. Two weeks after germination, the second true leaf was harvested and DNA isolated by following the NaOH protocol. The markers NI27 and T079, flanking *Rpi-cap1*, and the markers NI27, T081 and ADG2, flanking *Rpi-qum1*, were applied on the freshly isolated DNA. 848 individuals for *Rpi-cap1* and 349 individuals for *Rpi-qum1* were screened with these markers. The F1 individuals recombined between the flanking markers were transplanted into pots. We later isolated CTAB DNA to confirm the recombination. All the

recombinant plants were tested for resistance phenotypes, using the detached leaf assay and isolate 90128. The recombinants were transferred to *in vitro* to be maintained and their phenotype was retested.

N-like profiling

The N-like profiling is based on the NBS profiling protocol as described by van der Linden et al. (2004). We designed a total of eight degenerate primers on N-like sequences, based on conserved regions from the TIR, NBS and LRR domains (see **Chapter 2**). These primers were used in combination with five restriction enzymes generating blunt ends. The analysis was performed on LI-COR DNA sequencer (Lincoln, Nebraska, USA) using fluorescent-labeled primers. An adapted BSA was carried out: bulks with non recombined plants: 10 R and 10 S, and bulks of plants with recombination on either side of the gene, also 10 R and 10 S. The identified linked markers were verified on the F1 individuals of the bulks and then applied on the complete population for validation and mapping.

Results

Resistance in *S. capsicibaccatum* and *S. circaeifolium* spp. *quimense* accessions

To identify resistant genotypes to be used as parents for crossing with susceptible plants, several accessions of the wild species *S. capsicibaccatum* and both *S. circaeifolium* subspecies were screened for resistance to *P. infestans* isolate 90128. At least two or more plants per accession were tested (**Table S1**). For *S. capsicibaccatum,* forty plants from eight accessions were tested. Two genotypes showed resistance to *P. infestans*, one was from the accession 536, and the other one from 334. For *S. circaeifolium*, 30 plants from six accessions were tested and the majority of these, 22, were resistant. It seems, therefore, that resistance to *P. infestans* is the rule in S. *circaeifolium* and the exception in *S. capsicibaccatum*, at least with respect to *P. infestans* isolate 90128.

Population development and spectrum of late blight resistance

The resistant genotypes cap536-1 and qum567-1 from *S. capsicibaccatum* and *S. circaeifolium* ssp. *quimense,* respectively, were crossed with the susceptible *S. circaeifolium* ssp. *circaeifolium* crc564-3 and crc564-4, respectively. A detached leaf assay performed on the F1 plants allowed an unambiguous classification of resistant and susceptible phenotypes (**Figure 1**). The segregation of the resistance in the two populations followed a 1:1 ratio ($p<0.05$) which is consistent with the presence of a single dominant *R* genes *Rpi-cap1* and *Rpi-*

qum1 in the resistant parental plants cap536-1 and qum567-1, respectively (**Table 1**). Both F1 populations are suitable to map the *R* gene derived from the resistant parent. The resistance spectra of the resistant parental genotypes cap536-1 and qum567-1 were determined by challenging them with 21 isolates of different complexity and geographic provenance (**Table 2**). Both genotypes were resistant to all 21 isolates tested, indicating that they have a similar and wide resistance spectrum. The *S. circaeifolium* genotypes used for the crosses that were susceptible for isolate 90128 still showed resistance to several other isolates (**Table 2**), which suggests that additional *R* genes are present in *S. circaeifolium.* As a consequence, to test the segregation of *Rpi-cap1* and *Rpi-qum1* in the two F1 populations, only the isolates that were virulent on the two *S. circaeifolium* ssp. *circaeifolium* genotypes (crc564-3 and -4) could be used.

Table 2. Resistance phenotypes caused by *Phytophthora infestans* isolates on resistant parents cap536-1 and qum567-1, susceptible parents crc564-3 and crc564-4, and 10 resistant and 10 susceptible F1 genotypes from the *Rpi-cap1* population in detached leaf assays. R indicates resistant, S susceptible, Nd not determined.

P.i. isolate	cap536-1	crc564- 3	10 R F1 (cap)	10 S F1 (cap)	qum567-1	crc564-4
VK98014	R	S	R	S	R	S
IPO428-2	R	S	R	S	R	S
NL01096	R	S	R	S	R	S
EC1	R	S	R	S	R	S
90128	R	S	Nd	Nd	R	S
NL00228	R	S	Nd	Nd	R	S
F95573	R	S	Nd	Nd	R	S
H30P04	R	R	Nd	Nd	R	R
USA618	R	R	Nd	Nd	R	R
IPO-C	R	R	Nd	Nd	R	R
PIC99183	R	R	Nd	Nd	R	R
PIC99177	R	R	Nd	Nd	R	R
PIC99189	R	R	Nd	Nd	R	R
IPO-0	R	Nd	Nd	Nd	R	Nd
UK7824	R	Nd	Nd	Nd	R	Nd
88133	R	Nd	Nd	Nd	R	Nd
91011	R	Nd	Nd	Nd	R	Nd
88069	R	Nd	Nd	Nd	R	Nd
NL05-194	R	Nd	Nd	Nd	R	Nd
CA-65	R	Nd	Nd	Nd	R	Nd
3128-A	R	Nd	Nd	Nd	R	Nd

Mapping and fine mapping of *Rpi-cap1*

In a previous study using the same F1 population of cap563-1 x crc564-3, *Rpi-cap1* was shown to be located at the northern distal end of chromosome 11 in the *N* cluster by Jacobs et al. (2010). They identified the map position by NBS profiling and could link the resistance phenotype to the CAPS marker Cp58 with one recombinant out of 20 F1 individuals. To confirm the mapping position and

construct a genetic map, we characterized the resistance phenotype to *P. infestans* isolate 90128 of 108 F1 individuals of the *Rpi-cap1* population. This population was used for the development of CAPS markers. The linkage of the resistance gene with the CAPS marker Cp58 was confirmed with two recombinants found in a set of 91 individuals. Markers previously mapped in the *N* cluster in *S. tuberosum* were also tested. Six out of seven primer combinations tested gave amplification on the wild genotypes, of which only two resulted in polymorphism. These were markers Nl27 (Marczewski et al. 2001), derived from the NL-27 protein, and M33 (Brigneti et al. 1997). In parallel, markers were developed using sequence information available from the RH physical map on chromosome 11. Primers, designed on these BAC sequences, were applied to the DNA of cap536-1 and crc564-3. Out of 17 primer pairs giving PCR amplification in RH, only five gave an amplification product on the parental genotypes, and only two resulted in CAPS markers: T179 and T081. **Table 3** summarizes the markers developed for the *Rpi-cap1* population.

Table 3. PCR markers for the two F1 mapping populations of *Rpi-cap1* and *Rpi-qum1*, with primer sequences based on BAC sequences located on the northern arm of chromosome 11 from the reference genotype RH or references for primers already published, annealing temperature in °C (TM), and restriction enzyme to identify a polymorphism for each population. *Sequences of primers designed in this study.

Marker	Sequence* or Reference	TM	Enzyme	
			for cap	for qum
Nl27	Marczewski et al. 2001	55	As.	XapI
M33	Brigneti et al. 1997	62	AluI	-
ADG2	Hämäläinen et al. 1998	55	-	PsuI
T081	F GTTGGGCAGGTACTCAATGG R ATTCAGGACGGGTCATTAGG	55	MseI	HpyF3I
T179	F CTAGCTCTGTCCCCGTCCAC R CCGTGTTTACACCTAACTCAACC	55	AluI	-
Tir300F-Hinc	NTAGTRAAGAYATGGAATGC	55	HincI	-
Nbs15F-BspL	ATGCATGAYTTRATWVAAGABATGGG	55	BspLI	-

The marker development work resulted in five CAPS markers (Cp58, Nl27, M33, T179 and T081) to be tested on the complete population of 108 individuals to construct the genetic map of the *Rpi-cap1* region. The markers Cp58 and Nl27 were distal, T081 and T179 were proximal to the gene, and M33 was cosegregating with *Rpi-cap1* (data not shown). The markers were at least two recombinants away out of 108 individuals from *Rpi-cap1,* which was sufficient to rely on the order of the markers. This allowed the construction of a genetic map and the identification of flanking markers, necessary for the recombinant screen.

To map *Rpi-cap1* at a higher resolution, a larger population was required in order to have more recombination events. A recombinant screen was performed on 848 individuals with the flanking markers Cp58, Nl27 and T179. The SCAR marker Nl27 was multiplexed with another primer pair as internal control to test for DNA quality and false negative amplification. In total 792 plants out of 848 could be scored for these three markers. Nine 96 well plates were screened and each plate is referred to as a series. In the first two series of screening, more recombinants were identified between the marker Nl27 and *Rpi-cap1*, than between Cp58 and *Rpi-cap1*, suggesting that Nl27 was further away from the gene than Cp58. So in the last seven series, only Nl27 was used. In total 85 recombinants were detected, phenotyped and re-genotyped after CTAB DNA isolation. Out of the 85 recombinants, only 14 F1 individuals were confirmed as true recombinants. False recombinants were mainly due to the SCAR marker Nl27. The 14 recombinants were brought and maintained *in vitro* and their resistance phenotype was later retested in detached leaf assays and could be confirmed.

The genetic map of *Rpi-cap1* obtained with a population of 900 individuals is presented in **Figure 2**. The closest flanking markers to *Rpi-cap1* are the markers Nl27 and M33, both 0.1 cM (1 recombinant) away on each side of the gene. In the population of 108 individuals, the distance between *Rpi-cap1* and Nl27 appeared larger because of false positives. The Cp58 marker mapped 0.9 cM (8 recombinants) distal to Nl27. The recombinant screen allowed the construction of a reliable high resolution map of *Rpi-cap1* which should have a sufficient resolution to isolate the gene by map-based cloning.

High resolution mapping of *Rpi-qum1*

Similarly as for *Rpi-cap1*, the mapping position of *Rpi-qum1* was hypothesized to be located on chromosome 11 because of an NBS linked marker with homology to N-like sequences (Dr. M. Jacobs, Wageningen UR plant breeding, personal communication). We characterized 53 individuals of the *Rpi-qum1* mapping population with the *P. infestans* isolate 90128. And, to confirm the putative mapping position, the same primer combinations as for the *Rpi-cap1* population, were tested on the *Rpi-qum1* population. The markers from the *Rpi-cap1* population (Cp58 and M33) were not polymorphic in the *Rpi-qum1* population. Only Nl27 was polymorphic and segregating in the *Rpi-qum1* population. An extra known marker ADG2 (Hämäläinen et al. 1998), not polymorphic in the *Rpi-cap1* population, was polymorphic and segregated in the *Rpi-qum1* population. The primers designed on the RH sequences had the same results as for the *Rpi-cap1*

population, most of them did not give any PCR products and only marker (T081) could be developed. The three markers Nl27, T081 and ADG2 revealed linkage with the resistance phenotype confirming that *Rpi-qum1* also maps in the *N* cluster on chromosome 11. The marker Nl27 was distal to the gene with one recombinant, T081 was proximal with two recombinants. ADG2 was proximal to the gene with six recombinants, in a population of 53 individuals. It shows a similar mapping position as *Rpi-cap1*.

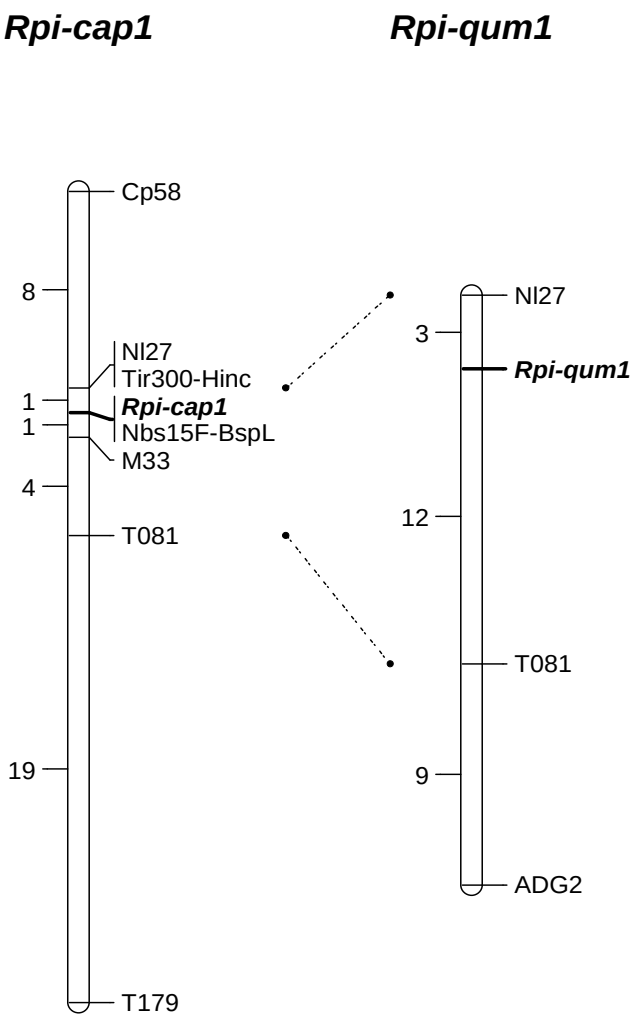

Figure 2. The genetic maps of *Rpi-cap1* and *Rpi-qum1* on chromosome 11. The numbers on the left indicate the number of recombinants out of 900 individuals for *Rpi-cap1* and 306 for *Rpi-qum1*. All the markers are CAPs markers except Nbs15F-BspL and Tir300-Hinc that are N profiling markers. The dotted lines connect the markers present in both populations.

A more extensive recombinant screen for *Rpi-qum1* was performed with the markers Nl27, T081 and ADG2 on 349 individuals. In total 306 plants could be scored with at least two markers. The same procedure was followed as for *Rpi-cap1*. In total, 21 recombinants were found, phenotyped and re-genotyped. The

16 confirmed recombinants between the markers Nl27 and ADG2 were brought in vitro and their resistance phenotype was retested and confirmed. The genetic map of *Rpi-qum1* resulting from that analysis is given in **Figure 2**. In the population of 360 individuals, the markers Nl27 and ADG2 were 6.7 cM away from each other (24 recombinants). The closest flanking markers were Nl27 and T081, which were located 0.8 cM (3 recombinants) distal and 2.5 cM (12 recombinants) proximal to *Rpi-qum1*, respectively.

Figure 3. An N-like profiling marker cosegregating with the resistance in 900 F1 individuals from the *Rpi-cap1* population. It was generated with the primer Nbs15F in combination with the enzyme BspLI. The phenotype is indicated with R for resistant and S for susceptible. On the left: Screen of the bulks of 10 resistant and susceptible F1 plants respectively, from left to right: the parents, bulks with non recombinant F1 plants, bulks with F1 plants recombined between the closest markers to the gene. On the right: Individual F1 plants including the ones with recombination events close to *Rpi-cap1*.

Development of cosegregating N-like profiling marker

Since *Rpi-cap1* and *Rpi-qum1* are located in a N-like gene rich cluster, we developed markers on the basis of N-gene specific sequences. We performed that study on *Rpi-cap1* as the population available was larger. The N-like profiling approach was done by following the classical NBS profiling protocol (van der Linden et al. 2004) and substituting the NBS degenerate primers for degenerate N-like primers (**Chapter 2**). From the 40 primer enzyme combinations tested, 21 markers were identified on the bulks and 10 were confirmed on the individuals of the bulks as linked with the resistance phenotype. Linked markers were identified on both sides of the gene, indicating that the *N* cluster is extending on both sides of the gene. Only the two closest linked markers, Tir300F-Hinc and Nbs15F-BspL, were added to the genetic map (**Figure 2**). Marker Tir300-Hinc, positioned distal to the gene and cosegregating with the marker Nl27, confirmed the single recombinant found between these markers and *Rpi-cap1*. Marker Nbs15F-BspL cosegregated with the resistance in 900 individuals (**Figures 2 and 3**). This confirmed that *N* homologous sequences are located in that cluster and suggests that the resistance genes *Rpi-cap1* and *Rpi-qum1* are likely to be homologous to the *N* gene.

Discussion

Resistance to *P. infestans* in the studied genotypes of wild Bolivian accessions of *Solanum* species *S. capsicibaccatum* and *S. circaeifolium* ssp*. quimense* is monogenic and is conferred by one dominant genes located in the *N* cluster on chromosome 11 in the *N* cluster: designated *Rpi-cap1* and *Rpi-qum1*. Segregating F1 populations, generated by crossing the selected resistant genotypes with susceptible genotypes, were characterized and CAPS markers from the *N* cluster were developed to construct a genetic map of the region where both *R* genes were located. Next step was to screen for individuals with recombination events between flanking markers to fine map the gene more precisely. The plants with the closest recombination events to *Rpi-cap1* were used to develop a N-like specific marker fully cosegregating with the resistance. This marker can be very useful for further cloning of *Rpi-cap1*. To date, the first functional potato resistance gene located on the northern arm of chromosome 11 in the *N* cluster has yet to be cloned (Hein et al. 2009a). RH BAC sequences and potato genome sequences (http://www.potatogenome.net) revealed that many *R* gene homologues are present in the *N* cluster, and since gene sequence extremities are variable, an allele mining approach is not feasible. A map based cloning approach is probably the most appropriate strategy to follow and should be successful as the phenotypic contrast between genotypes is large even though the levels of polymorphism and heterozygosity are not very high. *Rpi-cap1* and *Rpi-qum1* have a very wide resistance spectrum as they are resistant to all 21 isolates tested so far, and are thus very interesting genes to include in breeding programs.

In the tested accessions of *S. capsicibaccatum* the occurrence of resistance to late blight isolates is an exception whereas in those of *S. circaeifolium* ssp*. quimense,* it is more the rule. These observations suggest that the occurrence of the resistance in *S. circaeifolium* ssp*. quimense* is well established. In addition to *Rpi-cap1* and *Rpi-qum1*, additional resistance was identified in *S. circaeifolium* spp. *circaeifolium*. Altogether these data suggest a stronger disease pressure in the locations where *S. circaeifolium* accessions were collected, especially for *S. circaeifolium* ssp*. quimense*. Plants containing *R* genes have a selective advantage in regions favorable for late blight infections and the gene is more easily maintained. It might also be an indication of the presence of more *Rpi* genes in the cluster within one genotype, which would occur after a duplication or hybridization of two genotypes containing different *R* genes in the same *N* cluster. As the resistance in the tested accessions of *S. capsicibaccatum* appears as an isolated event, the probability that a single *R* gene is present in the cluster is

higher than in *S. circaeifolium* ssp*. quimense,* so *Rpi-cap1* is an easier and thus maybe better choice to continue with the cloning. If several *R* genes are present in *S. circaeifolium* ssp*. quimense*, they could be discriminated with different isolates or with the use of effectors (**Chapter 4**). This could provide a better understanding of the resistance in these wild *Solanum* species, as recently described by Lokossou (2010) and Champouret (2010).

The wild diploid species *S. capsicibaccatum* and *S. circaeifolium* ssp. *quimense* are not closely related to *S. tuberosum*. However, hybridization with diploid genotypes of *S. tuberosum* is possible resulting in F1 individuals with different ploidy levels (Louwes et al. 1992). Therefore, in this study the resistant genotypes were crossed with susceptible genotypes from the Circaeifolia series. Marker development was hampered because of a large difference between the *S. tuberosum* sequences used to design the primers and the genomic DNA of the *S. circaeifolium* group on which these primers were applied. Crosses, although inter-specific, are within the Circaeifolia series, which in this case implied a low level of polymorphism. So, from the large number of primer combinations designed on the *S. tuberosum* RH sequences, the relatively few primer combinations that gave amplification products on the wild species were often not polymorphic. The small phylogenetic distance within the *Circaeifolia* species renders the development of markers difficult because of absence of a sufficient amount of polymorphism. The large phylogenetic distance between these wild species and *S. tuberosum* limits the use of recently available information such as the physical mapping and genome sequences that normally could speed up the cloning process. Here the RH physical map (Borm 2008) and available sequences were of limited use to get closer to the genes, as is needed for a map based cloning approach.

Limitations in marker development would make the chromosome walking step of the map based cloning approach quite challenging. So the development of a marker cosegregating with the resistance in a large population is very helpful and could be used for BAC landing. The *Rpi-cap1* population was more suitable for that analysis as the available population was larger. The N-like profiling is an approach used to identify markers closely linked to *R* genes in the *N* cluster on chromosome 11, and was also successfully applied for *Rpi-avl1* from *S. avilesii* and *Rpi-end3* from *S. x edinense* (**Chapters 2 and 4**). It is based on NBS profiling with family specific primers for the targeted cluster. The objective is to get close to the gene of interest to be able to do BAC landing. The profiling markers can efficiently be used for BAC library screening, by diluting the

restriction ligation reaction for the PCR reaction, due to less complexity in the DNA template. Specific gene profiling can be very useful to speed up the physical mapping, shorten chromosome walking or achieve BAC landing.

The very similar mapping positions of *Rpi-cap1* and *Rpi-qum1,* the small phylogenetic distance between the two species, their resistance spectrum and their common geographic origin suggest that they could be the same gene or alleles of the same gene. *Rpi-avl1* from the wild species *S. avilesii* from the series *Tuberosa* also maps on chromosome 11 in the same *N* cluster (**Chapter 2**). *R4* from *S. demissum* which is also found in *S. x edinense* also maps on chromosome 11 in the *N* cluster (**Chapter 4**). Both *R* genes have a more narrow resistance spectrum than *Rpi-cap1* and *Rpi-qum1*. It shows that *Rpi* genes mapped in the same cluster can have different specificities. It has also been observed in the *R3* cluster with all the *S. demissum R* genes (Huang 2005) and in the *R2* cluster with the R2 family and *Rpi-mcd1* (Lokossou 2010). Two major genes conferring resistance to *P. infestans* from wild *Solanum* species can now be added to the *N* cluster, a hot spot for resistance to many pathogens on chromosome 11 (Gebhardt and Valkonen 2001). Because of their map position in the *N* cluster, *Rpi-cap1* and *Rpi-qum1* can be hypothesized to belong to the TIR-NBS-LRR class, similar to the *N* gene. They would be the first major genes conferring resistance to *P. infestans* from that class, as all cloned *Rpi* genes so far belong to the CC-NBS-LRR class (Hein et al. 2009a).

The *R* genes present in the wild *S. capsicibaccatum* and *S. circaeifolium* ssp. *quimense* appear to be very useful for resistance breeding programs applying a cisgenic approach (Jacobsen and Schouten 2007; Schouten and Jacobsen 2008). Cisgenes are defined as natural genes with their expression elements that originate from the same plant species or from crossable species, introduced by marker free plant transformation. The Circaeifolia series was suggested to be a rather primitive isolated series (Hawkes and Hjerting 1989), and was reported to only rarely intercross with species from other series (Louwes et al. 1992). Successful crosses with other series, *Tuberosa* in particular, have been observed: the species *S. capsicibaccatum* and *S. circaeifolium* ssp. *quimense* could both successfully be crossed with *S. lignicaule* from the series *Tuberosa* and with di(ha)ploid clones from *S. tuberosum* and resulted in true hybrids (Chavez et al. 1988). Therefore, after cloning, *Rpi-cap1* and *Rpi-qum1* would qualify as cisgenes.

Acknowledgement

We like to thank Hendrik Rietman for testing parental genotypes with some *P. infestans* isolates and Mirjam Jacobs for the NBS profiling marker linked to *Rpi-qum1.*

Supplementary material

Table S1. List of accessions tested from the wild *Solanum* section *Circaeifolia* species *S. capsicibaccatum* (cap) and *S. circaeifolium* spp. *quimense* (qum). Individual genotypes were tested in detached leaf assay for their resistance to *P. infestans* isolate 90128. The accessions from which the resistant genotypes of this study originated are indicated in bold.

Species	Accession	Gene bank	Number of genotypes tested	Number of resistant genotypes
cap	535	CGN[1]18254	5	0
cap	538	CGN18265	4	0
cap	335	CGN18268	9	0
cap	261	BGRC[2]35377	5	0
cap	534	CGN18291	5	0
cap	334	CGN18297	5	1
cap	**536**	**CGN22388**	**2**	**1**
cap	566	CGN22767	5	0
crc	564	CGN18133	5	0
crc	563	BGRC27058	5	5
qum	340	CGN18127	3	1
qum	341	CGN18128	9	8
qum	**567**	**CGN18158**	**3**	**3**
qum	565	CGN20643	5	5

[1] Center for Genetic Resources, The Netherlands (CGN, http://www.cgn.wur.nl/uk/)
[2] Braunschweig Genetic Resource Center (BGRC)

Table S2. *Phytophthora infestans* isolates with virulence factors and geographic origin.

