Mapping, Isolation and characterization of genes responsible for Late Blight Resistance in Potato

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This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

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Thesis

Submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. dr. M. J. Kropff,

in the presence of the

Thesis committee appointed by the Academic Board

to be defended in public

on Friday 21 May 2010

at 1.30 p.m. in the Aula

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PhD Thesis Wageningen University, The Netherlands, 2010
With references – with summaries in English, Dutch and French

ISBN 978-90-8585-636-8

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CHAPTER 1

General Introduction

$$[T+L]^R \times P^2$$

$$[T+L]^R \times P^2$$

$$[T+L]^R \times P^2$$

T: tuber

L: leaf

R: resistance

P: potato / *Phytophthora*

GENERAL INTRODUCTION

The sixth framework program named BioExploit aims to provide alternatives for fungicide use in the two main European food crops, wheat and potato, by exploiting natural variations in host plant resistance. Since the 1950s fungicides have become the foundation of disease control in major arable crops in Europe. In the late nineties, growing concerns on the fungicide residue levels in food and environmental risks associated with fungicide use lead to a withdrawal of 53% of the active substances for fungicides. Strikingly, a recent survey by EUROSTAT (2002, The use of plant protection products in the European Union. Data 1992-1999. Pp. 132) and FAOSTAT (http://apps.fao.org/page/collections) on the use of fungicide in the EU-15 actually shows an increase in the total amount of active ingredients applied to crops. This is due to the lack of good alternatives to fungicides. Of all the major food crops in the EU the cultivation of cereals and potatoes require the highest amounts of fungicides (EUROSTAT 2002) for the chemical control of fungal diseases. Therefore, reducing the application of fungicides in cereals and potato crops would directly improve the quality and safety of food for all consumers throughout the EU.

This thesis focuses on late blight resistance in the Potato- *Phytophthora infestans* pathosystem.

Potato crop

The potato plant, *Solanum tuberosum*, was first domesticated and cultivated in the region of the Andes in South America about 8,000 years ago. During the 16th century, Spanish conquistadors came in touch with potato plants and brought it to Europe. The English word *potato* is derived from the Spanish *patata* (the name used in Spain). Although it was initially feared to be poisonous, the potato became an important staple crop in northern Europe. Then it spread around the globe and became one of the most important crops in the world. After maize (791 Mt), rice (659 Mt) and wheat (605 Mt), potato (309Mt) is the fourth crop in term of production and the third one in order of nutritional importance consumed by men. The economic value of the potato crop resides in its tubers largely used for human consumption and starch derived products used in the paper, textile, detergent, pharmaceutical, cosmetical and other industries (www.potato2008.org).

Potato belongs to the botanical family *Solanaceae*, comprising a number of crop species such as tomato (*Lycopersicum esculentum*), tobacco (*Nicotiana tabacum* and *N. benthamiana*), eggplant (*Solanummelongena*), and pepper (*Capsicum annum*). The tuber bearing subsection

Potato of the genus *Solanum*, is classified in 16 "series"(Jacobs 2008). The domesticated species grown worldwide are *Solanum tuberosum* L., an auto-tetraploid with 48 chromosomes. Varieties of this former species, such as cv. Bintje, cv. Spunta, cv. Russet Burbank, cv. Nicola and cv. Desiree, are widely cultivated.

Potato tuber

Although potato tubers have a significant economic value, the molecular factors involved in tuber development are not yet fully understood. A potato tuber is formed from an underground stem called a stolon. Under short day conditions (inductive), the stolon grows until swelling to form a potato tuber. However, under long day conditions (non inductive), the stolon will not swell. Several environmental and hormonal factors affecting tuberization were reviewed by Jackson (1999) and Rodríguez-Falcón, M. et al. (2006). It was reported that high nitrogen levels or high temperatures inhibit tuberization whereas high light, high concentrations of sucrose or short day conditions promote tuberization. However, the transmissible signal produced under inductive conditions enabling the stolon to swell is unknown and thought to be hormonal, transcriptional or epigenetic.

A potato tuber contains different types of tissues (Figure 1). The potato skin or periderm is made up of three types of cells: phellem (suberized cells), phellogen (cork cambium) and phelloderm tissues (Lulai and Freeman 2001). The periderm forms a protective barrier at the surface of the tuber. The cortex tissue is delimited by the periderm and the vascular bundle. The medulla, found within the vascular bundle, is divided in perimedullar zone and inner medulla or pith (Van Eck, 2007).

Potato tuber cells contain amyloplasts which are responsible for the synthesis and storage of starch granules, through the polymerization of glucose . They also convert this starch back into sugar when the plant needs energy. The potato tuber contains vitamins and minerals that have been identified as vital to human nutrition, as well as an assortment of phytochemicals, such as carotenoids and polyphenols. Nutritionally, the potato is best known for its completeness and balance of starch/protein/vitamins content. The predominant form of this carbohydrate is starch. It represents between 16 and 20% of the total tuber weight. The other compounds found in tubers are water (72-75%), protein (2-2.5%), fiber (1-1.8%) and fatty acids (0.15%) (www.potato2008.org).

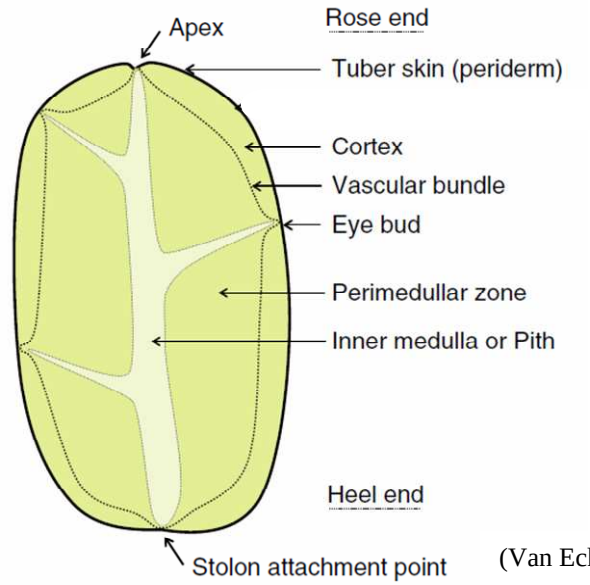

Figure 1. Cross section of a potato tuber where the different types of tissues are indicated. Reprinted with permission etc (Van Eck 2007)

Plant immunity

Plants have developed complex defense mechanisms to protect themselves against various pathogens (viruses, fungi, nematodes, bacteria). Upon pathogen's attack, these defense mechanisms are activated leading to a resistance response. For a given plant-pathogen system, a resistance phenotype can be referred to as non host resistance if the entire pathogen's species is unable to infect the plant or to host resistance if certain isolates of the pathogen's species are able to infect the plant (Heath 2000). In essence, there are two subdivisions within the plant immune system. One involves a transmembrane recognition patterns that interact with microbe- or pathogen- associated patterns (MAMPs or PAMPs) such as flagelin (Zipfel and Felix 2005). The second type comprises NBS-LRR proteins encoded by plant resistance (*R*) genes (Dangl and Jones 2001) involved in race specific recognition of pathogen avirulence (*Avr*) genes. When corresponding *Avr* and *R* genes are present in the pathogen and the plant respectively, a resistance response is triggered resulting in a hyper-sensitive response (HR) causing cell death at the infection site (Dangl et al. 1996). If one of these components is missing the plant will be susceptible. This sophisticated plant-pathogen interaction is known as the gene-for-gene interaction (Flor 1971). During infection, the pathogen injects into the plant cells a wide range of molecules or effectors reprogramming plant cells (Huitema et al. 2004). Upon direct or indirect recognition of one of these effectors by an *R* protein, the plant is able to trigger a resistance response activating plant defense-related genes. Indirect recognition is referred to as the guard cell hypothesis devoting a surveillance role to the *R*

protein “guarding” the virulence target of the pathogen (Dangl and Jones 2001; Van der Biezen 1998). In this guard model, the *R* protein is meant to be part of a protein complex including proteins targeted by the pathogen. *Avr* proteins target specific host proteins which are potential partners of *R* proteins. Change in the configuration of the targets leads to the activation of *R* protein triggering a resistance response in the plant.

The ‘guard’ hypothesis has been illustrated in three papers describing the protein RIN4 (RPM1 interacting 4) from Arabidopsis (Axtell and Staskawicz 2003; Mackey et al. 2003; Mackey et al. 2002). RIN4 was shown to mediate interactions between the RPM1 (resistance to *P. syringae pv. aculicola* 1) CC-NBS-LRR protein and the AvrB and AvrRpm1 type III effector proteins from the bacterium *P. syringae* but also between the RPS2 (resistance to *P. syringae pv.* tomato 2) CC-NBS-LRR protein and the AvrRpt2 type III effector protein also from *P. syringae*. RPM1 and RPS2 form a complex with RIN4. RIN4 gets phosphorylated by AvrB and AvrRpm1 causing the activation of RPM1 resistant protein. AvrRpt2 causes the degradation of RIN4, leading to a loss of function of RPM1, but activating RPS2.

Resistance genes

R genes are classified in three main classes: Nucleotide Binding Site Leucine Rich Repeat (NBS-LRR), LRR Receptor like kinase (LRR-RLK) and LRR Receptor like protein (LRR-RLP). The NBS-LRR class is the most abundant in all plant species so far. It is estimated that at least 200 different NBS-LRR genes are present in Arabidopsis representing up to 1% of its genome (Meyers et al. 1999). Characterization of 149 *R* loci from the *Columbia* ecotype of *Arabidopsis*, showed that NBS-LRR were often organized as clusters or singletons (Meyers et al. 2003). The NBS-LRR class has been subdivided into two subclasses based on motifs located in the N terminal part. One subclass codes for a TIR domain (Toll-Interleukin receptor-like region) sharing homology to the *Drosophila Toll* and mammalian *Interleukin-1* receptors (Qureshi et al. 1999). The second subclass codes for a coiled-coil (CC) structure which is sometimes in the form of a leucine zipper (Lz) (Baker et al. 1997; Lupas 1996; Pan et al. 2000,2001).

To counteract fast evolving pathogens generating new effectors, plants have to evolve new *R* gene specificities. *R* genes are often found in clusters facilitating genetic mechanisms such as equal or unequal crossing over (Devos et al. 2002; Leister 2004; Wicker and Yahiaoui 2007) leading to domain swapping or deletion/duplication of gene respectively. The most studied plantmodel, *Arabidopsis*, provides several examples of *R*-genes diversification. The *RPP8* gene characterized as a CC-NB-LRR gene in the Landsberg erecta accession confers

resistance to *Peronospora parasitica* (McDowell et al. 1998). *RPP8* gene is tightly linked to a *R*-gene homologue. Interestingly, the susceptible accession has only one copy (*rpp8*) at the same locus which seems to be derived from an unequal crossing over between ancestral alleles of *RPP8* and its tightly linked homologue. In contrast, the *RPP13* gene seems to have evolved via multiple duplications as it is separated from its two homologues by 17 genes (Bittner-Eddy et al. 2000). Several other genetic mechanisms such as gene conversion, point mutations, retrotransposon activity and illegitimate recombination (IR) are involved in *R*-gene diversification. The IR mechanism was first described by Wicker et al. (2007) leading to size variation in the LRR domain of *R*-genes. A stretch of 2-10 bp serves as template for an IR leading to either deletion or duplication of the sequence segment between two recombination sites. The lineages of *Cf-2, RGH2*, *Pm3* and *Xa1* genes were resolved and showed complex repeat arrays in the four RGA lineages by tracing them back to initial simple duplications caused by IR. Interestingly, most of the IR events resulted in duplications in the LRR domain. Only a few lead to deletions.

Late Blight disease

Late blight, one of the world's most devastating plant diseases, is caused by the oomycete *Phytophthora infestans (P. infestans*), leading to significant economic losses in potato growing areas worldwide. Disease management still relies on the application of fungicides (metalaxyl combined with carbamate or Cymoxanil and Mancozeb combination) (Mukerji 2004) which is expensive and damaging for the environment and probably for human health as well.

Phytophthora infestans

The origin of *P. infestans* was initially considered to be in Mexico but recent studies suggested South America as its center of origin (Gómez-Alpizar et al. 2007; Grünwald and Flier 2005). *P. infestans* belong to the oomycete class which forms a diverse group of fungus-like eukaryotic microorganisms. It includes saprophytes as well as pathogens of plants, insects, crustaceans, fish, vertebrate animals, and various microorganisms. Species from the genus *Phytophthora*, meaning "plant destroyer" in Greek, are the most devastating pathogens on dicotyledonous plants. The host range of *P. infestans* encompasses at least 90 plant species (Erwin and Ribeiro 1996). Most of them belong to the Solanaceae family. The hemi-biotrophic life style of *P. infestans* has been extensively studied (Figure 3) (Judelson et al. 2007). It comprises a sexual and asexual reproduction cycle producing different forms of

spores: oospores and zoospores respectively. Sexual reproduction occurs between mating types A1 and A2. In the 1950s the mating type A2 was discovered in Mexico and spread all over the world in the 1970s (Fry et al. 1992).

During the early stages of infection, *P. infestans* secretes a wealth of effectors into the plant extracellular space and cytoplasm. Apoplastic effectors interact with extracellular targets such as membrane receptors or secreted plant proteins. They are classified in three classes: enzyme inhibitors, small cysteine rich proteins and the Nep1-like (NPL) family. Cytoplasmic effectors are delivered inside the plant cell via a haustorium and are represented by two classes: RXLR protein family and the Crinkler (CRN) protein family (Kamoun 2006). So far, all the effectors identified as being the matching avirulence gene (*Avr1*, *Avr2*, *Avr3a*, *Avr4*, *Avr-blb1* and *Avr-blb2*) of an *Rpi*-gene (resistance to *P. infestans*) belong to the RXLR protein family (Armstrong 2005; Oh et al. 2009; van Poppel 2008; Vleeshouwers et al. 2008; J.G Morales et al., unpublished results; Govers et al. unpublished results). This RXLR motif, Arg-X-Leu-Arg (in which X represents any amino acid), characterizes a domain which was shown to be involved in the translocation of the effectors into host cells (Birch et al. 2006; Whisson et al. 2007). The genomes of *P. sojae* and *P. ramorum* revealed that the RXLR effectors family were abundant and evolved rapidly generating over 370 members for each species (Jiang et al. 2008). The recent completion of the sequence of the *P. infestans* genome (Haas et al. 2009) showed that the genomes of *P. ramorum* (65Mb) and *P. sojae* (95Mb) were several fold smaller than the *P. infestans* genome (240Mb). This comparison revealed that *Phytophthora* genomes underwent a rapid turnover and extensive expansion of specific families of secreted disease effector proteins which are located in highly dynamic and expanded regions of the *Phytophthora* genomes.

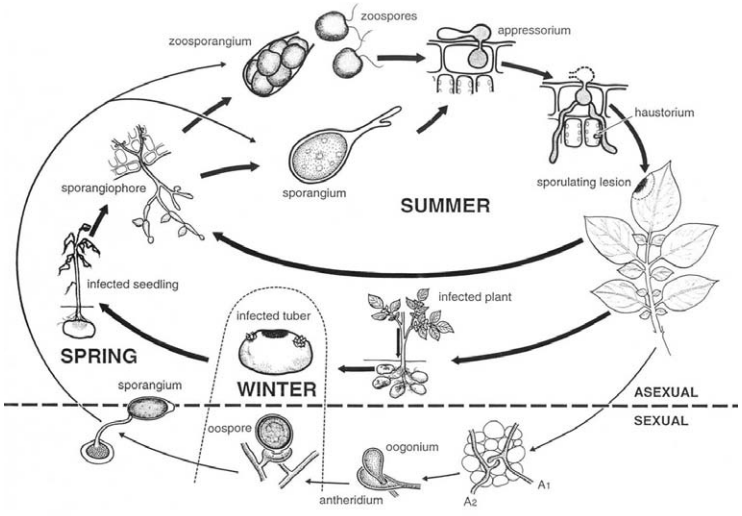

Figure 3. Life cycle of *P. infestans*

Foliar resistance against late blight

A recent comprehensive survey (Vivianne Vleeshouwers, personal communication) of wild tuber bearing *Solanum* species demonstrated that the genus *Solanum* harbors a wealth of late blight resistance sources that have yet to be exploited. Several studies explored the genetic basis of late blight resistance in *S. demissum* from which the first resistance genes were introgressed into potato cultivars in the nineteen fifties. These studies showed that eight of the eleven known specificities, *R3* (now known to be *R3a* and *R3b*), *R5*, *R6*, *R7*, *R8*, *R9*, *R10* and *R11*, are located close to each other on chromosome *11* (Bradshaw et al. 2006a; El Kharbotly et al. 1994; Huang et al. 2004,2005). Other mapped *R* genes from *S. demissum* include *R1* on chromosome *5* (El Kharbotly et al. 1994; Leonards-Schippers et al. 1992) and *R2* on chromosome *4* (Li et al. 1998).

Late blight resistance has been identified in other wild *Solanum* species. The described *R* loci include *Rpi-ber1* (initially named R_{ber}) from *S. berthaultii* on chromosome *10* (Ewing et al. 2000), *RB*/*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* on chromosome *8*, *6* and *4*, respectively (Van der Vossen et al. 2003, 2005; Lokossou et al. 2009), *Rpi-pnt1* initially named *Rpi1* from *S. pinnatisectum* on chromosome *7* (Kuhl et al. 2001), *Rpi-mcq1* and *Rpi-phu1* on chromosome *9* from *S. mochiquense* and *S. phureja* respectively (Smilde et al. 2005; Sliwka et al. 2006). Functional homologs of *Rpi-blb1* have been identified in *S. stoloniferum* and *S. papita*: *Rpi-sto1* and *Rpi-pta1* respectively (Vleeshouwers et al. 2008). Lokossou et al. (2009) have described one of the major late blight resistance gene clusters on chromosome 4 in which *R2*, *R2*-like, *Rpi-blb3* and *Rpi-abpt* were identified. In addition to *R* genes, several quantitative trait loci (QTL) involved in resistance to late blight have been reported, both in cultivated potato (reviewed in (Gebhardt and Valkonen 2001) and in wild species, e.g. *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008), *S. paucissectum* (Villamon and Spooner 2005) and *S. phureja* on chromosome *7* and *12* (Ghislain and Trognitz 2001).

Tuber resistance

Most of the research conducted on potato late blight resistance focuses on foliar resistance. However *P. infestans* is able to infect all parts of the plant comprising leaves, stems, inflorescences and tubers. Surprisingly, few studies were conducted to investigate and characterize tuber resistance. Vascular regions of potato stems and tubers were found to be significantly more resistant to *P. infestans* than the other organs of the plant mainly due to stronger physical barriers (Kassim et al. 1976). In the late eighties, Pathak (1987) observed necrosis which were similar in appearance to the hypersensitive response (HR), located in the

outermost 20-30 cell layers of the cortex. More than one cell was involved in this type of necrosis. He also hypothesized that tuber resistance could be attributed to three major components of the tuber: the periderm, the cortex cell layers and the medulla. These results may address the question of tissue specificity for late blight resistance. Interestingly, QTLs conferring foliar and/or stem resistance have been identified by (Danan et al. 2009).

The inheritance of tuber late blight resistance was explored by Wastie et al. (1987) who pointed out a correlation between foliage and tuber resistance. In 1992 Stewart et al. showed that indeed foliage and tuber resistance were correlated in certain cultivars such as cv. *Stirling*. However a recent study suggested that different genetic mechanisms control foliar and tuber resistance (Liu and Halterman 2009). One major QTL for tuber late blight resistance was mapped on chromosome 5 linked to GP179 (Oberhagemann et al. 1999). Later on, the *R1* gene was found to be located in the same region (El-Kharbotly et al. 1994). Recent inheritance studies of tuber late blight resistance showed that some *R*-genes were foliage specific (*R3a* and *Rpi-abpt*), whereas some other *R*-genes were foliage and tuber specific (*R1* and *Rpi-phu1*) (Park et al. 2005b; Sliwka et al. 2006). Millett and Bradeen (San Diego 2005) investigated the specificity of the *RB* gene to see whether it could confer tuber resistance or not. The *RB* gene was found to be constitutively expressed in leaf and tuber but conferred foliar resistance only. So far, no *R*-genes were found to be tuber specific.

Breeding for late blight resistance

Breeding for late blight resistance was stimulated in the mid 19th century by the disastrous consequences of *Phytophthora* epidemics in the USA and Europe. In the early 20th century, potato late blight resistance research started using wild *Solanum* species from South and Central America in breeding programs. Initially special attention was given to *S. demissum* as the main resistance source (Black and Gallegly 1957; Malcolmson and Black 1966). To date at least 11 specificities (*R1-R11*) have been identified in *S. demissum*, four of which (*R1, R2, R3* and *R10*) have been introgressed by breeders up to the cultivar level, but races of the pathogen that were able to overcome these genes emerged within a few years after market introduction (Turkensteen 1993). By the end of the 1950s, most breeders focused their efforts on the use of sources of germplasm with partial/quantitative resistance thought to be *R* gene independent and assumed to be of polygenic nature, and thus more durable (Turkensteen 1993). However, Tan et al. (2008) hypothesized that quantitative blight resistance conferred by *Rpi-mcd1* in *S. microdontum* mapped on chromosome *4* belong to the family of NBS-LRR genes. Many minor quantitative trait loci have been identified (Bradshaw et al. 2006b;

Collins et al. 1999; Costanzo et al. 2005), but stacking of these QTLs has proven to be very difficult. Moreover, this partial/quantitative resistance is strongly correlated with late maturity under long day conditions (Howard 1970). Finally, Flier et al. (2003a and b) showed that quantitative resistance was apparently also prone to erosion. That is why breeders have reconsidered the use of qualitative *R*-genes based resistance, preferably through stacking of complementary genes.