P.i. isolate	Country of origin	Race
VK98014	The Netherlands	1,2,4,11
IPO428-2	The Netherlands	1,3,4,7,8,10,11
NI01096	The Netherlands	1,3,4,7,8,10,11
EC1	Ecuador	1,3,4,7,10,11
90128	The Netherlands	1,3,4,7,8,10,11
NL00228	The Netherlands	1,2,4,7
F95573	The Netherlands	1,3,4,7,10,11
H30P04	The Netherlands	3[a], 7,10,11
USA618	Mexico	1,2,3,6,7,10,11
IPO-C	Belgium	1,2,3,4,5,6,7,10,11
PIC99183	Mexico	1,2,3,4,5,7,8,10,11
PIC99177	Mexico	1,2,3,4,7,9,11
PIC99189	Mexico	1,2,5,7,10,11
IPO-0	Unknwon	3b,4,7,10,11
UK7824	United Kingdom	1,2,3,6,7
88133	The Netherlands	1,3,5,7,11
91011	The Netherlands	3,4,5,11
88069	The Netherlands	1,3,4,7
NL05-194	The Netherlands	Nd
CA-65	Unknown	Nd
3128-A	Unknown	Nd

Chapter 4

Natural stacking of at least three *R* genes for wide spectrum resistance to *Phytophthora infestans* in *S. x edinense*

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Abstract

S. x edinense is a natural hybrid between the Mexican *Solanum demissum* and the South American *S. tuberosum* spp. *andigena*. Studying the genetic basis of the high level of resistance to *Phytophthora infestans* observed in that species revealed the presence of multiple *R* genes in different accessions. Using an F1 mapping population, three *R* genes were identified on different chromosomes. The use of SSR markers and NBS profiling, combined with phenotyping for effector response in a F1 population allowed the identification of an *R2* homologue on chromosome 4, a second *R* gene on the long arm of chromosome 9, and $R4^{Ma}$ on the Northern arm of chromosome 11. Although each *R* gene has been overcome by some *P. infestans* isolates, the resistance spectrum of *S. x edinense* covers all the isolates tested except for one. This natural stacking *R* genes from different origins, clusters, and resistance spectra serves as an example which should be followed in breeding programs.

Introduction

The Oomycete *Phytophthora infestans* is the causal agent of late blight in *Solanum*. The pathogen evolves quickly which renders the breeding for resistance relying on single *R* genes so far unsuccessful. A number of *R* genes from *Solanum* species conferring resistance to *P. infestans* have been identified. Cloning and stacking of these *R* genes in potato varieties could help to protect the potato crop against this devastating pathogen. In order to make map-based cloning a feasible and successful approach, it is required to have a single dominant *R* gene. Single dominant *R* genes are the easiest and the most studied examples of resistance genes, but the presence of several *R* genes in a single genotype has been reported and might be a common natural situation in many *Solanum* species. *S.*

demissum, *S. bulbocastanum* and *S. venturii* are examples of wild *Solanum* species in which genotypes that contain several *R* genes occur. In *S. demissum*, the first wild species to be extensively used as source of resistance, at least 11 different *R* genes have been identified: *R1* to *R11* (Gebhardt and Valkonen 2001). The mapping position of these *R* genes has been determined except for *R4*. Nine of them are located in the *R3* cluster on chromosome 11: *R3a, R3b, R5, R6, R7, R8, R9, R10* and *R11* (Bradshaw et al. 2006; ElKharbotly et al. 1996; Huang 2005). Some of the *demissum R* genes (*R1, R2, R3, R4, R7, R8*) have been found to occur simultaneously in a single genotype from the *S. demissum* accession CGN17810 (Huang et al. 2004). The fine mapping of *R3* revealed the presence of 2 functional *R* genes, *R3a* and *R3b*, 0.4 cM apart from each other, with different specificities (Huang et al. 2004). In *S. bulbocastanum*, three *R* genes mapping on distinct chromosomes have been cloned from different accessions: *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3 (Lokossou et al. 2009;van der Vossen et al. 2003;van der Vossen et al. 2005)*. Allele mining of these genes in *S. bulbocastanum* genotypes revealed that natural stacking of at least two of these three genes in a single genotype occurs with relative high frequency (Lokossou 2010). *S. venturii* is another example of the presence of several *R* genes with different specificities in a single genotype (Pel 2010). Stacking several *R* genes in a single genotype appears to be a feasible strategy to achieve high level and durable protection against potential pathogens. Pyramiding of *R* genes is still controversial and it is not known whether it is a durable approach (McDowell and Woffenden 2003; Pink and Puddephat 1999; Pink 2002). The pyramiding of *Rpi-ber1* (Rauscher et al. 2006), an *R* gene with a strong effect, and *Rpi-mcd1* (Tan et al. 2008), an *R* gene with a weak effect, revealed an additive effect on the resistance level (Tan et al. 2010). Observing natural pyramiding of *R* genes strengthens the idea that plants can benefit from combining individual *R* genes, even including some with weaker effect (Pink 2002). In breeding, pyramiding *R* genes could be facilitated by marker assisted selection (Mohan et al. 1997; Pink 2002). The mapping position of the *R* gene must be known and flanking or closely linked markers must be available.

S. x edinense P. Berthault, a pentaploid (2n=5x=60) potato species from Mexico, is a natural hybrid between *S. demissum* Lindl. (2n=6x=72) and *S. tuberosum andigena* (2n=4x=48) (Serquen and Hanneman 2002). It has been used in breeding programs and has revealed good field resistance to *P. infestans* (Van Soest et al. 1984). Two functional *R* genes have been cloned from one *S. x edinense* genotype (edn151-3): *Rpi-edn1.1* and *Rpi-edn1.2* also known as *R2-like*

(Champouret 2010). They were identified by allele mining of the *R2* family. Both are located in the *R2* cluster on chromosome 4. Both *R* genes recognize AVR2 (Champouret 2010; Lokossou et al. 2009) and their resistance is not effective against all *P. infestans* isolates, including IPO-C (Lokossou et al. 2009). Some *S. x edinense* genotypes are resistant to IPO-C, so they may contain additional resistance genes. The *S. x edinense* genotype edn151-3 responds to AVR4, indicating that it carries an *R4-like* resistance gene (N. Champouret, Wageningen UR Plant Breeding, unpublished data). $R4^{Ma}$ is one of the 11 *R* genes that were described from *S. demissum* (Mastenbroek 1953). Since $R4^{Ma}$ does not confer resistance to the isolate IPO-C, we hypothesized the presence of a third gene in *S. x edinense* edn151-3 and possibly other *S. x edinense* genotypes.

In the present study, we intended to identify the mapping position of the *R* gene responsible for the strong level of resistance to *P. infestans* in *S. x edinense,* for further map based cloning. To test the hypothesis of the presence of several *R* genes stacked in a single genotype and develop a mapping population, we screened two segregating populations from different *S. x edinense* genotypes (edn151-1 and edn150-4) crossed with cv. Concurrent. They were tested with different isolates and effectors that could discriminate between the different *R* genes (Champouret 2010; Oh et al. 2009; Vleeshouwers et al. 2008). SSR markers, NBS profiling (van der Linden et al. 2004) and CAPS markers were used to link the segregation of the resistance to a chromosomal position. Gene family targeted profiling was developed for different *R* genes and successfully applied to obtain markers that are closely linked to those *R* genes. We determined the resistance spectrum of the individual *R* genes to a diverse set of *P. infestans* isolates to understand the role played by each gene and how they complement each other.

Materials and methods

Plant material and mapping populations

S. × *edinense* P. Berthault accessions were provided by the Potato Collection Gross Lüsewitz, Germany (GLKS). The accessions were collected from an area near Toluca de Lerdo in Mexico (Sol*R*gene database, http://www.plantbreeding.wur.nl/phytophthora/). Fifteen genotypes from three *S. x edinense* accessions (GLKS 25492, GLKS 25493 and GLSK 25494) were screened for resistance to *P. infestans*. Two resistant genotypes were selected and crossed with the susceptible cv. Concurrent to generate F1 mapping

populations. The recombinant F1 genotypes of interest were transferred to *in vitro* culture to be maintained and multiplied.

***Phytophthora isolates* and disease tests**

Plants were tested for resistance by three different disease assays: an *in vitro* assay (Huang 2005), a detached leaf assay, (Vleeshouwers et al. 1999), and a field experiment. The *in vitro* assay was performed once on five plantlets with the *P. infestans* isolate 90128. In the detached leaf assay, one leaf between the third and the fifth fully developed leaves was collected from five weeks old plants, and inoculated with the two isolates 90128 and IPO-C. The leaves were scored after six days as resistant (R) due to a hypersensitive response (HR), susceptible (S) if a sporulating lesion appeared or as quantitative (Q) for a response not clearly resistant or susceptible. Two field trials, including *S. x edinense* genotypes, were performed in the summer of 2005 and 2007, in Wageningen, the Netherlands. Each field trial consisted of two randomized blocks, and within the blocks, genotypes were represented as four-plant subplots which were treated as single experimental unit as described by Colon and Budding (1988). For comparisons between years, standard cultivars Ostara, Bildtstar, Eersteling, Pimpernel, Robijn and Biogold were included. Spreader rows consisted of potato cultivar Bintje, the border rows consisted of potato cultivar Nicola. For the inoculum production, a large number of potato cultivar Bintje leaves were inoculated in detached leaf assay with isolate IPO-C. After 6 days, spores were washed off to prepare a spore suspension in large containers. Zoospore release was induced by incubating the containers at 10°C. At nightfall, the zoospore suspension was sprayed on the potato field using a tractor with two spraying arms. Disease assessments were made at weekly intervals. The percentage of leaf area covered with late blight lesions was estimated for each plot (Colon and Budding 1988). From these readings the area under the disease progress curve (AUDPC) was calculated (Fry 1978) and subsequently, the AUDPC values were transformed to a 1 (susceptible) -9 (resistant) scale (Sol*R*gene database).

The F1 individuals from the mapping populations were inoculated with isolates 90128, IPO-C, PIC99189 and UK7824 (**Table 1**). Isolates PIC99189 and UK7824 were used as they express *Avr4* and are avirulent on *R4* plants (van Poppel et al. 2008). The detached leaf assay on the F1 individuals was carried out twice and a single leaf per isolate was inoculated. The resistance spectra of the resistant parents and F1 individuals containing one of the three *R* genes were determined by a detached leaf assay with 16 isolates.

Table 1. *Phytophthora infestans* isolates used to phenotype the two segregating populations. The column effector indicates the avirulent effector present in the isolates recognized by the *R* gene to induce resistance.

Isolate	Country of Origin	Race	Effector
90128	The Netherlands	1,3,4,7,8,10,11	*PiAvr2*
IPO-C	Belgium	1,2,3,4,5,6,7,10,11	Unknown
PIC99189	Mexico	1,2,5,7,10,11	*Avr4*
UK7824	United Kingdom	1,2,3,6,7	*Avr4* and *Avr10*

Marker development

Young leaf tissue was collected from plants grown in the greenhouse. Genomic DNA was isolated by following the CTAB protocol with the Retsch machine in a 96 well format. Several marker technologies were used in this study: CAPS markers, SSR markers, NBS profiling markers (van der Linden et al. 2004) and *R* gene family targeted profiling markers that represent particular *R* gene families (**Chapters 2 and 3**).

A set of approximately 80 SSR markers, covering the potato genome (Collins et al. 1999;Feingold et al. 2005; Bakker et al., manuscript in preparation;Ghislain et al. 2004), was applied to determine the chromosomal position of segregating *R* genes. Parental genomic DNA and 11 resistant and 11 susceptible F1 individuals from the mapping populations were used for the SSR marker screen. PCR reactions for the SSR markers were performed using a single PCR profile: an initial cycle at 95°C for 2 min; then 30 cycles of 95°C for 30s sec, 56°C for 30 sec, using a ramp of 1°C/min, and 72°C for 45 sec, using a ramp of 1°C/min; and a final step at 72°C for 3 min. Subsequently the PCR products were visualized by electrophoresis on acrylamide gels run on the LI-COR DNA sequencer (Lincoln, Nebraska, USA).

To confirm the mapping position and obtain PCR markers linked to the *R* genes, known CAPS markers from the SGN database (http://solgenomics.net/), and the SH x RH genetic map (van Os et al. 2006), located close to the *R* gene clusters, on the identified chromosome arm were tested.

Gene specific profiling was used to develop markers closely linked to the *R* gene. It was performed as NBS profiling previously described (van der Linden et al. 2004) by replacing the NBS primers by gene family specific primers. For three *R* gene families R2, Tm2 and N, sequences available from NCBI (http://www.ncbi.nlm.nih.gov/) and sequences from allele mining studies performed in our laboratory were collected and aligned. Primers were designed on

conserved sequences for each family on the different domains of the gene: CC or TIR, NBS and LRR (**Table S1**). Some degenerate primers were designed especially for the N profiling. This analysis was combined with a bulk segregant analysis (BSA, Michelmore et al. 1991) on the F1 populations. Eight F1 individuals giving a resistant or susceptible phenotype were pooled and screened with the primer/enzyme combinations. The PCR products were visualized by electrophoresis on acrylamide gels. The fragments identified to be associated with the resistance were cut out of the gel and sequenced.

Effector analyses

In the binary PVX assays, recombinant *A. tumefaciens* GV3101 strains carrying pGR106-Avr4$_{1-287}$ with signal peptide and pGR106-Avr4$_{25-287}$ without signal peptide (van Poppel et al. 2008), pGR106-CRN2 as positive control (Torto et al. 2003) or the pGR106 empty vector, were used to characterize the plants. The experiment was performed as previously described (Vleeshouwers et al. 2006). Local symptoms were visually scored after 10 days and followed until 15 days post inoculation.

The agroinfiltrations were carried out on young plants three weeks after transplanting from *in vitro* multiplication. Three leaves were infiltrated with the following constructs: *Avr4* (van Poppel et al. 2008), *PiAvr2* (Lokossou et al. 2009), *R3a* and *Avr3a* (Bos et al. 2006) as positive control, and empty pGrab as negative control. The *Avr4* and *PiAvr2* sequences were inserted in the destination vector pK7FWG2.0 (Karimi et al. 2002). All the plasmids were introduced into *A. tumefaciens* strain AGL1 (Lazo et al. 1991) in combination with the helper plasmid pBBR1MCS-5.virGN54D (Van Der Fits et al. 2000). The agroinfiltration experiments of the recombinant *A. tumefaciens* were performed as described by van der Hoorn et al. (2000) with some adaptations.

Agrobacterium tumefaciens cultures were grown in 3ml of LB medium supplemented with antibiotics to select for the *A. tumefaciens* strains (carbenicilin), the binary vector (kanamycin or spectinomycin) and the helper plasmid (chloramphenicol). The next day, the cultures were transferred to 15 ml of YEB medium supplemented with antibiotics to select for the vector and the helper plasmid. On the third day, the cells were harvested and re-suspended in MMA solution supplemented with acetosyringone to a final OD600 of 0.2. Leaves of 4- to 5- weeks old plants from *Solanum* were infiltrated with this suspension with a 2-ml syringe. Responses were scored from 3 to 8 days post-infiltration.

Results

Screen for resistance to *P. infestans* in *S. x edinense* accessions

To identify a resistant genotype for *R* gene mapping and cloning, in total 15 genotypes from three *S. x edinense* accessions were tested for resistance to *P. infestans.* The 15 individuals were first tested by an *in vitro* assay with isolate 90128. Fourteen genotypes gave a strong level of resistance and one genotype had a lower level of resistance (**Table 2**). From each accession two strongly resistant genotypes per accession were selected. Their resistance to isolate 90128 was confirmed in a detached leaf assay, and inoculation with an additional isolate, IPO-C resulted also in resistant phenotypes. Two field experiments in 2005 and 2007 confirmed the strong resistance to IPO-C in all tested genotypes. Two resistant genotypes edn150-4 and edn151-1 were chosen to generate F1 populations.

Table 2. Resistance to two *Phytophthora infestans* isolates of 15 *S. x edinense* genotypes from three different accessions under three different assays and : in vitro assay, detached leaf assay (DLA) and field trial in two years. Resistance phenotype is characterized on the scale from 1 (susceptible) to 9 (resistant). Edn for *S. x edinense* and dms for *S. demissum*. In shadow grey, the genotypes used in DLA and field experiments, in bold the genotypes used in this study. (GLKS 25492: edn151; GLSK 25493: edn150; GLSK 25494: edn152).

Genotypes		In vitro	DLA		Field 2005	Field 2007
		90128	90128	IPO-C	IPO-C	IPO-C
edn	150-1	7	nd	nd	nd	nd
edn	150-2	9	9	9	9	nd
edn	150-3	9	nd	nd	nd	nd
edn	**150-4**	**9**	**9**	**9**	**9**	**9**
edn	150-5	9	nd	nd	nd	nd
edn	**151-1**	**9**	**9**	**9**	**9**	**9**
edn	151-2	9	nd	nd	nd	nd
edn	151-3	9	9	9	9	9
edn	151-4	9	nd	nd	nd	nd
edn	151-5	9	nd	nd	nd	nd
edn	152-1	9	9	8	9	nd
edn	152-2	9	9	7	9	nd
edn	152-3	9	nd	nd	nd	nd
edn	152-4	9	nd	nd	nd	nd
edn	152-5	5	nd	nd	nd	nd
Bintje		2	2	2	2	2
dms	344-14	9	9	9	nd	nd
dms	344-18	nd	9	9	9	9

Segregation of resistance in the mapping populations

The genotypes edn150-4 and edn151-1 were crossed with cv. Concurrent to generate F1 mapping populations. The F1 individuals were phenotyped for their resistance to four different *P. infestans* isolates in a detached leaf assay. 159

individuals from the edn150-4 x cv. Concurrent population and 125 from the edn151-1 x cv. Concurrent population were tested. The resistance to each of the four isolates segregated in the two populations (**Table 3**). In both populations, the response to each isolate segregated independently (**Table 4**). These results suggest the presence of up to four dominant *R* genes.

Table 3. Description of the F1 populations and their response to the different *Phytophthora infestans* isolates in detached leaf assay. *The number of F1 individuals phenotyped with isolate 90128 was smaller than for the other isolates (71 from the 159 plants were scored for edn150-4 x cv. Concurrent population and 57 from the 125 for edn151-1 x cv. Concurrent population).

R parent	S parent	Pop size	90128*			IPO-C			PIC99189			UK7824		
			R	S	Q	R	S	Q	R	S	Q	R	S	Q
edn150-4	cv. Concurrent	125	37	24	10	37	52	36	40	64	21	54	27	44
edn151-1	cv. Concurrent	159	27	17	13	70	50	39	51	76	32	66	34	59

Table 4. Segregation of the resistance in two F1 populations to three different isolates: IPO-C, PIC99189 and UK7824. Percentage of the number of plants showing a particular combination of resistance to each of the three isolates compared to total number of plants for which we have complete data. All possible resistant combinations are indicated here but not all are observed.

Combinations	IPO-C	PIC99189	UK7824	edn150-4 x concurrent (%)[1]	edn151-1 x concurrent (%)[2]
1	R	R	R	30	31
2	S	R	R	23	24
3	S	S	S	20	22
4	R	S	S	9	2
5	S	S	R	1	4
6	R	S	R	17	17
7	R	R	S	0	0
8	S	R	S	0	0

[1] 70 individuals in total. [2] 54 individuals in total (Excluding the unclear phenotype Q).

The resistance to 90128 segregated in the two populations. The segregation pattern of the resistance to 90128 was different from the segregation pattern of the resistance to the other isolates. Champouret (2010) cloned two *R2* homologues (*Rpi-edn1.1* and *R2-like*) in *S. x edinense* genotype edn151-3. *R2* confers resistance to 90128 and susceptibility to the other isolates tested on the population. We hypothesized that the *R* gene, *Rpi-edn1*, conferring resistance to 90128 is located in the *R2* cluster.

The segregation of the resistance to IPO-C in both populations also followed a different pattern than the segregation of the resistance to isolates PIC99189 and UK7824 (**Table 4**). This result indicates the presence of three more *R* genes located on different loci segregating in the populations, other than the *R2* cluster. The segregation of the resistance to the isolates PIC99189 and UK7824 was

expected to be similar as they both contain *Avr4*, but some differences were observed. All the plants resistant to PIC99189 were also resistant to UK7824, but around half the plants susceptible to PIC99189 were resistant to UK7824 (**Table 4**). This suggests the presence of an *R4*-like gene, giving resistance to PIC99189 and UK7824, that we propose to call *Rpi-edn3*. The difference of segregation of the resistance to the two isolates containing *Avr4* indicates the presence of another *R* gene in the populations inducing resistance after the recognition of an effector present only in UK7824. Interestingly, the race of UK7824 indicates that it contains *Avr10* (**Table 1**), and as cv. Concurrent is resistant to VK98014, which also contains *avr10* (**Table S2**), it indicates the possible presence of *R10* in cv. Concurrent. Therefore, *R10* might be the gene responsible for the resistance to isolate UK7824 segregating in the F1 populations.

In summary, the two F1 populations showed similar segregation ratios for resistance and susceptibility (**Table 3**) to three isolates (90128, 99189 and UK7824) that were independent between isolates (**Table 4**). The segregation of the resistance to IPO-C is slightly skewed in both populations. But the number of resistant F1 plants is higher for the population edn151-1 x cv. Concurrent whereas the number of susceptible F1 plants is higher for the population edn150-4 x cv. Concurrent. It can be speculated that the same set of four *R* genes is present in both *S. x edinense* parental genotypes. Therefore, the rest of the study focused only on one F1 population: edn150-4 x cv. Concurrent.

Marker development

The population edn150-4 x cv. Concurrent was used to map the four *R* genes segregating in the population. The mapping position of the two genes *Rpi-edn1* and *R10* are known. So we only tested markers mapped in the locus of interest. For *Rpi-edn1.1*, located in the *R2* cluster on chromosome 4, *R2* gene family profiling was performed. *R10* maps on chromosome 11 in the *R3* cluster (Bradshaw et al. 2006), so CAPS markers from the *R3* cluster were tested. The mapping position of the other two genes was unknown, and a genome wide screen was performed. SSR screening and NBS profiling were carried out to determine the map position of the *R* gene giving resistance to IPO-C and the *R* gene giving resistance to PIC99189. A subset of F1 individuals resistant or susceptible to all isolates was selected for that purpose. The DNA of the F1 individuals was kept separate for the SSR screening and bulked for the NBS profiling.

Table 5. Markers used for mapping of *Rpi-edn1, Rpi-edn2, Rpi-edn3* and *R10* in the F1 population edn150-4 x cv. Concurrent. TM =55°C for all markers. (*Nbs15F is a degenerate primer)

Type	Marker	Primer sequence or reference	Enzyme	Chr. (cluster)
NBS profiling	NBS5a	Van der Linden et al., 2004	RsaI	11(N)
NBS profiling	GLPL6	Van der Linden et al., 2004	MseI	11 (N)
CAPS	Ct182	Brigneti et al., 1997	HpyF10VI	11 (N)
CAPS	Gp163	Brigneti et al., 1997	MseI	11 (N)
N profiling	Nbs15F-Mse*	atgcatgayttratwvaagabatggg	MseI	11 (N)
SSR	Stm021	Collins et al.,1999	a.s.	9
Tm2 profiling	Tm19F-Mse	actgccaaattgtatggtg	MseI	9
R2 profiling	R2ch4F4-Rsa	tgtgcagtgataacagcttca	RsaI	4 (R2)
CAPS	Gp283	F tactcaaggagtctgcatgg R aacttcctgtccgaatgtcc	RsaI	11 (R3)

***Rpi-edn1* from the *R2* cluster is present in edn150-4**

The homologues *R2-like* and *Rpi-edn1.1* have been cloned from the genotype edn151-3, which was derived from the same accession as edn151-1 (Champouret 2010). We determined whether this gene would also occur in edn150-4. Seven R2 profiling primers (**Table S1**) were designed on several conserved regions of the *R2* gene family. The primers were tested in combination with RsaI, which cuts frequently in the *R2* sequence on the parental and F1 bulked DNA. Each primer revealed at least one fragment showing association with the resistance in the bulks. The primer (R2ch4F4) giving the largest number of polymorphic bands was tested on the individuals of the bulks and on the whole population. The resulting NBS marker R2ch4F4-Rsa (fragment of 400 bp) was linked to the resistance to 90128 with 10 recombinants out of 45 individuals (~20cM). Agro-infiltration assay with *PiAvr2* was performed on a subset of the population to confirm these mapping results. The response to *PiAvr2* cosegregated with the resistance to 90128 in 40 F1 individuals (**Figure 1 and 2**). The presence of *Rpi-edn1.1* (*R2-like* or *Rpi-edn1.1* or both) on chromosome 4 in the *R2* cluster in edn150-4 is thus confirmed.

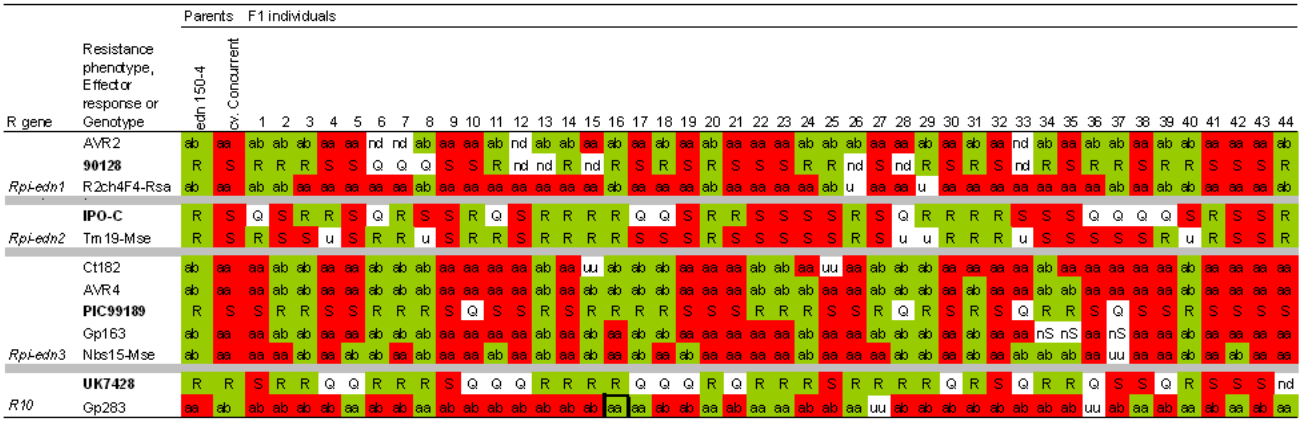

Figure 1. Graphical genotyping of the edn150-4 x cv. Concurrent population. A subset of the F1 individuals is represented. Indicated are the response to the four *Phytophthora infestans* isolates (90128, IPO-C, PIC99189 and UK7824), the response to effectors AVR2 and AVR4 linked to the resistance to 90128 and PIC99189, respectively, and the genotype score for one or two markers linked to the individual *R* gene loci. R: resistant (green), S: susceptible (red), Q: unclear phenotype, ab: presence of fragment, aa: absence of fragment, nd: not determined. The grey horizontal lines separate the *R* gene loci. The F1 individual number 16 contains the three *Rpi-edn* and potentially R10 from cv. Concurrent.

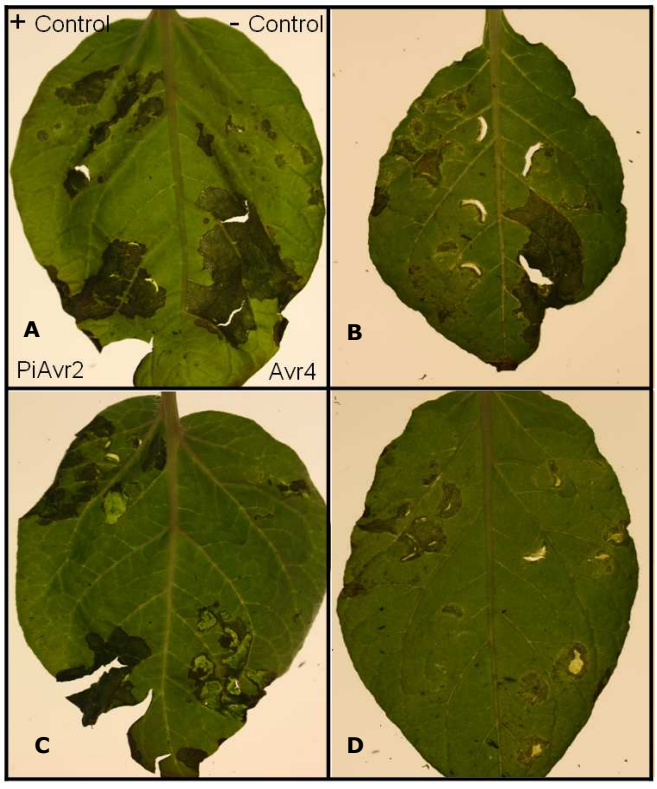

Figure 2. Agroinfiltration experiment with the AVR2 and AVR4 effectors on the F1 individuals of the edn150-4 x cv. Concurrent population. The response to the two effectors segregates independently in the population. A: Responding to AVR2+ AVR4, B: Responding to AVR4 only, C: Responding to AVR2 only, D: Responding to none of the two effectors. + control: R3a + Avr3a, - control: empty pGRAB vector.

Cv. Concurrent contributes to the resistance

In order to confirm that the *R* gene giving resistance to UK7824 came from cv. Concurrent, we tested markers mapping in the *R3* cluster. *R10* maps in the *R3* cluster on chromosome 11 (Bradshaw et al. 2006). To test that hypothesis, we tested for association between markers in the *R3* cluster and the resistance to UK7824. Since UK7824 contains *Avr4* and *Avr10*, resistant plant individuals used for the mapping had to be resistant to UK7824 and susceptible to PIC99189. One CAPS marker Gp283, linked to the resistance to isolate UK7824, was identified (**Table 5**). The segregating fragment was present in cv. Concurrent and absent in edn150-4 and was located 15 recombinants out of 50 individuals (~ 30cM) to the resistance to UK7824 (**Figure 1**). This result confirms that the resistance to UK7824 is contributed by cv. Concurrent, and strongly suggests that it is due to *R10*.

***Rpi-edn2* maps on chromosome 9**

The screen of the set of approximately 80 SSRs applied on the parents and 24 F1 individuals resulted in one linked marker associated with the resistance to IPO-C. This marker, Stm021, (**Table 5**) is located on chromosome 9 (Bakker et al., manuscript in preparation). The linkage with resistance to IPO-C was confirmed with 17 recombinants out of 116 individuals (~15cM). We propose to call this gene *Rpi-edn2*, the *R* gene conferring resistance to IPO-C, located on the long arm of chromosome 9 (**Figure 2**). Marker Stm021 is located between two known *R* gene clusters on chromosome 9: the cluster containing the *R* genes from *S. venturii*, *Tm-2²* homologues (Foster et al. 2009; Pel et al. 2009) and the cluster containing *Rpi-mcq1,* also homologous to *Tm-2²* (Smilde et al. 2005; patent WO2009013468). *Tm-2²* is an *R* gene from tomato located on the long arm of chromosome 9, conferring resistance to Tobacco Mosaic Virus (Lanfermeijer et al. 2003). More markers were needed to determine whether *Rpi-edn2* may be located in one of these clusters. The development of CAPS markers from that region of the genome was not successful as none of the 13 primer combinations tested revealed linkage. So, in order to develop a closely linked marker and determine the exact position of the *R* gene, a *Tm-2²* gene family profiling was performed. Twelve *Tm-2²* specific primers (**Table S1**) were designed and tested in combination with two enzymes RsaI and MseI on the parental and F1 bulked DNA. Two primer/enzyme combinations revealed association with the resistance to IPO-C in the bulks, but only one marker was confirmed. The marker Tm19F-Mse was linked to *Rpi-edn2* with 6 recombinants out of 107 individuals (~6cM). The fragment, of 70 bp, showing association with the phenotype, was cut out

from the gel and sequenced. The comparison of this sequence with the *Rpi-vnt1* and *Rpi-mcq1* genes could not reveal the cluster to which *Rpi-edn2* belongs. PCR reaction with the start and stop codon primers used for the cloning of *Rpi-vnt1* did not give any amplification product on either of the *S. x edinense* genotypes. In spite of unsuccessful attempts to identify which of the two clusters on the long arm of chromosome 9 contains *Rpi-end2*, we postulated that it is most likely homologous to *Tm-2*^{2}, and might be located in the *Rpi-mcq1* cluster (**Figure 3**).

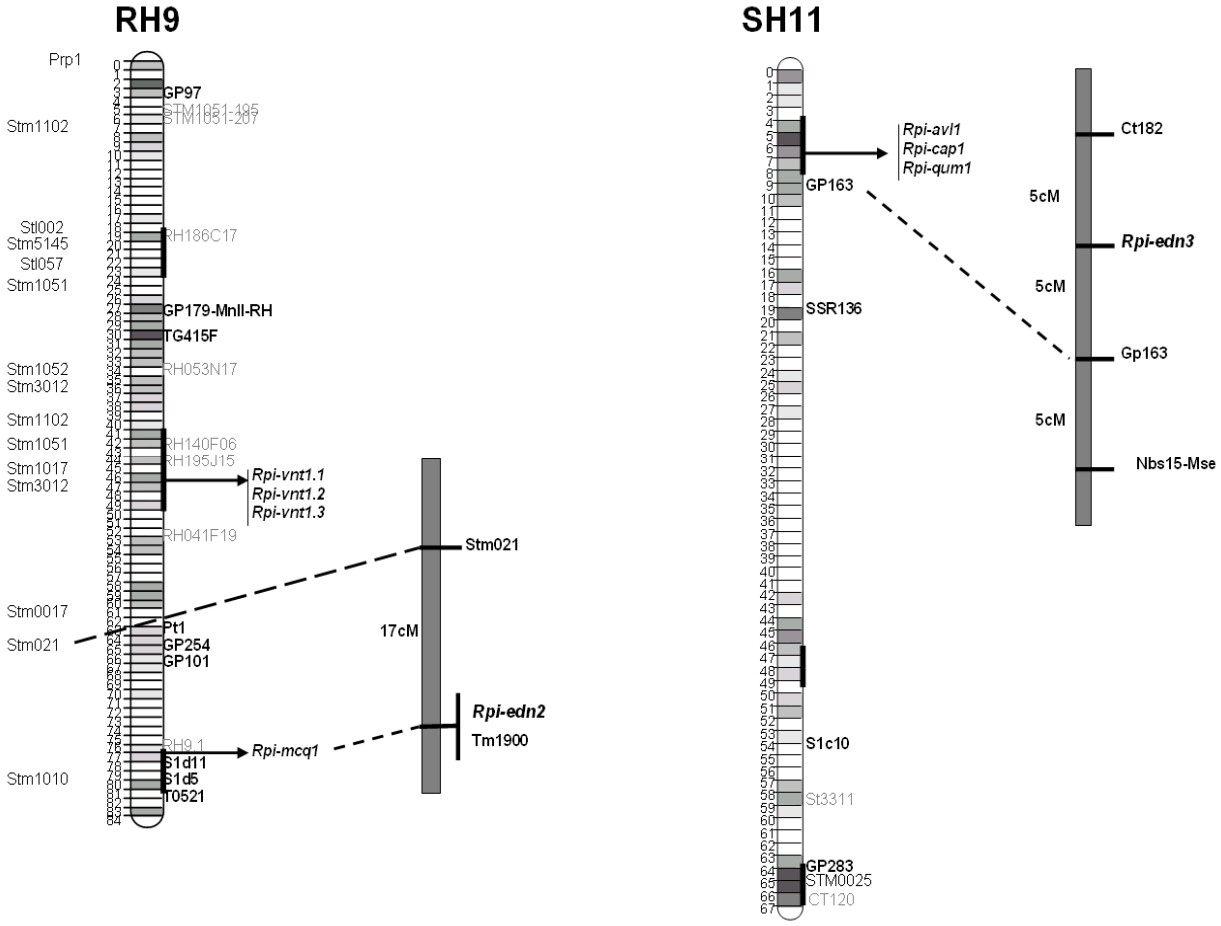

Figure 3. Genetic maps of the *Rpi-edn2* and *Rpi-edn3* genes segregating in edn150-4 x cv. Concurrent population, mapping on chromosome 9 and 11, respectively. The genetic maps are compared to the SH x RH UHD reference genetic map. The vertical black bars are representing the known *R* gene clusters. The mapping position of *Rpi-edn2* is still hypothetical and here arbitrarily represented in the *Rpi-mcq1* cluster, while it could also be 17cM above the SSR marker Stm021, and approximately in the *Rpi-vnt1* cluster.

Rpi-edn3 co-localizes with *R4*^{Ma} on chromosome 11

NBS profiling screen with 25 primer/enzyme combinations on the parental and bulked F1 DNA resulted in five fragments that differentiated between the bulks. Two markers could be confirmed in the complete population to be linked to the resistance to isolate PIC99189. These two markers (NBS5a/RsaI and GLPL6/ MseI,

Table 5) were cosegregating and located 17cM from the resistance gene (n=64). The sequencing of the fragments of these two markers indicated a high identity score with *R3a* homologues. The *R3* cluster resides in the distal part on the southern side of chromosome 11 (Huang et al. 2004). CAPS markers from chromosome 11 were developed and markers from the northern side of chromosome 11 (Ct182 and ADG2, **Table 5**) revealed stronger linkage with the resistance to PIC99189 (**Figure 1**). These markers suggest that *Rpi-end3,* the *R* gene conferring resistance to PIC99189, maps on the northern arm of chromosome 11 (**Figure 3**). To test the hypothesis that *Rpi-edn3* is located in the N cluster and is an N-like gene, we performed N gene family profiling (**Chapters 2 and 3**). Eight primers, designed on N-like sequences, were tested in combination with two enzymes MseI and RsaI on the parental and bulked F1 DNA. It resulted in one marker Nbs15F-Mse (**Table 5**) located 10 cM from *Rpi-edn3* (**Figure 1**). This indicates the presence of N-like sequences still 10 cM below the *Rpi-edn3* locus and suggests that *Rpi-edn3* could be an N-like gene.

For effector analysis, two main assays are available, PVX agroinfection and agroinfiltration. The PVX inoculation (Vleeshouwers et al. 2006) is quick but has the risk of missing a responsive plant because of extreme response which does not produce any macroscopically visible necrosis (Vleeshouwers and Rietman 2009). Agroinfiltration (Van der Hoorn et al. 2000) has the disadvantage of inducing background response to *A. tumefaciens* in some *Solanum* species. A PVX screen was performed in our laboratory, 55 putative RXLR effectors were tested on 24 resistant *Solanum* genotypes, belonging to 18 wild species from eight taxonomic series, to identify specific plant effector interactions (Champouret 2010; Vleeshouwers et al. 2008). *Avr4*, the avirulence gene corresponding with $R4^{Ma}$ from *S. demissum*, (van Poppel et al. 2009), was identified to induce HR on some wild species. Further screen on 100 genotypes revealed that edn151-3 respond to *Avr4*. This specific response to *Avr4* was confirmed by agroinfiltration in *S. x edinense* edn150-4 (**Table 6**). A subset of the F1 population was tested for the segregation of the response to *Avr4*, and co-segregation between the resistance to PIC99189 and the response to *Avr4* was observed in a population of 44 individuals (**Figures 1 and 2**). This indicates that the genes *Rpi-edn3* and $R4^{Ma}$ both map on the northern side of chromosome 11, probably in the N cluster (**Figure 3**), and might be homologues to the *N* gene. It can be hypothesized that Rpi-edn3 recognizes Avr4 and that this recognition induces defense response and resistance to PIC99189 and UK7824.

Table 6. Wild Solanum species recognizing AVR4, PVX screen and agro-infiltration (ATTA).

Solanum species	Gene Bank	Country of origin	Series (according to Jacobs, 2008)	PVX	ATTA	Other R genes*
S. stoloniferum	sto836-1	Mexico	Longipedicellata	+	nd	
S. stoloniferum	sto835-3	Mexico	Longipedicellata	+	+	
S. papita	pta767-1	Mexico	Longipedicellata	+	+	
S. papita	pta767-8	Mexico	Longipedicellata	+	+	*Rpi-pta1*
S. papita	pta369-1	Mexico	Longipedicellata	+	+	
S. papita	pta370-5	Mexico	Longipedicellata	+	+	
S. cardiophyllum	cph541-2	Mexico	Polyadenia Pinnatisecta Bulbocastana Morelliformia	+	+	*Rpi-edn1.1* *R2-like*
S. edinense	edn151-3	Mexico	Acaulia *S. demissum*	+	+	
S. gourlayi	grl607-1	Argentina	Longipedicellata	+	nd	
S. okadae	oka366-8	Argentina	Tuberosa Megistacroloa	+	nd	

Nd: not determined, +: positive response to AVR4 with positive and negative controls as expected.
*(Vleeshouwers et al. 2008), (Champouret 2010)

All three postulated *R* genes in *S. x edinense* have been overcome by *P. infestans*

In order to understand the contribution conferred by each of the three genes to the wide and strong resistance observed in the *S. x edinense* resistant genotypes, the resistance spectrum of F1 individuals from the edn150-4 x cv. Concurrent population containing single *R* genes was determined. The F1 individuals were selected based on their resistance phenotype to the four isolates used to characterize the population, and on the genotype of the linked marker if available. Sixteen isolates were tested on 16 potato genotypes including 12 *S. x edinense* genotypes, two containing *Rpi-edn1*, two containing *Rpi-edn2*, five containing *Rpi-edn3*, and three free of any of these three *R* genes, and four genotypes were included as controls: A03-142 (containing *Rpi-blb3, R2* homologue), mcq717-3, $R4^{Ma}$ and cv. Bintje (**Figure 4**). Plants containing only *R10* were not tested in this experiment since it originates from cv. Concurrent. The marker Gp283 was used to indicate the presence of *R10,* especially necessary for *Rpi-edn3*. As the marker is distant (~30cM) from *R10* it can only give an indication. The results are summarized in **Figure 4**. The plants were characterized as susceptible (S), resistant (R) or showing quantitative response (Q). For each *Rpi-edn* gene, at least two different F1 plants were tested with all 16 isolates with the detached leaf assay, and the spectrum of resistance of the gene was deduced from the response of the F1 individuals. The Qs were taken into consideration, and when one of the F1 plants was susceptible to a specific isolate, the *R* gene was concluded not to be effective against that isolate. The resistance observed in the other F1 individuals could come from the presence of other *R* genes, like *R10* or other *R* genes present in the parents but not identified.

The two F1 individuals used to characterized *Rpi-edn1* revealed a similar spectrum. The spectrum of resistance of *Rpi-edn1* was compared to the spectrum of resistance of the genotype A03-142, a cv. Desiree transformant with *Rpi-blb3,* which also recognizes PiAVR2 and has the same spectrum as *R2* (Lokossou et al. 2009). By excluding the not informative unclear responses (Q), the spectra of resistance of the two *R* genes were very similar. That result was to be expected since two *R2* homologues were cloned from the *S. x edinense* genotype number 3 from the accession number edn151 (Champouret 2010).

Chromosome	Genotypes	IPO-0	91011	VK98014	NL00228	UK7824	IPO428-2	IPO-C	H30P04	NL050194	PIC99189	USA618	90128	PIC99183	NL01096	3128-A	PIC99177	Gp283
	edn 150-4	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	aa
	cv. Concurrent	R	R	R	R	R	S	S	S	S	S	S	S	S	S	S	R	ab
4	A03-142	R	R	R	R	Q	R	S	R	Q	S	S	R	R	R	Q	S	-
	Rpi-edn1	R	R	R	R	S	R	S	R	R	S	Q	R	R	R	S	S	-
	F1-83	R	R	R	R	S	R	S	R	R	S	Q	R	R	R	S	S	ab
	F1-123	R	R	R	R	Q	R	Q	R	R	S	Q	R	R	Q	Q	Q	ab
9	mcq 717-3	R	R	R	R	S	R	R	R	Q	R	S	R	R	S	S	S	-
	Rpi-edn2	R	R	S	S	S	S	R	R	Q	S	S	S	S	S	R	S	-
	F1-104	R	R	S	S	S	S	R	R	Q	S	S	S	S	Q	Q	R	ab
	F1-148	R	R	R	R	S	S	R	R	Q	S	S	S	Q	S	R	S	ab
11	*R4^{Ma}*	R	S	S	S	R	S	S	R	R	R	R	S	Q	S	S	S	-
	Rpi-edn3 / R4	R	S	R	R	R	S	S	R	R	R	R	S	S	S	S	S	-
	F1-90	R	S	R	R	R	S	S	R	Q	R	R	S	S	S	S	Q	ab
	F1-62	R	R	R	R	R	S	S	R	R	R	R	Q	R	S	S	S	aa
	F1-74	R	R	R	R	R	Q	S	R	R	R	R	Q	R	S	S	S	aa
	F1-73	R	R	R	R	R	S	S	R	R	R	R	S	S	S	S	S	aa
	F1-107	R	R	R	R	R	S	S	R	R	R	R	S	S	Q	R	R	ab
None	Bintje	S	S	S	S	S	S	S	Q	S	S	S	S	S	S	S	S	-
	No R genes	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	-
	F1-30	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	ab
	F1-151	Q	S	S	S	S	S	Q	S	nd	S	S	S	S	S	Q	Q	ab
	F1-150	R	R	Q	R	S	Q	Q	Q	S	Q	S	S	S	S	Q	R	aa

Figure 4. Resistance spectrum of the parents edn150-4 and cv. Concurrent and F1 individuals with one of the three *Rpi-edn* genes. R: resistant (green), S: susceptible (red), Q: unclear (white). For each *R* gene, several F1 individuals were tested, and the spectrum of the *R* gene deduced from them. The row with *Rpi-edn1*, *Rpi-edn2* and *Rpi-edn3* indicate the deduced spectrum for each gene. For each *R* gene a control plant from the same cluster was included for comparison. A03-142 is a cv. Desiree transformant with *Rpi-blb3*. cv. Bintje was included as negative control. In the last column is the CAPS marker Gp283 linked 10cM to *R10* is added to indicate the presence or absence of *R10* when the F1 plants are resistant to PIC99189. In grey the isolates used to differentiate between the genes.

For *Rpi-edn2*, two genotypes were characterized and the deduced resistance spectrum was compared to the resistance spectrum of the genotype mcq717-3. For three isolates, the F1 individuals revealed opposite phenotypes. The resistance spectrum of *Rpi-edn2* is different from that of *Rpi-mcq1.* The latter

showed resistance to six more isolates than *Rpi-edn2*, and *Rpi-edn2* is resistant to one isolate virulent on *Rpi-mcq1*. The spectrum of resistance of *Rpi-edn2* is restricted to a small number of isolates, at least from the set of isolates used in this study (**Figure 4**).

For *Rpi-edn3*, the deduced resistance spectrum from five F1 individuals was compared to the resistance spectrum of $R4^{Ma}$. For four isolates, the responses of the different F1 individuals varied. Four of the five F1 individuals showed extra resistance to four different isolates to which at least one other F1 was susceptible. The extra resistance observed could partly be explained by the presence of *R10* but it could not explain all the extra resistance, for two main reasons. There is variation among the F1 individuals and three F1 individuals show resistance to two isolates virulent on cv. Concurrent (PIC99183 and 3128-A). The F1 individuals 148 and 150 show extra resistance to the isolates VK98014 and/or NL00228, which indicates that the extra gene(s) segregating in the population confers resistance to these two isolates and could explain the extra resistant phenotypes. Screening of more F1 individuals containing *Rpi-edn3* alone could help to draw firm conclusions on its exact resistance spectrum. For the other 12 isolates (excluding the four with variation among the F1 individuals), all F1 individuals containing *Rpi-edn3* gave the same resistance phenotype. So the deduced spectrum is reliable for these 12 isolates. Differences between *Rpi-edn3* and $R4^{Ma}$ were observed. Two virulent isolates on $R4^{Ma}$ were avirulent on *Rpi-edn3* (VK98014 and NL00228). These two isolates were also avirulent on cv. Concurrent. Although it cannot be excluded that *Rpi-edn3* confers resistance to these two isolates, it can be hypothesized that the presence of *R10* or another *R* gene explained the resistance observed in these F1 individuals. However, it can also be postulated that the resistance spectrum of *Rpi-edn3* is similar to the resistance spectrum of $R4^{Ma}$.

Three F1 genotypes were selected and confirmed not to contain any of the four *R* genes segregating in the population (**Figure 4**). One of them (number 30) showed complete susceptibility to all isolates. Another one (number 151) showed some intermediate level of susceptibility to three isolates. And number 150 showed an intermediate level of susceptibility to six isolates and resistance to three isolates. The Gp283 marker indicates that the third individual (150) probably contains *R10*, which in this case can alone explain the extra resistant phenotypes. This result confirms the presence of other *R* genes segregating in the F1 population.

The identification of the resistance spectrum of the individuals *Rpi-edn* genes, revealed that each individual *R* gene has been overcome by at least some isolates of *Phytophthora infestans*, and that their combination confers resistance to all the isolates tested, except 99177. The results also strongly indicate the presence of other *R* genes from *S. x edinense* segregating in the population.

Stacking in *S. x edinense* population

The pentaploid resistant parent contains at least three *R* genes on different chromosomes, that segregate independently. In total four F1 individuals from the edn150-4 x cv. Concurrent population revealed the presence of the three *R* genes from edn150-4 (number: 3, 16, 29 and 31 in **Figure 1**). The presence of the fourth *R* gene originating from cv. Concurrent could not be determined by the resistance phenotype to UK7824, since the F1 individuals resistant to PIC99189 are resistant to UK7824, but it could be hypothesized with the marker Gp283. This marker can be a useful indicator of the presence of the *R* gene from cv. Concurrent in the F1 individuals resistant to PIC99189. The marker is not tightly linked to the *R* gene so recombinations introduce a bias to the marker indication. One plant (number 16 in **Figure 1**) contains the three *Rpi-edn* genes probably together with *R10* since the marker Gp283 indicates the presence of the *R* gene from cv. Concurrent. The model presented in **Figure 5** summarizes all the findings and illustrates the possible accumulation of four *R* genes in a single genotype. This F1 individual is an example of a genotype containing at least four functional *R* genes in its genome.