Two technologies are available to improve the efficiency of late blight resistance breeding in potato: marker assisted selection (MAS) and genetic modification. Marker assisted selection is based on molecular makers used for indirect selection of a genetic trait of interest. This strategy requires markers tightly linked with the trait of interest or within the gene of interest in the case of a single locus. Although this strategy may render efficiency to the breeding process, introgression of *R*-genes may face linkage drag issues in which non desirable traits cannot be removed via successive back crosses. These limitations can be overcome using a genetic modification strategy based on *Agrobacterium tumefaciens* transformation. Recent work on plant genetic transformation has developed a marker-free transformation system (Vetten et al. 2003) avoiding the use of selection markers (resistance genes to antibiotics). Promotion of genetically modified organisms (GMOs) is rather difficult among consumers because of public concern. Resistance genes to *Phytophthora infestans* (*Rpi* genes) with their native promoter derived from sexually compatible species, so called cisgenesis (Schouten et al. 2006) can be introduced using a marker-free system generating cisgenic plants with only the gene(s) of interest and without linkage drag (Jacobsen and Schouten 2007).

Scope of the thesis

The aim of this thesis is to clone and characterize a new resistance gene locus on chromosome 9 from *S. venturii*, identify its counterpart effector gene from *P. infestans* and better understand the genetic and molecular mechanisms of tuber blight resistance.

We applied a map-based cloning strategy combined with a homology based mining strategy to clone *R* alleles (*Rpi-vnt1.1* and *Rpi-vnt1.3*) sharing 75% homology with the $Tm2^2$ gene conferring resistance to Tomato Mosaic Virus (TMoV) (**Chapter 2**). In parallel, a 'classical' map-based cloning strategy was used to clone *Rpi-vnt1.1* and *Rpi-vnt1.2* (**Chapter 3**). Both strategies appeared to be complementary to achieve an efficient cloning (homology based mining strategy) and robust physical mapping (classical map-based strategy).

Allele mining of *Rpi-vnt1* alleles within 196 different species represented by 5 accessions containing 5 individual genotypes showed that *Rpi-vnt1* alleles were not widely

spread among wild potato species. Only *S. mochiquense* and *S. weberbaueri* carried one of the three alleles, *Rpi-vnt1.1*. Phylogenetic study and sequence analysis revealed that each of the *Rpi-vnt1* alleles were monophyletic and may have evolved through illegitimate recombination (**Chapter 4**). Extensive phenotyping and genotyping of *S. venturii* accessions identified a second single dominant resistance gene named *Rpi-vnt2*, complementing the resistance spectrum of *Rpi-vnt1* alleles. *Rpi-vnt2* has not been localized yet.

The corresponding effector of *Rpi-vnt1* alleles, *Avr-vnt1*, was identified by using an efficient and high throughput effector screen in resistant wild potato species (**Chapter 5**). *Avr-vnt1* has the typical RXLR-EER motif and is silenced in the virulent strain EC1.

For the first time an attempt was made to study the correlation of foliage and tuber blight resistance in the same genetic background (cv Desiree) carrying different *R* genes in their tubers (*R1*, *Rpi-blb3*, *R3a*, or *Rpi-vnt1.1*; **Chapter 6**). Transient expression of the corresponding effector (*Avr1*, *Avr2*, *Avr3a* and *Avr-vnt1* respectively) in transgenic tuber slices triggered a hypersensitive response (HR) demonstrating that the *R*-genes were functional in tubers. For a given *R*-/*Avr*- gene pair we showed that tuber blight resistance was *R*-gene based by association of transcript level with phenotypic data obtained from a panel of transformants. Moreover, the expression ratio of a given *R*-gene/effector pair seemed to determine resistance.

In the general discussion (**Chapter 7**), all presented experimental data are placed in a broader context. The strategies to clone *R*-genes are discussed as well as the dynamics of *R*-gene clusters, *R*-gene based deployment strategies and other leads to achieve late blight resistance such as recessive *R*-genes and non host resistance to *P. infestans*.

CHAPTER 2

Mapping and cloning of late blight resistance gene from *Solanum venturii* using an interspecific candidate gene approach

Pel, M. A., Foster, S. J., Park, T.-H., Rietman, H., van Arkel, G., Jones, J. D. G., Van Eck, H. J., Jacobsen, E., Visser, R. G. F., and Van der Vossen, E. A. G.

Molecular Plant-Microbe Interactions (2009) 22:601-615.

Potato leaves from *S. venturii* infected with a virulent and/or avirulent *P. infestans* strains to *Rpi-vnt1.1* gene

Mapping and cloning of late blight resistance genes from *Solanum venturii* using an interspecific candidate gene approach

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ABSTRACT

Late blight (LB), caused by the oomycete *Phytophthora infestans,* is one of the most devastating diseases on potato. Resistance (*R*) genes from the wild species *Solanum demissum* have been used by breeders to generate late blight resistant cultivars, but resistance was soon overcome by the pathogen. A more recent screening of a large number of wild species has led to the identification of novel sources of resistance, many of which are currently being characterized further. Here we report on the cloning of dominant *Rpi* genes from *S. venturii*. *Rpi-vnt1.1* and *Rpi-vnt1.3* were mapped to chromosome *9* using NBS profiling. Subsequently, a *Tm-2*^{2} based allele mining strategy was used to clone both genes. *Rpi-vnt1.1* and *Rpi-vnt1.3* belong to the CC-NBS-LRR class of plant *R* genes and encode predicted peptides of 891 and 905 amino acids, respectively, which share 75% amino acid (aa) identity with the ToMV resistance protein Tm-2^2 from tomato. Compared to Rpi-vnt1.1, Rpi-vnt1.3 harbors a 14 aa insertion in the N-terminal region of the protein and two different aa in the LRR domain. Despite these differences, *Rpi-vnt1.1* and *Rpi-vnt1.3* genes have the same resistance spectrum.

Keywords: *Rpi-vnt1.1*, *Rpi-vnt1.3*, disease resistance, *Phytophthora infestans*, *Solanum venturii*, late blight, potato.

Accession numbers: *Rpi-vnt1.1* and *Rpi-vnt1.3* sequences are deposited in genbank FJ423044 and FJ423046 respectively

INTRODUCTION

Late blight, one of the world's most devastating plant diseases, is caused by the oomycete *Phytophthora infestans*, causing an estimated yearly economic loss of $3.25 billion in potato growing areas worldwide. Despite more than 150 years of resistance breeding, disease management still relies on the enormous application of fungicides. Breeding for late blight resistance was stimulated in the mid 19th century by the disastrous consequences of *Phytophthora* epidemics in the USA and Europe. Only a few of the many existing landraces survived and these formed the basis for variety development. However, until 1910 the reduced aggressiveness of the pathogen since 1850 probably played a more prominent role in the survival of the potato crop in Europe than the first breeding activities. The rediscovery of the Mendelian laws of genetics subsequently directed the focus of potato resistance research on the use of wild *Solanum* species grown in South and Central America. Initially special attention was given to *S. demissum* as the main resistance source (Black and Gallegly 1957; Malcolmson and Black 1966). To date at least 11 specificities (*R1-R11*) have been identified in *S. demissum*, four of which (*R1, R2, R3* and *R10*) have been introgressed by breeders up to cultivar level, but races of the pathogen that were able to overcome these genes emerged within a few years after market introduction (Turkensteen 1993). By the end of the 1950s, most breeders switched to the use of sources of germplasm with partial/quantitative resistance, the underlying paradigm being that this type of resistance is *R* gene independent and assumed to be of polygenic nature, and thus more durable (Turkensteen 1993). Extensive research in this area has lead to the identification of many minor quantitative trait loci (Bradshaw et al. 2006b; Collins et al. 1999; Costanzo et al. 2005) but stacking of these QTLs has proven to be very difficult, due to the out breeding nature of cultivated potato. Moreover, this type of resistance is strongly correlated with late maturity under long day conditions (Howard et al. 1970). This, together with the finding that quantitative resistance is apparently also amenable to erosion (Flier et al. 2003a and b), has stimulated breeders to reconsider the use of *R* genes, preferably through stacking of complementary genes.

A recent comprehensive survey of wild tuber bearing *Solanum* species revealed that the genus *Solanum* harbors a wealth of late blight resistance sources that have yet to be exploited. Recent studies into the genetic basis of late blight resistance in *S. demissum* showed that eight of the eleven known specificities, *R3* (now known to be *R3a* and *R3b*), *R5, R6, R7, R8, R9, R10* and *R11*, are located close to each other on chromosome *11* (Bradshaw et al. 2006a; El Kharbotly et al. 1994; Huang et al. 2004, 2005). Other mapped *R* genes from *S. demissum*

include *R2* on chromosome *4* (Li et al. 1998) and *R1* on chromosome *5* (El Kharbotly et al. 1994; Leonards-Schippers et al. 1992). Besides *S. demissum*, other wild *Solanum* species have been recognized as sources of late blight resistance. The described *R* loci include *Rpi-ber1* (initially named R_{ber}) from *S. berthaultii* on chromosome *10* (Ewing et al. 2000), *RB*/*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* on chromosome *8*, *6* and *4*, respectively (Van der Vossen et al. 2003, 2005; Lokossou et al 2009), *Rpi-pnt1* (initially named *Rpi1*, (Kuhl et al. 2001) from *S. pinnatisectum* on chromosome *7*, *Rpi-mcq1* (initially named *Rpi-moc1*, Smilde et al. 2005) from *S. mochiquense* on chromosome *9* and *Rpi-phu1* from *S. phureja* on chromosome *9* (Smilde et al. 2005; Sliwka et al. 2006). In addition, several quantitative trait loci (QTL) involved in resistance to late blight have been reported, both in cultivated potato reviewed by Gebhardt and Valkonen (2001) and in wild species, e.g. *S. microdontum* (Sandbrink et al. 2000; Tan et al. 2008), *S. paucissectum* (Villamon and Spooner 2005) and *S. phureja* on chromosome *7* and *12* (Ghislain and Trognitz 2001).

In contrast to introgression breeding, isolation of *R* genes from *Solanum* species and their stable transformation into existing potato varieties is by far the fastest means of exploiting potentially durable late blight resistance present in the *Solanum* gene pool. Cloning of *R* genes is typically done through a positional cloning strategy. Currently four *R* genes for late blight resistance have been cloned and all belong to the CC-NBS-LRR class of plant *R* genes: *R1* (chromosome *5*) and *R3a* (chromosome *11*) from *S. demissum* (Ballvora et al. 2002b; Huang et al. 2005a) and *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* on chromosomes 8 and 4, respectively (Van der Vossen et al. 2003, 2005). Once a functional gene is cloned from a specific *R* locus, one can try to clone functional alleles from the same or different species in order to determine allele frequency and allelic variation at a given locus. This was illustrated by the cloning of *Rpi-sto1* and *Rpi-pta1* from *Solanum stoloniferum* and *S. papita*, respectively, which were shown to be functional alleles of *Rpi-blb1* (Vleeshouwers et al. 2008). Here we demonstrate that NBS profiling (Linden et al. 2004), when combined with bulked segregant analysis (BSA) (Michelmore et al. 1991), is a powerful tool to generate candidate gene markers. They can predict the position of the *R* locus under study and upon sequence information, form a starting point for cloning of the gene through a functional allele mining strategy, avoiding the map based cloning approach. Depending on the resolution of relevant genetic mapping studies and the size of the candidate gene family, an allele mining approach can generate many candidate genes which need to be functionally analyzed.

To date functional analysis of candidate *R* gene homologues (RGH) typically requires stable transformation of a susceptible genotype for complementation purposes. This is a time

consuming and inefficient approach as it takes at least several months to generate transgenic plants that can functionally be analyzed. In the current study, we have exploited the finding that *Nicotiana benthamiana* is susceptible to *P. infestans* (Becktell et al. 2006), to develop a transient complementation assay for *R* genes that confer resistance against *P. infestans* (Rietman et al. in preparation).

Here we present an example of how we used the above described approaches/techniques to clone two functionally equivalent *R* genes from *S. venturii*, a species with its origin in the Andean region of South America.

RESULTS

Genetic basis and spectrum of late blight resistance in S. venturii.

To determine the genetic basis of late blight resistance in *S. venturii* (vnt), 14 vnt accessions were screened in detached leaf assays (DLA) with the *P. infestans* isolate IPO-C. Resistant genotypes selected from the vnt accessions CGN18108 and CGN18000 were used to generate the mapping populations 7698 and 7663 respectively. Following DLA's with 52 F1 progeny plants of population 7698, 30 were scored as resistant and 22 as susceptible, suggesting the presence of a single dominant *R* gene (*Rpi-vnt1.1*). Of the 60 F1 progeny plants screened from population 7663, 24 were scored as resistant and 36 as susceptible, suggesting that also vnt365-1 contained a single dominant *R* gene (*Rp-vnt1.3*). Resistance genes were named in agreement with the concurrent work of Foster et al. (2009) who identified in the *S. venturii* accessions CGN18108, CGN18279 and CGN18000 three single dominant *R* genes, *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* respectively.

The resistance spectra of *Rpi-vnt1.1* and *Rpi-vnt1.3* were analyzed by challenging them with several isolates of different complexity and aggressiveness (Table 1). Both genes were only overcome by strain EC1, suggesting that they share the same resistance spectrum.

Table 1. Description of *Phytophthora infestans* isolates used to determine the specificity of *Rpi-vnt1.1* and *Rpi-vnt1.3*

Isolate ID	Country of Origin	Isolation year	Race	*Rpi-vnt1.1* and *Rpi-vnt1.3* phenotypes
90128	Geldrop, The Netherlands	1990	1.3.4.7.(8)	Resistant
H30P04	The Netherlands	unknown	7	Resistant
IPO-C	Belgium	1982	1.2.3.4.6.7.10.11	Resistant
USA618	Toluca Valley, Mexico	unknown	1.2.3.6.7.11	Resistant
VK98014	Veenkolonien, The Netherlands	1998	1.2.4.11	Resistant
IPO-428-2	The Netherlands	1992	1.3.4.7.8.10.11	Resistant
NL00228	The Netherlands	2000	1.2.4	Resistant
Katshaar	Katshaar, The Netherlands	Unknown	1.3.4.7.10.11	Resistant
F95573	Flevoland, The Netherlands	1995	1.3.4.7.10.11	Resistant
89148-09	The Netherlands	1989	0	Resistant
EC1	Ecuador	Unknown	3.4.7.11	Susceptible

Mapping of Rpi-vnt1.1 and Rpi-vnt1.3 to chromosome 9

In an attempt to develop markers linked to *Rpi-vnt1.1* and *Rpi-vnt1.3*, we carried out a bulked segregant analysis (BSA) in combination with NBS profiling in both mapping populations. This led to the identification of nine bulk specific markers for *Rpi-vnt1.1* in 7698 and eight for *Rpi-vnt1.3* in 7663. On the full offspring, only two resistant bulk specific fragments, one generated with the NBS2/*RsaI* primer-enzyme combination and the other with NBS3/*HaeIII*, cosegregated with resistance in both initial 7698 and 7663 populations of 52 and 60 F1 progeny plants, respectively. These fragments were therefore cloned and sequenced. When subjected to a BLAST analysis, both sequences turned out to be highly similar to the *Tm-2*^{2} gene on chromosome *9* of tomato (Lanfermeijer et al. 2003). The cloned NBS2/*RsaI* and NBS3/*HaeIII* fragments were 350 and 115 bp in size and shared 88.3% and 80.3% DNA sequence identity with *Tm-2*^{2}, suggesting that *Rpi-vnt1.1* and *Rpi-vnt1.3* were *Tm-2*^{2} related and thus could lie on chromosome *9*. Genetic linkage of the NBS fragments to the *R* genes under study was verified by developing fragment specific SCAR markers in the *Rpi-vnt1.1* and *Rpi-vnt1.3* mapping populations. In this way, the NBS3 derived marker NBS3b was

mapped relative to the chromosome *9* derived markers TG35, TG551, TG186, CT183 or T1421 (Figure 1). Linkage of TG35 and TG186, and TG35 and TG551, to *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively, indicated that both genes were indeed derived from similar regions on chromosome *9* (Figure 1). These findings were in line with the result of concurrent work of Foster et al. (2009), who followed a map-based cloning approach to clone *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* genes.

In order to develop flanking markers to screen a large offspring for recombinant events, markers linked to TG35 or TG551 were selected from Solanaceae Genomics Network (SGN) and screened in both populations. Despite low levels of polymorphism, EST based markers U276927 and U270442 have been developed and mapped in populations 7698 and 7663, respectively (Table 2a and Figure 1). U276927 could be mapped 2cM north of *Rpi-vnt1.1* whereas U270442 was mapped 3.5cM south of *Rpi-vnt1.3*. Subsequently, recombinant offspring was identified using 500 individuals of population 7698 and 1005 individuals of population 7663, using the flanking markers U276927 / TG186 and NBS3B / U270442, respectively. This resulted in the mapping of *Rpi-vnt1.1* and *Rpi-vnt1.3* within a genetic intervals of 4cM and 3.7cM, respectively (Figure 1).

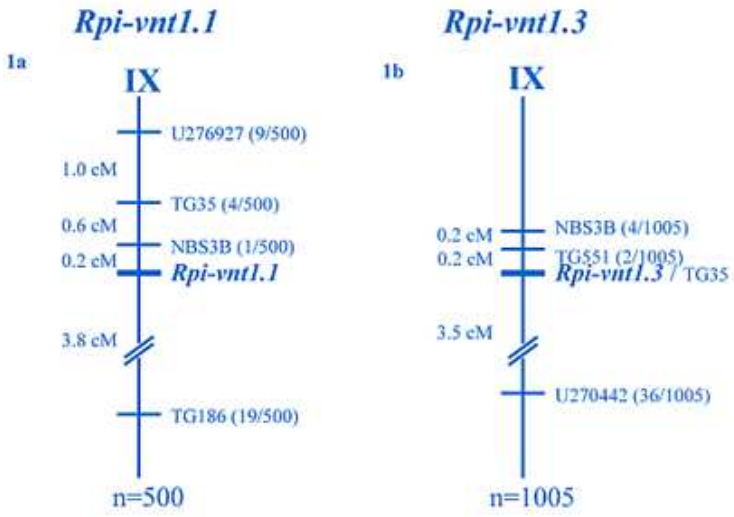


Figure 1. Genetic linkage maps of chromosome *9* of the *Rpi-vnt1.1* (a) and *Rpi-vnt1.3* (b) loci mapped in the populations 7698 and 7663 respectively. Numbers on the left side indicate genetic distances (cM). Relative positions of mapped loci are indicated by horizontal lines. The letter n represents the size of each population.

Table 2A. Markers used to map *Rpi-vnt1.1* and *Rpi-vnt1.3*

Marker	Primer orientation	Primer sequence	Annealing temperature	Enzyme
NBS3B	F	ccttcctcatcctcacatttag	65	a.s.
	R	gcatgccaactattgaaacaac		
TG35	F	cacggagactaagattcagg	60	HhaI / XapI (c)
	R	taaaggtgatgctgatgggg		
TG551	F	ccagaccaccaagtggttctc	58	TaqI (c)
	R	aactttcagatatgctctgcag		
TG186	F	aacggtgtacgagattttac	58	HphI (c)
	R	acctacatagatgaacctcc		
U270442	F	ggatattatcttgcaacatctcg	55	XapI (r)
	R	cttctgatggtatgcatgagaac		
U276927	F	gcattagcgcaattggaatccc	58	HphI (c)
	R	ggagagcattagtacaggcgtc		

a.s.: allele specific
(c) coupling phase
(r) repulsing phase

Tm-2^2 based allele mining

In view of the expected high DNA sequence homology between *Rpi-vnt1.1*, *Rpi-vnt1.3* and *Tm-2^2*, we adopted a homology based allele mining strategy to clone the former two genes. The first step was to design degenerated primers incorporating the putative start and stop codons of candidate *Tm-2^2* gene homologs (*Tm2*GH). Based on an alignment of all the available potato and tomato derived *Tm-2^2*-like sequences in public sequence databases, we designed primers ATG-Tm2F and TGA-Tm2R (Table 2b). However, no amplicons of the expected size were generated when this primer set was tested on the parental genotypes of both mapping populations. As the ATG-Tm2F primer sequence was present in the cosegregating NBS profiling derived marker sequences, three new reverse primers (REV-A, -B and -C) were designed 100bp upstream of the initial TGA-Tm2R primer site, in a region that was conserved in all the aligned *Tm-2^2*-like sequences. When combined with either ATG-Tm2F or NBS3BF, a single amplicon of approximately 2.5kb was specifically amplified from the resistant parental genotypes only, i.e., vnt7014-9 and vnt365-1. These fragments were cloned into the pGEM®-T Easy vector and approximately 96 individual clones from each genotype were sequenced using a primer walk strategy. All the obtained sequences shared 75-80% similarity to *Tm-2^2*. A total of 5 different classes could be distinguished within the vnt7014-9 derived sequences whereas the vnt365-1 derived sequences fell into only 3 different classes. These different classes were subsequently named NBS3B-like or non-NBS3B-like based on the degree of homology to the NBS3B sequence .

Table 2B. Primers used for various experiments as described in this manuscript

Marker	Primer orientation	Primer sequence	Annealing temperature	Experiments
NBS-GSP1	F	tccaaatattgtcgagttggg	Touch down	Genome Walking
NBS-GSP2	F	gctttggtgcagacatgatgc	Touch down	Genome Walking
REV-A	R	ggttgtcgaagtaacgtgcac	55	*Tm2*-based allele mining
REV-B	R	tgcacggatgatgtcagtatgcc	55	*Tm2*-based allele mining
REV-C	R	caacttgaagttttgcatattc	55	*Tm2*-based allele mining
ATG-Tm2F	F	atggctgaaattcttctcacagc	55	*Tm2*-based allele mining
TAA-8bisR	R	ttatagtacctgtgatattctcaac	55	*Tm2*-based allele mining
ATG2-Tm2F	F	atgaattattgtgtttacaagacttg	55	*Tm2*-based allele mining
TGA-Tm2R	R	tgatattctcaactttgcaagc	55	*Tm2*-based allele mining
GSP1-5race	R	gaacactcaaattgatgacagacatgcc	67	5' RACE
GSP2-5race	R	cccaaaccgggcatgccaactattg	67	5' RACE

In order to retrieve the missing C-terminal part of the amplified *Tm2*GH's a 3'-genome walk was performed using primers NBS-GSP1 and NBS-GSP2 (Table 2), which were designed approximately 100bp upstream of the REV-A, -B and –C primers, in order to generate an overlap of 100bp between the cloned NBS3B-like sequences and clones generated with the genome walk. Three amplicons of ~200bp were obtained from vnt7014-9 and a single one of ~1kb from vnt365-1. Following cloning, sequencing and alignment to the cloned *Tm2*GH's, all four clones seemed to fit to clone *Tm2*GH-vnt8b, as the overlapping 100bp were an exact match. To be able to subsequently amplify full-length *Tm2*GH's from the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci we designed a novel reverse primer (TAA-8bR) (Table 2) based on the alignment of the full-length *Tm2*GH-vnt8b sequence with the *Tm-2*^{2} sequence from tomato (Figure 2). As the original TGA stop codon was not present in the *Tm2*GH-vnt8b sequence we included the next in-frame stop-codon (TAA) which was situated 12bp downstream.