Discussion

***S. x edinense* source of *R* genes**

The pentaploid *S. x edinense* had been identified as an interesting source of resistance to *P. infestans* already in 1908 by Salaman and was included in breeding programs by Brioli in 1914 (Pavek and Corsini 2001; Toxopeus 1964). It was named after the Edinburgh Botanic Garden (Glendinning 1983), where its hybrid characteristic was first described. In this study, we partly unraveled the genetic basis of the late blight resistance observed in *S. x edinense*. At least three genes with different specificities and located on different loci are present in one genotype, edn150-4. A mapping population derived from another plant genotype (edn151-1) that was derived from a different *S. x edinense* accession showed a very similar segregation pattern to four *P. infestans* isolates. It is likely that the same combination of *R* genes is present in that other accession from the same

area. This multiple *R* gene combination may be responsible for the wide late blight resistance spectrum found in the two *S. x edinense* genotypes studied here.

New approaches to facilitate mapping

Many *R* gene clusters in *Solanum* have already been identified. A fruitful approach to map novel *R* genes is to search for association with known *R* gene clusters. This simplifies *R* gene mapping, since they should lie in one of the *R* gene clusters listed in several reviews (Gebhardt and Valkonen 2001; Grube et al. 2000; Pan et al. 2000). The identification of the mapping position of a new potato *R* gene is still not a routine task, especially in tetraploid plants. The first step is to identify a susceptible plant for the cross with the resistant genotype. The second step is to choose a *P. infestans* strain that will allow the characterization of the resistance in the F1 individuals. The next step is to confirm that the resistance phenotype is clear in the population, that the resistance is segregating and that it follows a 1:1 ratio. Once all the conditions are met, the identification of the mapping position can begin. To be successful this step requires a sufficient level of heterozygosity from the resistant parent and an optimal level of polymorphism in the population. For most of these aspects, little can be done to increase the chance of mapping a new *R* gene. To improve the scoring of the phenotype or the development of linked markers, new approaches have shown to be successful in this study. The response to effectors can be used instead of the resistance phenotype to *P. infestans* to score the population. SSR markers and general or specific profiling approaches can also be useful for the development of diagnostic markers and the identification of the *R* genes chromosomal position.

A single *P. infestans* strain can contain several effectors and a single genotype can contain several *R* genes. A good example of this complexity is the isolate UK7824 that contains at least two known *Avrs* (*Avr4* and *Avr10*), and the two *S. x edinense* genotypes that contain at least three *R* genes. This complexity can make *R* gene mapping a difficult task. For mapping, the gene of interest should be dominant and occur in simplex, or at least be scored as single trait. The use of effectors to score the segregation of the *R* genes in the F1 population allows the scoring of a single *R* gene recognizing a specific effector. The responses to Avr2 and Avr4 segregating in the F1 population was associated with resistance to 90128 and PIC99189, respectively. It shows that effector responses can be used to phenotype an F1 population and map an *R* gene, which can be very useful in the presence of multiple *R* genes in a single genotype. Knowing the avirulence

gene to *R10* (*Avr10*) would allow the scoring of the plants for the presence of *R10* and simplify its mapping.

SSR marker screening and NBS profiling are the two approaches used in this study to identify *R* genes map position. NBS profiling identified the mapping position of *Rpi-edn3* on chromosome 11 and SSR marker the one of *Rpi-edn2* on chromosome 9. Both marker technologies are suitable for polyploids and are complementary to each other. SSR marker screening is an addition to the NBS profiling to identify *R* gene map position. A large set of SSR markers that cover the potato genome is now available (Collins et al. 1999; Feingold et al. 2005; Ghislain et al. 2004; Bakker et al., manuscript in preparation). Bakker et al., (manuscript in preparation) have developed the largest and most useful set of SSR markers for *R* gene mapping purposes because the primers were designed from BAC sequences selected with *R* gene analogue (RGA) probes and mapped in *R* gene clusters in the SH x RH UHD genetic map (van Os et al. 2006). These SSR markers allow the identification of a position of a gene for novel resistance on a chromosomal arm. For mapping purposes, the SSR marker screen approach has several technical advantages. The presence of polymorphism and its segregation in the population is determined directly with one PCR. One primer combination shows several alleles, which means that the probability to determine which allele is associated with the resistance, especially for polyploid populations, is higher than with other marker approaches. Another advantage of the SSR marker approach for *R* genes mapping is that the mapping position of each marker is already known. So the identification of a marker associated with a novel resistance will directly assign the *R* gene to a particular chromosome arm, and hence a probable *R* gene cluster.

NBS profiling was designed to specifically target *R* genes but it can easily be adapted to target other conserved gene families. It was adapted for peroxidase profiling in barley to map peroxidase clusters on the genome and correlate them with resistance QTL map position (González et al. 2010). In this study, we adapted the NBS profiling to specific *R* gene families and showed its success for three *R* gene families: R2, Tm2 and the N gene family. *R* genes from the same cluster usually have similarities in their sequences not shared with other *R* genes (McDowell and Simon 2006; Meyers et al. 2005) so it is possible to design specific primers for a particular *R* gene cluster. Sequence information on *R* genes is largely available and more sequences will become available with potato genome sequencing. This approach could be developed for each *R* gene cluster and could

be an addition to the standard NBS profiling or SSR marker screen for *R* gene mapping purposes.

*R4^Ma^* in *S. x edinense* and in other *Solanum* species

*R4^Ma^*, which was originally identified in *S. demissum* (Malcolmson et al. 1966;van Poppel et al. 2009), was mapped in *S. x edinense* by scoring of the response to the effector *Avr4* in the segregating F1 population. The response to *Avr4* was associated with resistance to isolates carrying *Avr4* (PIC99189 and UK7824). The linkage group containing the *R* genes was identified by NBS profiling and confirmed with flanking CAPS markers. *R4^Ma^* maps on chromosome 11 in the N cluster, in the same cluster as *Rpi-avl1*, *Rpi-cap1* and *Rpi-qum1* (**Chapters 2 and 3**). This result is in contradiction with the putative map position of *R4^Ma^* on chromosome 12 in the *Rx/Gpa2* cluster (van Poppel 2009) or it must be the first example in potato that a specific Avr is reacting with *R* genes from different clusters. The identified NBS profiling marker used in the study done by van Poppel (2009) was not confirmed with other markers. *R4^Ma^* could be a member of the N gene family, which belong to the TIR-NBS-LRR class of *R* genes (Whitham et al. 1994). As *Avr4* is cloned, the cloning of *R4^Ma^* would provide an interesting opportunity to compare TIR-NB-LRR to CC-NB-LRR resistance mechanism against *P. infestans*. It could be compared to the *R3a* and *Avr3a* interaction (Armstrong et al. 2005; Huang et al. 2005). Differences in the induction of the defence response between these two types of NB-LRR type of *R* genes are still unclear (Dangl and Jones 2001; Meyers et al. 2003). A diploid genotype containing *R4^Ma^* should be identified for population development to simplify marker development for the map-based cloning.

Avr4 responding species are found in three different taxonomic *Solanum* groups and reveal two different regions of occurrence (Mexico and Argentina; see **Table 6**). We speculate that *R4^Ma^* has an ancestral origin and was maintained in some *Solanum* species after geographical separation, as for *R3a* (Champouret 2010). Interestingly, *S. stoloniferum* and *S. papita,* two species with response to *Avr4,* were identified to also contain *Rpi-blb1. Rpi-blb1* is located on chromosome 8 and confers broad resistance spectrum (van der Vossen et al. 2003). It was first cloned by map-based cloning from *S. bulbocastanum* (van der Vossen et al. 2003) and then cloned by allele mining in *S. stoloniferum (Rpi-sto1)* and *S. papita* (Rpi-pta1; Vleeshouwers et al. 2008). The accession CGN17607 from *S. stoloniferum* was shown to contain the two indicated *R* genes (Table 6; Lokossou 2010). More recently, two *P. infestans* strains PIC99189 and 99177 originating from Mexico were found to break *Rpi-blb1, -sto1 and pta1* resistance

(Champouret et al. 2009). However, the isolate PIC99189 contains *Avr4* and is therefore avirulent on $R4^{Ma}$. From these observations, it can be speculated that $R4^{Ma}$ has a spectrum which is complementary to *Rpi-sto1* and the presence of $R4^{Ma}$ could be essential for plants to remain free of infection as also observed in the same material by Zhu et al. (2010). Despite the limited resistance spectrum of $R4^{Ma}$, it might be under positive selection because of the complementarities of its resistance spectrum with that of other *R* genes. It is another example of natural stacking of *R* genes in wild *Solanum* species.

***S. x edinense*, a lesson from nature on *R* gene stacking**

S. x edinense shows high level of resistance in the different assays and the resistance seems well established in the natural population and effective to a wide range of *Phytophthora infestans* isolates. Breeders would very much like to introduce such a level of wide spectrum resistance in their varieties. This study revealed that the resistance observed in two *S. x edinense* genotypes is explained by the presence of at least three genes that each has been overcome by some *P. infestans* strains. Each *Rpi-edn* gene causes resistance to an isolate to which none of the other *Rpi-edn* genes confer resistance. This suggests a natural stacking of *R* genes that may be caused by selection pressure to keep all three *R* genes together in most genotypes of the species.

The second aspect that could explain the level of resistance in *S. x edinense* is the provenance of the stacked *R* genes. *S. x edinense* is a natural hybrid between *S. demissum* and *S. tuberosum* ssp. *andigena* (Serquen and Hanneman 2002). *S. demissum* originates from Mexico (Watanabe and Peloquin 1991) and *S. tuberosum* ssp. *andigena* from Bolivia where it was domesticated (Van Soest et al. 1984). The centre of origin of *P. infestans* still has not been determined. Some studies brought evidence favoring a Mexican origin, and more recent studies suggest a South American origin (Gomez-Alpizar et al. 2007). Presently, Mexico and South America are both considered as centers of diversity of *P. infestans*. This implies a co-evolution between the pathogen and the plant host in both regions, so *R* genes have evolved in both places, and may have evolved differently. Wild *Solanum* species originating from a center of diversity should be a valuable source for resistance and for stacking (Goodwin 1994). As presented in **Figure 5**, it can be postulated that the resistance in *S. x edinense* is the result of a combination of *R* genes from the two centers of diversity of *P. infestans*. *Rpi-edn1* (*R2* homologue) and *Rpi-edn3* (*R4* homologue) originate from the Mexican *S. demissum* species. *Rpi-edn2* could come from South-American *S. tuberosum* spp. *andigena*, since the other *Solanum R* genes in the Tm2 cluster also occurred in

Solanum species that originated so far only from South America: *Rpi-vnt1* and *Rpi-mcq1* (Foster et al. 2009; Pel et al. 2009).

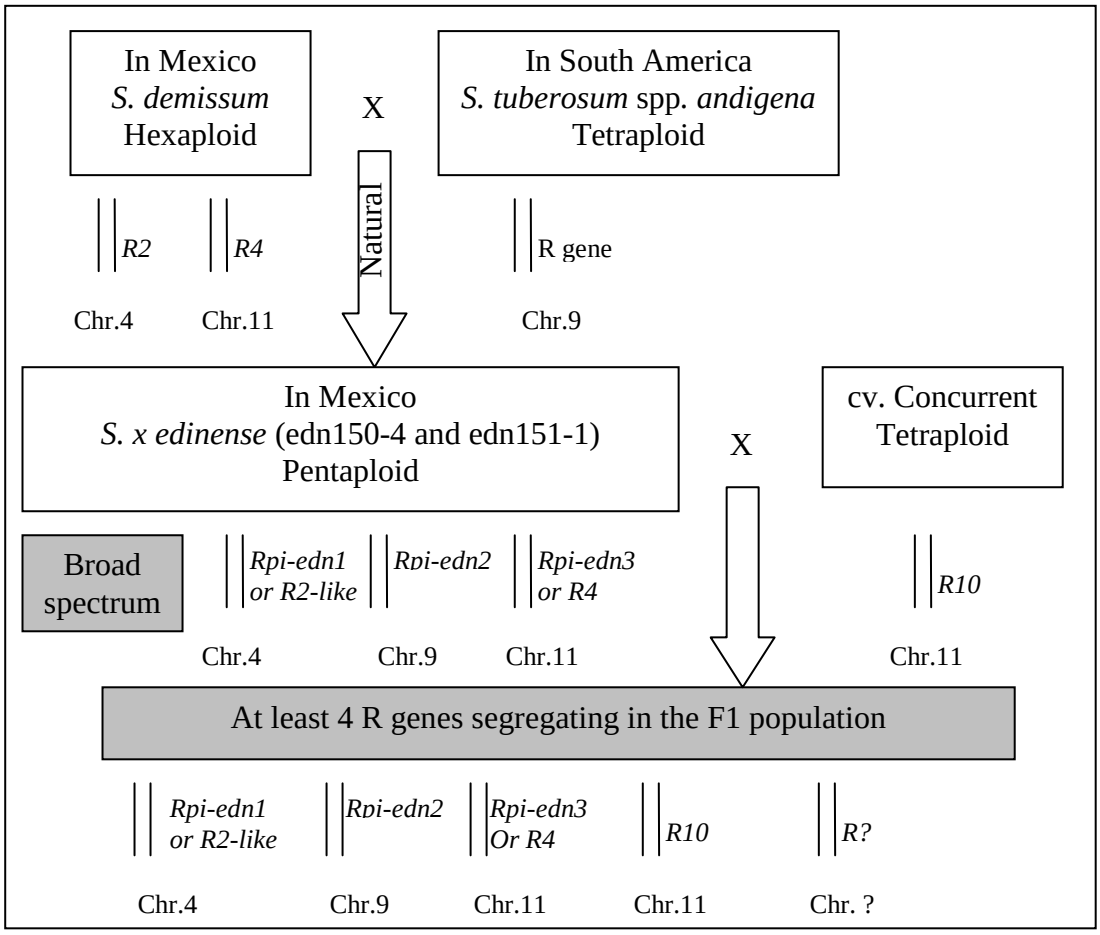

Figure 5. Model for the resistance present in *S. x edinense,* cv. Concurrent and in the mapping population. *R?* indicates the additional *R* genes segregating in the population.

In conclusion, the investigation of inheritance of stacked *R* genes in potato genotypes can be made more easy by using *Avr* genes from the complementing *R* genes and *R* gene cluster specific markers. If that is not possible, more generations of backcrossing are needed to unravel the nature of the different *R* genes involved. The natural stacking of broken *R* genes located on different clusters and originating from geographically distinct centers of diversity of *P. infestans* confers a strong level of resistance in *S. x edinense*. This study shows that stacking of *R* genes does occur in nature and seems to be a successful strategy to fend off the pathogen. It may be taken as a natural proof of principle, and applied in an agricultural context as a strategy to achieve durable resistance.

Acknowledgement

We like to thank Dirk Jan Huigen for technical assistance in the greenhouse and Francine Govers for providing *Agrobacterium tumefaciens* clones expressing *Avr4*.

Supplementary material

Table S1. R2, Tm2 and N-like profiling primers.

Profiling	Primer name	Sequence
R2	R2ch4-F1	TGTTTGAGATCAACTCTATTGCTAATG
R2	R2ch4-F2	CAATTGTTGTATTGAGCGGACT
R2	R2ch4-F3	GGAAAGATGTTGACCCTGTTG
R2	R2ch4-F4	TGTGCAGTGATAACAGCTTCA
R2	R2ch4-R2	GCTGCTAATGTTGTTTAGGGAGT
R2	R2ch4-R3	TGGATCGAAGAACATAATTGACC
R2	R2ch4-R4	AATGACTCTGCTTCCATTCTTG
Tm2	Tm1-R	CATTTCTCTCTGGAGCCAATC
Tm2	Tm1-F	GAGAGAAATGAGACACATTCG
Tm2	Tm3-F	GCGGATGAGTTTGCTATGGAG
Tm2	Tm3-R	CTCCATAGCAAACTCATCCGC
Tm2	Tm6-F	TGTTTCMATAGTTGGCATGCC
Tm2	Tm15-F	AGTTTGTGTGTGGACTTGGC
Tm2	Tm15-R	GTAACAAGTCATGTATGCGAC
Tm2	Tm19-F	GCCAAATAGTATTGTCAAGCTC
Tm2	Tm19-R	GAGCTTGACAATACTATTTGGC
Tm2	Mcq19-F	ACTGCCAAATTGTATGGTG
Tm2	Mcq21-R	ATTGGTGCAACAATCTCGCC
Tm2	Mcq23-F	GAATGTTTGCGGAAGAATGCG
N	Nbs13-R	AAGAARCATGCDATATCTARAAATAT
N	Nbs12-R	YTTSARSGCTAAAGGRAGRCC
N	Nbs12-F	CTTTAGCBYTSAARKTGTKKGG
N	Nbs15-F	ATGCATGAYTTRATWVAAGABATGGG
N	Tir270-F	TATGCTACRTCDAGNTGGTGC
N	Tir300-F	NTAGTRAAGAYATGGAATGC
N	Lrr3050-R	YGATGGTGGAACCAHCTTGGG
N	Lrr3150-R	CAGAGTAACATACARCAAATCCC

Table S2. Phytophthora infestans isolates with virulence factors and origin (Champouret et al. 2009).

Isolate	Origin	Race
IPO-0	Unknown	3b,4,7,10,11
91011	The Netherlands	3,4,5,10
VK98014	The Netherlands	1,2,4,11
NL00228	The Netherlands	1,2,4,7
IPO428-2	The Netherlands	1,3,4,7,8,10,11
IPO-C	Belgium	1,2,3,4,5,6,7,10,11
H30P04	The Netherlands	3[a],7,10,11
NI050194	The Netherlands	Nd
USA618	Mexico	1,2,3,6,7,10,11
PIC99183	Mexico	1,2,3,4,5,7,8,10,11
NL01096	The Netherlands	1,3,4,7,8,10,11
3128-A	Unknown	Nd
PIC99177	Mexico	1,2,3,4,7,9,11

Nd: not determined.

Chapter 5

Cloning of *Rinf1-mcd*, encoding an extracellular LRR receptor from *Solanum microdontum* that responds with cell death to INF1 elicitin from *Phytophthora infestans*

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Cloning of *Rinf1-mcd*, encoding an extracellular LRR receptor from *Solanum microdontum* that responds with cell death to INF1 elicitin from *Phytophthora infestans*

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Abstract

The INF1 elicitin from *Phytophthora infestans* has many characteristics of a pathogen-associated molecular patterns (PAMP) effector that elicits basal defense responses in plants. Here, we describe the map-based cloning of *Rinf1-mcd,* the gene responsible for the INF1-mediated cell death observed in the late blight susceptible *Solanum microdontum.* The *Rinf1-mcd* locus was mapped on the long arm of chromosome 12, proximal to the *Rx/Gpa2* cluster. Fine mapping was facilitated by the use of the potato and tomato physical maps and sequences. By molecular analysis of the bacterial artificial chromosome (BAC) clones covering the *Rinf1-mcd* locus, a cluster of 13 genes belonging to the eLRR-TM-sCT type of RLP genes was identified. With flanking markers the interval containing *Rinf1-mcd* was restricted to two RLP candidates, and one of these was able to complement the INF1-mediated cell death phenotype. The absence of correlation between resistance to *P. infestans* and the INF1 response still remains to be elucidated. Understanding the molecular basis of PAMP recognition will offer new perspectives for engineering of a potential durable and broad spectrum late blight resistance in *Solanum*.

Introduction

The plant immune system is currently represented as a multi-phased zigzag model (Jones and Dangl 2006). In the first phase, pathogen-associated molecular patterns (PAMPs) are recognized by transmembrane pattern recognition receptor (PRRs), which leads to PAMP-triggered immunity (PTI) that can arrest colonization of the pathogen (Nürnberger et al. 2004). In the second phase, successful

pathogens deploy effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS). In the following phase, effectors may be recognized by cytoplasmic nucleotide binding leucine-rich repeat (NB-LRR) resistance (R) genes (Martin et al. 2003), leading to effector-triggered immunity (ETI). ETI is often associated with a hypersensitive response (HR) that leads to resistance. ETI is considered an amplified PTI (Jones and Dangl 2006).

Extracellular LRR (eLRR) resistance proteins (Chisholm et al. 2006) are able to perceive pathogens in the apoplast with their LRR domain (Jones and Takemoto 2004). Two major subclasses of eLRR proteins have been identified (Chisholm et al. 2006). The first subclass represents the receptor-like kinases (RLK) composed of an eLRR, a TM domain, and a cytoplasmic kinase, and is also referred to as eLRR-TM-kinases (Hammond-Kosack and Parker 2003). Well-characterized examples of the RLK family include Fls2 and Xa21, which induce non-host and race specific resistance, respectively (Gómez-Gómez and Boller 2000; Hammond-Kosack and Parker 2003; Song et al. 1995). The second subclass represents the receptor-like proteins (RLP) composed of an eLRR, a transmembrane (TM) domain and a short cytoplasmic tail lacking motifs for intracellular signaling, except for endocytosis motifs found in some members (Kruijt et al. 2005). This subclass of eLRR can also be named according to the endocytotic motif present in cytoplasmic tail: eLRR-TM-sCT for single cytoplasmic tail, and eLRR-TM-PEST-ECS for PEST and ECS motifs (Hammond-Kosack and Parker 2003). A number of RLPs were shown to be involved in race specific disease resistance. From tomato, various *Cf* genes that confer race-specific resistance to *Cladosporium fulvum* are summarized in recent reviews by Wulff et al. (2009), Stergiopoulos and de Wit (2009) and de Wit et al. (2009).

Elicitins are a family of structurally related extracellular protein elicitors that are ubiquitously secreted by most species of the genus *Phytophthora*, and are able to induce cell death in a restricted number of plants, particularly in the genus *Nicotiana* (Kamoun 2006). *Inf1* from *Phytophthora infestans* is highly expressed in mycelium (Kamoun et al. 1997) and encodes a 10kDa cystein rich elicitin. INF1 is produced in almost all *P. infestans* isolates, except for some US-1 isolates from the former German Democratic Republic (Kamoun et al. 1998). In *Nicotiana* species, INF1 induces an HR and was described as an avirulence factor in the interaction between *N. benthamiana* and *P. infestans* (Kamoun et al. 1998). In tomato, INF1 elicitin activates jasmonic acid- and ethylene-mediated signaling pathways but does not induce a cell death response (Kawamura et al. 2009). In

Solanum, cell death responses to INF1 were detected in *S. microdontum* and *S. huacabambense*. However, the response to INF1 was not correlated with the resistance to *P. infestans,* and therefore INF1 does not have avirulence (Avr) activity in these plants (Vleeshouwers et al. 2006). INF1 was classified as PAMP, as its characteristics fit into the definition: "PAMPs/MAMPs are defined as invariant epitopes within molecules that are fundamental to the pathogens' fitness, widely distributed among different microbes, (…) and recognized by a wide array of potential hosts" (Schwessinger and Zipfel 2008). The biological role of elicitins is hypothesized to be related to lipid binding and/or processing as so called sterol scavengers, since *Phytophthora* species do not synthesize their own sterols (Jiang et al. 2005; Tyler 2002). Because these compounds are vital for *Phytophthora,* INF1 can be considered as fundamental to the pathogens' fitness. Similar to other PTI responses, the response to INF1 in *N. benthamiana* is dependent on BAK1, a transmembrane receptor thought to interact with the receptor and to play a role in signal transduction (Heese et al. 2007; Zipfel 2008). The response to flagellin (Flg22) is also Bak1 dependent (Chinchilla et al. 2007). Following the zigzag model, PAMP responses can be inhibited by effectors (ETS), and indeed, Avr3a and two other *P. infestans* RXLR effectors are able to suppress the INF1-mediated cell death response in *N. benthamiana* (Bos et al. 2006; Oh et al. 2009). INF1-meditated cell death is also suppressed by a venom allergen protein from *Globodera rostochiensis* (Gr-vap1) hypothesized to interfere with different host defense response pathways in *N. benthamiana* (Lozano et al. 2009). A potential INF1 receptor has been identified in *N. benthamiana*: NbLRK1, a lectin-like receptor kinase protein (Kanzaki et al. 2008). This cytoplasmic kinase domain interacts with INF1 elicitin and mediates INF1 induced cell death.

Understanding the mechanism of the INF1 response and the absence of correlation of the INF1 response with resistance to *P. infestans* in *Solanum* will contribute to more knowledge on PAMP response and innate immunity. In this study, we aimed to identify the gene causing the cell death response to INF1 in *S. microdontum*, through map-based cloning. An F1 population segregating for the response to INF1 was used to genetically localize *Rinf1-mcd* on the long arm of chromosome 12. A bacterial artificial chromosome (BAC) library was constructed for the physical mapping which led to the identification of a novel gene family showing homology to the RLP resistance genes in tomato (De Wit et al. 2009; Stergiopoulos and De Wit 2009; Wulff et al. 2009). By fine mapping, the candidate genes were restricted to two candidates that were tested by agroinfiltration. One candidate, *Rinf1-mcd,* was able to complement the non-

responding phenotype in *S. microdontum*. *Rinf1-mcd* belongs to the eLRR-TM-sCT class of RLP.

Materials and methods

Plant material and mapping population

Two accessions of *S. microdontum,* provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands, were used in this study. The two diploid genotypes, INF1 responding *S. microdontum* mcd360-1 (CGN 17596) and INF1-non-responding *S. microdontum* mcd714-1 (CGN 23050) were crossed to generate a mapping population (Sol*R*gene database, http://www.plantbreeding.wur.nl/phytophthora/). The intraspecific cross resulted in more than 4000 seeds.

INF1 assays

Culture filtrates of *P. infestans* isolates 88069 and PY23 were produced as described previously (Kamoun et al. 1998). Isolate 88069 secretes INF1 in the medium, and PY23 is an antisense transformant of 88069 that does not produce INF1 (Kamoun et al. 1998). Recombinant INF1 protein from *Pichia pastoris* was produced as described in Vleeshouwers et al. (2006). The infiltration of the purified INF1 protein was side by side with the infiltration of culture filtrates from *P. infestans.*

PVX agroinfection assays were performed as described in (Vleeshouwers et al. 2006). Recombinant *A. tumefaciens* GV3101 strains carrying pGR106-INF1 (Kamoun et al. 1999a), were toothpick-inoculated in leaves of individual F_1 plants with two plant replicas. The pGR106 empty vector and pGR106-CRN2 (Torto et al. 2003) were included as positive and negative controls, respectively, and the local symptoms were visually scored from 10 - 15 days post inoculation.

Marker development

A screen with a set of SSR markers covering the potato genome was performed to identify the mapping position of *Rinf1-mcd.* About 80 SSR markers were applied as described in **Chapter 4**. SSR PCR products were separated on a LI-COR DNA sequencer (Lincoln, Nebraska, USA).

CAPS markers were used to confirm the mapping position and for fine mapping. Several sources were used to develop CAPs markers for both purposes. To confirm the mapping position, known markers from literature and sequence

information from the Gabi primary database (https://gabi.rzpd.de/) were used. For fine mapping purposes, primers were designed on specific sequences from the chromosome region of interest from several origins (chromosome 12). Primers were designed on RH89-039-16 (RH) (http://www.potatogenome.net/) BAC end sequences from contig 167 mapped in the cluster of interest, on tomato BAC sequence from the SGN database (Mueller et al. 2005), and on MCD BAC sequence (this study). The PCR amplifications obtained with these primers on the parents and F1 population were cleaved with a selection of enzymes to identify segregating polymorphisms linked with the INF1 response phenotype (**Table 1**). The two CAPS markers used for the recombinant screen were converted into high resolution melting curve analysis (HRM) markers (Gundry et al. 2003). The PCRs were performed using the following cycle profile: an initial cycle at 98°C for 30 sec then 40 cycles of 98°C for 5 sec, 60°C for 5 sec, 72°C for 15 sec; followed by several final steps: 72°C for 30 sec, 94°C for 30 sec and 25°C for 30 sec. The resulting PCR products were visualized and analyzed with the LightScanner (Idaho Technology).

Recombinant screen

A total of 3600 F1 individuals were screened for recombination events using the flanking markers p17 and IPM5. The seeds were sown in trays in 96 well format. Genomic DNA was isolated following the quick NaOH protocol (Wang et al. 1993) and used for PCR amplification. The recombinant genotypes of interest were transplanted into pots, multiplied by vegetative propagation, and characterized for INF1 response through binary PVX assay. The important recombinant genotypes were transferred *in vitro* and maintained.

Bacterial artificial chromosome (BAC) library construction

The INF1-responding mcd360-1 genotype was used as source of DNA for the construction of a *S. microdontum* BAC library (MCD). High molecular weight DNA preparation and BAC library construction were carried out as described by Rouppe van der Voort et al. (1999). Approximately 110,000 clones with an average size of 110 kb, which corresponds to 10 genome equivalents, were obtained. A total of approximately 110,000 clones were individually stored at -80°C. Marker screening of the BAC library harboring the individually stored BAC clones was carried out as described in Rouppe van der Voort et al. (1999).

DNA sequencing and analysis

The DNA sequences of the BAC clones were determined by shotgun sequence analysis which was performed by Macrogen (www.macrogen.com). Approximately 1000 subclones of 2kb were produced and sequenced, which resulted in a 6x coverage of each BAC clone. The annotation and identification of putative genes was performed by using GENSCAN (Burge and Karlin 1997) and Fgenesh (Softberry, Salamov and Solovyev 2000). Homology searches were performed with the BLAST program looking in the DNA and protein databases for similarity (http://www.ncbi.nlm.nih.gov/BLAST/). Motifs were identified using the SMART program (http://smart.embl-heidelberg.de/). DNAstar (Lasergene, Madison, WI, USA) software package was used for sequence alignment and comparison.

Plasmid constructions

The candidate genes were cloned behind a 35 promoter. ORF of the two gene candidates were predicted with Fgenesh (Softberry). Primers were designed on the start and stop codon of both ORFs to test the candidates in over-expression assays (**Table 2**). The fragments were amplified by long-range PCR (LR-PCR) with the Phusion high-fidelity DNA polymerase (New England Biolabs) in a 20-µl reaction mixture, using the BAC clones as template. The mixture contained 50 ng of genomic DNA, 1 µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM), 0.8 µl of dNTPs (5 mM each), 5 µl of 10× buffer, or 1 µl of Phusion (NEB). The following PCR program was used: 94°C for 3 min, 94°C for 30 s, 55°C for 30 s, 72°C for 4 min, and 72°C for 5 min during 30 cycles.

PCR products were purified using the QIAEX II gel extraction kit (Qiagen). Purified PCR products were cloned using the Gateway technology (Invitrogen, SanDiego, CA, USA). They were first cloned into the donor plasmid pDONR 221 in a BP reaction (Untergasser 2006a). The inserts were confirmed through complete sequencing, and subsequently cloned into the destination vectors via a Classic LR-Reaction II (Untergasser 2006b). The ORFs of the candidate gene were cloned into the destination vector pK7FWG2.0 with 35S regulatory elements (Karimi et al. 2002). LR clones were selected by comparing the digestion pattern with the in silico pattern.

The plasmid p35S-INF1 is a fusion between the signal peptide of tobacco PR1a and the *P. infestans* INF1 gene driven by the 35S promoter of the Cauliflower mosaic virus, as described in Kamoun et al (1999a).

Table 1. Markers used for the mapping of *Rinf1-mcd*. Markers above the grey line were used for the mapping and markers below were developed for the fine and physical mapping.*SSR marker ramp for TM (see Materials and methods).

Name	Type	Sequence or reference	TM	Enzyme
Rh192P22	SSR	Dr. E. Bakker (Laboratory of Nematology, WUR)	56*	a.s.
GB1755	CAPS / HRM	F ttaacgaactagcagtttatagacgc R ttgcttggactcttcataaaaca	52	DdeI
Ct129	CAPS	Bendahmane et al., 1997	52	XapI
111R	CAPS	Rouppe van der Voort et al., 1999	52	MspI
IPM5	CAPS / HRM	Bendahmane et al., 1997	52	DdeI
IPM4	CAPS	Bendahmane et al., 1997	52	RsaI
77R	CAPS	Rouppe van der Voort et al., 1999	52	Hin1I
IPM3	CAPS	Bendahmane et al., 1997	52	XapI
Gp34	CAPS	Bendahmane et al., 1997	52	HaeIII
Gp178	CAPS	F tgcactttaagagaggagaaaaga R ctgcagcttactcggaatgc	52	MwoI
Gp306	CAPS	F cgttgctaggtaagcatgaaga R ctgcaggttggattttgtga	52	AluI
Rhl8	CAPS	F cttccaaatttcccggattgg R gtaacattggctctgagcctc	55	MnlI
Rhr0	CAPS	F ttgggtaagtggagcaggg R ggcttggaatctcggactatg	55	MnlI
LBC	CAPS	F tgaatcagctgaagcagtcg R tgttgaacatcttcttaacagca	45	ScrFI
C95	CAPS	F tgagccaccagtaggtaggg R aaaccaaaaagcccaaagt	57	Hin1II
T85	CAPS	F ggttccattgaagcctagca R agccctctttttccctacca	57	MwoI
C12	CAPS	F cactcggattgacctttctg R tgaatcgggactgatgaaca	58	ScrFI
T207	CAPS	F ataattactggcagataaacc R gtacttacagatatgagagcg	55	a.s.

Table 2. Primers used for the cloning of *Rinf1-mcd* candidate genes.

Construct	**Insert**	**Primer**	**Sequence**	**Destination vectors**
p35S-85	ORF-85	85st-F 85st-R	ATGGTCATGAGTCTGTTTTTCTTTTAT TTAAGTCCTTCGTCTCTGAGCTC	pK7FWG2.0
p35S-207-1	ORF-207-1	207-1st-F 207-1st-R	ATGGTGAGTTTGTTTTTCTTTTATTCA TTAAGACCGTCGTCTCTGAGCT	pK7FWG2.0

***A. tumefaciens* strains and culture conditions.**

The three plasmids (p35S-85, p35S-207-1 and p35S-INF1) were introduced by electroporation into *A. tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG (pBBR1MCS-5.virGN54D, Van Der Fits et al. 2000). *A. tumefaciens* was grown at 28°C in Luria-Bertani (LB) media using the appropriate antibiotics.

Agro-infiltration assays

The agroinfiltrations were carried out on young F1 individuals three weeks after transplanting from *in vitro* culture. Three leaves were infiltrated with the constructs: INF1, ORF85, ORF207-1, the combination of INF1 with the gene candidates, and of R3a with Avr3a as positive control (Armstrong et al. 2005). The agroinfiltration experiments of the recombinant *A. tumefaciens* were performed as described by van der Hoorn et al. (2000) with some adaptations as described in **Chapter 4**. Leaves from *S. microdontum* species were infiltrated with the suspensions.

Results

Development of the mapping population

A first and crucial step towards map-based cloning is the development of a mapping population segregating for the trait of interest. The *S. microdontum* genotype mcd360-1, identified as responding to INF1, was crossed with the INF1 non-responding genotype mcd714-1 to generate a mapping population, and more than 4000 seeds were produced.

To determine whether response to INF1 was segregating in the population, a first batch of seeds were sown and 59 F1 individuals were tested for response to INF1 in two repeated experiments. Recombinant INF1 fused to the His and Flag epitope tags were expressed and purified from cultures of *Pichia pastoris.* Also culture filtrates of *P. infestans* isolates 88069 and PY23 were produced, representing INF1-containing and INF1-lacking samples, respectively. The two culture filtrates and the *Pichia*-produced INF1 sample were infiltrated in leaves of mature *Solanum* plants, in two independent experiments, and the symptoms were scored after two days. 25 F1 plants showed cell death responses to *Pichia*-produced INF1 and culture filtrate of 88069, but no response to culture filtrate of PY23. These plants were scored as INF1-responding. 26 other plants did not show any response to the *Pichia*-produced INF1 nor the culture filtrates, and these were scored as INF1-non-responding. The phenotype of eight plants was inconclusive, because of some background responses perhaps from the culture medium or

other elements secreted by *P. infestans,* and these plants were excluded from further analysis. We concluded that response to INF1 segregates in the F1 population following a 1:1 ratio. This indicates that *Rinf1-mcd*, the gene responsible for the cell death response to INF1 infiltration, is a single dominant gene.

Rinf1-mcd maps on chromosome 12 above the *Rx Gpa2* cluster

To identify the mapping position of *Rinf1-mcd*, a genome-wide SSR markers screen was performed (**Chapter 4**). Around 80 SSR markers were tested for linkage with the INF1 response on the parental genotypes and 20 individuals of F1 population, i.e. 10 INF1-responding and 10 INF1-non-responding. The marker RH192P22 was linked to the INF1 response with two recombinants out of 51 F1 individuals. This marker maps in bin 70/71 at the bottom of chromosome 12 on the SH83-92-488 (SH) genetic map (van Os et al. 2006), indicating that *Rinf1-mcd* is located on the southern side of chromosome 12 (**Figure 1**).

In order to confirm and fine map *Rinf1-mcd,* CAPS markers from chromosome 12 were tested on the 51 F1 plants. Ten CAPS markers were polymorphic and segregated in the population (**Table 1**). A genetic map of the *Rinf1-mcd* locus was constructed with the 11 (10 CAPS and 1 SSR) markers (**Figure 1**). Gp178 was the only proximal marker to *Rinf1-mcd*. All the markers from the *Rx/Gpa2* cluster were distal to the gene. With these data, the mapping position of *Rinf1-mcd* on chromosome 12 is confirmed, and defined more precisely proximal to the *Rx/Gpa2* cluster at about 6 cM. This indicates that *Rinf1-mcd* is probably not a homologue of the *Rx/Gpa2* family.

Presence of a cluster of RLP at the *Rinf1-mcd* locus

In order to fine-map the gene, recombinants in the *Rinf1-mcd* locus needed to be identified and more closely linked markers needed to be developed. F1 individuals identified as recombinants between the flanking markers GB1755 and IPM5 were tested for their response to INF1 (**Figure 2**). PVX agroinfection with pGR106-INF1 was performed on three vegetatively propagated plants per genotype, and clear phenotypes were obtained for all recombinants.

We made use of the SH x RH UHD genetic map (van Os et al. 2006) and of the RH physical map (Borm 2008) to develop closely linked markers. The marker Gp34, 6 cM distal to *Rinf1-mcd*, maps in bins 67-71 of the RH genetic map (**Figure 1**). So we looked for contigs mapped around bin 67, and interestingly the BAC clone RH195L21 from contig 167 maps in bin 67 (Tang et al. 2009). Primers

were designed on the BAC end sequences of the BAC clones at the extremities of contig 167. Two markers, Rhl8 and Rhr0, from opposite ends of contig 167, were successfully developed (**Table 1**).

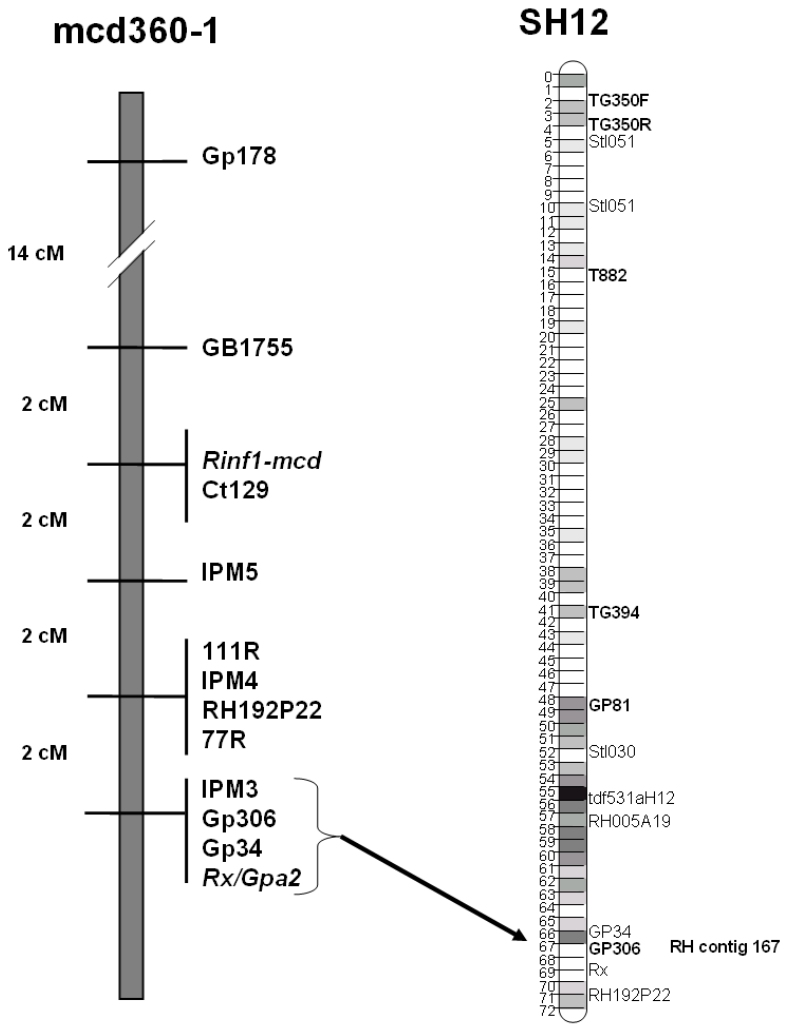

Figure 1. Genetic map of the *Rinf1-mcd* locus (n=51) compared with the chromosome 12 of the SH genetic map (van Os et al. 2006).

pGR106-INF1

pGR106-INF1

pGR106

pGR106-CRN2

Figure 2. PVX agroinfection experiment on F1 individuals. This assay was performed to phenotype the F1 plant with recombination between the flanking markers. On the left is an example of an INF1- responding genotype and on the right a INF1 non-responding genotype.

The markers Rhl8 and Rhr0 were applied on the recombinants. In a population of 3600 individuals, the markers Rhl8 and Rhr0 were flanking *Rinf1-mcd*: Rhl8 was proximal to *Rinf1-mcd* with five recombinants, and Rhr0 was also distal to the gene with five recombinants **(Figure 3A**). These results indicate that the RH contig 167 covers the *Rinf1-mcd* interval (**Figure 3B**). BlastN of the RH BAC end sequences from contig 167 revealed homology with a tomato BAC clone LE_HBa0146I19 of 120 kb. Primers designed on the tomato sequence resulted in the LBC marker, which mapped two recombinants away proximal to *Rinf1-mcd* (**Table 1 and figure 3A**).

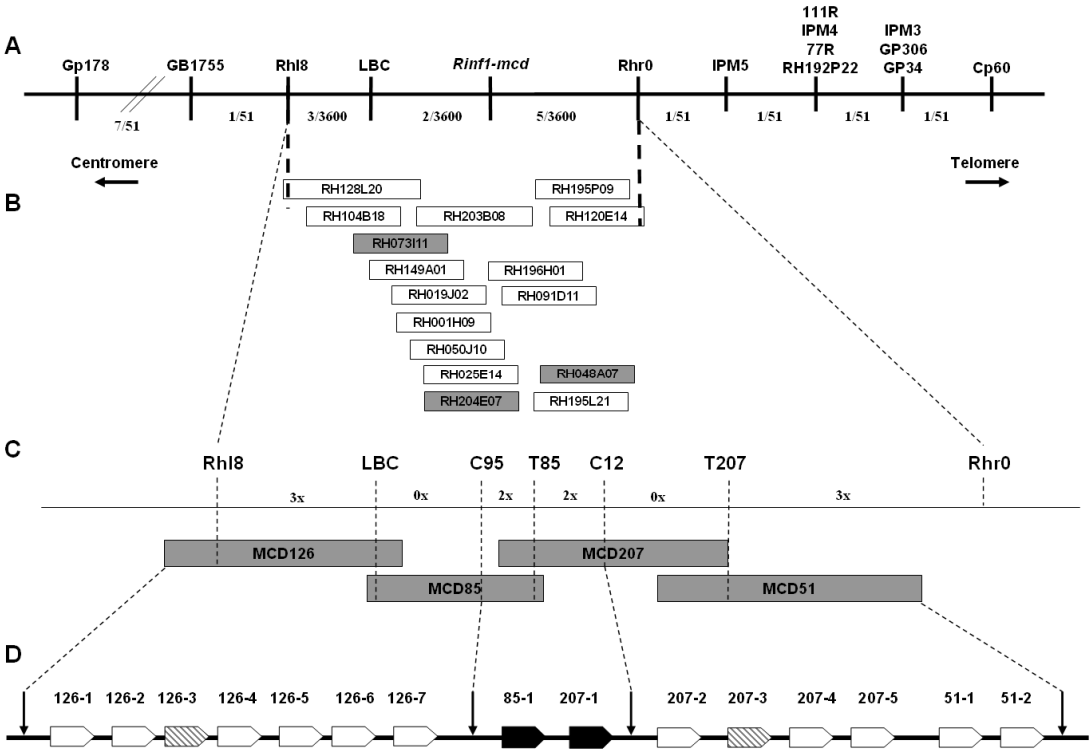

Figure 3. High resolution genetic and physical map of the *Rinf1-mcd* locus. A. Genetic map of the *Rinf1-mcd* locus. B. RH contig 167 covering the locus and used to design primers. In grey, the BAC clones with BAC end sequences homologous to *Cf*-like sequences. Physical map with MCD BAC clones covering the locus. D. Relative position of the RLP candidate genes. They were named according to the BAC clone on which they are located followed by a number relative to the number of *Cf* homologue on the BAC clone. In white the RLP genes, in black: RLP genes left in the interval, hatched box: pseudo RLP genes.

BlastX of the RH BAC end sequences of the contig 167 and of the tomato BAC clone LE_HBa0146I19 sequence showed the presence of genes with homology to the RLP resistance genes in tomato and with *Cf* genes conferring resistance to *Cladosporium fulvum.* Interestingly, the physical mapping of *Gpa2,* resistance gene to *Globodera pallida*, revealed on BAC end sequences of northern BAC

clones the presence of sequences with homology to RLP (*Cf*) genes (van der Vossen et al. 2000). This indicates the presence of a cluster of RLP in the *Rinf1-mcd* interval and implies that *Rinf1-mcd* might be a RLP. Since INF1 is an apoplastic elicitor, extracellular receptors are more likely gene candidates than cytoplasmic NBS LRR genes like the *Rx/Gpa2* family members.

Two RLP are gene candidates for *Rinf1-mcd*

For physical mapping of *Rinf1-mcd*, a BAC library of the INF1 responding mcd360-1 was constructed. The MCD BAC library consisted of 110592 clones with an average insert size of 110 kb. In order to build a contig across the *Rinf1-mcd* locus, the MCD BAC library was screened with the CAPS markers Rhl8, LBC and Rhr0. Rhl8 identified BAC clones MCD126, MCD89 and MCD185, LBC identified BAC clones MCD85, MCD86, MCD170, MCD126 and MCD185, and Rhr0 identified BAC clone MCD73. BAC clones MCD126 and MCD 185 were both identified by markers Rhl8 and LBC, indicating they are closely linked. Only the two BAC clones 126 and 85 were in coupling phase with *Rinf1-mcd*, so they were selected for further analysis. Sequencing the BAC ends of these BAC clones led to the development of one coupling phase marker, T85, co-segregating with *Rinf1-mcd* (**Figure 3C**). In a second BAC library screen, the marker T85 identified a coupling phase BAC clone MCD207. BAC end marker development resulted in the marker T207, a coupling phase SCAR marker, located two recombinants away from the gene. Two BAC clones from the BAC library were positive for that marker: BAC clone MCD51 and MCD19. The BAC clone MCD51 was selected for further analysis because of its larger insert size. The four MCD BAC clones covered completely the *Rinf1-mcd* interval between the closest flanking markers Rhl8 and Rhr0.

The four BAC clones MCD126, MCD85, MCD207 and MCD51 were sequenced. Sequence annotation revealed a large RLP cluster with a total of 13 RLP (**Figure 3D**): seven were identified on BAC clone MCD126, one on MCD85, four on MCD207 and two on MCD51, excluding two pseudogenes (**Figure 3D**). To allow distinction between all these RLP, primer pairs were designed between the different candidate genes to develop markers. The markers C95 and C12 (**Table 1**) were the closest flanking markers, each two recombinants away from the gene, and restricted the *Rinf1-mcd* interval to around 100 kb. In this interval, two RLPs, 85-1 and 207-1, were detected.