Full-length amplification of *Tm2*GH's from vnt7014-9 and vnt365-1 was subsequently pursued with high fidelity *Pfu* Turbo polymerase using primers ATG-Tm2F and TAA-8bR. Amplicons of ~2.6kb were cloned into the pGEM®-T Easy vector and sequenced. Three different types of clones were obtained from vnt7014-9, one of which harbored an ORF of the expected size (*Tm2*GH-vnt1b). All the clones obtained from vnt365-1 were identical to each other and contained the expected ORF. Clone *Tm2*GHvnt1.9 was chosen together with *Tm2*GH-vnt1b for further genetic analysis.

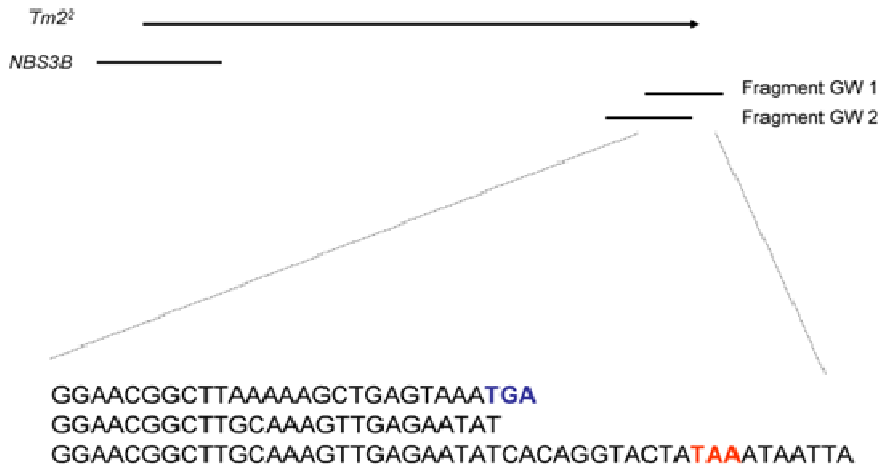


Figure 2. Alignment of the *Tm2*^{2} ORF with two PCR fragments obtained with a Genome Walking kit (GW1 and GW2). The stop codons of the *Tm2*^{2} gene and *Rpi-vnt1.1 / vnt1.3* are presented in blue and red respectively.

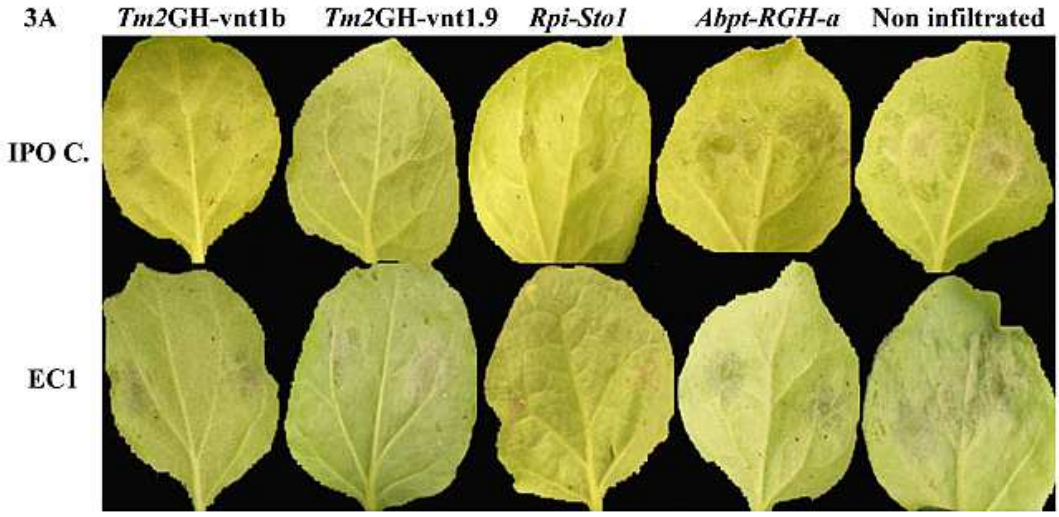
Before targeting *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 for complementation analysis, we needed to confirm that the selected *Tm2*GH's indeed mapped to the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci. When tested as SCAR markers in the initial mapping populations, both markers cosegregated with resistance. Upon amplification with ATG-Tm2F and TAA-8bisR in the set of recombinants which defined the *Rpi-vnt1.1* and *Rpi-vnt1.3* loci, amplicons of the expected size were indeed only generated from late blight resistant recombinants, confirming that both *Tm2*GA's were indeed good candidates for *Rpi-vnt1.1* and *Rpi-vnt1.3*. However, there were resistant recombinants, two in the *Rpi-vnt1.1* mapping population and one in the *Rpi-vnt1.3* mapping population, which did not give the expected PCR product, suggesting that both loci could in fact harbor a tandem of two functional *R* genes.

Transient complementation using Agrobacterium Transient Transformation Assays (ATTA) in Nicotiana benthamiana

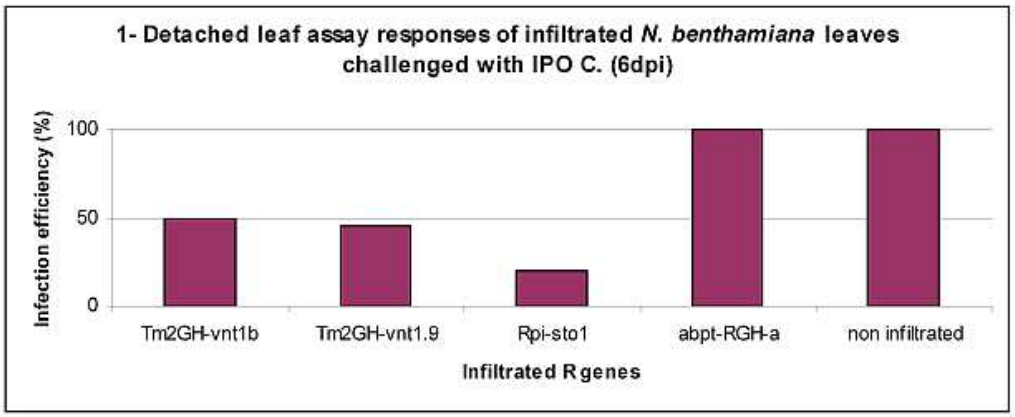
To investigate whether *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 were functional *R* genes, we inserted them into a Gateway® binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. 2009). *N. benthamiana* leaves were then infiltrated with *A. tumefaciens* cultures containing the relevant clones. Two days post infiltration the leaves were challenged with *P. infestans* in a detached leaf assay. Two different isolates were used, EC1 and IPO-C, which show a differential response to *Rpi-vnt1.1* and *Rpi-vnt1.3*. Both genes confer resistance to IPO-C but allow for a compatible interaction in the case of EC1. Three independent transient complementation assays were carried out in triplicate with both isolates. For each replicate, leaf numbers 4, 5 and 6 when counting from the bottom of the plant, were

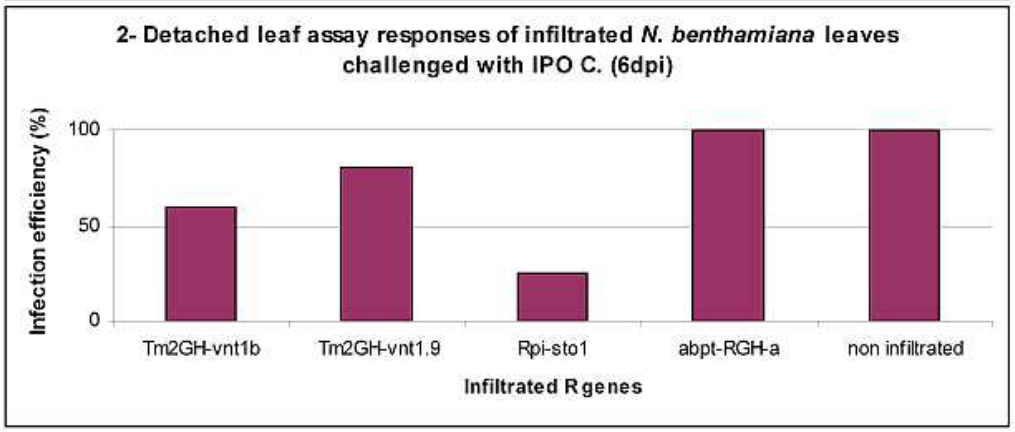
agro-infiltrated and subsequently challenged with *P. infestans*. At six days post inoculation with IPO-C, leaves transiently expressing *Tm2*GH-vnt1b or *Tm2*GH-vnt1.9 displayed an infection efficiency between 40-60% (Figure 3a and b). The resistant control plants transiently expressing the functional *Rpi-sto1* gene (Vleeshouwers et al., 2008) showed a significant lower infection efficiency ranging between 10-20%, (80-90% of the challenged leaves showed an HR response). In contrast, leaves expressing *abpt*GH-a, a non-functional paralogue of *Rpi-abpt* (Lokossou et al. 2009) were fully susceptible (Figure 3a and b). In case of EC1, all agro-infiltrated leaves were susceptible except for those infiltrated with *Rpi-sto1*, which confers resistance to EC1 (Figure 3a and c). These data matched with the resistance spectrum of *Rpi-vnt1.1* and *Rpi-vnt1.3*, suggesting that *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 indeed represented *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively.

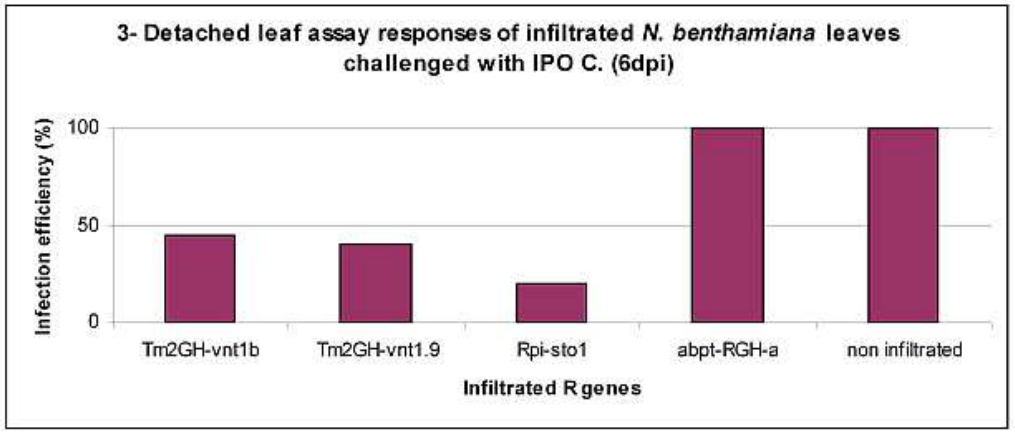
3A


Tm2GH-vnt1b
Tm2GH-vnt1.9
Rpi-Sto1
Abpt-RGH-a
Non infiltrated
IPO C.
EC1

3B


1- Detached leaf assay responses of infiltrated N. benthamiana leaves challenged with IPO C. (6dpi)
Infection efficiency (%)
100
50
0
Tm2GH-vnt1b
Tm2GH-vnt1.9
Rpi-sto1
abpt-RGH-a
non infiltrated
Infiltrated R genes


2- Detached leaf assay responses of infiltrated N. benthamiana leaves challenged with IPO C. (6dpi)
Infection efficiency (%)
100
50
0
Tm2GH-vnt1b
Tm2GH-vnt1.9
Rpi-sto1
abpt-RGH-a
non infiltrated
Infiltrated R genes


3- Detached leaf assay responses of infiltrated N. benthamiana leaves challenged with IPO C. (6dpi)
Infection efficiency (%)
100
50
0
Tm2GH-vnt1b
Tm2GH-vnt1.9
Rpi-sto1
abpt-RGH-a
non infiltrated
Infiltrated R genes

3C

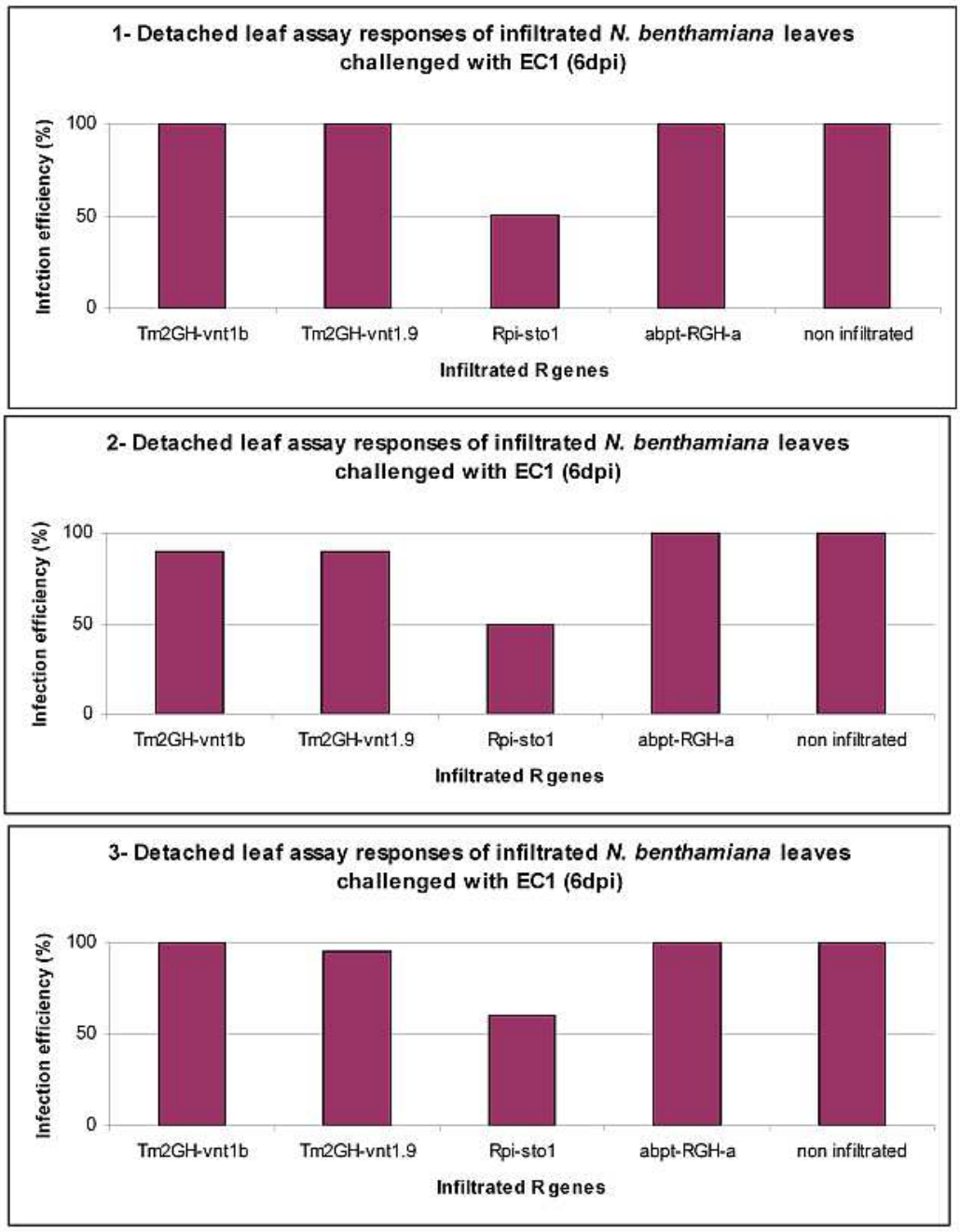


Figure 3. Transient complementation assays in *Nicotiana benthamiana.* **A.** Typical detached leaf assay responses of *N. benthamiana* leaves infiltrated with either *Tm2*GH-vnt1b, *Tm2*GH-vnt1.9, *Rpi-sto1* (resistant control) or *abpt*GH-a (susceptible control). The top row shows the response to IPO-complex (non-virulent isolate) whereas the bottom row shows the response to EC1 (virulent isolate). *Rpi-sto1* gives resistance to both isolates as expected. Pictures were taken 6 days post inoculation. **B**. and **C.** Quantification of infection efficiency (see Materials and Methods) in the transient complementation assay as illustrated in A. *Tm2*GH-vnt1b and *Tm2*GH-*vnt1.9* showed a higher infection efficiency, ranging from 40 to 60%, than the resistant control *Rpi-sto1* construct during an incompatible interaction with IPO Complex isolate.

Complementation analysis through stable transformation of cv. Desiree

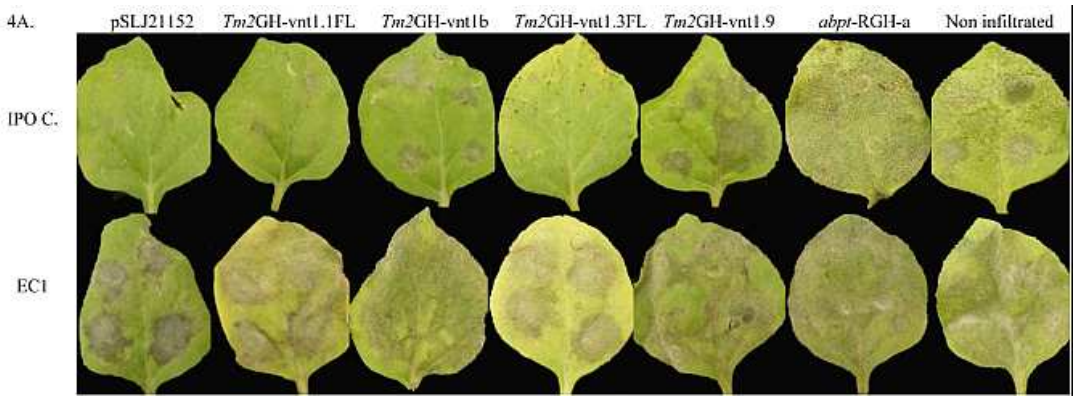
To confirm the results obtained with the transient complementation assays in *N. benthamiana,* the binary Gateway constructs harbouring *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 were transferred to the susceptible potato cultivar Desiree through *Agrobacterium* mediated transformation. As resistant control we also transformed cv. Desiree with construct pSLJ21152, a binary construct harbouring a 4.3 kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF and terminator sequence (Foster et al. 2009). Primary transformants harbouring the transgenes of interest were tested for resistance to *P. infestans* in detached leaf assays. Surprisingly, only the genetic construct harbouring the 4.3 kb *Rpi-vnt1.1* fragment was able to complement the susceptible phenotype; eight out of nine primary transformants were resistant. All twenty two *Tm2*GH-vnt1b and seventeen *Tm2*GH-vnt1.9 containing primary transformants were susceptible to *P. infestans*.

Alignment of the *Tm2*GH-vnt1b and *Tm2GH-vnt1.9* sequences to the 4.3 kb *Rpi-vnt1.1* fragment revealed the presence of an additional ATG start codon 99 nt upstream from the start codon that was used as basis for the PCR based allele mining experiments exploiting $Tm2^2$ homology. This finding, together with the negative complementation results obtained with the *Tm2*GH -vnt1b and *Tm2GH-vnt1.9* and the positive complementation result with 4.3 kb *Rpi-vnt1.1* fragment suggest that the 5' most upstream start codon represents the actual start of the functional *Rpi-vnt1.1* and *Rpi-vnt1.3* genes.

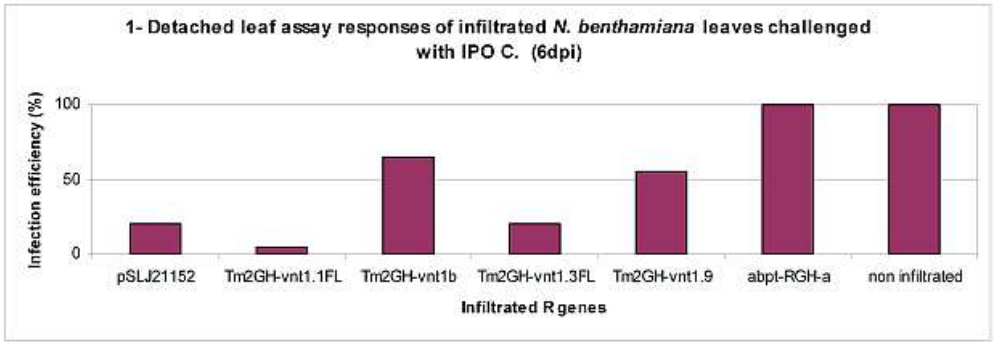
Transient complementation assays using extended allele mining products

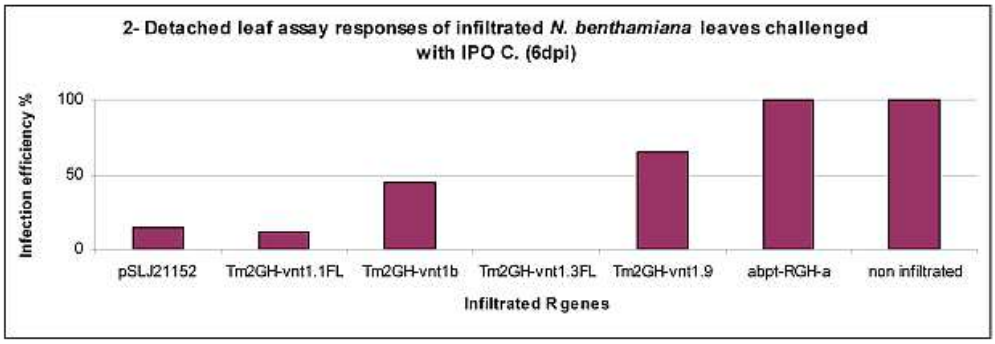
In an attempt to PCR-amplify the putatively full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from vnt7014-9 vnt365-1, respectively, genomic DNA of both genotypes was subjected to long range PCR using the primers ATG2-Tm2F and TAA-8bR (Table 2b). Amplicons of the expected size were cloned into the pGEM®-T Easy vector and sequenced. Clones obtained from vnt7014-9 were all the same and identical to the corresponding sequence in pSLJ21152 (Foster et al. 2009). Clones obtained from vnt365-1 were also all identical but contained an insertion of 42 nt in the 5'extended region compared to those obtained from vnt7014-9. Both sequences were subsequently inserted into the Gateway® binary expression vector in between the regulatory elements of the *Rpi-blb3* gene (Lokossou et al. 2009) and targeted for transient complementation analysis in *N. benthamiana*, together with the original *Tm2*GH-vnt1b and *Tm2GH-vnt1.9* constructs and pSLJ21152. Both full-length genes showed comparable

infection efficiency to the 4.3 kb genomic clone harbouring the *Rpi-vnt1.1* gene (10-30% of the challenged leaves showed typical symptoms of late blight), whereas the shorter gene constructs again displayed significantly higher infection efficiency (65-75% of infected leaves), indicating that the full-length amplicons derived from vnt7014-9 and vnt365-1 represent *Rpi-vnt1.1* and *Rpi-vnt1.3*, respectively (Figure 4a, b and c).