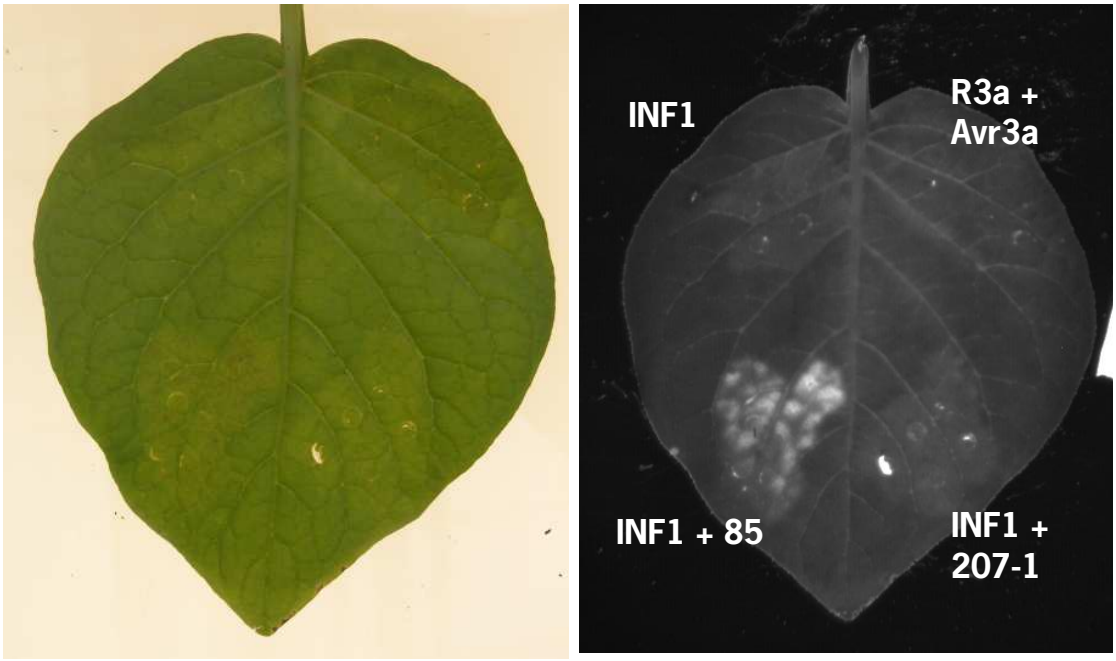

Figure 4. Transient transformation of a F1 individual under normal and UV light. Co-infiltration of the two RLP candidates with INF1, the INF1 alone and the positive control.

Complementation analyses

To determine whether one of the two candidate genes complements the cell death response to INF1, we cloned both ORFs in the vector pK7FWG2.0 behind a 35S promoter. The two constructs p35S-85 and p35S-207-1 were subsequently transformed to *A. tumefaciens* for transient expression in plants. To choose an INF1 non-responding genotype that is suitable for agroinfiltration assays, we screened F1 plants from the population for response to INF1, and included R3a-Avr3a co-infiltrations as positive control. Only one out of six tested F1 plants, genotype 117, showed expected responses to the controls, whereas the other plants gave background necrosis to *A. tumefaciens* or showed too low transformation efficiency. *A. tumefaciens* carrying p35S-INF1 was mixed with strains carrying p35S-85 or p35S-207-1 in a 1:1 ratio, and the mixtures were infiltrated side-by-side with individual strains and the R3a-avr3a positive control on the leaves of genotype 117 (final OD600 = 0.2). Co-infiltration of p35S-85 with p35S-INF1 resulted in a cell death response (**Figure 4**), whereas co-infiltration of p35S-207-1 with p35S-INF1 did not elicit any visible cell death (**Figure 4**). The observation of the infiltrated leaves under UV light revealed strong autofluorescence for the coinfiltration of p35S-INF1 with p35S-85 (**Figure 4**). Autofluorescence is associated with accumulation of phenolic compounds as part of resistance responses (Nicholson and Hammerschmidt 1992; Van Den Burg et al. 2008). The autofluorescence for INF1-p35S-85 was stronger than for the positive control R3a-Avr3a. We concluded that candidate gene 85 is responsible

for the cell death response to INF1, and this RLP was therefore designated as the *Rinf1-mcd* gene.

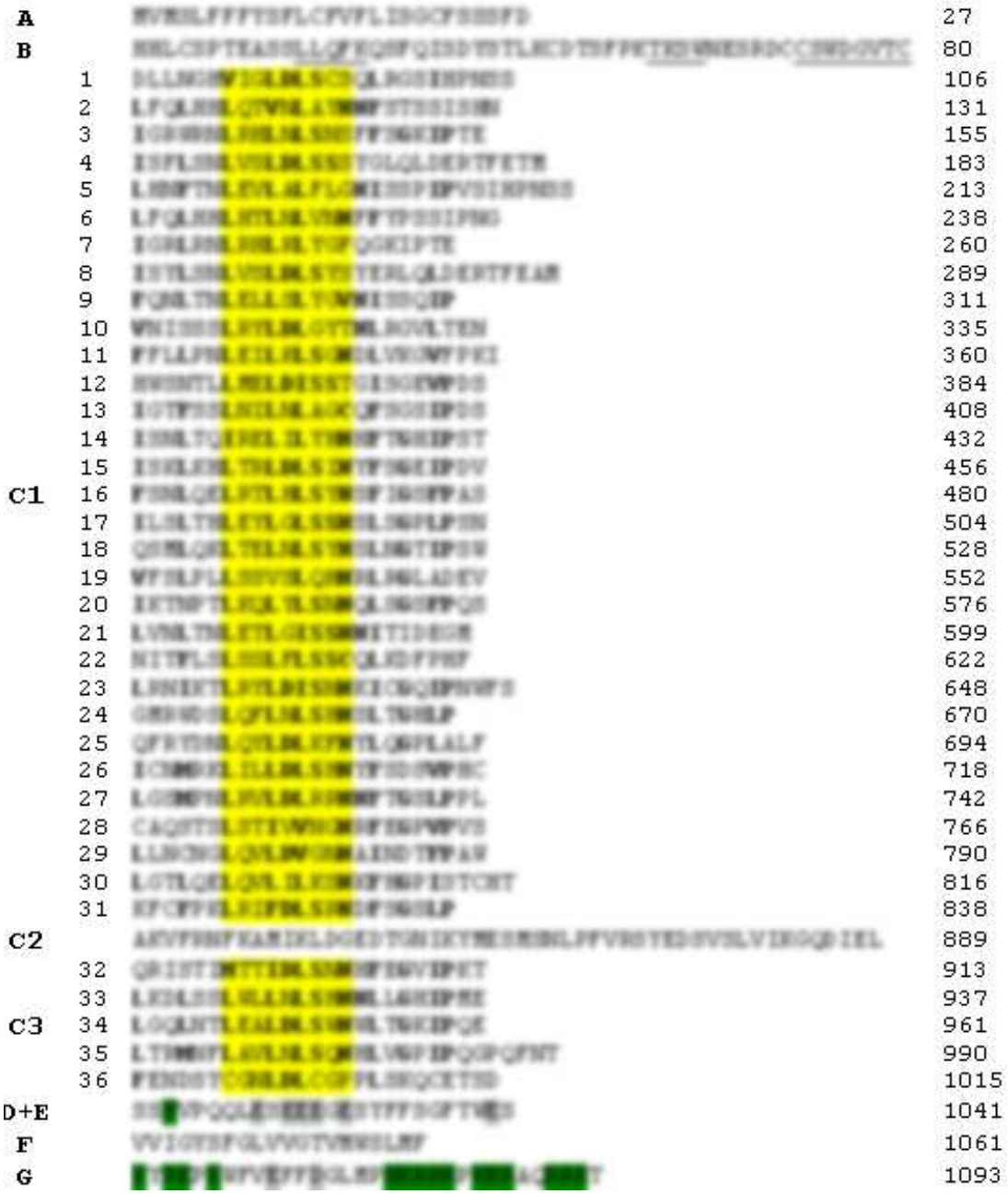

Figure 5. *Rinf1-mcd* deduced protein sequence. The protein is divided into eight domains from A to G as described in Wang et al. (2008) and Wulff et al. (2009) A: a putative signal peptide, B: a Cys-rich or mature N-terminus C: LRR domain subdivided into three subdomains, where C1 and C3 are LRR region and C2 a non-LRR island, D: a spacer, E: an acidic domain, F: a transmembrane domain and G: a short cytoplasmic region. The LRR motif is LxxLxxLxxLDLSSNNLxGxIPxx (Jones et al., 1994) where the conserved L of the LRRs is often replaced by V, F, I, or M. Highlighted in yellow the conserved motif LXXLXLSSN, in grey the acidic amino acids and in green the basic amino acids. Underlined in the B domain the three conserved motifs LLxxK, LssW and CxWxGVxC (van der Hoorn et al., 2005). (The sequence is blurred for confidentiality reasons)

***Rinf1-mcd* Gene structure and putative amino acid sequence**

The deduced ORF of the *Rinf1-mcd* gene encodes a predicted polypeptide of 1094 amino acids from a single ORF. The Rinf1-mcd protein has 30% identity with other RLP such as LeEix1 and LeEix2 (Ron and Avni 2004), 37% identity with Cf-2/Cf-5 and EILP from *N. tabacum* (Takemoto et al. 2000) and 45% identity with Cf-9/Cf-4. It shares only 11% identity with the lectin-like receptor kinase protein (NbLRK1) identified in *N. tabacum* as interacting with INF1 (Kanzaki et al. 2008). Rinf1-mcd consists of the same domains as the other RLP (**Figure 5**). Domain A contains a putative signal peptide of 23 amino acids (SignalP). In domain B, the three conserved structural motifs present in RLPs and RLKs are found namely LLxxK, LssW and CxWxGVxC (Rivas and Thomas 2005; Van der Hoorn et al. 2005). The third domain (C) is the largest and consists of 36 imperfect repeats of the consensus sequence LxxLxxLxxLDLSSNNLxGxIPxx (Jones et al. 1994). The consensus sequence containing the amino acid Gly fits with the prediction of an extracytoplasmic LRR region (Jones et al. 1994; Song et al. 1995). Domains D+E represent the part between the LRR domain and the transmembrane domain, in which domain E contains acidic amino acids. Domain F is the transmembrane domain. It is followed by a short cytoplasmic tail (domain G) that does not contain any known motif related to endocytosis present in the *Cf* homologues like the PEST, YXXø or E/DxxxLø motifs (Geldner and Robatzek 2008). Sequence alignment with homologous sequences from the *Rinf1-mcd* cluster and other RLPs reveals that domain G is conserved among the homologues from the *Rinf1-mcd* cluster, but different from other RLPs (data not shown).

Discussion

Development of late blight resistant potato cultivars is a challenge for breeders and still relies on *R* genes which are normally not very durable. In this study, our intentions were to identify possible alternatives to achieve durable resistance. We and others postulate that better understanding the molecular basis of *P. infestans* PAMP recognition in *Solanum* can offer new perspectives for engineering of durable late blight resistance in *Solanum* (Ellis 2006). In contrast to fast-evolving RxLR effectors (Haas et al. 2009; Jiang et al. 2008; Schornack et al. 2009; Win et al. 2007) that normally interact with the typical, non-durable *Rpi* genes, PAMPs are thought to be slow-evolving (Jones and Dangl 2006), and therefore perhaps confer a more durable type of resistance. PAMP receptors can be functional when transferred to other plant families, indicating that PTI signaling pathways must be similar among plant families (Lacombe et al. 2010). Therefore, transferring the receptor of a *P. infestans* PAMP from *Solanum* species or non-hosts, such as *N.*

tabacum or *Arabidopsis thaliana,* to potato may induce a certain level of broad resistance to *P. infestans* based on PAMP defense responses. With INF elicitins to be considered as oomycete PAMPs (Hein et al. 2009b; Vleeshouwers et al. 2006), transferring the INF receptor from *Nicotiana* to potato would be a way to introduce PAMP-based resistance. However, molecular cloning of INF receptors is hampered by the absence of genetic variation in response to elicitins in *Nicotiana*, yet the genetic variation for response to INFs in *S. microdontum* (Vleeshouwers et al. 2006) set the stage for a successful map-based cloning of the putative INF1 receptor.

In order to identify the gene responsible for INF1-mediated cell death, we produced an F1 segregating population and followed a map-based cloning approach. *Rinf1-mcd* is located on chromosome 12 proximal to the *R* gene cluster containing *Rx* and *Gpa2*. At the *Rinf1-mcd* locus a relatively high recombination frequency was noted, as also observed in the *Cf-4/Cf-9* cluster on chromosome 1 and in the *Mi-3* region on chromosome 12 of tomato (Bonnema et al. 1997; Yaghoobi et al. 2005). Physical mapping of the *Rinf1-mcd* locus revealed sequences comprising a large cluster of eLRR-TM-sCT type of RLP genes (Hammond-Kosack and Parker 2003). Among these, one candidate gene was able to induce cell death when co-expressed with INF1 and this RLP was designated *Rinf1-mcd*. Rinf1-mcd was found to have at least 70% identity on amino acid level to other RLP homologues found in *S. microdontum*, and tomato, but had less than 50% identity with the homologues from the two *Cf* clusters in tomato (*Cf-2/Cf-5* and *Cf-4/Cf-9*). Thus, we postulate that the RLP cluster on chromosome 12 represents a novel gene family in *Solanum*. In the syntenic region of tomato and pepper, various *R* genes and QTLs occur, namely *Lv* conferring resistance to *Leveillula taurica* (Chunwongse et al. 1997) and three QTLs conferring resistance to powdery mildew, *Ol-qtl2, Ol-qtl3* and *Lt-9.1* (Bai et al. 2003; Lefebvre et al. 2003). These genes might belong to the same RLP gene family and be homologous to *Rinf1-mcd*.

Host and non-host resistance to *P. infestans* is associated with the HR response (Kamoun et al. 1999b; Vleeshouwers et al. 2000). INF1 can induce an array of plant defense responses, including an HR (De Wit et al. 2002) in *Nicotiana* species (Ponchet et al. 1999; Sasabe et al. 2000), a specific cell-death response in some *Solanum*, *Raphanus* and *Brassica* species (Takemoto et al. 2005; Vleeshouwers et al. 2006) and jasmonic acid- and ethylene-mediated signaling pathways in tomato (Kawamura et al. 2009). Although some plants induce cell death upon

INF1 infiltration, this response is not necessarily conferring resistance to *P. infestans* in *Solanum* and in *N. benthamiana* (Becktell et al. 2006; Vleeshouwers et al. 2006). In *N. benthamiana,* various RxLR effectors such as Avr3a, PexRD8 and PexRD36$_{45\text{-}1}$ were shown to suppress INF1-mediated cell death (Bos et al. 2006; Oh et al. 2009). There is no evidence however that these effectors also suppress the HR response in *Solanum*. Another explanation for absence of a correlation between response to INF and resistance to *P. infestans* is the fact that INF1 is down-regulated during potato infection, and thereby perhaps evading recognition (Kamoun et al. 1997). Down-regulation of a PAMP can be a strategy for the pathogen to avoid recognition, and gain virulence. *NbLRK1*, the lectin-like receptor kinase protein receptor interacting with INF1 from *N. benthamiana* only shares 11% sequence identity with *Rinf1-mcd* and belongs to a different class of receptors (Kanzaki et al. 2008). Perhaps both genes are different components of the protein complex that recognizes INF1 in both plant species. Alternatively *N. benthamiana* and *Solanum* species may have evolved different receptors to detect INF1 and induce different pathways.

The long-term objective of the identification of the putative INF1 receptor from *S. microdontum* was to design a strategy for developing potato varieties with broad and durable resistance to late blight. Since *Solanum* is much less sensitive to INF1 than *Nicotiana* species (Vleeshouwers et al. 2006), we hypothesize that despite the down-regulation of INF1 expression during potato infection, overexpression of the INF1 receptor in potato could contribute to the induction of cell death and thus confer the plant with broad resistance to *P. infestans*.

Acknowledgement

We thank Sophien Kamoun for providing the p35S-INF1 plasmid and *Agrobacterium* expressing pGR106-INF1. We thank Jack Vossen for his support and technical advice, and Jan de Boer for information on the RH physical map.

Chapter 6

General discussion

General discussion

Introduction

The research described in this thesis was performed to achieve one major objective: find a way to control potato late blight, caused by the devastating *Phytophthora infestans* pathogen. Our work resulted in 1. the mapping of genes conferring resistance to *P. infestans* (*Rpi*) from three Bolivian wild *Solanum* species, *viz*. *S. avilesii*, and *S. capsicibaccatum* and *S. circaeifolium* spp. *quimense,* 2. the discovery of natural stacking of at least three *Rpi* genes in the natural hybrid *S. x edinense* from Mexico, and 3. the cloning of, *Rinf1-mcd*, a putative pathogen-associated molecular patterns (PAMP) receptor from *S. microdontum*. *Rinf1-mcd* may serve as basis for engineering of broad spectrum resistance. In total, six *Rpi* genes were identified and mapped in different clusters (**Table 1**). In addition, this study revealed new tools and strategies that contribute to a better understanding of the potato/*P. infestans* interaction and increased the possibility of controlling the disease. Two main aspects of this research, common to several chapters are discussed here: the *N* cluster and the map-based cloning approach. Finally perspectives for cloning of the mapped genes and implications for resistance breeding are discussed.

Table 1. Summary of all the *Rpi* genes mapped in this thesis with the species, country of origin, name, chromosomal position and gene cluster.

Species	Country	*R* genes	Chro.	Cluster
S. avilesii	Bolivia	*Rpi-avl1*	11	N
S. capsicibaccatum	Bolivia	*Rpi-cap1*	11	N
S. circaeifolium spp. *quimense*	Bolivia	*Rpi-qum1*	11	N
S. x edinense	Mexico	*Rpi-edn1*	4	R2
		Rpi-edn2	9	Tm2
		Rpi-edn3/R4^{Ma}	11	N

N cluster on chromosome 11

Rpi gene mapping in this research was performed on four different wild *Solanum* species *S.* a*vilesii*, *S. capsicibaccatum, S. circaeifolium* spp. q*uimense* and *S. x edinense*. Initially nothing was known about the resistance to *P. infestans*

occurring in these species. So it was remarkable that in each of these four species, one *R* gene was mapped in the *N* cluster, which is located on the northern side of chromosome 11. A comparative genetic map of the four *Rpi* genes is presented in **Figure 1**. We call it *N* cluster because of the high sequence homology of the sequences found in that cluster with the *N* resistance gene against tobacco mosaic virus in tobacco (**Chapters 2 and 3**, Whitham et al. 1994). Thus, we hypothesized that the *Rpi* genes located in that clusters are homologous to the *N* gene.

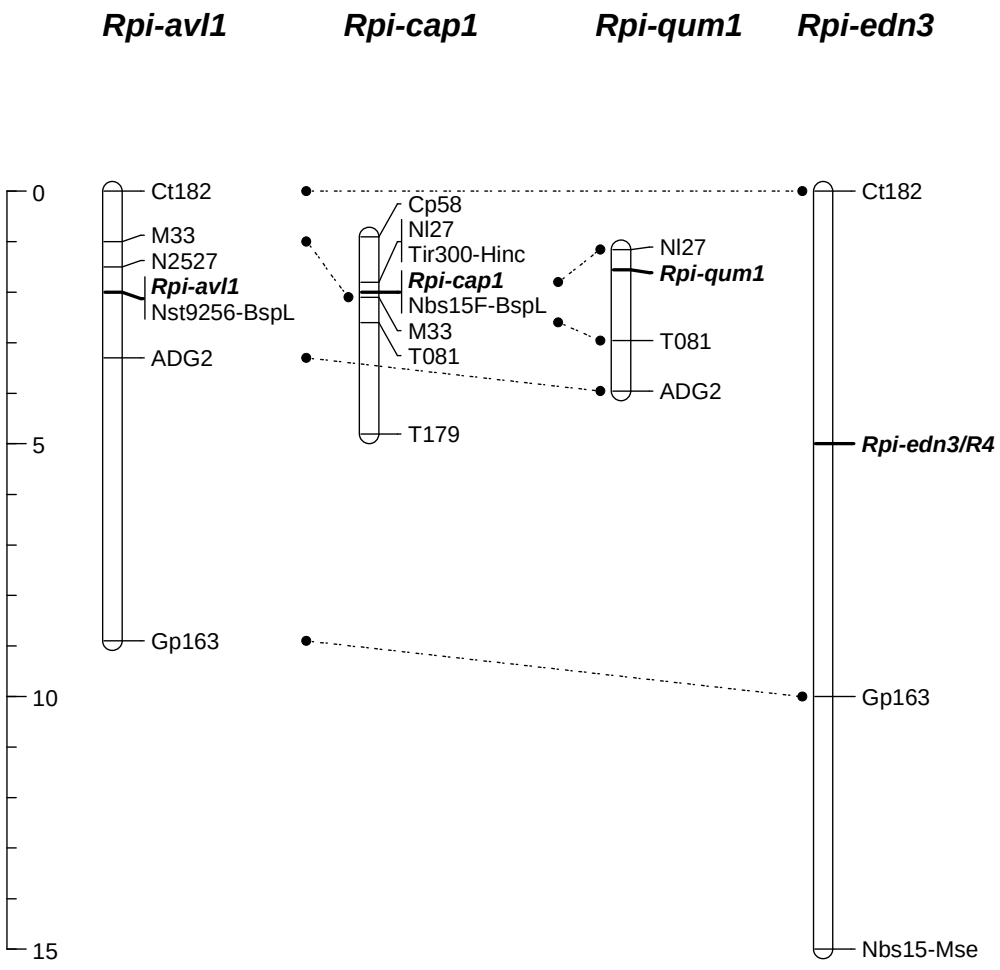

Figure 1. Comparative genetic maps of the four *Rpi* genes belonging to the *N* cluster on chromosome 11.

Characteristics of the *N* cluster

The *N* cluster is considered as a "hot spot" for resistance at least in potato, since it contains resistance to almost all pathogens: viruses, bacteria, fungi, nematodes and oomycetes (**Chapters 2 and 3**). One singularity of *N*-like R genes is that it contains a Toll/interleukin-1 receptor (TIR) domain, followed by the NBS and LRR domains in stead of the coiled coil (CC) domain, like all the present cloned *Rpi*

genes that are of the CC-NBS-LRR class (CNL). Other characteristics that are different from other *R* gene families are that the *N* gene is composed of several exons, which are distributed over the different domains (Vidal et al. 2002; Whitham et al. 1994). In addition to the TIR-NBS-LRR (TNL) R protein, studies in *Arabidopsis* and other plant genomes revealed the presence of two other families of TIR-containing proteins: TIR-X domain (TX) or the TIR-NBS domain (TN). Analyses suggest that these TX and TN genes encode functional proteins, but their function remains unknown (Meyers et al. 2002). TX and TN sequences were also found in the late blight susceptible diploid potato genotype RH89-039-16. The N protein starts with the following amino acids: one Methionine, one Alanine and six Serines (MASSSSSS). Interestingly the translated sequences found in the RH sequence show a similar start with a variable number of Serine residues, but the nucleotide sequence is variable. Serine and Leucine are the only amino acids for which six different codons result in the same amino acid. This explains why, although the translated sequences are similar, the nucleotide sequences are different and do not allow allele mining, and hence prevent accelerated cloning of *R* genes. Since *R* gene cloning by allele mining is not feasible, the only alternative strategy to clone *R* genes in this cluster is map based cloning. So far, no *R* gene has been cloned in potato by this approach in the *N* cluster, probably because of its complexity. The *N* cluster can be considered as complex because of the large number of *N*-like sequences and the presence of repetitive and repeated sequences. Despite these difficulties, cloning an *Rpi* gene that belongs to the TNL class should be very interesting. Differences between the two classes still remain unclear and variation in downstream signaling pathways has been identified (Glazebrook 2001; Hammond-Kosack and Parker 2003; Meyers et al. 2003). Although cloned *R* genes belonging to both CNL and TNL subclasses have been cloned in other pathosystems (e.g. *Arabidopsis/Pseudomonas*), it would be important to clone a TNL gene in potato conferring resistance to *P. infestans* (Hulbert et al. 2001). From a scientific point of view, it would be interesting as it would allow determining whether also in potato CNL genes against late blight differ in their mode of action from TNL genes. From a practical point of view, it would be useful to combine the two *Rpi* gene classes (CNL and TNL) in cultivated potato, as the defense pathway they induce might be complementary and might confer more durable and higher levels of resistance.

South American origin of the *N* cluster?

It is remarkable that in all four wild species, analyzed in this study, at least one *Rpi* gene mapped in the *N* cluster. Three out of the four species originate from Bolivia (**Chapters 2 and 3**), and the fourth one has an unclear origin (**Chapter 4**). This fourth species is *S x edinense,* originating from Mexico, is a hybrid between *S. demissum* that comes from Mexico and *S. tuberosum* spp. *andigena* that comes from South America. *Rpi-edn3*, the *R* gene from *S. x edinense* mapping in the *N* cluster, co-localizes with $R4^{Ma}$ that was originally identified in the Mexican species *S. demissum*. These observations lead to several possible hypotheses. 1. The *N* cluster is not specific to South America and expanded independently in both centers of diversity i.e. Mexico and South America. The *Solanum* species with *R* genes mapped in the *N* cluster have origins in both regions. 2. The *N* cluster so far mainly expanded in South America. The presence of $R4^{Ma}$ in the *N* cluster in Mexican species can be explained by various hypotheses: *Solanum* species containing $R4^{Ma}$ may have migrated from South America to Mexico and hybridized with *S. demissum* (Hawkes 1990). This possibility is supported by the response to AVR4 in two other *Solanum* species originating from Argentina, which could be an indication for the presence of $R4^{Ma}$ (**Chapter 4**). It is also possible that *Rpi-edn3* and $R4^{Ma}$ belong to different but rather closely linked clusters since the comparative genetic map indicated a possible proximal mapping position (**Figure 1**).