4B





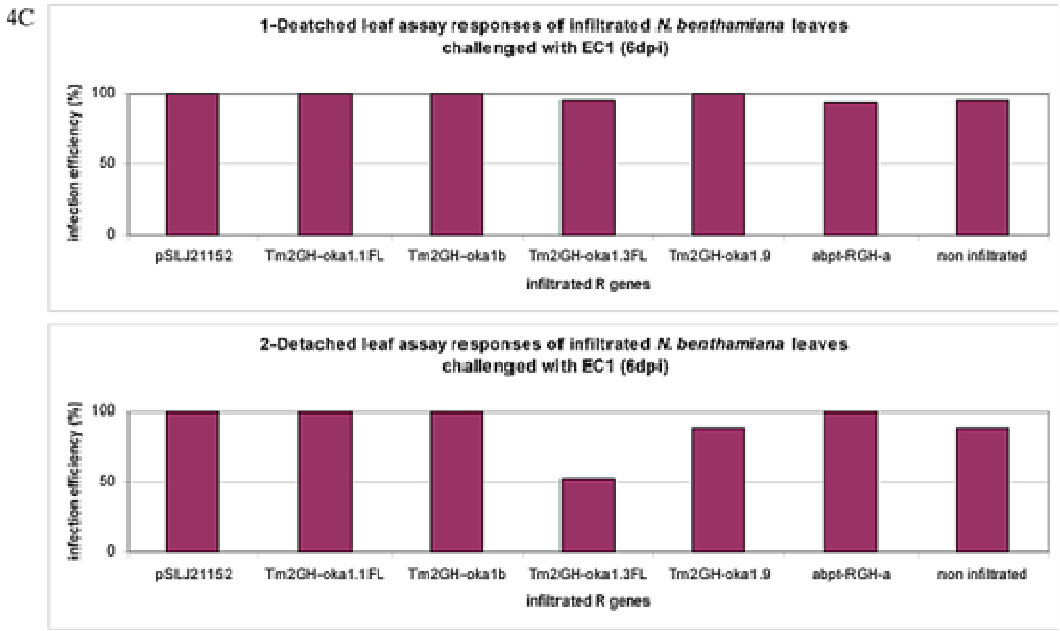


Figure 4. Functional comparison of the N-terminal part of the truncated and full length *Rpi-vnt1.1* and *Rpi-vnt1.3* gene constructs. **A.** Transient complementation assays in *Nicotiana benthamiana* were repeated to compare infection efficiency of the full length candidate genes *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL with the truncated ones, *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9. The construct pSLJ21152, harbouring a 4.3kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF and terminator sequence was used as resistant control. The non-functional resistance gene *abpt*-RGH-a was chosen as susceptible control. Candidate genes *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL show an infection efficiency as low as the resistant control (construct pSLJ21152) whereas the truncated candidate genes show a higher infection efficiency. **B** and **C.** Quantification of infection efficiency (see Materials and Methods) in the transient complementation assay as illustrated in A. *Tm2*GH-vnt1.1FL (full length of *Rpi-vnt1.1* candidate gene) and *Tm2*GH-vnt1.3FL (full length of *Rpi-vnt1.3* candidate gene) showed similar level of infection as the resistant control, pSLJ21152 (4.3kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF and terminator sequence).

Complementation analysis through stable transformation of cv. Desiree with the full length PCR products

To confirm the results obtained with the second transient complementation assays in *N. benthamiana,* the binary Gateway constructs harbouring the full length candidate genes *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL were transferred to the susceptible potato cultivar Desiree through *Agrobacterium* mediated transformation. Primary transformants harbouring the transgenes of interest were tested for resistance to *P. infestans* in detached leaf assays. As expected, both construct were able to complement the susceptible phenotype (Figure 5). Thirteen out of fithteen and fourteen out of twenty primary transformants with *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL respectively were resistant. This result confirmed the results observed in the transient assay. Therefore the cloned PCR products *Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL were demonstrated to be *Rpi-vnt1.1* and *Rpi-vnt1.3* respectively.

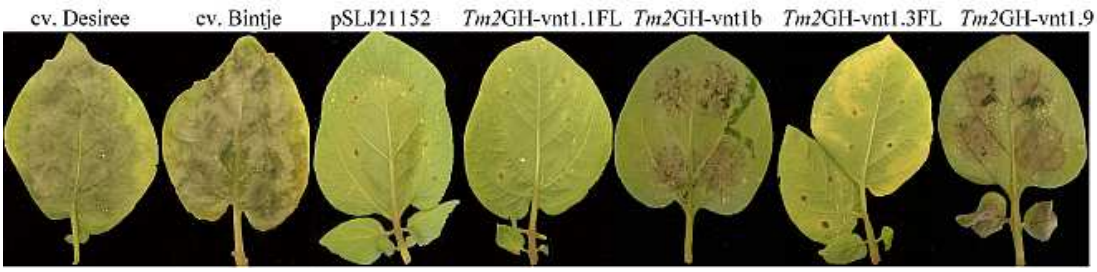


Figure 5. Genetic complementation of cv. Desiree using full length versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* (*Tm2*GH-vnt1.1FL and *Tm2*GH-vnt1.3FL, respectively). cv. Bintje, cv. Desiree, *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 were used as susceptible controls and pSLJ21152 (4.3kb fragment carrying the putative *Rpi-vnt1.1* promoter, ORF and terminator sequences) as resistant control. Primary transformants were challenged with IPO-Complex (non-virulent isolate) and scored six days post inoculation. *Tm2*GH-vnt1.1FL primary transformant -16 and *Tm2*GH-vnt1.3FL primary transformant -15 were chosen to illustrate DLA results. As expected cv. Bintje, cv. Desiree, *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9 showed late blight symptoms whereas pSLJ21152 and the full length primary transformants were fully resistant to IPO-Complex.

Gene structure of Rpi-vnt1.1 and Rpi-vnt1.3

The 5'-terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3* was determined by comparing the genomic sequences with cDNA fragments generated by 5' rapid amplification of cDNA ends (RACE). For *Rpi-vnt1.1* and *Rpi-vnt1.3* genes RACE identified 5' cDNA fragments comprising 5' untranslated regions of 83 and 43 nucleotides (nt) respectively. The open reading frames of *Rpi-vnt1.1* and *Rpi-vnt1.3* encode predicted peptides of 891 and 905 amino acids, respectively. In addition to the 14 amino acid insertion in the N-terminal region of Rpi-vnt1.3, only two other amino acids differ between Rpi-vnt1.1 and Rpi-vnt1.3. At position 548 and 753, Rpi-vnt1.1 harbours an asparagine and arginine residue whereas the corresponding residues in Rpi-vnt1.3 are tyrosine and lysine, respectively (Figure 6). However, the substituted residues have the same characteristics. Asparagine and tyrosine belong to the group of hydrophobic residues whereas arginine and lysine are positively charged residues. The protein sequences of both genes harbor several conserved motifs of the CC-NBS-LRR class of R proteins (Figure 6). A coiled-coil (CC) domain is located in the N-terminal parts of the proteins between amino acids 1 and 183 for Rpi-vnt1.1 and between 1 and 198 for Rpi-vnt1.3. In the first 183 or 198 residues 2 pairs of putative heptad motifs composed of hydrophobic residues could be recognized in Rpi-vnt1.1 and Rpi-vnt1.3 sequences respectively. A NBS-ARC (nucleotide-binding site, apoptosis, *R* gene products, CED-4) domain could be recognized in the amino acid stretch between residues 183 or 198 and 472 or 486 respectively (P-loop, Kinase-2, GLPL(Van der Biezen 1998). The C terminal part of *Rpi-vnt1.1* and *Rpi-vnt1.3* comprises a series of 15 LRR motifs of irregular size that can be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where x

is any amino acid;(McHale et al. 2006). A PROSITE analysis (Hofmann et al. 1999) identified 4 N-glycosylation sites, 7 Casein kinase II phosphorylation sites, 10 protein kinase C phosphorylation sites, 6 N-myristoylation sites and 1 cAMP- and cGMP-dependent protein kinase phosphorylation site.

At the protein level, Rpi-vnt1.1 and Rpi-vnt1.3 share 73% amino acid sequence identity with Tm-2^2. Interestingly, the lowest percentage of similarity was found in the LRR domain where Rpi-vnt1.1 and Rpi-vnt1.3 share 62% amino acid sequence identity with Tm-2^2. In contrast, the coiled-coil and NB-ARC domains of Rpi-vnt1.1, Rpi-vnt1.3 share 86.5% amino acid sequence identity with the corresponding regions of Tm-2^2.

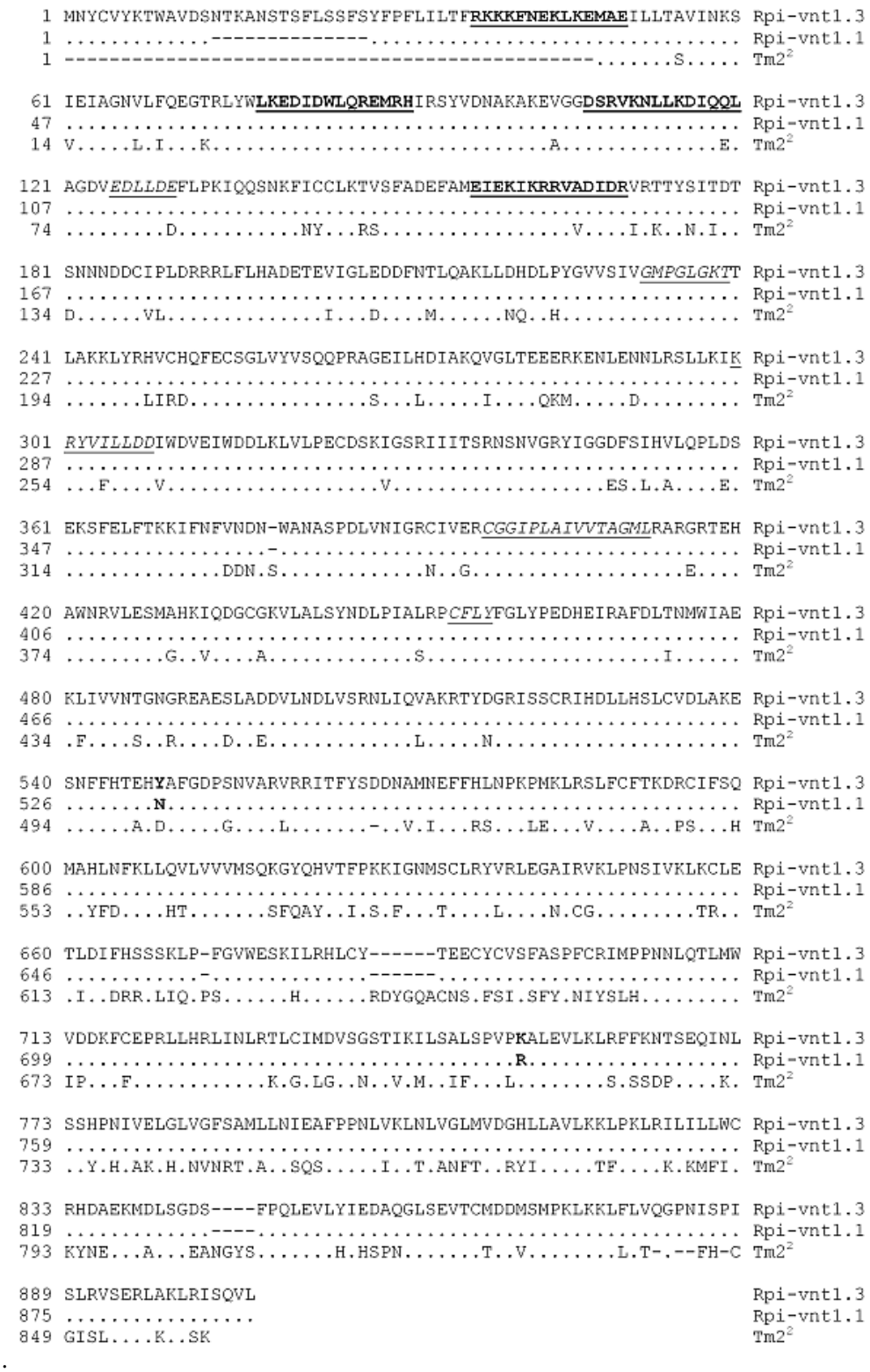


Figure 6. Alignment of Rpi-vnt1.1, Rpi-vnt1.3 and Tm-2^2 protein sequences. In the CC domain four putative heptad motifs and the conserved motif EDVDID are underlined in bold or in italic respectively. Conserved motifs within the NBS-ARC domain are underlined in italic. Residues in bold represent the differences between *Rpi-vnt1.1* and *Rpi-vnt1.3* in the LRR domain.

Mapping of Tm2GH in the ultra dense genetic map of potato

As previously described, two groups of *Tm2*GH were found based on the degree of homology to the NBS3B sequence: NBS3B-like and non-NBS3B-like. The *Rpi-vnt1.1* and *Rpi-vnt1.3* candidate genes *Tm2*GH-vnt1b and *Tm2*GH-vnt1.9, respectively, were from the NBS3B-like group and segregated with resistance. In an attempt to determine the chromosomal positions of other *Tm2*GH belonging to the non-NBS3B-like sequences group, we used the primers ATG-Tm2-F combined with REV-C to develop *Tm2*GH specific markers in the diploid reference mapping population SHxRH, which was previously used to generate the ultra dense genetic map of potato (Isidore and van 2003; van Os et al. 2006). Following digestion of the PCR amplicons derived from SH, RH and 5 F1 progeny plants, with different restriction enzymes, we identified four enzymes to generate CAPS markers that segregated from RH (*Cfr*131, *Hin*1II, *Alu*I and *Hha*I) and one that segregated from SH (*HpyF10IV*). Subsequent mapping of these markers in the complete SHxRH mapping population positioned them at the bottom of chromosome 9, a region of the chromosome that is similar to the late blight *R* gene *Rpi-mcq1* (Figure 7a and b) (Lanfermeijer et al. 2003; Sliwka et al. 2006; Smilde et al. 2005; Trognitz and Trognitz 2005). The mapped fragments shared between 85 and 90% homology (DNA level) with *Rpi-vnt1.1*, *Rpi-vnt1.3* and *Tm-2*^{2} revealing the presence of a third *Tm2*^{2}*-like* cluster at the distal end of the long arm of the chromosome *9*. Alignment of mapped fragments shows that they all cluster with *Tm2*GHs in a different group than *Rpi-vnt1.1* and *Rpi-vnt1.3*.Therefore *Rpi-mcq1* is likely to be a *Tm2*GH as well.

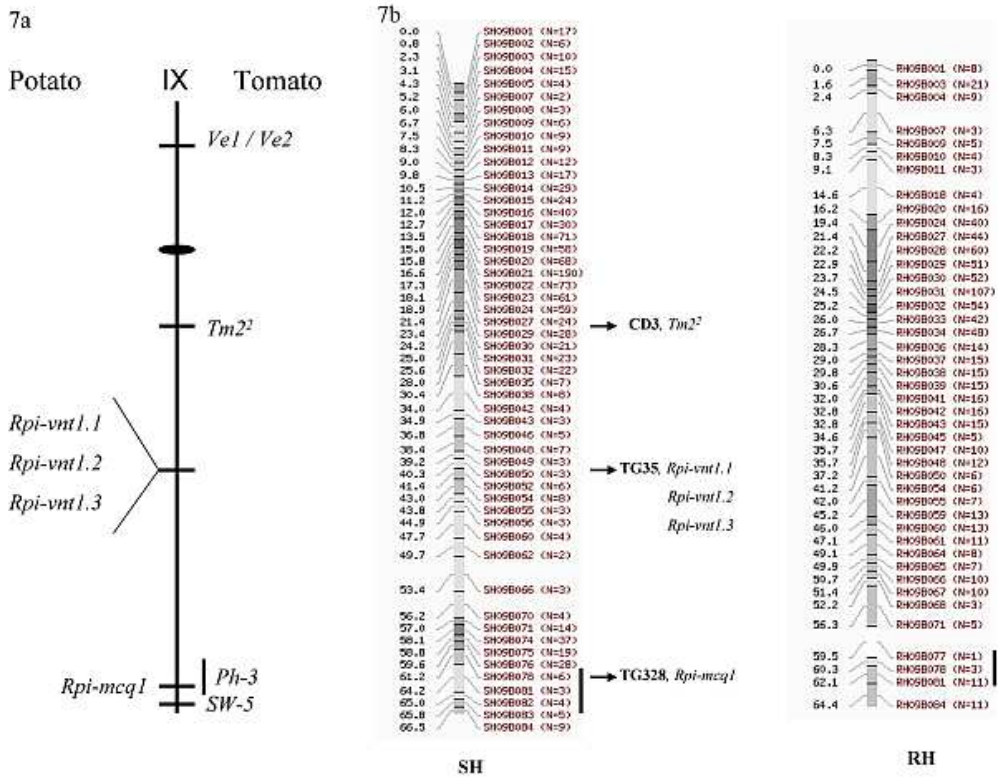


Figure 7. Relative genetic positions of *Tm2*GH in potato and tomato. **A.** Schematic linkage maps of potato and tomato showing five resistance gene clusters. **B.** Ultra Dense potato genetic map of chromosome *9* divided in BINs from the parents SH and RH. The thick vertical bars show the location of *Tm2*GHs belonging to the non-NBS3B-like group in the genome of SH and RH. The distal end of the long arm of the chromosome *9* from SH and RH harbours *Tm2*GHs in BINs 78-82 and BINs 77-81, respectively. This region coincides with the late blight resistant loci *Rpi-mcq1* and *Ph-3* from potato and tomato, respectively.

DISCUSSION

Here we report on the cloning of two putatively allelic late blight *R* genes *Rpi-vnt1.1* and *Rpi-vnt1.3* from the wild potato species *S. venturii*, using a candidate gene allele mining approach. The candidate gene family was identified through NBS profiling (Van der Linden et al. 2004) in combination with a BSA approach. Sequences of NBS marker bands genetically linked to the resistance phenotypes suggested that the target genes were located in an area of the potato genome that harboured *R* gene homologues that were highly homologous to the tomato mosaic virus resistance (ToMV) gene *Tm-2*^{2} from tomato, which resides on the long arm of chromosome *9* (Ganal et al. 1989; Young et al. 1988). Putative genomic location of *Rpi-vnt1.1* and *Rpi-vnt1.3* allowed for the targeted selection of chromosome *9* specific markers (e.g. TG35 and/or TG551) to align both linkage groups to potato and tomato chromosome *9*. Subsequently, all publicly available *Tm-2*^{2} homologous sequences were aligned to design a set of primers with which we could PCR-amplify putatively full-length gene candidates from relevant resistant genotypes. Candidate genes that were genetically closely linked to the *R* loci of interest were cloned in between the promoter and terminator sequences of the recently cloned *Rpi-blb3* gene (Lokossou et al. 2009) and targeted for complementation analyses, either through *Agrobacterium* Transient Transformation Assays (ATTA) in *N. benthamiana* or by stable *Agrobacterium* mediated transformation of the susceptible potato cv. Desiree. Results of transient assays carried out with the initial truncated amplicons in *N. benthamiana* using the appropriate differential isolates, suggested that we had indeed cloned two functional *R* genes. However, transgenic Desiree plants transformed with the same gene constructs were fully susceptible to *P. infestans*, whereas those transformed with a 4.3 kb genomic fragment, which was subcloned by Foster and co-workers from a BAC clone that spanned the *Rpi-vnt1.1* locus (Foster et al. 2009), displayed the expected resistance spectrum. Alignment of the initial truncated amplicon sequences to those of the functional genomic fragment revealed the presence of an additional in frame ATG start codon 99 nt upstream of the start codon that was initially used as basis for the PCR-based allele mining experiments, suggesting that the functional Rpi-vnt1.1 protein had an extended N-terminus of 33 amino acids compared to Tm-2^2. Subsequent analysis of the full-length *Rpi-vnt1.1* and *Rpi-vnt1.3* genes from the relevant parental genotypes revealed that *Rpi-vnt1.3* contained an additional 42 nt insertion in the 5' extended region, compared to *Rpi-vnt1.1*. Both full length versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* were shown to confer resistance using transient and stable complementation

assays. Although the 5' truncated versions of *Rpi-vnt1.1* and *Rpi-vnt1.3* were not able to confer resistance when expressed as stable transgenes, they were able to induce significant levels of resistance when expressed transiently in ATTA experiments. We speculate that absence of the N-termini of the R proteins may be compensated by the relatively high expression inherent to ATTA experiments. Rpi-vnt1.1 and Rpi-vnt1.3 belong to the CC-NBS-LRR class of plant R proteins. The CC domain, typically containing two or more alpha helices, which interact to form a super coil structure, is involved in protein-protein interactions (Liu et al. 2006). In the case of R proteins, it seems that the CC domain is involved in downstream signaling rather than in recognition (Van der Biezen 2002; Warren et al. 1999). Alignment of several R protein sequences, recently lead to the identification of a conserved EDVID motif among CC-NBS-LRR proteins. This motif was shown to be required for interaction between the CC domain and NBS-ARC or LRR domain (Rairdan et al. 2008). Moreover, this interaction is dependent on a wild type P-loop motif (Moffett et al. 2002a). A model, proposed by Takken and co-workers (2006), suggests that the CC domain plays a role as an interactive platform for downstream signaling partners. Upon binding of ATP to the NB domain, a conformational change occurs in the CC domain, which releases the LRR's signaling potential. Although the CC domain plays a role in downstream signaling, it cannot trigger the HR response on its own. Another model proposed by Moffett and coworkers (2002), suggests that the CC domain may bind to an effector molecule, which is released upon conformational changes within the R protein leading to the activation of downstream partners. The N-termini of Rpi-vnt1.1 an Rpi-vnt1.3 harbor 4 putative alpha helices that could interact to form two dimers. The first helice is present in the additional 99nt upstream of the start codon that corresponds to the start codon of *Tm-2*^{2} gene. The truncated versions of Rpi-vnt1.1 and Rpi-vnt1.3 lack this first helice, thereby destabilizing a possible interaction of the CC domain with the NB or LRR domain leading to less efficient downstream signaling. Over expression of the truncated protein may override the negative effect of destabilized intramolecular interactions and thus only have minor effects on the triggering of the HR response upon pathogen attack. This is in line with previous findings that over expression of functional *R* genes, e.g. *Rx*, *RPM1* and *RPS2*, using the 35S promoter, can lead to cell death responses even in the absence of the pathogen (Belkhadir et al. 2004; Tao et al. 2000).