Resistance present in the *N* cluster compared to other MLB clusters

The resistance spectrum of the mapped *Rpi* genes is another characteristic that can be compared in order to understand better the peculiarities of the *N* cluster. The four *Rpi* genes represent three different spectra of resistance (**Chapters 2, 3 and 4**). *Rpi-cap1* and *Rpi-qum1* both confer resistance to all the isolates tested, so although their resistance spectrum cannot be differentiated, they might be different (**Chapter 3**). With four different *Rpi* genes, the *N* cluster can be considered as a major late blight (MLB) cluster. It is located on the northern arm of chromosome 11, and would be the third MLB cluster indentified, after the *R2* cluster on chromosome 4 (Li et al. 1998), and the *R3* cluster on the opposite arm of chromosome 11 (El-Kharbotly et al. 1994). The latter two clusters both evolved in Mexico and are present in *S. demissum*. Comparison of the diversity in resistances present in the three clusters reveals differences. In the *N* cluster, genes conferring resistance to other pathogens have also been mapped in potato (**Table 2**), and in the syntenic region of tomato, pepper and tobacco. The *R2* cluster presents resistance to a diversity of pathogens and contains a large

number of *R2* homologues identified through allele mining (Champouret 2010; Lokossou et al. 2009), with little diversity in *Rpi* genes (**Table 2**). In the *R3* cluster, the resistance to *P. infestans* is more diverse, and the number of *R* genes to other pathogens identified so far seems smaller (**Table 2**). In comparison, the *N* cluster contains resistance to a wide diversity of pathogens. The number of *Rpi* genes mapped in the *N* cluster is smaller than in the *R3* cluster, but those four that were found show high diversity in their resistance spectrum. The evolution of the *N* cluster seems to differ from that of the other two clusters and recognition specificities to a wider range of pathogens may have been created.

Table 2. Comparison between three major late blight (MLB) clusters in potato, including resistance to other pathogenic organisms.

***R* gene clusters**	**Oomycetes**	**Virus**	**Bacteria**	**Nematodes**	**Fungi**
R2	*R2* and homologues *Rpi-mcd1*	Ny_{tbr}*	*Eca*-QTL	*Gpa4*- QTL	
R3	*R3a* *R3b* *R5* *R6* *R7* *R8* *R9* *R10* *R11* *Pi*-QTL		*Eca*-QTL	*Gro1.3*-QTL	
N	*R4* *Rpi-edn3* *Rpi-avl1* *Rpi-cap1* *Rpi-qum1* *Pi*-QTL	Na_{adg} Ry_{adg}	*Eca*-QTL	R_{Mc1}	*Sen1*

References can be found in Gebhardt and Valkonen (2001) *(Celebi-Toprak et al. 2002)

The presence in the *N* cluster of genes for resistance to many different pathogens could be an indication that this cluster is older than the other MLB clusters (*R2* and *R3*), since it has evolved new *R* genes with a wide range of diversity for a longer time. Gene recombination and point mutation are two mechanisms for *R*

gene evolution (Hulbert et al. 2001). Meiotic recombination, which can be an equal or an equal exchange, is the principal factor in *R* gene evolution (Ellis et al. 2000; Michelmore and Meyers 1998). The structure of the *N* genes and the fact that resistance to a diversity of pathogens is found in the cluster suggest that distorted genetic recombination events must be an important factor in the *N* cluster in the development of new alleles. Once we have cloned and understood the functioning of the *Rpi* genes with their different domains from the *N* cluster, we may be able to engineer new *Rpi* genes, and use the wide sequence diversity present in the cluster to create new specificities.

Map-based cloning

Most of the work performed in this research project was toward map-based cloning of *Rpi* genes. Not all the work performed during this four-year project is included in the thesis. We started working on the genetic mapping of *Rpi* genes from three additional wild species *S. huacabambense*, *S. verrucosum* and *S. microdontum* spp. *gigantophyllum,* but did not continue since their chromosomal position could not be identified. Valuable information and lessons can be learned from those cases and from the presented work, which illustrates the difficulties that can be met and stresses the points that require special attention.

For map based cloning, the first essential element is that the gene of interest confers clear resistance phenotypes and is segregating in the population as a monogenic and dominant trait. The population used for the mapping should also fulfill three essential conditions: 1. a sufficient level of heterozygosity within the resistant parental genotype, 2. a sufficient level of polymorphism between the two parents and 3. a sufficient recombination frequency in the chromosome region of the *Rpi* gene locus. The first two conditions are especially important for marker development and thus for the mapping and can be characterized quickly. Marker development is much easier in diploid compared to polyploid populations (**Chapter 4**), so it is advised to choose for diploid wild *Solanum* species. The third characteristic is essential for the fine mapping and, only during the actual fine mapping, it can be determined whether the recombination frequency in the *R* gene region is high enough. A suboptimal situation for at least one of the first two conditions hampers marker development. It could lead to a paucity of markers developed on the chromosome arm containing the gene of interest leading to the impossibility of identifying the mapping position. This is the most probable reason for the failure in mapping the *Rpi* genes occurring in resistant genotypes from the *Solanum* species *S. huacabambense* and *S. verrucosum*. Another very important

characteristic limiting *Rpi* gene mapping, is unclear phenotypes in the segregating population. The mapping population originating from a resistant genotype from the wild species: *S. microdontum* spp. *gigantophyllum*, did not reveal a distinct qualitative segregation for resistance but rather a quantitative segregation with many intermediate phenotypes. Consequently, the gene could not be mapped precisely.

The work performed on *S. capsicibaccatum* and *S. circaeifolium ssp. quimense* (**Chapter 3**) showed the importance of polymorphism and heterozygosity. A limited number of markers could be developed in these populations due to a low level of polymorphism as well as heterozygosity. The resistance phenotype segregating in the populations was very clear and easy to score, which allowed reliable high resolution mapping despite the difficulties with marker development. During the cloning of *Rinf1-mcd* (**Chapter 5**), the relatively high recombination frequency was a good example allowing fine mapping and quick completion of the physical mapping. In addition, the level of polymorphism and heterozygosity was close to optimal. A large number of markers could be developed to saturate the region for mapping, and for physical mapping to narrow down the window with the gene of interest to only two candidates. The clear phenotypic segregation also provided a good basis for accurate mapping. In the high resolution mapping of *Rpi-avl1* (**Chapter 2**), the three characteristics were intermediate between the two previous examples regarding the level of polymorphism, heterozygosity and recombination frequency. Marker development and fine mapping was thus relatively easy. On the other hand, the phenotypic segregation in the population was not always very clear. So, special attention had to be given to that aspect to ensure accurate fine mapping.

The future of *Rpi* map-based cloning in potato will benefit from two main developments: progress in the understanding and application of effectors and new sequencing technologies. The use of effectors to phenotype segregating populations (**Chapter 4**) and complementation analysis of candidate genes by ATTA will allow more efficiency in the cloning process. The availability of the *P. infestans* genome sequence (Haas et al. 2009) is highly facilitating effector research. Apart from cloning, effectors are and will be very useful in classical and GM-breeding and in deployment of *Rpi* genes (Schornack et al. 2009). The new sequencing technologies and the pending release of the potato genome sequence will also largely contribute to accelerate the mapping process. Availability of the potato genome sequence makes a tremendous difference in the way of working

and could significantly speed up the cloning work. It will be especially useful for fine mapping, and physical mapping through the development of closely linked markers. Moreover having access to easier (cheaper and faster) sequencing facilities will also influence the sequencing of other sequences such as BAC clones or gene candidate constructs, and probably one day, of the complete genome of the genotype of interest.

Perspectives for *Rpi* gene cloning and resistance to P. infestans in potato

This research provides important results that can be applied in breeding programs. We have mapped two *Rpi* genes, which will hopefully be cloned in a very near future: *Rpi-avl1* and *Rpi-cap1*. We developed diagnostic markers that can already be used in marker assisted selection to select for these two genes. In *S. x edinense*, a natural example of *Rpi* gene stacking was unraveled, offering a strategy for creating strong and potentially durable resistance. It leads the way for *Rpi* gene stacking by a cisgenic approach. And finally, because all *Rpi* genes can be overcome by the pathogen, for example, two isolates have been identified to overcome *Rpi-blb1* resistance (**Chapter 4**), we also studied alternative possibilities with the cloning of a PAMP receptor.

PAMP receptor an alternative to *R* genes?

To develop late blight resistant potato varieties, breeders are essentially relying on *Rpi* genes. Since *Rpi* genes can be overcome by the quickly evolving *P. infestans*, it is necessary to search for alternative approaches to achieve durable resistance. For that reason, a more fundamental question was addressed to cell death response induced by the INF1 elicitin in *S. microdontum* (**Chapter 5**). The objective was to better understand basal resistance, with the hope to engineer broad spectrum durable resistance to late blight in potato. More studies will still be necessary to achieve the final objective. For example, it will be tested whether over-expression of *Rinf1-mcd* provides enhanced resistance to *P. infestans*. In addition to the map-based cloning work presented in the thesis, the response to INF1 in *Arabidopsis* was studied (data not shown). Since INF1 does not induce any visible response in *Arabidopsis*, two bioassays already developed in *Arabidopsis* for PAMP responses were tested: PAMP-triggered immunity (PTI) marker gene expression and a reactive oxygen species (ROS) assay. The identification of a bioassay showing the response to INF1, would allow the screen of *Arabidopsis* mutated for putative PAMP receptors and the identification of the

INF1 receptor in *Arabidopsis*. This would allow us to test the hypothesis that transformation of potato with the *Arabidopsis* INF1 receptor may confer enhanced resistance to *P. infestans* in potato . The ROS assay was negative probably due to an insufficient concentration of the INF1 protein. The PTI marker gene expression could not be repeated because of time limitation. So, unfortunately, final conclusions on the *Arabidopsis* response to INF1 could not be drawn. Another type of experiment was performed in order to gather more information about the INF1 response. The identified receptor like protein (RLP) gene, responsible for the INF1 mediated cell death response, was used in a yeast two-hybrid assay to determine the nature of the interaction between the receptor and the INF1 elicitin. Preliminary results, from the yeast two-hybrid assay, indicate a possible direct interaction, but more experiments are necessary to draw firm conclusions on the molecular basis of this RLP-INF1 interaction.

Future cloning of *Rpi-avl1* and *Rpi-cap1*

The cloning process of *Rpi-avl1* and *Rpi-cap1* is already quite advanced and is continuing. The complexity of the *N* cluster has hampered the easy cloning. The development of cosegregating markers was necessary for successful cloning, and was a prerequisite for the identification of BAC clones close to the *Rpi* genes. It still has to be determined whether the contigs formed by these BAC clones cover the interval between the closest flanking markers, and whether the candidate *R* genes present on the BAC clones are able to complement the resistance phenotype. The identification of the *Avr* genes of *Rpi-avl1* and *Rpi-cap1* could help to accelerate the complementation work, by co-agroinfiltration of the candidate genes and the corresponding *Avr* in *N. benthamiana*. The set of over 100 effectors, present in our laboratory based on the genome sequence of the *P. infestans* isolate T30-4 (Haas et al. 2009), has been screened on both wild species. The identified candidate *Avr* genes still have to be confirmed in the mapping populations for co-segregation between effector response and resistance phenotype. Knowing the *Avr* gene and its variants can also be useful to evaluate and monitor the pathogen population, which could allow speculation on the working spectrum of the *Rpi* gene in the field and thus on the relevance of including it into breeding programs.

***Rpi* gene stacking in nature: an example to follow?**

In order to rely on *Rpi* genes, breeders know they have to introduce several *Rpi* genes into a variety to hamper adaptation by the pathogen, since multiple mutations to virulence would then be required. Introduction of several *Rpi* genes into a variety can be done through pyramiding in classical breeding programs or by cisgenesis. The study of the genetic basis of the wide spectrum resistance occurring in *S. x edinense* revealed a natural example of *Rpi* gene stacking of *R* genes with different characteristics of origin, chromosome position, spectrum of resistance and putative gene structure. This example of *Rpi* gene stacking from *S. x edinense* corresponds to the advice Nelson gave to breeders in 1978 (Nelson 1978): "Go back young man and gather up your weary and defeated genes of the past, take your currently successful genes, find new ones if you can and build yourself a pyramid." He said that even defeated *Rpi* genes should be included suggesting they play a role in the total level of resistance. The presence of $R4^{Ma}$ also suggests that defeated *Rpi* genes can contribute to a wider spectrum of resistance (**Chapter 4**). This advice given by Nelson is probably still valid and useful to follow in the actual breeding strategies that rely on *Rpi* genes.

The threat caused by *P. infestans* is not decreasing. Its world wide occurrence and thus economic impact increased during the last years. The emergence of the clonal lineage "blue 13" that appears more aggressive than other pathogen genotypes is rather worrying (Hein et al. 2009a). More efforts still have to be done to find a way to control the disease and limit the risk of a devastating epidemic, which is real everywhere when chemical control is not effective anymore.

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Summary

Resistance and susceptibility to late blight in *Solanum*:
gene mapping, cloning and stacking.

The potato late blight disease, caused by the oomycete *Phytophthora infestans*, is a major threat for potato production worldwide. To breed potato varieties with durable resistance against *P. infestans*, it is necessary to combine two or more resistance (*R*) genes. Single *R* genes are easily overcome by the rapidly evolving pathogen, whereas the presence of several *R* genes could probably prevent gain of virulence from a single mutation in the pathogen. The large gene pool available within wild potato species offers sufficient possibilities to identify new and diverse *R* genes conferring resistance to *P. infestans* (*Rpi*). Map-based cloning is the most suitable strategy to isolate such new *Rpi* genes. The objective of this research was mapping, if possible, followed by cloning of *Rpi* genes from wild *Solanum* species. In addition, we initiated studies on another type of defense system that is not based on the typical *R* genes, namely the response of *Solanum* to INF1 elicitin.

This thesis describes the work performed for the identification of the genetic basis of resistance to *P. infestans* by major *Rpi* genes from four different wild *Solanum* species. The final objective of this research was the cloning of these *Rpi* genes. Here, we describe the work on the mapping of such a gene in **Chapter 4** and the fine mapping of such genes in **Chapters 2 and 3**. In **Chapter 5**, we describe the mapping of *Rinf1-mcd*, that causes a hypersensitive reaction of plant tissue upon challenge with INF1, but is not associated with late blight resistance. The initial experiments carried out for genetic mapping were similar in all four chapters and are described here in general terms. It started with the identification of a late blight resistant (or INF1 responding) genotype for crossing with a susceptible (or INF1 non-responding) genotype. To identify resistant genotypes, wild *Solanum* accessions were screened for resistance to different specific *P. infestans* isolates. The F1 mapping populations, resulting from the crosses, were tested and

phenotyped as resistant or susceptible to *P. infestans*, or as inducing cell death or not upon INF1 treatment, respectively. The mapping positions of the *Rpi* (and *Rinf1-mcd)* genes of interest were determined by screening a set of 80 SSR markers covering the whole potato genome on 10 resistant and 10 susceptible individuals, or by performing NBS profiling on bulked resistant or susceptible F1 individuals. Known CAPS markers from several sources (databases and literature) were used to confirm the mapping position of the different genes.

Rpi genes were found in three wild *Solanum* species, *viz. S. avilesii*, *S. capsicibaccatum* and *S. circaeifolium* spp. *quimense*, all originating from Bolivia. *S. avilesii* is resistant to a relatively large number of different *P. infestans* isolates (**Chapter 2**), whereas *S. capsicibaccatum* and *S. circaeifolium* spp. *quimense* are resistant to all isolates tested so far (**Chapter 3**). Mapping of the resistance to *P. infestans* occurring in these species resulted in the identification of three *Rpi* genes, *Rpi-avl1*, *Rpi-cap1* and *Rpi-qum1*, respectively. These three *Rpi* genes are all localized in the *N* gene cluster on the northern side of chromosome 11 (**Chapters 2 and 3**). The *N* cluster is considered a "hot spot" for resistance containing many *R* genes to different pathogens, and now also counts major *R* genes conferring resistance to *P. infestans*. Three genetic maps with high resolution were constructed after screening for recombination events between the closest markers flanking the genes of interest. Finally a gene family targeted marker approach was carried out and resulted in the development of co-segregating markers for *Rpi-avl1* and *Rpi-cap1*.

In **Chapter 4**, the resistant pentaploid *S. x edinense,* a natural hybrid originating from Mexico, was investigated. Unlike in the three wild *Solanum* species mentioned above, in *S. x edinense* three different *Rpi* genes were found segregating in the F1 mapping populations. Each *Rpi* gene mapped on a different chromosome: *Rpi-edn1* in the *R2* cluster on the short arm of chromosome 4, *Rpi-end2* on the long arm of chromosome 9 and *Rpi-edn3* co-segregated with $R4^{Ma}$ in the *N* cluster on chromosome 11. The screen of several *P. infestans* isolates on selected F1 plant individuals that contained one of the three *Rpi* genes revealed that each *Rpi* gene was overcome by some *P. infestans* isolates. The combination of the three *Rpi* genes with complementary resistance spectra conferred resistance to all isolates tested except one. The observations on this natural hybrid provide support for the strategy of stacking of *Rpi* genes in breeding programmes to achieve wide spectrum and durable resistance to potato late blight.

Current models describe that the recognition of pathogen-associated molecular patterns (PAMP) is followed by the induction of defense responses that lead to PAMP-triggered immunity (PTI). INF1 belongs to the elicitin gene family, which are small proteins that are secreted by *P. infestans*, and it fits into the definition of a PAMP. INF1-mediated cell death occurs in *S. microdontum* genotypes, however, response to INF1 is not associated with resistance to *P. infestans*. To investigate this further, we aimed at cloning the gene encoding for the INF1 receptor (**Chapter 5**). The *Rinf1-mcd* gene was localized on the long arm of chromosome 12 proximal to another *R* gene cluster containing the genes *Rx* and *Gpa2* coding for potato virus X (PVX) and cyst nematode resistance. A physical map of the *Rinf1-mcd* locus was constructed and the analysis of the sequences between the two closest flanking markers revealed the presence of two receptor-like protein (RLP) genes. One RLP gene was able to induce cell death upon INF1 treatment and was identified as *Rinf1-mcd*. The long term objective of cloning *Rinf1-mcd* is to gain more knowledge on the response induced by INF1 in potato and to study the possibilities to engineer durable late blight resistance.

The fact that four new *Rpi* genes from four different wild *Solanum* species with different resistance spectra all genetically localize in the *N* cluster reveals the presence of another major late blight (MLB) cluster in the potato genome. Differences between this *N* MLB mainly from Bolivian *Solanum* species and previously described *R2* and *R3* MLB from Mexican species are discussed (**Chapter 6**). The map-based cloning work performed in this research highlighted the importance of an optimal level of heterozygosity and polymorphism for the development of markers, and the necessity of a phenotype that can be easily scored. The availability of *P. infestans* and potato genome sequences promises to facilitate further *Rpi* gene cloning that can contribute to breeding durable late blight resistance in potato.

Samenvatting

Genetisch kartering, klonering en stapeling van genen betrokken bij resistentie en vatbaarheid tegen *P. infestans* in aardappel

De aardappelziekte, veroorzaakt door de oomyceet *Phytophthora infestans*, vormt overal ter wereld een ernstige bedreiging voor de aardappelteelt. Voor de veredeling van aardappelrassen met een duurzame resistentie tegen *P. infestans* is het nodig om twee of meer resistentie (R) genen voor resistentie in combinatie toe te passen. Enkelvoudige *R* genen worden gemakkelijk doorbroken door het snel evoluerende pathogeen, maar de aanwezigheid van verscheidene *R* genen zou de kans op een dergelijke ontwikkeling van virulentie sterk kunnen reduceren. Het grote arsenaal aan wilde aardappel (*Solanum*) soorten biedt voldoende mogelijkheden om nieuwe en gevarieerde *R* genen voor resistentie tegen *P. infestans* (*Rpi*) te vinden. Op positie gebaseerde isolatie (map-based cloning) is de meest geëigende strategie om dergelijke nieuwe *Rpi* genen in handen te krijgen. Het doel van dit onderzoek was het in kaart brengen van *Rpi* genen in wilde *Solanum* soorten, en deze, waar mogelijk, te kloneren. Bovendien maakten we een begin met onderzoek naar een ander type van afweer van planten, niet gebaseerd op *R*-genen maar op een reactie in *Solanum* op het INF1 elicitine.

Dit proefschrift behandelt het werk dat verricht werd aan het ontrafelen van de genetische basis van resistentie tegen *P. infestans* door *Rpi* hoofdgenen in vier wilde *Solanum* soorten. Het uiteindelijke doel van dit onderzoek was de klonering van deze *Rpi* genen. Hier beschrijven we de kartering van zo'n gen in **Hoofdstuk 4** en de fijnkartering van dergelijke genen in **Hoofdstukken 2 en 3**. In **Hoofdstuk 5** beschrijven we de kartering van *Rinf1-mcd*, dat een overgevoeligheidsreactie van plantweefsel veroorzaakt als INF1 in dat weefsel wordt ingebracht, maar het gen veroorzaakt geen resistentie tegen de aardappelziekte. De experimenten die beoogden genen te karteren waren in alle hoofdstukken soortgelijk. We beschrijven ze hier in algemene termen. Het begon met het identificeren van een aardappelherkomst met resistentie tegen de

aardappelziekte of met een respons tegen INF1. Een collectie wilde *Solanum* soorten en herkomsten werd met verschillende *P. infestans* isolaten getoetst op resistentie of getoetst op het optreden van plantencelnecrose als reactie op een behandeling met INF1. Een geselecteerde herkomst werd gekruist met een vatbare of niet- INF1-responderende aardappelherkomst. De F1 karteringspopulaties die uit de kruisingen ontstonden werden getoetst op resistentie tegen *P. infestans*, of op reactie op behandeling met INF1. De kaartposities van de interessante *Rpi* (en *Rinf1-mcd)* genen werden vastgesteld door van tien resistente en tien vatbare individuen de merker allelen te bepalen van een set van 80 SSR merkers die het hele genoom van aardappel dekken. Een alternatief was de toepassing van "NBS profiling" op samengevoegde DNA monsters van resistente en van vatbare F1 individuen. Bekende CAPS merkers van verschillende bronnen (literatuur en gegevensbestanden) werden gebruikt om de kaartpositie van de verschillende genen te bevestigen.

Rpi genen werden gevonden in drie wilde *Solanum* soorten, namelijk *S. avilesii*, *S. capsicibaccatum* en *S. circaeifolium* spp. *quimense*, alle afkomstig uit Bolivia. *S. avilesii* is resistent tegen een redelijk groot aantal verschillende *P. infestans* isolaten (**Hoofdstuk 2**), terwijl *S. capsicibaccatum* en *S. circaeifolium* spp. *quimense* resistent zijn tegen alle tot dusver geteste isolaten (**Hoodstuk 3**). Kartering van de resistentie tegen *P. infestans* in deze soorten leidde tot de ontdekking van drie *Rpi* genen, te weten *Rpi-avl1*, *Rpi-cap1* and *Rpi-qum1*. Deze drie *Rpi* genen liggen alle in het *N* gen cluster aan de noordzijde van chromosoom 11 (**Hoofdstukken 2 and 3**). Het *N* cluster wordt beschouwd als een "hot spot" voor resistentie, waar vele *R* genen gelegen zijn tegen verschillende pathogenen, en nu dus ook *R* genen voor resistentie tegen *P. infestans*. Het zoeken naar recombinaties tussen de merkers die de doelgenen flankeerden leverden drie genetische kaarten op met een hoge resolutie. Tenslotte ontwikkelden we merkers die gebaseerd waren op basensequenties die karakteristiek zijn voor een bepaalde genfamilie. Dit leverde merkers op die co-segregeerden met de genen *Rpi-avl1* en *Rpi-cap1*.

In **Hoofdstuk 4** onderzochten we de resistente pentaploide *S. x edinense.* Dit is een natuurlijke hybride die afkomstig is uit Mexico. In tegenstelling tot bij de drie wilde *Solanum* soorten die hierboven werden genoemd, werden in *S. x edinense* drie verschillende *Rpi* genen gevonden, die uitsplitsten in de F1 karteringspopulatie. Elk *Rpi* gen was gelegen op een ander chromosoom: *Rpi-edn1* ligt in het *R2* cluster op de korte arm van chromosoom 4, *Rpi-end2* op de

lange arm van chromosoom 9, en *Rpi-edn3* co-segregeerde met $R4^{Ma}$ en ligt in het *N* cluster op chromosoom 11. Het toetsen van verscheidene *P. infestans* isolaten op geselecteerde F1 planten die elk maar één van de drie *Rpi* genen bezaten wees uit, dat elk *Rpi* gen doorbroken was door sommige van de *P. infestans* isolaten. De combinatie van drie *Rpi* genen met complementaire resistentiespectra resulteerde in resistentie tegen op één na alle isolaten. De bevindingen verkregen in deze studie aan een natuurlijke hybride ondersteunen het idee dat de strategie van het accumuleren cq stapelen van *Rpi* genen in veredelingsprogramma's kan leiden tot breed-spectrum en duurzame resistentie tegen de aardappelziekte.