Complementation analysis of candidate *R* genes is usually done by stable transformation of susceptible potato cultivars. This approach is time consuming, requiring several months to confirm the function of the candidate genes. In case of an allele mining approach, one can expect to identify many paralogous candidate resistant genes, which calls for a quick and

efficient complementation assay. Previous studies reported that resistance observed on *N. benthamiana* to *P. infestans* was mediated by the recognition of the elicitor protein INF1 (Kamoun et al. 1998). A transgenic *P. infestans* line, engineered to silence INF1 (West et al. 1999), was shown to be virulent on *N. benthamiana*. However, recent screens with a diverse set of *P. infestans* isolates showed that most isolates are in fact able to infect *N. benthamiana* (Rietman et al., in preparation). Previous studies, focused on potato-*Phytophthora* gene-for-gene interactions, showed that co-infiltration of cognate avirulence (*Avr*) and *R* genes in *N. benthamiana* leaves leads to the triggering of an HR response typical for incompatible interactions (Bos et al. 2006), indicating that all the necessary *P. infestans* resistance signaling components are present in *N. benthamiana.* We have exploited these findings to develop a transient complementation assay for late blight *R* genes using ATTA in combination with detached leaf assays. Despite the successful use of the transient assay in the cloning of the functional *Rpi-blb1* orthologs *Rpi-sto1* and *Rpi-pta1* (Vleeshouwers et al. 2008), the results obtained with the truncated *Rpi-vnt1.1* and *Rpi-vnt1.3* genes indicate that one needs to be careful with the interpretation of the results, and that stable transformation of a susceptible potato variety remains the ultimate functional assay of a gene.

Chromosomes *9* of tomato and potato seem to be hot spots for resistance as several *R* genes, conferring resistance to a broad range of pathogens, are located on this chromosome. In tomato, in addition to *Tm-2*^{2}, four other *R* genes have been mapped on chromosome *9*; *Sw5* at the distal end of the long arm, conferring resistance to tomato spotted wilt virus (Spassova et al. 2001), *Ve1* and *Ve2* conferring resistance to *Verticillium dahliae* (Simko et al. 2004), and *Frl* conferring resistance to *Clavibacter michiganensis* ssp. *michiganensis* (Vakalounakis et al. 1997). Interestingly, in tomato three major QTLs for resistance to *P. infestans* have been described, one of which, *Ph3*, is located at the distal end of the long arm of chromosome *9*) (Chunwongse and Black 2002). In potato, *Nx-Phu*, a potato gene for hypersensitive resistance to potato virus X (Tommiska et al. 1998c), the late blight *R* genes *Rpi-mcq1* (Smilde et al. 2005) and *Rpi-phu1* (Sliwka et al. 2006) reside on chromosome *9*. In the current study we mapped *Tm2*GHs at the distal end of the long arm of chromosome *9*, a region that coincides with the *Rpi-mcq1* locus (Figure 6), suggesting that these two genes are likely be *Tm2*GHs. *Rpi-phu1* is also likely to be *Tm2*GHs as it was mapped in the region harboring *Tm2GH* clusters. In addition to the above described *R* loci, several QTLs have also been described on chromosome *9* of potato, that confer foliage resistance to *P. infestans* (*Pin9a*), resistance to the root cyst nematode *Globodera pallida* (*Gpa6*), and tuber and leaf resistance to *Erwinia*

carotovora ssp *atroseptica* (*Eca9A*) (Gebhardt and Valkonen 2001). To what extent *Tm2*GH are also involved in quantitative resistance remains unclear.

Alignment of the Rpi-vnt1.1 and Rpi-vnt1.3 protein sequences to those of the currently cloned genes *R1, R3a, Rpi-blb1, Rpi-blb2* and *Rpi-blb3* (Ballvora et al. 2002; Huang et al. 2005); Lokossou et al. 2009), revealed very little homology to those genes, although all belong to the CC-NBS-LRR class of plant *R* genes. This is the first time that *Tm2*GHs are shown to be involved in late blight resistance. Alignment of the protein sequences of the chromosome *9* derived *R* genes (*Tm-2*^{2}, *Sw5a, Sw5b, Ve1*, and *Ve2*), suggest the presence of three distinct gene families, *Tm2*-like, *Sw5*-like, and Ve like. The *Ve* genes are located on the short arm of chromosome *9* whereas the *Sw5* genes are located at the distal end of the long arm (Figure 6). *Tm-2*^{2} and the cloned and/or mapped *Rpi* genes reside at three *Tm2*GH clusters that are spread across the long arm of chromosome *9* (Figure 6), suggesting that *Tm2*GH is the pre-dominant gene family on this chromosome arm. The overall homology between the identified *Tm2*GHs ranges between 74% to 99.8% amino acid identity, suggesting that they have a common origin and that they have arisen through gene duplication events. Gene duplication and sequence exchange between RGHs are major mechanisms that shape *R* gene diversity in plants (Kuang et al. 2004; Meyers et al. 1999; Michelmore and Meyers 1998). Comparison of the protein sequence of *Tm-2*^{2} with those of *Rpi-vnt1.1* and *Rpi-vnt1.3* revealed a higher homology within the CC and NB-ARC domains (86.5%) than the LRR domain (62%), which is in line with the fact that they confer resistance to completely different pathogens, ToMV and *P. infestans*, respectively, and that the LRR domain primarily determines recognition specificity and is thus subject to diversifying selection (Ellis et al. 2000). The functions of the CC and NBS domains, on the other hand, are likely to be more conserved, encompassing the fine tuning of intra-molecular interactions (Moffett et al. 2002) and the regulation of downstream signaling (Van der Biezen 2002; Warren et al. 1999; Belkhadir et al., 2004).

The rapid break down of the first introgressed *Rpi* genes from *S. demissum* previously stimulated breeders to reconsider their breeding goals and subsequently, efforts towards improving late blight resistance were focused on increasing partial resistance by using race-nonspecific sources of resistance. However, under long day conditions, breeders using this strategy have achieved little progress. The major draw-back being the correlation between foliage resistance and late foliage maturity. We nevertheless anticipate that breeding for late blight resistance in potato, aiming at substantially contributing to disease management, requires, by one way or another, the deployment of *Rpi* genes. As more and more *Rpi* genes are identified and cloned, the chances increase that new *Rpi* genes reside at known and well-

characterized loci, enabling the use of comparative genomics, and thus the development of efficient allele mining strategies. Moreover ongoing potato and tomato genome sequencing projects by international consortia are providing a (complete) survey of the distribution of *R* gene clusters in the *Solanaceae,* enabling even faster cloning of *Rpi* genes. Challenges that remain are how to predict *Rpi* gene durability and how to introduce durable combinations of *Rpi* genes into existing and future varieties in the most efficient and sustainable manner. We believe that knowledge of effector diversity may provide clues about the putative durability of *Rpi* genes (Birch et al., 2008; Vleeshouwers et al. 2008). The recent discovery of a common RXLR motif in oomycete Avr effector proteins (Birch et al. 2006; Rehmany et al. 2005) promises to accelerate the discovery and functional characterization of late blight *Rpi* genes and of *P. infestans* Avr genes and thus the engineering of durable late blight resistant potato varieties in the future. Efficient stacking of *Rpi* genes from one or several species is essential, but in practice this will enlarge the linkage drag problems considerably. Their introduction by genetic modification (GM) is a much more efficient way to improve resistance in one step and in a short period. It can even be applied to existing varieties with a long history of safe use. Currently, *Rpi* genes of natural origin derived from sexually compatible species, so called cisgenes (Schouten et al. 2006) can be introduced using marker-free transformation systems (Vetten et al. 2003) leading to cisgenic plants with only the gene(s) of interest and without linkage drag (Jacobsen and Schouten 2007). It implies an environmental benefit and will therefore hopefully meet the consumer acceptance.

MATERIALS AND METHODS

Plant material

Accessions of *S. venturii* were provided by the Centre of Genetic Resources (CGN) in Wageningen, The Netherlands. Following screening with *P. infestans*, resistant genotypes, vnt367-1 and vnt367-1, from accessions CGN18108 and CGN18000, respectively, were used to generate inter or intra-specific mapping populations. The *Rpi-vnt1.1* mapping population 7698 was generated by crossing [vnt7014-9 (resistant) (vnt367-1 (resistant) x vnt366-8(susceptible)) x nrs735-2(susceptible)]. The *Rpi-vnt1.3* mapping population 7663 was generated by crossing the resistant plant [vnt365-1(resistant) x nrs735-2(susceptible)]. The genes observed in CGN18108 and CGN18000 (initally called *Rpi-oka1* and *Rpi-nrs1*, respectively, unpublished results) were named after the species *S. okadae* and *S. neorossi*. DNA fingerprinting of a wide range of wild *Solanum* species however showed that the genebank accessions CGN18108 and CGN18000 clustered with other accessions of *S. venturii* (Jacobs et al. 2008). Morphological inspection of the CGN18108 and CGN18000 accessions confirmed that the labels *S. okadae* and *S. neorossi* should indeed be replaced with the label *S. venturii*. Furthermore, all available genebank accessions of *S. okadae* and *S. neorossi* were susceptible and did not amplify a PCR product with our *Rpi-vnt1.1/1.3* specific primers (manuscript in preparation, Pel et al.).

Disease assays and Agroinfiltration

Detached leaf assays (DLA) on the *Solanum* species were carried out as described by Vleeshouwers *et al*. (1999). Leaves were inoculated with 10µl droplets of inoculum ($5x10^4$ zoospores/ml) on the under side and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16h/8h day/night. At 6 days post inoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant.

Agrobacterium Transient Transformation Assays (ATTA) were performed in *N. benthamiana* followed by DLA's using appropriate *P. infestans* isolates. Four week old plants were infiltrated with a solution of *Agrobacterium tumefaciens* strain COR308 (Hamilton and Frary 1996), harboring putative *R* gene candidates. Agro-infiltration of the lower side of *N. benthamiana* leaves was carried according to (Van der Hoorn et al. 2000), using a OD_{600} of

0.1. Two days post infiltration, a DLA was performed as mentioned above. The phenotyping was done between 4 and 7 days post inoculation.
In ATTA assays, for each construct tested, three leaves of three different *N. benthamiana* plants were infiltrated with either *Tm2*GH-vnt1b (truncated *Rpi-vnt1.1* candidate gene), *Tm2*GH-vnt1.9 (truncated *Rpi-vnt1.3* candidate gene), *Rpi-sto1* (resistant control) or *abpt*-RGH-a (non-functional resistance gene). Assays were repeated three times. Infection efficiency was quantified by scoring 36 inoculation spots for each infiltrated constructs (three times one plant with three infiltrated leaves harbouring each 4 inoculated spots).

Marker development

Candidate gene markers were developed through NBS profiling as described by Van der Linden *et al*. (2004). Templates were generated by restriction digestion of genomic DNA using the restriction enzymes *MseI*, *HaeIII*, *AluI*, *RsaI* or *TaqI*. Adapters were ligated to restriction fragments. PCR fragments were generated by radioactive-labeled primers (nbs1, nbs2, nbs3, nbs5a6 or nbs9) designed on conserved domains of the NBS domain (P-loop, Kinase-2 and GLPL motifs ;(Calenge 2005; Syed and Sorensen 2006). Additional markers from appropriate chromosomal positions were selected from the Solanaceae Genomics Network (SGN) database and subsequently developed into polymorphic markers in each of the relevant mapping populations.

PCR amplification of candidate R genes

Long range PCR with Taq-polymerase or *Pfu* Turbo polymerase in a 50µl reaction-mixture was prepared containing 50ng of gDNA, 1µl of the forward primer (10µM), 1µl of the reverse primer (10µM), 0.8µl dNTPs (5mM each), 5µl 10X buffer, 5 units of Taq-polymerase (Perkin Elmer) or 1µl of *Pfu* Turbo (Invitrogen). The following PCR program was used: 94°C for 3 mins, 94°C for 30 sec, 55°C for 30 sec, 72°C for 4 mins, 72°C for 5 mins during 29 cycles.

Genome walking

Marker sequences were extended by cloning flanking DNA fragments with the ClonTech Genome Walker kit according to the manufacturer's instructions using a blunt adapter, comprising the complementary sequences 5'GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGA and 5'PO_4-TCCAGCCC and the adapter specific primers AP1

(TAATACGACTCACTATAGGGC) and AP2 (5'ACTATAGGGCACGCGTGGT). A simultaneous restriction-ligation was performed followed by two rounds of PCR. A 50µl restriction-ligation (RL) mixture was prepared containing 250ng of genomic DNA, 5 units of blunt cutting enzyme (*Bsh1236I*, *AluI*, *DpnI*, *HaeIII*, *RsaI*, *HincII*, *DraI*, *ScaI*, *HpaI* or *SspI*), 1µl genome walker adapter (25µM), 10mM ATP, 10µl of 5X RL buffer, 1 unit of T4 DNA ligase (Invitrogen 1U/µl). The digestion mix was incubated at 37°C for 3 hours. Samples were diluted 50 times prior to PCR. For the first PCR round, a 20µl reaction-mixture was prepared containing 5µl of diluted RL DNA, 0.6µl specific forward primer 1 (10µM), 0.6µl AP1 (10µM), 0.8µl dNTPs (5mM each), 2µl 10X buffer (Perkin Elmer), 5 units *Taq* polymerase (Perkin Elmer). The first PCR was performed using the following cycle program: 30-sec at 94°C as denaturation step, 30-sec at 56°C as annealing step and 60-sec at 72°C as extension step. 35cycles were performed. A second PCR using the same conditions as the first one was performed using specific primer 2 and AP2 and 5µl of 50 times diluted product from the first PCR. 5µl of the second PCR product was checked on gel (1% agarose) and the largest amplicons were cloned into the pGEM®-T Easy Vector from Promega and sequenced.

Gateway® cloning of candidate R genes into a binary expression vector

The Gateway® cloning technique was used according to the manufacturer's instructions to efficiently clone candidate genes together with appropriate promoter and terminator sequences into the binary Gateway® vector pKGW-MGW. In plasmid pKGW the gateway cassette was exchanged against a multiple gateway cassette amplified from pDESTr4r3 resulting in pKGW-MGW. In this study we used the promoter and terminator of *Rpi-blb3* (Lokossou et al. 2009) which were cloned into the Gateway® pDONR vectors pDONRP4P1R and pDONRP2RP3, respectively, generating pENTR-Blb3P and pENTR-Blb3T. PCR amplicons generated with *Pfu* Turbo polymerase were cloned into pDONR221 generating pENTR-RGH clones, and subsequently cloned together with the *Rpi-blb3* promoter and terminator fragments into pKGW - MGW using the multiple Gateway® cloning kit (Invitrogen). The pENTR clones were made by carrying out a BP-Reaction II overnight (http://www.untergasser.com/lab/protocols/bp_gateway_reaction_ii_v1_0.shtml). DH5α competent cells (Invitrogen) were transformed by heat shock with 5µl of the BP Reaction II mixture. Cells were selected on LB medium containing 50mg/ml of Kanamycine. Colonies were checked for the presence of the relevant inserts by colony PCR. DNA of appropriate pENTR clones was extracted from *E. coli* and used to perform a multiple Gateway® LR cloning reaction to generate the final binary expression clones (pVNT1 and pNRS1)

(http://www.untergasser.com/lab/protocols/lr_multiple_gateway_reaction_v1_0.shtml). DH5α competent cells (Invitrogen) were transformed by heat shock with 5µl of the LR reaction mixture. Cells were selected on LB medium containing 100mg/ml of spectinomycine. Colonies were checked by PCR for the presence of the correct inserts. Positive colonies were grown overnight in LB medium supplemented with 100mg/ml of spectinomycine to extract the final expression vector. The final expression vector was transferred to *Agrobacterium tumefaciens* strain COR308 through electropration. Colonies were selected on LB medium supplemented with 100mg/ml of spectinomycine and 12.5mg/ml of tetracycline overnight at 30ºC.

Stable transformation

Binary vectors carrying *Tm2*GH-vnt1b, *Tm2GH-vnt1.9*, pSLJ21152, *Tm2*GH-vnt1.1FL or *Tm2*GH-vnt1.3FL were used for Agrobacterium mediated transformation in strain COR308. Internodia with a size of 2-5 mm were cut from explants of cv Desiree and transferred to R3B medium plates containing two sterile filter papers. The next day internodia were incubated for 5-10 mins in the bacteria solution, dried and transferred back to R3B plates and placed at 24ºC with a light period of 16h. Two days later, internodia were transferred to selective medium containing kanamycine (100 mg/l), zeatine (1 mg/l), claforan (200 mg/l) and vancomycine (200 mg/l). Every two weeks internodia were transferred to fresh selective medium. Green shoots emerging from calli were harvested and transferred to MS30 medium supplemented with kanamycine (100 mg/l) to regenerate plantlets.

5' Rapid Amplification of cDNA Ends

The GeneRacer™ Kit from Invitrogen was used according to the manufacturer's instructions to determine the 5' terminal structure of *Rpi-vnt1.1* and *Rpi-vnt1.3*. Two gene specific primers (GSP1-5race and GSP2-5race, Table 2b) were designed to perform a nested PCR using cDNA template from relevant parental genotypes and transgenic plants and High Fidelity Platinum® *Taq* DNA Polymerase (HT Biotechnology Ltd). PCR products were cloned into the pGEM®-T Easy Vector (Promega) and sequenced.

Sequencing

Cloned fragments or PCR products generated either with *Taq*-polymerase (Perkin Elmer) or *Pfu* Turbo polymerase (Invitrogen) were sequenced as follows: 10µl sequencing reaction

mixtures were made using 5µl of PCR product or 5ng of plasmid, 3µl of buffer, 1µl of DETT (Amersham) and 1µl of forward or reverse primer. The PCR program used was 25 cycles of 94ºC for 20sec, 50ºC for 15sec, 60ºC for 1min. The sequences were generated on ABI 3730XL sequencers.

Mapping of Tm2GHs in the ultra dense genetic map of potato

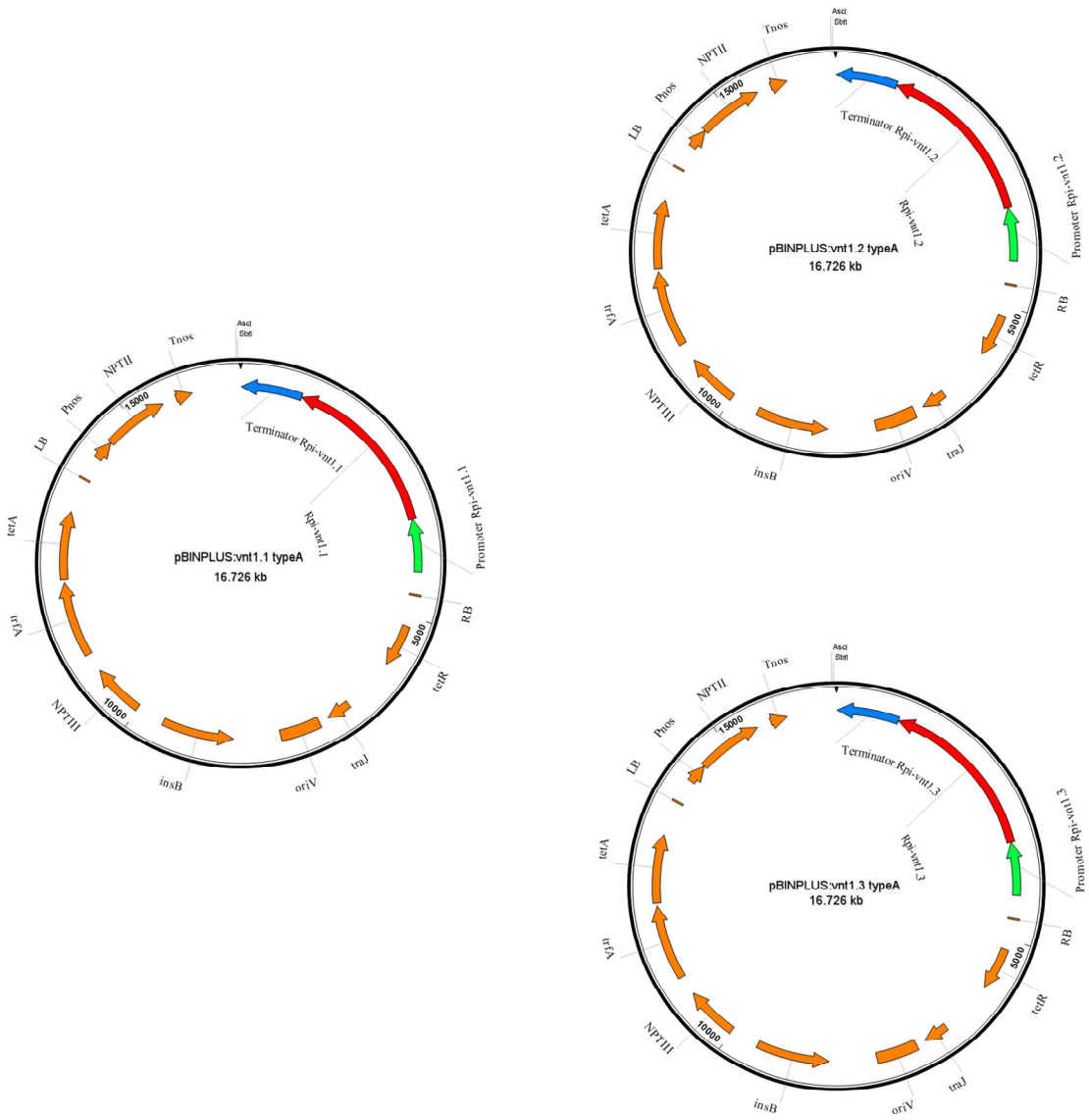
The Ultra Dense genetic map of potato (http://www.plantbreeding.wur.nl/potatomap), which was previously made with a mapping population consisting of 130 F1 progeny derived from a cross between the diploid potato genotypes SH83-92-488 (SH) and RH89-039-16 (RH) (Rouppe van der Voort J. N. A. M. 1997) was used to map *Tm2*GHs. Genomic DNA from the parents (SH and RH) were PCR amplified using the primers ATG-Tm2-F and Rev-C (Table 2). Amplification products were digested with restriction enzymes to detect polymorphisms between the parents. A sub-population of 50 F1 individuals was subsequently analysed through PCR amplification and appropriate restriction digestion. 20µl PCR reaction-mixtures contained 50ng of genomic DNA, 1µl of the forward primer (10µM), 1µl of the reverse primer (10µM), 0.8µl dNTPs (5mM each), 5µl 10X buffer, and 5 units of *Taq* polymerase (Perkin Elmer). The following PCR program was used: 94°C for 3mins, 94°C for 30 sec, 55°C for 30 sec, 72°C for 4mins, 72°C for 5mins during 29 cycles. 5µl of PCR product was digested at the required temperature for 3 hours with the appropriate restriction enzymes. Segregating polymorphisms were mapped in the ultra dense potato map, relative to 10.000 AFLP markers.

ACKNOWLEDGMENTS

We thank A. Untergasser for providing pKGW-MGW and Paul Birch for providing the *P. infestans* strain EC1. The presented work was financially supported by the FP6 programme BioExploit Food-CT-2005-513959.

CHAPTER 3

Rpi-vnt1.1, a *Tm-2*^{2} homologue from *Solanum venturii* confers resistance to potato late blight



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Mololecular Plant-Microbe Interactions (2009) 22:589-600.

Expression vectors carrying *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* genes

Rpi-vnt1.1, a *Tm-2*^{2} homologue from *Solanum venturii* confers resistance to potato late blight

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Nucleotide sequence data for the *Solanum venturii* late blight resistance genes *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* are available in the GenBank database under accession numbers FJ423044, FJ423045 and FJ423046 respectively.

ABSTRACT

Despite the efforts of breeders and the extensive use of fungicide control measures, late blight still remains a major threat to potato cultivation worldwide. The introduction of genetic resistance into cultivated potato is considered a valuable method to achieve durable resistance to late blight. Here we report the identification and cloning of *Rpi-vnt1.1,* a previously uncharacterised late blight resistance gene from *Solanum venturii*. The gene was identified by a classical genetic and physical mapping approach and encodes a CC-NB-LRR protein with high similarity to *Tm-2*^{2} from *S. lycopersicum* which confers resistance against *Tomato mosaic virus* (ToMV). Transgenic potato and tomato plants carrying *Rpi-vnt1.1* were shown to be resistant to *Phytophthora infestans*. Of 11 *P. infestans* isolates tested only isolate EC1 from Ecuador was able to overcome *Rpi-vnt1.1* and cause disease on the inoculated plants. Alleles of *Rpi-vnt1.1* (*Rpi-vnt1.2* and *Rpi-vnt1.3*) which differed by only a few nucleotides were found in other late blight resistant accessions of *S. venturii*. The late blight resistance gene *Rpi-phu1* from *S. phureja* (Śliwka et al. 2006) is shown here to be identical to *Rpi-vnt1.1,* suggesting either that this strong resistance gene has been maintained since a common ancestor, due to selection pressure for blight resistance, or that genetic exchange between *S. venturii* and *S. phureja* has occurred at some time.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is the fourth most important crop and the most important non-cereal food crop in the world (Lang 2001). The major biotic factor which limits potato crop yields is late blight caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary. Famous as the cause of the Great Irish Potato Famine of the mid 19th Century (Large 1940), this devastating disease can result in complete loss of crop yield unless effective control measures are applied (Świezyński and Zimnoch-Guzowska 2001). Fungicide treatment is currently the most common method for controlling late blight. However, sprays can be required every four days during severe epidemics and the high cost of fungicide application is a major burden to growers, especially in developing countries. Moreover, because fungicide application can impact on health and the environment, the use of many chemicals is becoming restricted. In addition, the pathogen evolves quickly and insensitivity/tolerance to commonly used fungicides can arise (Day and Shattock 1997; Goodwin 1997). Therefore, the introduction of genetic resistance into cultivated potato is considered a valuable method to achieve durable resistance to late blight.

Two main types of resistance to late blight have been described in potato, field resistance and resistance (*R*) gene-mediated resistance (Umaerus and Umaerus 1994). Field resistance (also referred to as general or quantitative resistance) is frequently attributed to major quantitative trait loci (QTL) and a few minor QTLs and generally results in partial resistance. Field resistance is considered by some to be more durable than resistance conferred by single *R* genes (Turkensteen 1993). However, partial resistance is also strongly correlated with maturity type and thus makes resistance breeding more difficult (Wastie 1991). Also the genetic positions of QTLs often correspond to regions of *R* gene clusters (Gebhardt and Valkonen 2001; Grube et al. 2000) raising the possibility that field resistance is due to the action of multiple weak *R* genes, which are also susceptible to defeat by the evolving pathogen. Specific resistance is based on major dominant *R* genes. In early breeding programs during the first half of the 20th Century, 11 *R* genes (*R1-R11*) were identified in *S. demissum*, a wild species originating from Mexico. The *S. demissum* genes R1, R3 and R10 have been heavily relied on for blight resistance in major breeding programmes within Europe since their introgression. As a result, the *R* genes introgressed from *S. demissum* to cultivated potatoes have been overcome as new pathogen strains evolve that are virulent on the previously resistant hosts (Fry and Goodwin 1997; Umaerus and Umaerus 1994; Wastie 1991). This ability of *P. infestans* to rapidly overcome *R* genes limits the durability of any

single *R* gene. Although some of the *S. demissum* genes such as R5, R8 and R9 have not been utilised in European cultivars, isolates of *P. infestans* which overcome these genes are known, albeit rare. However, it is possible that by deploying multiple *R* genes as a mixture in an otherwise genetically uniform crop, the ability of *P. infestans* to overcome these genes may be impaired (Jones 2001; Pink and Puddephat 1999).

Recent efforts to identify late blight resistance have focused on major *R* genes conferring broad-spectrum resistance derived from diverse wild *Solanum* species. Beside *S. demissum*, other wild *Solanum* species such as *S. acaule*, *S. chacoense*, *S. berthaultii*, *S. brevidens*, *S. bulbocastanum*, *S. microdontum*, *S. sparsipilum*, *S. spegazzinii*, *S., stoloniferum*, *S. sucrense*, *S. toralapanum*, *S. vernei* and *S. verrucosum* have been reported as new sources for resistance to late blight (reviewed by Hawkes (1990) and Jansky (2000).

Much progress has been made in the identification, mapping and cloning of *R* genes against late blight. Of the *S. demissum R* genes, *R3*, *R6* and *R7* have been mapped to a complex locus on chromosome XI (El-Kharbotly et al. 1994, 1996; Huang et al. 2004). The *R3* locus transpired to contain 2 distinct closely linked genes *R3a* and *R3b* (Huang et al. 2004, 2005). *R5*, *R8*, *R9*, *R10* and *R11* appear to be allelic versions of the *R3* locus (Huang 2005) although *R10* is reported to behave more like a QTL than a dominant *R* gene (Bradshaw et al. 2006a). *R1* has been mapped to chromosome V (El-Kharbotly et al. 1994; Leonards-Schippers et al. 1992) and *R2* to chromosome IV (Li et al. 1998). Of these, *R1* and *R3a* have been cloned (Ballvora et al. 2002; Huang et al. 2005). Of the resistances identified in wild *Solanum* species, three *R* genes, *RB*/*Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* have been mapped to chromosomes VIII, VI and IV, respectively (Park et al. 2005a; Van der Vossen et al. 2003, 2005). *Rpi-abpt*, probably from *S. bulbocastanum*, has been localized on chromosome IV (Park et al. 2005a). *Rpi1* from *S. pinnatisectum* on chromosome VII (Kuhl et al. 2001), *Rpi-mcq1* (formerly *Rpi-moc1*) from *S. mochiquense* (Smilde et al. 2005) and *Rpi-phu1* from *S. phureja* on chromosome IX (Śliwka et al. 2006) have also been identified. Thus far *Rpi-blb1* (*RB*) and *Rpi-blb2* are the only late blight *R* genes reported to have been successfully cloned from wild *Solanum* species (Song et al. 2003; Van der Vossen et al. 2003, 2005).

Here we report the identification and cloning of the chromosome IX *Rpi* gene *Rpi-vnt1.1*, a previously uncharacterised late blight resistance gene from *S. venturii*, an Argentinean wild species with an endosperm balance number (EBN) of 2. The gene was identified by a classical genetic and physical mapping approach and encodes a CC-NB-LRR protein with high

similarity to *Tm-2*^{2} from *S. lycopersicum* (formerly *Lycopersicon esculentum*) which confers resistance against *Tomato mosaic virus* (ToMV).

RESULTS

Variation for resistance to P. infestans in CGN accessions

Accessions of *S. venturii* and *S. okadae* were obtained from the Centre for Genetics Resources in Wageningen, the Netherlands (CGN). The *S. venturii* accessions were originally listed as *S. okadae* in the CGN database but have recently been re-classified based on work using AFLP markers to study the validity of species labels in Solanum section Petota (Jacobs 2008; Jacobs et al 2008). Species classification of accession CGN18279 is at present unclear as conflicting positions in the species dendrogram were obtained for this accession (Jacobs 2008). Screening of the accessions using *P. infestans* isolates 98.170.3 and 90128 in detached leaf assays showed phenotypic variation for resistance in four of the *S. venturii* accessions, *S. okadae* accession CGN18129 and the unclassified accession CGN18279. Resistance was evident as a complete lack of sporulation on leaf tissue whereas extensive mycelial growth was evident on leaves of sensitive individuals from 5-6 days post inoculation. Sensitive leaves often turned completely black by seven days post inoculation.All individuals tested from the remaining accessions were sensitive to isolates 90128 and 98.170.3, despite CGN data indicating that at least three of these accessions were moderately or very resistant to *P. infestans*.

Development of S. venturii mapping populations

Resistant individuals from five of the *S. venturii* accessions were crossed with sensitive individuals from either the same or different accessions (Table 2). In each of the crosses, resistance to *P. infestans* segregated 1:1 in the resulting progeny indicating the presence of potentially five *Rpi* genes in the resistant heterozygous parents (Table 2).

Table 2. Crosses within *S. venturii* and late blight resistant (R) and sensitive (S) segregants in their progenies

Population	Population parents[a]		Segregating progeny[b]		*Rpi* gene identified
	Female	Male	R	S	
Svnt014	A618, CGN18108, R	A613, CGN18108, S	26	24	*Rpi-vnt1.1*
Svnt012	A622, CGN18279, S	A618, CGN18108, R	18	23	*Rpi-vnt1.1*
Svnt013	A624, CGN18279, R	A613, CGN18108, S	18	25	*Rpi-vnt1.2*
Svnt040	A606, CGN17998, R	A628, CGN18279, S	25	23	*Rpi-vnt1.3*
Svnt241	D986, BGRC08237, R	B419, Svnt012, S	24	26	*Rpi-vnt1.4*
Svnt184	D403, CGN17999, R	D401, CGN17999, S	24	21	*Rpi-vnt1.5*

[a]Plant identifier number followed by its accession number and reaction to *P. infestans* inoculation: CGN Centre for Genetic Resources in the Netherlands; BGRC, Braunschweig Genetic Resources Center.

[b]Number of plants showing resistant (R) or sensitive (S) phenotypes

Mapping Rpi genes in S. venturii

Bulked segregant analysis (BSA; Michelmore et al. 1991) was carried out on DNA pools comprising DNA from each of 10 resistant and susceptible progeny from the populations Svnt014 (*Rpi-vnt1.1*), Svnt013 (*Rpi-vnt1.2*) and Svnt040 (*Rpi-vnt1.3*) to find AFLP markers linked to these resistance loci. A total of 72 primer combinations were tested for each population and potentially linked markers were confirmed by checking for co-segregation of the marker with the resistance gene in the individual progeny that made up the bulks. A number of linked AFLP markers were placed on the *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* linkage maps (Fig. 1), including SvntM2.9L (*Rpi-vnt1.1*), Svnt13M5.17, P12M44_103, P13M42_228 and P17M33_472 (*Rpi-vnt1.2*) and M6.44 (*Rpi-vnt1.3*). Of these, SvntM2.9L (6 cM from *Rpi-vnt1.1*), Svnt13M5.17 (6.5 cM from *Rpi-vnt1.2*) and M6.44 (23 cM from *Rpi-vnt1.3*) were successfully converted into PCR-based markers.

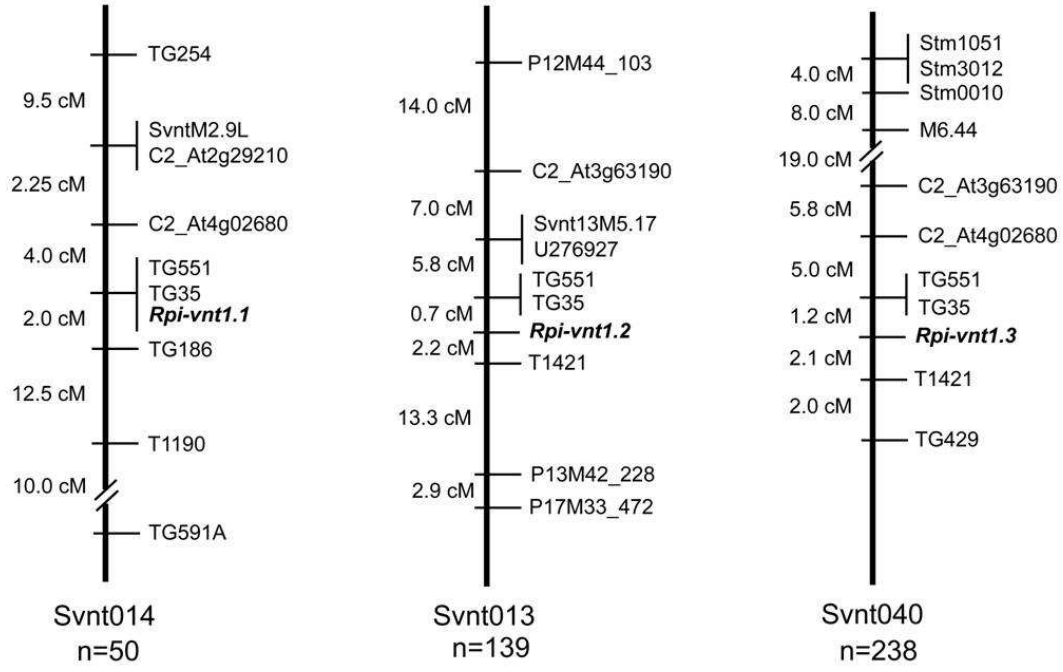

Figure. 1. Genetic linkage maps for *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* on chromosome IX of *Solanum venturii*. Marker names and genetic distance in cM are indicated on the left and right of each map, respectively. For each map, the number of individuals on which the mapping is based is given by n.

The *Rpi-vnt1.1* marker SvntM2.9L was also found to be polymorphic (by *Alu*I digestion) between *S. lycopersicum* (formerly *Lycopersicon esculentum*, Le) and *S. pennellii* (formerly

L. pennellii, Lp), the parents of the Le/Lp introgression lines (Eshed and Zamir 1994). Screening of the individual introgression lines (IL) showed that this marker was located in IL 9.2, indicating that the marker could be on either arm of chromosome IX. The polymorphism was not present in IL 9.1, which overlaps IL 9.2 substantially, suggesting that the marker was situated proximal to the centromere on either chromosome arm. Further evidence for the location of the *Rpi-vnt* locus on chromosome IX came from the finding that polymorphisms in three chromosome IX SSR markers (Stm0010, Stm 1051 and Stm 3012; (Milbourne et al. 1998)) were linked to *Rpi-vnt1.3* (Fig. 1).

Additional chromosome IX markers were developed by designing PCR primers from known chromosome IX RFLP marker sequences within the SGN database, sequencing the PCR products amplified from both resistant and sensitive parental DNA and identifying SNPs that could be used to develop cleaved amplified polymorphic sequence (CAPS) markers (Table 3). In this way, *Rpi-vnt1.1* was mapped to a 6.0 cM region of chromosome IX, delimited by markers C2_At4g02680 and TG186. *Rpi-vnt1.2* and *Rpi-vnt1.3* also mapped to the same location as *Rpi-vnt1.1* (Fig. 1). Although the markers TG551, TG35, T1421, C2_At3g63190 and C2_At4g02680 are bridging markers between the maps of at least two of the *Rpi* genes, the SNPs present between the parents of the respective populations were not identical (as illustrated by the restriction enzymes used to reveal the polymorphisms, Table 3), suggesting that each of the genes resides on a distinct haplotype. The markers TG551, TG35 and T1421 were also found to be polymorphic in the *Rpi-vnt1.4* and *Rpi-vnt1.5* populations. However, the SNPs present in the markers were identical to those of the *Rpi-vnt1.1* haplotype and thus we assumed that the haplotype present in *S. venturii* accessions BGRC08237 and CGN17999 was identical to that of CGN18108 from which *Rpi-vnt1.1* is derived.

Table 3. PCR based markers used for mapping of *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*

Marker	Primer sequence (5'-3')	Tm (°C)	Type of marker[a]		
			Rpi-vnt1.1	*Rpi-vnt1.2*	*Rpi-vnt1.3*
vntNBSHae	f: cttactttccctttcctcatcctcac	60	as	as	*Mae*III
	r: tgaagtcatcttccagaccgatg				
vnt1Long	f: agttatacaccctacattctactcg	60	as	as	as
	r: ctttgaaaagaggcttcatactccc				
vnt1ORF	f: gggctcgagcgaaataccagctaacaaaagatg[b]	60	as	as	as
	r: ggcggatcctagtacctgtgatattctcaactttgc[c]				
TG254	f: agtgcaccaagggtgtgac	60			
	r: aagtgcatgcctgtaatggc				
At2g38025	f: atgggcgctgcatgtttcgtg	55			*Tsp509*I [R]
	r: acacctttgttgaaagccatccc				
Stm1051	f: tccccttggcattttcttctcc	55			SSR
	r: tttagggtggggtgaggttgg				
Stm3012	f: caactcaaaccagaaggcaaa	55			SSR
	r: gagaaatgggcacaaaaaaca				
Stm0010	f: tccttatatggagcaagca	50			SSR [R]
	r: ccagtagataagtcatccca				
M6.44	f: attgaaagaatacacaaacatc	55			*Dde*I
	r: attcatgttcagatcgtttac				
At3g63190	f: ttggtgcagccgtatgacaaatcc	55		*Eco*RI	*Tsp509*I
	r: tccatcattatttggcgtcatacc				
SvntM2.9L	f: acaaacctatgttagcctcccacac	60	*Dde*I		
	r: ggcatcaagccaatgtcgtaaag				
At2g29210	f: agcaggacactcgattctctaataagc	55	*Nco*I		
	r: tgcactaagtagtaatgcccaaagctc				
Svnt13M5.17	f: ctgaggtgcagccaataac	55		as	
	r: ccagtgagaaacagcttctc				
U276927	f: gatgggcaacgatgttgttg	60		*Hpy188*I	
	r: gcattagtacagcgtcttggc				
At4g02680	f: gtgaagaaggtctacagaaagcag	55	*Mse*I		*Nhe*I
	r: gggcattaatgtagcaatcagc				
TG551	f: catatcctggaggtgttatgaatgc	60	*Mwo*I	*Taq*I	*Taq*I
	r: aactttcagatatgctctgcagtgg				
TG35	f: cacggagactaagattcagg	55	*Hha*I	*Alu*I	*Tsp509*I
	r: taaaggtgatgctgatgggg				
T1421	f: catcaattgatgcctttggacc	60		*Bsl*I	*Rsa*I
	r: ctgcatcagcttcttcctctgc				
TG186	f: aatcgtgcagtttcagcataagcg	60	*Dra*I [R]		
	r: tgcttccagttccgtgggattc				
TG429	f: catatggtgacgcctacag	55			*Mse*I
	r: ggagacattgtcacaagg				
T1190	f: gttcgcgttctcgttactgg	55	as		
	r: gttgcatggttgacatcagg				
TG591A	f: ctgcaaatctactcgtgcaag	60	as		
	r: ctcgtggattgagaaatccc				
266I9F	f: gtatgtttgagttagtcttcc	55		*Hinf*I	
	r: tataataggtgttcttgggg				

266I9R	f: aaggtgttgggagtttttag r: tatcttcctcattttggtgc	55	*Hind*III	*Hind*III
185L21R	f: gattgagacaatgctagtcc r: agaagcagtcaatagtgattg	55	*Bsl*I	*Rsa*I
148P20R	f: aagattctttttcctccttag r: aaagatgaagtagagttttgg	58	*Hpy*CH4IV	

[a] Restriction enzymes indicate that marker is a CAPS marker, as indicates allele-specific markers, [R] indicates that marker is linked in repulsion phase, SSR indicates that marker is a simple sequence repeat marker, blank indicates that the marker was either not polymorphic or not tested for that *Rpi* gene.