Huidige modellen gaan ervan uit dat de herkenning van pathogeen-geassocieerde moleculaire patronen (PAMP) afweerreacties op gang brengt, die leiden tot PAMP-geïnduceerde immuniteit (PTI). INF1 behoort tot de familie van elicitine eiwitten. Dat zijn kleine eiwitten die afgescheiden worden door *P. infestans*, en die voldoen aan de definitie van een PAMP. INF1-geïnduceerde celnecrose komt voor in *S. microdontum* genotypen, maar een dergelijke respons leidt niet tot resistentie tegen *P. infestans*. Om de rol van INF1 verder te onderzoeken, stelden we ons tot doel om het INF1 receptor-gen te kloneren (**Hoofdstuk 5**). Het *Rinf1-mcd* gen bleek te liggen op de lange arm van chromosoom 12, proximaal van een andere cluster van *R* genen, waartoe ondermeer de genen *Rx* voor resistentie tegen het aardappelvirus X (PVX) en *Gpa2* voor resistentie tegen het aardappelcystenaaltje behoren. Een fysische kaart van het *Rinf1-mcd* locus werd ontwikkeld, en de analyse van de sequenties tussen de twee meest nabije flankerende merkers wees uit dat er twee genen voor een receptor-achtige eiwit (Receptorlike proteins, RLP) gelegen zijn. Eén RLP gen induceerde celnecrose na toediening van INF1, en werd daardoor geïdentificeerd als *Rinf1-mcd*. Het uiteindelijke doel van de klonering van *Rinf1-mcd* is om meer inzicht te krijgen in de respons die optreedt tegen INF1 in aardappel, en de mogelijkheid te bestuderen om de INF1-receptor te gebruiken om duurzame resistentie tegen de aardappelziekte te verkrijgen.

Het feit dat vier nieuwe *Rpi* genen uit vier verschillende wilde *Solanum* soorten met verschillende resistentiespectra alle in het *N* cluster liggen betekent dat dit een nieuw en belangrijk cluster is in het aardappelgenoom voor resistentiegenen tegen de aardappelziekte. Verschillen tussen dit belangrijke *N* cluster in Boliviaanse *Solanum* soorten en de eerder beschreven clusters *R2* en *R3* in Mexicaanse soorten worden besproken in de algemene discussie (**Hoofdstuk 6**). Het werk aan de op kaartpositie gebaseerde klonering dat in dit proefschrift wordt

besproken maakt duidelijk hoe belangrijk het is dat het materiaal een optimaal niveau van heterozygotie en polymorfisme heeft, en dat het fenotype gemakkelijk vastgesteld kan worden. De beschikbaarheid van de genoomsequenties van *P. infestans* en aardappel zullen bijdragen aan een efficiëntere klonering van *Rpi* genen, waardoor een bijdrage geleverd kan worden aan de ontwikkeling van aardappelrassen met duurzame resistentie tegen de aardappelziekte.

Résumé

Résistance et sensibilité au mildiou de la pomme de terre chez *Solanum*:
Cartographie, clonage et combinaison de gènes

Le mildiou de la pomme de terre, engendré par l'oomycete *Phytophthora infestans*, est une menace majeure pour la production de pomme de terre au niveau mondial. Pour sélectionner des variétés de pomme de terre ayant une résistante durable à *P. infestans*, il est nécessaire de combiner au moins deux gènes de résistance (R). Un seul gène de résistance est facilement contourné par le pathogène capable d'évoluer rapidement, donc la présence de plusieurs gènes de résistance pourrait probablement empêcher le gain de virulence obtenu suite a une simple mutation du pathogène. Le pool de gènes disponible au sein des espèces sauvages de pomme de terre offre suffisamment de possibilités pour identifier de nouveaux et différents gènes de résistance á *P. infestans* (*Rpi*). Le clonage positionnel est la stratégie la plus appropriée pour l'isolation de nouveaux gènes *Rpi*. L'objective de ce projet était la cartographie, si possible suivi du clonage de gènes *Rpi* provenant d'espèces sauvages de *Solanum*. De plus, nous avons initié l'étude d'un autre type de système de défense qui ne dépend pas des gènes de résistance typiques, à savoir la réponse chez *Solanum* à l'élicitine INF1.

Cette thèse décrit le travail réalisé pour identifier la base génétique de la résistance à *P. infestans* causé par la présence de gènes majeurs provenant de différentes espèces sauvages de *Solanum*. L'objectif final de cette recherche était le clonage de ces gènes *Rpi*, cependant ici, nous présentons le travail réalisé pour la cartographie de tels gènes dans le **Chapitre 4**, et pour cartographie fine de tels gènes dans les **Chapitres 2 et 3**. Dans le **Chapitre 5**, nous décrivons la cartographie de *Rinf1-mcd*, responsable de l'induction de mort cellulaire des tissus de la plante suite au traitement avec INF1, mais qui n'est pas lié à la résistance au mildiou. Les premières expériences réalisées pour la cartographie étaient similaires dans les quatre chapitres et sont ici décrites de façon générale. La première étape était l'identification d'un génotype résistant au mildiou (ou répondant à INF1) pour le croiser avec un génotype sensible (ou ne répondant

pas à INF1). Pour identifier des génotypes résistants, plusieurs accessions de *Solanum* sauvages ont été testées pour leur résistance à différents isolats de *P. infestans.* Les populations, résultantes des croisements entre parents résistants et sensibles, ont été testées et phénotypées comme résistant ou sensible à *P. infestans*, ou comme engendrant une mort cellulaire ou non après traitement avec l'élicitine INF1. Les positions cartographiques des gènes *Rpi* d'intérêt (et *Rinf1-mcd*) ont été déterminées suite a l'évaluation de 10 plantes résistantes et 10 plantes sensibles avec un série de 80 marqueurs SSR répartis sur l'ensemble du génome de la pomme de terre, ou en appliquant le « NBS profiling » sur le regroupement des plantes résistantes ou sensibles. Des marqueurs CAPS déjà connus provenant de différentes sources (bases de données et littérature) ont été utilisés pour confirmer la position des différents gènes *Rpi*.

Des gènes *Rpi* ont été identifiés dans trois espèces de *Solanum* sauvages, *viz. S. avilesii*, *S. capsicibaccatum* et *S. circaeifolium* spp. *quimense*, toutes provenant de Bolivie. *S. avilesii* est résistant à un relativement grand nombre d'isolats de *P. infestans* (**Chapitre 2**), alors que *S. capsicibaccatum* et *S. circaeifolium* spp. q*uimense* sont résistants a tous les isolats testés jusqu'à maintenant (**Chapitre 3**). La cartographie de la résistance à *P. infestans* présente dans ces espèces a permis l'identification de trois gènes *Rpi: Rpi-avl1*, *Rpi-cap1* and *Rpi-qum1*. Ces trois gènes *Rpi* sont localisés dans le cluster de gènes *N* situé sur la partie nord du chromosome 11 (**Chapitres 2 et 3**). Le cluster *N* est considéré comme un «hot spot» de résistance avec la présence de gènes de résistance contre différents pathogènes, et compte maintenant des gènes majeurs de résistance a *P. infestans*. Trois cartes génétiques de haute résolution ont été construites suite à la recherche d'événements de recombinaison entre les marqueurs les plus proches flanquant les gènes d'intérêt. Finalement une approche de marqueurs basée sur le dessin d'amorces spécifiques pour une famille de gènes a été utilisée et a permis le développement de marqueurs en coségrégation avec les gènes *Rpi-avl1* et *Rpi-cap1.*

Dans le **Chapitre 4**, des génotypes résistants de l'espèce pentaploid *S. x edinense*, un hybride naturel provenant du Mexique, a été étudié. A l'inverse des trois espèces sauvages de *Solanum* mentionnées précédemment, trois gènes différents étaient en ségrégation dans les populations de *S. x edinense*. Chaque gène *Rpi* est situé sur un chromosome différent : *Rpi-edn1* dans le cluster *R2* sur le bras court du chromosome 4, *Rpi-end2* sur le bras long du chromosome 9 et *Rpi-edn3* en coségrégation avec $R4^{Ma}$ dans le cluster *N* sur la partie nord du

chromosome 11. Le test de plusieurs isolats de *P. infestans* sur les individus de la population contenant un seul des trois gènes *Rpi* a révélé que chaque gène *Rpi* avait été contourné par un certain nombre d'isolats de *P. infestans.* La combinaison des trois gènes *Rpi,* ayant un spectre de résistance complémentaire, procure de la résistance à tous les isolats à l'exception d'un isolat. Les observations sur cet hybride naturel apporte un soutien à la stratégie de combiner des gènes *Rpi* au sein des programmes de sélection variétale afin d'obtenir une résistance de large spectre et durable contre le mildiou de la pomme de terre.

Les modèles actuels indiquent que la reconnaissance des «pathogen-associated molecular patterns» (PAMP) est suivie de l'induction de réponse de défense par la plante qui conduit à « PAMP-triggered immunity » (PTI). INF1 appartient à la famille des élicitines, qui sont des protéines de petite taille qui sont secrétées par *P. infestans*, et rentre dans la définition des PAMPs. La mort cellulaire induit par INF1 survient chez les génotypes de l'espèce *S. microdontum*, cependant, la réponse à INF1 n'est pas associée à la résistance à *P. infestans*. Pour étudier ce phénomène en profondeur, nous cherchons à cloner le gène codant pour le récepteur à INF1 (**Chapitre 5**). Le gène *Rinf1-mcd* est situé sur le bras long du chromosome 12 proximal à un autre cluster de gènes de résistance incluant *Rx* and *Gpa2* conférant la résistance contre le virus X de la pomme de terre (PVX) et contre le nematode cyst, respectivement. Une carte physique du locus de *Rinf1-mcd* a été construite et l'analyse des séquences entre les deux plus proches marqueurs flanquant le gène a révélé la présence de deux gènes du type: «receptor-like protein» (RLP). L'un des deux gènes RLP a induit une réponse de mort cellulaire suite au traitement avec l'élicitine INF1 et a été identifié comme *Rinf1-mcd.* L'objectif à long terme du clonage de *Rinf1-mcd* est l'accumulation de connaissances sur la réponse induite par l'élicitine INF1 chez la pomme de terre et l'étude des possibilités de concevoir une résistance durable contre le mildiou de la pomme de terre par le génie génétique.

Le fait que quatre nouveaux gènes *Rpi* provenant de différentes espèces sauvages de *Solanum* ayant des spectres de résistance différents soient tous génétiquement situés dans le cluster *N* révèle la présence dans le génome de la pomme de terre d'un autre majeur cluster (major late blight: MLB) procurant résistance contre le mildiou. Nous discutons des différences entre le *N* MBL provenant d'espèces originaires de Bolivie et les autres MBL, *R2* et *R3* précédemment décrits, provenant principalement d'espèces sauvages originaires

du Mexique (**Chapitre 6**). Le travail de clonage positionnel réalisé lors de cette recherche a souligné l'importance d'avoir un niveau optimum d'hétérozygosité et de polymorphisme pour le développement de marqueurs, et la nécessité d'un phénotype facile à évaluer. La disponibilité des génomes de *P. infestans* et de la pomme de terre promet de faciliter les clonages ultérieurs de gènes *Rpi* qui pourraient contribuer à la sélection de pomme de terre avec une résistance durable au mildiou.

Resumen

Resistencia y susceptibilidad al tizón tardío en *Solanum*:
Mapeo, clonación y combinación de genes

El tizón tardío, causado por el oomycete *Phytophthora infestans*, es una gran amenaza para la producción de papas a nivel mundial. Para mejorar las variedades de papas con resistencia durable a *P. infestans*, es necesario combinar al menos dos genes de resistencia (R). Un solo gene de resistencia es fácil de vencer por el patógeno, que evoluciona rápidamente, entonces la presencia de varios genes de resistencia podría probablemente prevenir que el patógeno obtenga virulencia debido a una simple mutación. El pool de genes disponibles dentro de las especies silvestres de papas ofrecen suficientes posibilidades para identificar nuevos y diferentes genes de resistencia a *P. infestans* (*Rpi*). La clonación basada en mapeo o clonación posicional es la estrategia más apropiada para el aislamiento de nuevos genes *Rpi*. El objetivo de este proyecto fue el mapeo, si fuese posible seguido de la clonación de genes *Rpi* provenientes de especies silvestres de *Solanum*. Además, iniciamos el estudio de otro tipo de sistema de defensa que no está basado en los genes de resistencia típicos, específicamente la respuesta de *Solanum* a la elicitina INF1.

La presente tesis describe el trabajo realizado para la identificación de la base genética de la resistencia a *P. infestans* causado por la presencia de genes mayores derivados de diferentes especies silvestres de *Solanum*. El objetivo final de esta investigación fue la clonación de esos genes *Rpi*. Aquí, se presenta el trabajo que se realizó para el mapeo de tal gene en el **Capítulo 4**, y para el mapeo fino de tal genes en los **Capítulos 2 y 3**. En el **Capítulo 5**, se describe el mapeo de *Rinf1-mcd*, que causa inducción de muerte celular de la planta después de tratamiento don INF1, pero no está asociado con la resistencia al tizón tardío, de igual manera se realizó un trabajo de mapeo. Los primeros experimentos realizados para el mapeo fueron similares en los cuatro capítulos y son descritos aquí de manera general. La primera etapa fue la identificación de un genotipo resistente al tizón tardío (o respondiendo a INF1) para cruzarlo con un genotipo

susceptible (o no respondiendo a INF1). Para identificar genotipos resistentes, accesiones de *Solanum* silvestres fueron probadas por su resistencia a diferentes aislamientos de *P. infestans*. Las poblaciones que resultaron de los cruces, fueron probadas y clasificadas por su fenotipo como resistentes o susceptibles a *P. infestans*, o como induciendo o no a una muerta celular después del tratamiento con la elicitina INF1. Las posiciones de mapeo de los genes *Rpi* de interés (y *Rinf1-mcd*) fueron determinadas con la evaluación de 10 plantas resistentes y 10 plantas susceptibles con una serie de 80 marcadores SSR distribuidos sobre todo el genoma de la papa, o aplicando el "NBS profiling" sobre el conjunto de plantas resistentes o susceptibles. Marcadores CAPS ya conocidos originados de diferentes fuentes (base de datos y literatura) fueron usados para confirmar las posiciones de los genes.

Los genes *Rpi* fueron encontrados dentro de tres especies silvestres de *Solanum* silvestres, *viz. S. avilesii*, *S. capsicibaccatum* y *S. circaeifolium* spp. q*uimense,* todas con origen en Bolivia. *S. avilesii* presenta resistencia a un numero relativamente grande de aislamientos de *P. infestans* (**Capítulo 2**), mientras *S. capsicibaccatum* et *S. circaeifolium* spp. q*uimense* presentan resistencia a todos los aislamientos probados hasta ahora (**Capítulo 3**). El mapeo de la resistencia a *P. infestans* presente en estas especies a resultado en la identificación de tres genes *Rpi*: *Rpi-avl1*, *Rpi-cap1* y *Rpi-qum1.* Esos tres genes están localizados en el grupo de genes *N* que está en la parte norte del cromosoma 11 (**Capítulos 2 y 3**). El grupo *N* está considerado como un "hot spot" para la resistencia con la presencia de genes de resistencia contra diferentes patógenos, y ahora cuenta con genes mayores de resistencia contra *P. infestans*. Tres cartas genéticas de alta resolución han sido construidas después de la búsqueda por eventos de recombinación entre los marcadores moleculares los más cercanos y flanqueando los genes de interés. Finalmente, un enfoque con marcadores basados en el diseño de iniciadores específicos por una familia de genes ha sido utilizada y ha permitido el desarrollo de marcadores en cosegregación con los genes *Rpi-avl1* y *Rpi-cap1.*

En el **Capítulo 4**, los genotipos resistentes de la especie pentaploide *S. x edinense*, un híbrido natural con origen en México, ha sido estudiado. Al contrario de las tres especies silvestres de *Solanum* mencionadas anteriormente, tres genes diferentes fueron encontrados en segregación en las poblaciones de *S. x edinense*. Cada gene *Rpi* esta localizado en un cromosoma diferente: *Rpi-edn1* en el grupo *R2* en el brazo corto del cromosoma 4, *Rpi-end2* en el brazo largo del

cromosoma 9 y *Rpi-edn3*, que estaba en cosegregación con $R4^{Ma}$ en el grupo *N* en la parte norte del cromosoma 11. La prueba de varios aislamientos de *P. infestans* en la plantas de F1 conteniendo uno de los tres genes *Rpi* ha revelado que cada gene *Rpi* ha sido vencer por una cierta cantidad de aislamientos de *P. infestans*. La combinación de los tres genes con espectro de resistencia complementario confiere resistencia a todos los aislamientos excepto por uno. Las observaciones realizadas en este híbrido natural ofrecen respaldo a la estrategia de combinar genes *Rpi* en programas de mejoramiento de variedades para alcanzar a obtener una resistencia contra el tizón tardío con amplio espectro y durable.

Los modelos actuales indican que el reconocimiento del "pathogen-associated molecular patterns" (PAMP) está seguido por la inducción de una respuesta de defensa de la planta que lleva a "PAMP-triggered immunity" (PTI). INF1 pertenece a la familia de las elicitinas, las cuales son proteínas de pequeño tamaño que son secretadas por *P. infestans*, y corresponde a las definiciones de los PAMPS. La muerte celular inducida por INF1 ocurre en los genotipos de la especie de *S. microdontum*, sin embargo, la respuesta a INF1 no está asociada con la resistencia a *P. infestans*. Para estudiar este fenómeno con más detalle intentamos clonar el gene que codifica para el receptor de INF1 (**Capítulo 5**). El gene *Rinf1-mcd* ha sido localizado en el brazo largo del cromosoma 12 proximal a otro grupo de genes de resistencia conteniendo *Rx* y *Gpa2* confiriendo resistencia al virus X de la papa y al nematodo del quiste, respectivamente. Un carta física del locus *Rinf1-mcd* ha sido construida y el análisis de la secuencias entre los dos más cercanos marcadores flanqueando el gene ha revelado le presencia de dos genes "receptor-like protein" (RLP). Uno de esos dos genes RLP ha podido inducir la muerte celular después del tratamiento con INF1 y ha sido identificado como *Rinf1-mcd*. El objetivo a largo plazo de la clonación de *Rinf1-mcd* es la acumulación de conocimiento sobre la respuesta inducida por INF1 en la papa y el estudio de las posibilidades de obtener una resistencia durable contra el tizón tardío a través de la ingeniería genética.

El hecho de que cuatro genes *Rpi* tengan su origen en diferentes especies silvestres de *Solanum* con diferentes espectros de resistencia y que estén todos genéticamente localizados en el mismo grupo *N* releva la presencia en el genoma de la papa de otro mayor grupo (major late blight :MLB) con resistencia al tizón tardío. Se discute las diferencias entre el MBL *N,* presente principalmente en especies con origen en Bolivia, con los MBLs *R2* y *R3* previamente descritos,

presentes en especies silvestres con origen en México (**Capítulo 6**). El trabajo de clonación posicional realizado durante esa investigación subrayo la importancia de un nivel óptimo de heterocigocidad y de polimorfismo para el desarrollo de marcadores, y la necesidad de un fenotipo fácil de evaluar. La disponibilidad de los genomas de *P. infestans* y de la papa promete facilitar ulterior clonación de genes *Rpi* que podrían contribuir a la selección de papas con resistencia durable al tizón tardío.

Acknowledgements

Four years later… and I am writing the last part of my PhD thesis. The part where I thank all the persons who from far or close contributed to the story of my life during these last years. It has been a succession of great, fun and also difficult times, which I will always remember, especially the good parts. "Everything is here! Except what's missing…" this sentence of mine is useful to many situations as illustrated here: All the work performed in four year does not fit within 100 pages, and all the persons who contributed to my PhD life story can not be mentioned within 3 pages. I here mention all the people who contributed to the thesis itself.

It is during my master thesis that I found out how great it was to do research and decided to continue my career with a PhD research. I am grateful to my supervisor Yuling who initiated me to molecular biology and research. She is now my group leader and I appreciate her for her kindness and concern about my feelings.

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Dank U!
Merci!
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About the author

Estelle Verzaux was born on May 25th 1981 in Rambouillet, Yvelines, France. After completion of her academic high school in the Institut Sainte Thérèse, she studied agriculture at the Institut Superieur d'Agriculture (ISA) in Lille (France) and graduated in November 2005.

In 2003 she went as an Erasmus student to Wageningen University to study plant breeding and stayed longer to complete the Master program. She performed her master thesis at the department of Plant breeding under the supervision of Yuling Bai and Pim Lindhout on resistance to mildew in tomato. In January 2006 she graduated from her Master in Plant Sciences and started her PhD research in the same department. The project financed by Avebe had for topic the cloning of resistance to *Phytophthora infestans* in wild *Solanum* species.

From July 2010, she will work as molecular geneticist at Van Rijn - KWS B.V., The Netherlands.

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Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Estelle Verzaux**
Date: **17 June 2010**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase		*date*
▶	**First presentation of your project**	
	Cloning of new Rpi-genes and Rinf1-mcd	April 06, 2006
▶	**Writing or rewriting a project proposal**	
	Cloning of new Rpi-genes and Rinf1-mcd	May 2006
▶	**Laboratory use of isotopes**	
	Radiation expert level 5B	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 Theme Symposia**	
	EPS theme 2 symposium 'interaction between Plants and Biotic Agents' Amsterdam	Feb 02, 2007
▶	**NWO Lunteren days and other National Platforms**	
	NWO-ALW Lunteren meeting	Apr 03-04, 2006
	NWO-ALW Lunteren meeting	Apr 02-03, 2007
	NWO-ALW Lunteren meeting	Apr 07-08, 2008
	NWO-ALW Lunteren meeting	Apr 06-07, 2009
▶	**Seminars (series), workshops and symposia**	
	Seminar: Rob Martienssen	Oct 23, 2006
	Seminar: Kurt H. Lamour	Nov 06, 20606
	Seminar: Cyril Zipfel	Feb 05, 2009
	Reconsidering Intellectual Property Policies in Public Research (1 day)	Apr 11, 2008
▶	**International symposia and congresses**	
	5th annual meeting Oomycte Molecular Genetics Research Network, Wagenignen	May 05-06, 2006
	XIII Congress on Molecualr Plant-Microbe Interactions	Jul 21-27, 2007
	5th Solanacae Genome Workshop	Oct 12-16, 2008
	XIV Congress on Molecualr Plant-Microbe Interactions	Jul 19-23, 2009
▶	**Presentations**	
	Poster presentation, 5th annual meeting Oomycte Molecular Genetics Research Network,	May 05-06, 2006
	Poster presentation, XIII Congress on Molecualr Plant-Microbe Interactions	Jul 21-27, 2007
	Progress meeting Avebe	Apr 04, 2006
	Progress meeting Avebe	Sep 09, 2006
	Progress meeting Avebe	Feb 15, 2007
	Progress meeting Avebe	Oct 01, 2007
	Progress meeting Avebe	Jan 21, 2008
	Progress meeting Avebe	Jun 05, 2008
	Progress meeting Avebe	Jan 22, 2009
	Progress meeting Avebe	Jun 23, 2009
	Progress meeting Avebe	Jan 14, 2010
▶	**IAB interview**	Dec 05, 2008
	Subtotal Scientific Exposure	*17.1 credits**
3) In-Depth Studies		*date*
▶	**EPS courses or other PhD courses**	
	Summerschool 'Signaling in Plant Development and Defence: towards Systems Biology'	Jun 19-21, 2006
	Summerschool 'Environmental signaling: Arabidopsis as a model'	Aug 27-29, 2007
	Summerschool 'On the evlution of plant pathogen interaction: from principles to practices'	Jun 18-20, 2008
▶	**Journal club**	
	Literature discussion group 'Plant Breeding'	2006-2009
▶	**Individual research training**	
	Sainsbury Laboratory in Norwich (1 month)	Oct 19-Nov 22, 2008
	Subtotal In-Depth Studies	*8.7 credits**
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▶	**Skill training courses**	
	Personnal efficacy	Sep 27, 2006
	Endnote	Sep 26, 2006
	Information Literacy	Oct 10-11, 2006
	Teaching and supervising thesis student	Jun 2006
	PhD competence assessment	2007
▶	**Organisation of PhD students day, course or conference**	
	Organisation of the Lab trip for the group 'Plant Breeding'	May 2006
	Subtotal Personal Development	*3.5 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**38.3**

Herewith the Graduate School declares that the PhD candidate has complied with the educational
** A credit represents a normative study load of 28 hours of study*

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Potato variety: Estela, selected by farmers from marginal communities of central and northern Ecuador

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