[b] Bold type indicates *Xho*I site included in 5' region for cloning purposes

[c] Bold type indicates *Bam*HI site included in 5' region for cloning purposes

Concurrent work by Pel et al (2009) used NBS-profiling (Van der Linden et al. 2004) to identify a candidate NBS marker (NBS3a) with homology to the ToMV *R* gene *Tm-2*^{2} (Lanfermeijer et al. 2003) which was closely linked to *Rpi-vnt1.1* in a population derived from the same *S. venturii* accession (CGN18108). From the sequence of this marker we designed allele-specific PCR primers (vntNBSHae-F/R, Table 3) and showed that this marker (vntNBSHae) co-segregated with *Rpi-vnt1.1* and *Rpi-vnt1.2* and mapped 1.2 cM distal to *Rpi-vnt1.3* (Fig. 1).

BAC library screening and contig construction

The *S. venturii* plant K39 which was used to construct the BAC library is a transheterozygote carrying both *Rpi-vnt1.1*, originally from the resistant parent plant A618 (accession CGN18108), and *Rpi-vnt1.2* from the resistant plant A624 (accession CGN18279). The *S. venturii* K39 BAC library consisted of 105,216 clones stored in 274 x 384-well microtiter plates. Insert size ranged from 60 to 165 Kb with an average of 103.5 Kb, based on pulsed-field gel analysis of *Not*I digested DNA from randomly selected clones. The haploid genome size of *S. venturii* is estimated to be about 1,000 Mb, therefore the library was predicted to represent a coverage of ~11 genome equivalents.

PCR markers TG551, TG35 and vntNBSHae linked to *Rpi-vnt1.1* and *Rpi-vnt1.2* were used to screen the *S. venturii* K39 BAC library. BAC clones identified using these markers were end sequenced and a contig was constructed using a BAC-end PCR strategy (Fig. 2). Marker vntNBSHae is an allele-specific marker linked to both *Rpi-vnt1.1* and *Rpi-vnt1.2* and consequently it was not possible to assign haplotype to the BAC clones identified using this marker. However, using many of the BAC-end markers, and the CAPS markers TG551 and TG35, BAC clones from the *Rpi-vnt1.1* and *Rpi-vnt1.2* haplotypes can be distinguished by

restriction digestion (Table 3). Two of the clones from the K39 library (K39_272N11 with *Rpi-vnt1.2* haplotype and K39_256M23 with *Rpi-vnt1.1* haplotype) had BAC-end sequences which were highly similar to each other and to the ToMV *R* gene *Tm-2*^{2} (Lanfermeijer et al. 2003) and were identical to the vntNBSHae marker.

As a parallel contig construction approach, BAC clones containing sequences homologous to the vntNBSHae marker were identified by hybridisation of the vntNBSHae probe to the pooled BAC DNA (274 pools of 384 clones each). A total of 67 pools were identified as containing BAC clones with homologous sequences. Clones from the positive pools were double-spotted at high density onto nylon membranes and hybridised with the vntNBSHae probe to identify individual BAC clones from the pools. A total of 85 BAC clones were identified. DNA from identified BACs was subjected to BAC SNaPshot fingerprinting (Luo et al. 2003), along with an additional 10 selected clones which were positive for the TG551 and/or TG35 markers. Analysis of the SNaPshot fingerprinting patterns using FPC v4.7 (Soderlund et al. 2000) showed that the BAC clones formed 9 distinct contigs containing between 1 to 22 clones. From the contigs generated, one contig contained the BAC clones identified both by PCR-based screening using the linked markers TG551, TG35 and vntNBSHae and also by hybridisation using the vntNBSHae marker as a probe (Fig. 2).

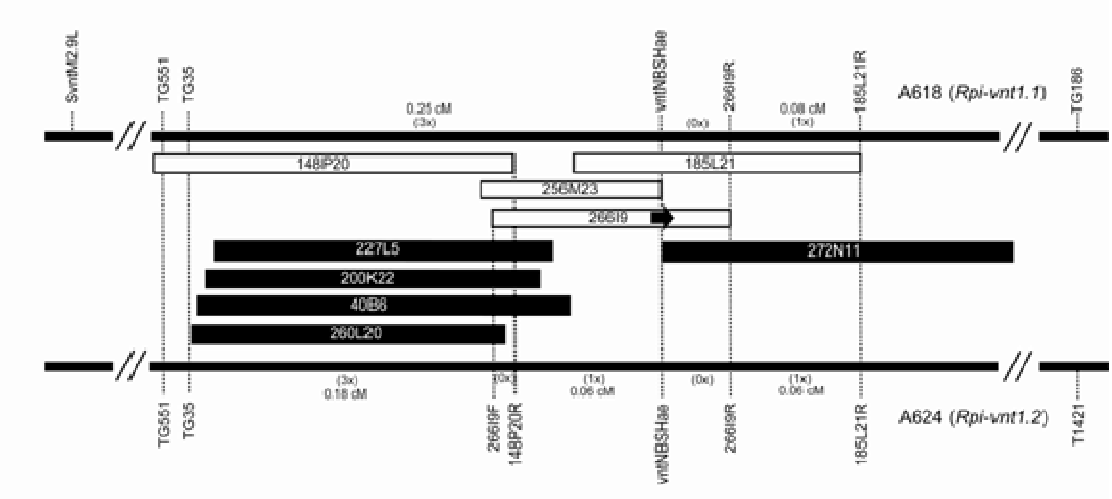

Figure. 2. High resolution genetic mapping and physical mapping of *Rpi-vnt1.1* and *Rpi-vnt1.2* using the K39 BAC library. Diagram shows the alignment of BAC clones from both the *Rpi-vnt1.1* and *Rpi-vnt1.2* haplotypes around the marker vntNBSHae which co-segregates with resistance. BAC clone 148P20 was identified using markers TG551 and TG35; clones 185L21, 256M23, 266I9 and 272N11 were identified using marker vntNBSHae as an allele-specific PCR marker and also as a hybridisation probe; clones 227L5, 200K22 and 40B6 were identified using the BAC end markers 266I9F and 148P20R; clone 260L20 was identified using the BAC end marker 266I9F. Open bars represent BAC clones from the *Rpi-vnt1.1* haplotype, black bars represent BAC clones from the *Rpi-vnt1.2* haplotype. The black arrow within BAC clone 266I9 illustrates the position of *Rpi-vnt1.1*. Vertical dotted lines indicate the positions of markers linked to resistance. Genetic distances are given in cM and the numbers in brackets represent the number of recombinants between markers. Markers SvntM2.9L, TG186 and T1421 are included to aid orientation of the physical map with the genetic map in Figure 1.

High resolution mapping and cloning of Rpi-vnt1.1, Rpi-vnt1.2 and Rpi-vnt1.3

PCR Primers were designed from BAC end sequences and used to amplify products from the parental genotypes of *Rpi-vnt1.1* and *Rpi-vnt1.2.* PCR products were sequenced and analysed for the presence of SNPs that allowed use of the PCR products as CAPS markers in the respective populations. Sixty recombinants between the closest flanking markers SvntM2.9L and TG186 were selected from the total *Rpi-vnt1.1* population of 1213 individuals and successfully converted BAC-end markers were used to construct a higher resolution genetic map for *Rpi-vnt1.1.* Similarly, 46 recombinants (between TG551 and T1421) from the *Rpi-vnt1.2* population of 1706 individuals were used to construct a higher resolution *Rpi-vnt1.2* genetic map. The positions of these markers in relation to marker vntNBSHae which co-segregates with both *Rpi-vnt1.1* and *Rpi-vnt1.2* are shown in Fig. 2.

Marker analysis indicated that *Rpi-vnt1.1* was located within a genetic interval of 0.33 cM delimited by the CAPS marker TG35 and the BAC end marker 185L21R (Fig. 2). By reference to the physical map constructed from PCR and SNaPshot fingerprinting analysis of BAC clones from the K39 library (Fig. 2) *Rpi-vnt1.1* was predicted to be present on a physical region covered by the BAC clones K39_148P20, K39_266I9 and K39_185L21 (Fig. 2). Low stringency Southern blotting of these clones revealed that a single fragment homologous to the vntNBSHae marker was present in both K39_266I9 and K39_185L21 indicating that there was potentially a single CC-NB-LRR gene located in the overlap region between these two clones. BAC clones K39_266I9 and K39_148P20 were sequenced and one candidate ORF was identified for *Rpi-vnt1.1* on K39_266I9. No candidate *R* genes were present on BAC clone K39_148P20.

Interestingly, 2 individuals from the pre-selected *Rpi-vnt1.1* recombinants were resistant despite genotyping results suggesting that they should be susceptible. The mapping data for these two genotypes suggests that there may be a second, closely-linked *R* gene located proximal to marker TG35.

High resolution mapping indicated that *Rpi-vnt1.2* was located within a genetic interval of 0.12 cM delimited by the BAC-end markers 266I9F and 185L21R (Fig. 2). By reference to the physical map constructed from PCR and fingerprinting analysis of BAC clones from the K39 library *Rpi-vnt1.2* was therefore predicted to be present on the same physical region as that identified for *Rpi-vnt1.1*. However, other than the BAC clone K39_272N11, one end of which contained a partial *Tm-2*^{2} homologue which was identical to the corresponding region in *Rpi-vnt1.1,* clones from the *Rpi-vnt1.2* haplotype which covered the same physical region

as *Rpi-vnt1.1* could not be identified within the library (Fig. 2). As an alternative approach, primers designed to amplify the complete *Rpi-vnt1.1* ORF (vnt1ORF-F/R; Table 3) were used to genotype the 5 recombinant individuals between markers TG551 and T1421 from the *Rpi-vnt1.2* population. The resulting PCR product co-segregated with resistance in the 3 resistant individuals from these recombinants. The PCR product amplified from plant A624, the resistant parent plant of the *Rpi-vnt1.2* population was cloned into pGEM-T Easy (Promega) and sequenced.

As *Rpi-vnt1.3* also mapped to the same genetic location as *Rpi-vnt1.1* and *Rpi-vnt1.2*, rather than initiate high-resolution mapping, the PCR primers designed against *Rpi-vnt1.1* were employed to amplify *Rpi-vnt1.3* candidates. The resulting PCR products were cloned into pGEM-T Easy (Promega) and sequenced. To confirm that the sequenced PCR product corresponded to *Rpi-vnt1.3*, seven recombinants between markers TG35 and T1421 (Fig. 1) were assessed by PCR. The candidate PCR product was only amplified from the resistant recombinants.

Analysis of Rpi-vnt1.1 and Rpi-vnt1.2 and Rpi-vnt1.3

The *Rpi-vnt1.1* ORF is 2673 bp long and translates into a protein sequence of 891 amino acids with a calculated molecular weight of 102 kDa and a pI of 8.05. The *Rpi-vnt1.2* ORF comprises 2715 bp and translates into a protein sequence of 905 amino acids with a calculated molecular weight of 103.6 kDa and a pI of 8.16. The sequence of *Rpi-vnt1.2* differs from *Rpi-vnt1.1* by an insertion of 42 nucleotides in the 5' end of the gene (Fig. 3). The resulting additional 14 amino acids present in the corresponding region of Rpi-vnt1.2 do not affect any of the predicted coiled coil domains. There are also 3 single nucleotide polymorphisms (SNPs) between *Rpi-vnt1.1* and *Rpi-vnt1.2*; A1501T, T1767C and G2117A (Fig. 3). These nucleotide differences result in two amino acid differences between Rpi-vnt1.1 and Rpi-vnt1.2 (Fig. 3). The difference at position 501 is at the end of the NB-ARC domain, just prior to the LRR region and results in the change of an asparagine in Rpi-vnt1.1 to a tyrosine in Rpi-vnt1.2. This amino acid change does not affect any of the characterised NB-ARC domains. At position 706, within the 9th LRR, an arginine in Rpi-vnt1.1 becomes a lysine in Rpi-vnt1.2; both of these residues are positively charged polar amino acids and hence this can be considered a synonymous change.

The Rpi-vnt1.1 proteins contain all the features characteristic of the coiled coil-nucleotide binding region-leucine rich repeat (CC-NB-LRR)-class of resistance proteins. Within the first

215 (229 for Rpi-vnt1.2) amino acids of the N-terminal part of the protein were 4 regions each with 3 predicted heptad repeat motifs typical of coiled coil domains (Fig. 3). All NB-ARC domains (van der Biezen and Jones 1998) are present in the amino acid sequence from 216-505 (230-519 in Rpi-vnt1.2). Following the NB-ARC domain is a region comprising of a series of 15 irregular LRR motifs that could be aligned according to the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where L can be L, I, M, V, Y or F and x is any amino acid; McHale et al. 2006).

Rpi-vnt1.1 and *Rpi-vnt1.2* share 80.9% and 79.7%, identity, respectively, with *Tm-2*^{2} at the nucleic acid level. At the amino acid level, this translates to 72.1% and 71.1% identity, respectively. As expected, given its role in recognition specificity, the percentage of similarity was lowest in the LRR domain where *Rpi-vnt1.1/1.2* and *Tm-2*^{2} share only 57.5% similarity. In contrast, the sequence similarity across the coiled-coil and NB-ARC domains of *Rpi-vnt1.1/1.2* and *Tm-2*^{2} is 81.8% and 79.7%; notably, within the conserved domains of the NB-ARC region, *Tm-2*^{2} and *Rpi-vnt1.1* are identical.

The primers vnt1long-F and vnt1long-R (Table 3) were used to amplify *Rpi-vnt1.1* homologous sequences from the parental material containing *Rpi-vnt1.3*. Resulting PCR products were cloned into pGEM-T and sequenced. The sequences obtained were identical to *Rpi-vnt1.2* with the exception of a single SNP in the 5' insertion region, relative to *Rpi-vnt1.1*, which resulted in a phenylalanine to serine amino acid substitution (Fig. 3).

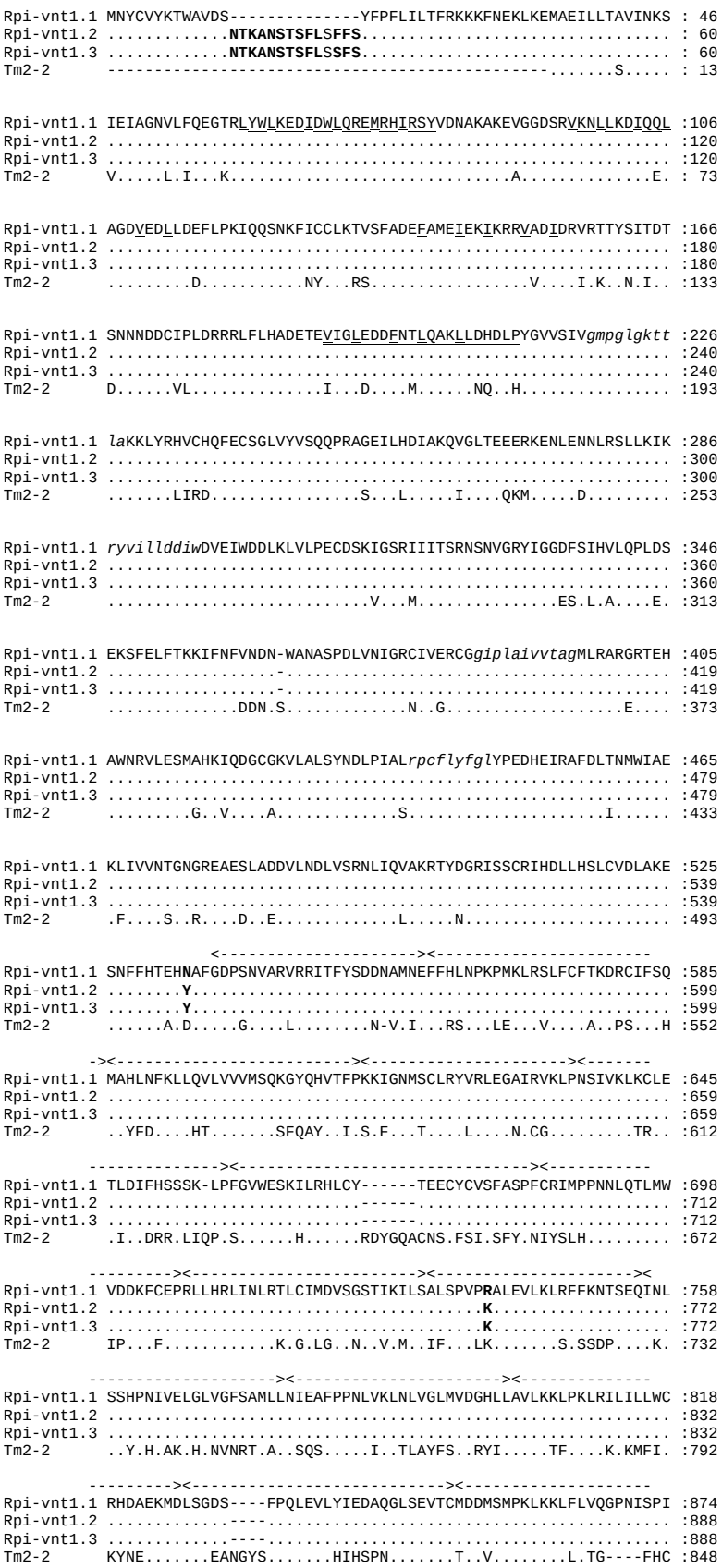

Figure. 3. Alignment of the deduced protein sequences of *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3* and *Tm-2*[2] (AAQ10736). The complete amino acid sequence of Rpi-vnt1.1 is shown and dots indicate identical residues in the other two proteins. Where residues from Rpi-vnt1.2 and Tm-2[2] differ from Rpi-vnt1.1, the residues in these proteins are given. Amino acid differences between Rpi-vnt1.1, Rpi-vnt1.2 and Rpi-vnt1.3 are indicated in bold type. Predicted coiled coil domains are underlined and the first and fourth hydrophobic residues of each heptad repeat are double-underlined. Conserved motifs within the NB-ARC domain are indicated in lower case italics. Putative leucine-rich repeats (LRRs) are indicated above the sequence line.

It was not possible to amplify full-length *Rpi-vnt1.1* paralogues from the susceptible *S. venturii* parent A613. This observation, together with the fact that the vntNBSHae marker could only be amplified from resistant genotypes could be taken to suggest that, at least in the susceptible parent used in this study, the susceptible phenotype is caused by an absence of *Rpi-vnt1.1* rather than a non-functional copy. An alternative explanation could be that the insertion of a transposable element into the susceptible allele has rendered it recalcitrant to PCR amplification or that sequence at the sites against which primers were designed is sufficiently different so as to prevent successful amplification.

Complementation analysis

Potato cultivar Desiree was transformed with plasmid pSLJ21152 which contained *Rpi-vnt1.1* under the control of its native promoter and terminator. A total of 37 *S. tuberosum* cv. Desiree plants capable of growth on kanamycin were selected as putative *Rpi-vnt1.1* transformants. Following transfer to the glasshouse, leaves from 29 plants were excised and used in a detached leaf assay with *P. infestans* isolates 90128 and BPC2006 3928A (super blight) to determine whether the transgene conferred blight resistance (Fig. 4). Of the 29 transformants tested, 24 were confirmed as being resistant and did not show any signs of blight infection. Some plants exhibited signs of a hypersensitive response localised to the inoculation site. The remaining 5 plants were susceptible to both isolates, as was the control (non-transformed Desiree). The phenotype of the transgenic plants correlated exactly with amplification of the *Rpi-vnt1.1* ORF by PCR; all plants from which *Rpi-vnt1.1* could be amplified were confirmed as resistant. Detached leaves of selected transgenic lines of potato cv. Desiree carrying *Rpi-vnt1.1* were inoculated with a range of *P. infestans* isolates to determine the range of isolates against which *Rpi-vnt1.1* confers resistance (Table 4). Of the 11 isolates tested, only isolate EC1 from Ecuador was able to overcome *Rpi-vnt1.1* and cause disease on the inoculated plants. The resistant parent from which *Rpi-vnt1.1* was isolated was also shown to be susceptible to isolate EC1, demonstrating that the specificity of *Rpi-vnt1.1* was retained in the transgenic plants and that the resistance phenotype was not due to constitutive activation of defence pathways by the transgene.

Transgenic tomato cv. Moneymaker plants carrying *Rpi-vnt1.1* were also shown to be resistant to *P. infestans* isolate 90128 (Fig. 4).

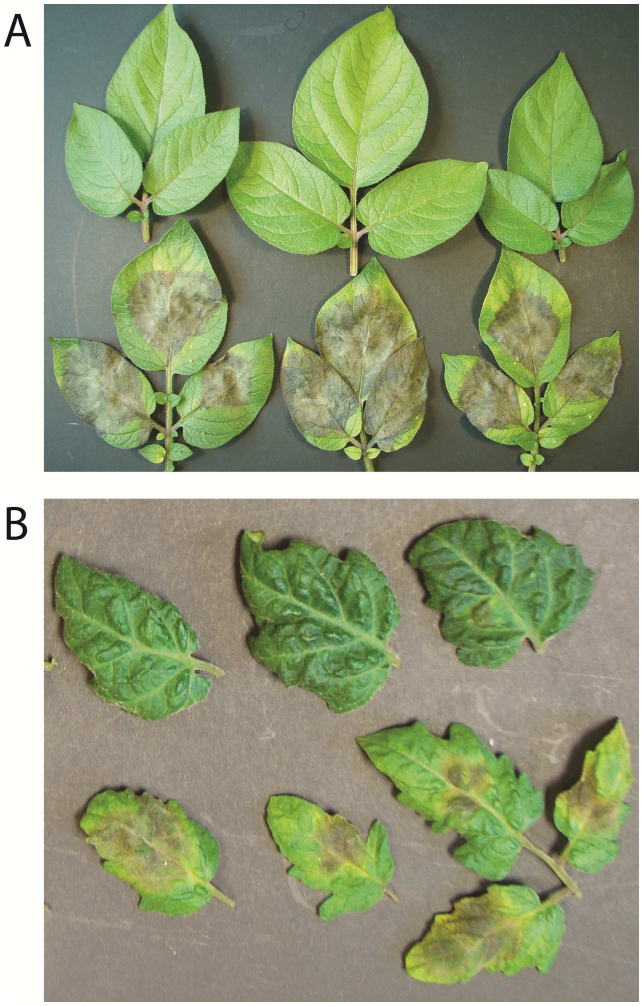

Figure. 4. *Rpi-vnt1.1* confers resistance to *P. infestans* upon transformation into susceptible potato and tomato cultivars. Detached leaves from plants of (A) *S. tuberosum* cv. Desiree containing (top) or lacking (bottom) *Rpi-vnt1.1* and (B) *S. lycopersicum* cv. Moneymaker containing (top) or lacking (bottom) *Rpi-vnt1.1* were inoculated at 5-6 points with 10 µl droplets of zoospore suspensions of *P. infestans* isolate 90128 and incubated at 16 °C, 16 h light/8 h dark. Photographs were taken 6 days post inoculation.

Rpi-vnt1.1 is also present in S. venturii accession CGN18000 and co-segregates with resistance in plants containing Rpi-phu1

We identified a *Rpi* gene in a segregating population derived from a resistant individual of the *Solanum* accession CGN18000 which also showed close linkage between the identified gene (originally named *Rpi-nrs1*) and marker TG551 and co-segregated with marker vntNBSHae, indicating that it was located in the same region as *Rpi-vnt1.1* (data not shown). The same genetic location for this gene was also determined by Pel at al (manuscript submitted). Although listed in the CGN collection as *S. neorossii*, AFLP fingerprinting work showed that accession CGN18000 clustered with accessions of *S. venturii* and not *S. neorossii* (Jacobs 2008; Jacobs et al 2008). Sequencing of a PCR product amplified using primers that amplify full-length *Rpi-vnt1.1* from resistant CGN18000 material showed that a sequence identical to *Rpi-vnt1.3* (i.e. with a single SNP in the 5' region compared with *Rpi-vnt1.2*) was present in this material and co-segregated with resistance in the CGN1800 mapping population. Resistant CGN18000 material also showed the same response to the panel of *P. infestans*

isolates as did *Rpi-vnt1.1*. Interestingly, genotypes from the CGN18000 mapping population were identified which were found not to contain *Rpi-vnt1.3* by PCR yet were resistant in detached leaf assays, suggesting the presence of a second, linked gene reflecting the situation with *Rpi-vnt1.1*.

Rpi-phu1 from *S. phureja* was also reported to map to this region (Śliwka et al. 2006). The *Rpi-vnt1.1* marker vntNBSHae-F/R co-segregated with resistance in a population of 148 diploid *S. tuberosum* plants segregating for *Rpi-phu1*. Full-length *Rpi-vnt1.1* paralogues were amplified from DNA of 3 resistant genotypes containing *Rpi-phu1*. In each case, a single PCR product was obtained and sequencing showed this to be identical to *Rpi-vnt1.1*. Resistant plant material containing *Rpi-phu1* was also shown to be resistant to the *P. infestans* isolates used in this study, with the exception of EC1.

DISCUSSION

Despite the efforts of breeders and the extensive use of fungicide control measures, late blight still remains a major threat to potato cultivation worldwide. The spread of new isolates, including the A2 mating type, from Mexico to Europe from 1984 onwards (Goodwin 1997) has resulted in a European late blight population which is capable of sexual reproduction and consequently has the potential for strains of the pathogen to evolve and overcome *R* genes present within current cultivated potato germplasm which were introgressed from *S. demissum* in the 1930s (Black et al. 1953; Malcolmson and Black 1966). Consequently there is a need to source new *R* genes and extend the repertoire available. The South American wild *Solanum* species are a valuable source of *R* genes which have potentially novel recognition specificities and thus may prove to be valuable tools in the fight against late blight (Bradshaw and Ramsay 2005). Here we have identified, mapped and cloned such a gene from *S. venturii* and shown that this gene can be used to transfer resistance to the susceptible cultivar Desiree.

Rpi-vnt1.1 was cloned using a genetic mapping and positional cloning approach. Bulked segregant analysis (Michelmore et al. 1991) together with AFLP (Vos et al. 1995) were proven to be a powerful combination of tools for efficiently mapping *Rpi-vnt1.1* and *Rpi-vnt1.2.* In addition, the tomato introgression lines (Eshed and Zamir 1994) were invaluable in allowing the rapid location of the chromosomal positions of these genes as was found previously for *Rpi-mcq1* (Smilde et al. 2005). *Rpi-vnt1.1* is located on the long arm of chromosome IX and shows highest similarity to *Tm-2*^{2} from *S. peruvianum* which confers resistance against ToMV and is located in a centromeric position on the long arm of chromosome IX (Lanfermeijer et al. 2003).

Both *Rpi-vnt1.1* and *Tm-2*^{2} belong to the largest family of *R* genes which contain a characteristic coiled coil (CC) domain in addition to the canonical NB-ARC and leucine-rich repeat (LRR) domain. The majority of *R* genes cloned from potato are members of this CC-NB-LRR family and all *Rpi* genes cloned thus far are of this class. A member of the TIR-NB-LRR family of *R* genes (characterised by the presence of a toll/interleukin 1 receptor (TIR) domain) has been shown to be responsible for resistance against the nematode *Globodera rostochiensis* in potato (Paal et al. 2004) but whether or not members of the TIR-NB-LRR family, confer resistance against *P. infestans* remains to be seen.

Mapping results from 2 recombinants within the *Rpi-vnt1.1* locus indicated that a second closely-linked *Rpi* gene may be located proximal to *Rpi-vnt1.1* in *S. venturii* accession CGN18108. This gene also confers resistance against *P. infestans* isolate 90128, but whether

this gene has exactly the same spectrum of recognition specificity as *Rpi-vnt1.1* remains to be seen. It was not possible to amplify either the marker vntNBSHae or the full length *Rpi-vnt1.1* ORF from these genotypes which suggests that the gene is somewhat different to *Rpi-vnt1.1* at the sequence level. Mapping using populations derived from these genotypes is required to investigate this gene further.

Although no other *R* genes were found within the 185 kb that comprised the BAC clones sequenced during the search for *Rpi-vnt1.1*, the presence of another closely-linked *R* gene or cluster of *R* genes would not be unprecedented. All of the *Rpi* genes cloned to date are members of complex loci comprised of *R* gene clusters. In the case of *Rpi-blb1*, *Rpi-blb2*, *R1* and *R3a*, the other *R* genes present in the clusters did not necessarily confer late blight resistance, although this possibility could not be ruled out (Ballvora et al. 2002; Huang et al. 2005; Song et al. 2003; van der Vossen et al. 2003, 2005). However, *R2*, *Rpi-blb3* and *Rpi-abpt* (all of which confer resistance against a different spectrum of races of *P. infestans*) are found together in a single complex locus on chromosome IV (Park et al. 2005a).

Although no paralogues are found in the immediate vicinity of *Rpi-vnt1.1*, chromosome IX harbours a number of *Tm-2*^{2} paralogues; in addition to *Rpi-vnt1.1*, a contig of BAC clones generated by SNaPshot fingerprinting of BACs from the K39 BAC library also contains paralogous sequences and maps to a region on the distal part of chromosome IX, in the region of marker TG591B (which is itself a *Tm-2*^{2} homologue). This contig contains an estimated 3 or 4 *Rpi-vnt1.1* paralogues according to Southern blotting results (data not shown). *Rpi-mcq1*, which has recently been shown to be a *Tm-2*^{2} homologue with high similarity to *Rpi-vnt1.1* also maps to this locus (Z. Chu, *unpublished data*). Thus it appears that there is a family of *Rpi-vnt1.1*/*Tm-2*^{2} paralogues distributed along the length of the long arm of chromosome IX. Additionally, screening of the *S. venturii* BAC library revealed the presence of at least 6 other contigs containing paralogues. The location of these on the genetic map is currently unknown.

A number of other resistance genes against pests and pathogens of potato and tomato are also found on chromosome IX. *Gm*, a gene which confers resistance against potato virus M (Marczewski et al. 2006) has recently been mapped to a region similar to that occupied by *Rpi-vnt1.1* but it is presently not sufficiently mapped to determine whether it occupies the same locus in *S. gourlayi*, the donor of *Gm*. Proximal to *Rpi-vnt1.1* and the aforementioned *Tm-2*^{2} are *Fr1* which confers resistance against *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato (Vakalounakis et al. 1997) and *Ve1* which confers resistance against *Verticillium dahliae* in both potato and tomato (Kawchuk et al. 2001). Distal to *Rpi-vnt1.1* are *Sw-5* which confers resistance against *Tomato spotted wilt virus* (TSWV, (Brommonschenkel et al. 2000;

Brommonschenkel and Tanksley 1997), *Nx* conferring resistance against *Potato virus X* (Tommiska et al. 1998) in *S. phureja*, and a QTL for resistance against the nematode *Globodera pallida* in potato designated *Gpa6* (Van der Voort et al. 2000). *Rpi-mcq1* (formerly *Rpi-moc1*; Smilde et al. 2005) lies distal to *Rpi-vnt1.1* at the end of the short arm of chromosome IX, in a similar region to that which *Ny-1*, conferring resistance to *Potato virus Potato virus* (PVY) has also been mapped (Szajko et al. 2008).

The late blight resistance gene *Rpi-phu1* (Śliwka et al. 2006) was mapped within a segregating diploid mapping population derived from complex *Solanum* hybrids of *S. tuberosum* with other wild species, including late blight resistant clones of *S. phureja* and *S. stenotomum.* This gene is shown here to be identical to *Rpi-vnt1.1*. Genotyping shows that *Rpi-vnt1.1* co-segregates with resistance in segregating progeny and the nucleotide sequence of an ORF amplified using the *Rpi-vnt1.1* specific primers is identical to *Rpi-vnt1.1*. This level of conservation is surprising although recent reports that highly conserved and putatively functional *Rpi-blb1* homologues have been found in *S. stoloniferum*, *S. polytrichon* and *S. papita* (Vleeshouwers et al. 2008; Wang et al. 2008) and also in *S. verrucosum* (Liu 2006) suggest that some *Rpi* genes belong to ancient loci that are conserved through the selection pressure imposed by *P. infestans*, or that genetic exchange (presumably infrequently) can occur between *Solanum* species. The fact that *Rpi-vnt1.1* and *Rpi-phu1* are identical suggests that the divergence event that led to the two species is relatively recent. This sequence conservation could also be a result of the fact that no other *Rpi-vnt1.1* paralogues are found within the immediate vicinity of *Rpi-vnt1.1*, thereby reducing the opportunity for sequence exchange which is thought to contribute to the evolution of *R* genes and appears to have been the mechanism by which *Rpi-blb1* arose by recombination between two paralogues (*RGA1-blb* and *RGA3-blb*) that reside in the same locus (van der Vossen et al. 2003).

The studies of Jacobs (2008) were not able to assign a species label to the accession CGN18279 due to it occupying conflicting positions within species dendrograms. However, the presence of an *Rpi* gene almost identical to *Rpi-vnt1.1* and the ability of plants from this accession to cross easily with *S. venturii* accessions indicate that it is most likely that CGN18279 is a *S. venturii* accession.

It was not possible to identify a BAC clone containing *Rpi-vnt1.2*. This may simply be due to the distribution of BAC clones in the library or it may be that neighbouring sequences in the *Rpi-vnt1.2* haplotype cause instabilities within the BAC vector that do not allow it to be propagated in *E. coli.* Due to the lack of a genomic clone of *Rpi-vnt1.2* we have not been able

to demonstrate that this gene confers resistance against *P. infestans* as we have for *Rpi-vnt1.1*. However, a number of lines of evidence lead us to postulate that *Rpi-vnt1.2* is responsible for the resistant phenotype of accession CGN18279. Firstly, mapping data presents conclusive evidence that *Rpi-vnt1.2* resides in the same genetic location as *Rpi-vnt1.1.* Secondly, the resistance proteins differ by only an insertion of 14 amino acids within the CC domain and a further 2 amino acids substitutions within the remainder of the protein; given that only one of these residues is in the LRR region which governs *R* gene specificity (Parniske et al. 1997), and that the substitution is synonymous with respect to physical properties, we expect that this gene is also functional and provides late blight resistance with the same specificity as *Rpi-vnt1.1*. We also show here that *Rpi-vnt1.3,* the amino acid sequence of which differs from Rpi-vnt1.2 at a single amino acid residue, maps to an identical region in the resistant *S. venturii* accession CGN18000. Pel et al (2009) have shown by both *Agrobacterium*-mediated transient assay in *Nicotiana benthamiana* and stable transformation of potato cultivar Desiree that this gene is functional and confers resistance against the same spectrum of *P. infestans* isolates as does *Rpi-vnt1.1*.

For a resistance gene to be of use in controlling late blight, it should be demonstrated that the resistance has an element of durability. Although we cannot determine durability of the *Rpi-vnt1.1* gene until it is deployed widely, information from tests against a wide range of isolates in Poland have shown that between 1999-2008, only one isolate (identified in 2008) capable of overcoming *Rpi-phu1* was found (Jadwiga Śliwka, unpublished data). Importantly, *Rpi-vnt1.1* confers resistance against an aggressive isolate (BPC2006 3928A) of genotype 13 known colloquially as superblight which is prevalent throughout the UK and is capable of overcoming the durable resistance present in the commercially valuable Stirling cultivar and which has been mapped to a QTL at the R2 locus on chromosome IV.

To introduce *Rpi* genes into cultivated varieties requires immense efforts by breeders. Potatoes are highly heterozygous out-breeding species and introgression of genes from wild *Solanum* relatives would require recurrent backcrosses to the cultivated parent which would result in inbreeding depression due to the selfing of the recurrent parent (Bradshaw and Ramsay 2005). Although this can be avoided by using different parents for the backcrossing, this would result in the loss of important agronomic and nutritional traits that had taken time and effort to select for. The use of genetic modification technologies to transfer single *Rpi* genes into cultivated varieties with combinations of traits that have taken considerable time and financial resources to develop is therefore attractive. Recently transgenic crops containing the *RB* (*Rpi-blb1*) gene from *S. bulbocastanum* were shown to be highly resistant to *P.*

infestans and had no negative effects on tuber size or yield, indicating that the expression of *Rpi* transgenes does not have a fitness cost or cause undesirable physiological traits (Halterman et al. 2008). We propose that the use of genetically uniform lines containing mixtures of *R* genes or, alternatively, mixtures of lines containing single *R* genes isolated from novel *Solanum* species is viable strategy for improving the resistance of current cultivated potato varieties and may provide a means to ensure that valuable *R* genes are not broken

MATERIALS AND METHODS

Solanum growing conditions

Seed of *Solanum* accessions (Table 1) was obtained from the Centre for Genetics Resources in Wageningen, the Netherlands (CGN). Seed was surface sterilised in 70 % ethanol for 1 minute, disinfected with 1.5 % hypochlorite for 5 minutes, rinsed 3 times in sterile distilled water and placed on solid MS (Murashige and Skoog) medium (2 % agarose) containing 3 % sucrose for germination. Germinated seedlings were transferred to glasshouse facilities and treated regularly with fungicides and pesticides to control thrips, aphids, spider mites, powdery mildew and early blight (*Alternaria solani*).

Phytophthora infestans strains, inoculation and pathogenicity scoring

P. infestans isolate 98.170.3 (race 1.3.4.10.11; Smilde et al. 2005) was provided by Dr David Shaw at Bangor University, UK. Isolates 90128, IPO-complex, IPO-0 and EC1 (Table 4) were provided by Dr Edwin van der Vossen at Plant Research International, Wageningen, The Netherlands. The BPC2006 3928A (superblight) isolate was provided by Dr Paul Birch, SCRI, Dundee, UK and is an isolate currently virulent on a large number of commercially grown potato cultivars in the UK and Europe. Isolates MP324 (Śliwka et al. 2006), MP717, MP778, MP674, MP622, MP618 and MP650 were obtained from IHAR, Poland.

Table 1. Reaction to *Phytophthora infestans* of *Solanum okadae* and *S. venturii* accessions used in this study

Accession[a]	Species[b]	Reference data		Fine screening[e]			
		Phenotype[d]	Source	R	MR	MS	S
CGN17998	*S. venturii*	Very resistant	CGN	2			7
CGN17999	*S. venturii*	Resistant	CGN	3			7
CGN18108	*S. venturii*	Very resistant	CGN	8		3	
CGN18109	*S. venturii*	Very resistant	CGN				10
CGN22703	*S. venturii*	Very sensitive	CGN	4	1		4
CGN18269	*S. venturii*	Sensitive	CGN				10
CGN18279	Unclassified	Very resistant	CGN	4			5
CGN18129	*S. okadae*	Sensitive	CGN		2	2	6
CGN20599	*S. okadae*	Sensitive	CGN				10
CGN18157	*S. okadae*	Moderately resistant	CGN				10
CGN22709	*S. okadae*	Very sensitive	CGN				8
BGRC27158	*S. okadae*[c]	Moderately resistant	CGN				1

[a]CGN, Centre for Genetic Resources in the Netherlands (http://www.cgn.wageningen-ur.nl); BGRC, Braunschweig Genetic Resources Center.

[b]Species designation according to Jacobs (2008).

[c]Was not included in the study of Jacobs (2008), hence original classification given.

[d]Blight resistance phenotype according to CGN data.

[e]Number of plants showing resistant (R) or sensitive (S) phenotypes.

Table 4. Response of transgenic potato plants containing *Rpi-vnt1.1* against a range of *P. infestans* isolates

Isolate	Country of Origin	Race	*Rpi-vnt1.1* phenotype
90128	The Netherlands	1.3.4.7.8.9.10.11	Resistant
IPO-0	Unknown	unknown	Resistant
IPO-Complex	Belgium	1.2.3.4.6.7.10.11	Resistant
BPC2006 3928A (superblight)	United Kingdom	unknown	Resistant
Hica	United Kingdom	unknown	Resistant
MP717	Poland	1.2.3.4.5.6.7.9.10.11	Resistant
MP778	Poland	1.3.4.5.6.7.9.10.11	Resistant
MP674	Poland	1.2.3.4.5.6.7.10.11	Resistant
MP622	Poland	1.3.4.7.8.10.11	Resistant
MP650	Poland	1.2.3.4.5.7.8.10.11	Resistant
EC1	Ecuador	2.4.10.11	Susceptible

The isolates were maintained at 18°C on Rye B agar (Caten and Jinks 1968). Fresh sporangia were produced in a two-weekly cycle by sub-culturing to fresh plates. Periodically, the ability of isolates to infect host material was confirmed on detached leaves of a suitable, sensitive plant. Mature, fresh sporangia were harvested after 10 days growth on Rye B medium by flooding the plate with sterile deionised water and allowing the harvested spore suspension to

stand for 20 minutes in a fresh Petri dish. After this time most sporangia are stuck to the plastic surfaces of the dish. Water from the original suspension was replaced by fresh cold water, the sporangia re-suspended and incubated at 4 °C for 1 to 4 hours to induce zoospore release.

A detached leaf assay was used to screen for resistance to *P. infestans* (modified from (Vleeshouwers et al. 1999). Two leaves per plant were detached, and placed onto moistened tissue paper in a 25 cm x 25 cm assay plate (Nunc, Rochester, NY; 6 genotypes/plate). Leaves were inoculated with 10 µl droplets of a zoospore suspension (50,000 zoospores ml^{-1}). Inoculated leaves were incubated for 7 to 12 days under controlled environmental conditions (16°C; 16 h light/8 h dark cycle) before scoring phenotypes. Plants with leaves showing sporulating lesions were scored as susceptible; plants with leaves showing no visible symptoms or limited necrosis in the absence of sporulation were scored as resistant. For unclear phenotypes, at least three independent inoculations were carried out. For clear phenotypes (either both leaves resistant or both sensitive), two independent rounds of inoculations were considered sufficient.

DNA isolation and sequencing

DNA was isolated from plant material using the protocol of Park et al. (2005b). BAC clone DNA was isolated using the Qiagen Midi Prep Kit (Qiagen, Hilden, Germany). BAC ends and PCR products were sequenced using the ABI PRISM Big Dye (v. 3.1) Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) according to the manufacturer's instructions.

AFLP and SSR analysis and PCR-based mapping

AFLP was performed essentially as described in Thomas et al. (1995) and Vos et al. (1995) on *Pst*I/*Mse*I-digested template DNA using a pre-amplification step with *Pst*I+0 and *Mse*I+1 primers and a selective amplification step using *Pst*I+2 and *Mse*I+3 primers. Informative AFLP bands were cut from the gel and rehydrated in TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). The gel slices were then transferred to fresh TE, crushed and the debris removed by centrifugation at 14000g for 1 min. For cloning, AFLP fragments were first re-amplified by PCR using 2 µl of the supernatant and the same primers as for the original amplification and cloned into pGEM-T Easy (Promega, Madison, Wisc.).

SSR PCR reactions were done in 25 µl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.4 mM each of dCTP, dTTP and dGTP, 0.012 mM non-labelled dATP, 370 kBq [□-^{33}P)]dATP (Amersham Biosciences, Bucks., UK), 0.4 µM of each primer, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and 100 ng template DNA. Thermal cycling conditions consisted of an initial denaturation step at 94 °C for 4 min, followed by a primer annealing step (either 50 °C or 55 °C depending upon the primer pair used; see Table 3) for 2 min and an extension step at 72 °C for 90s. Subsequent cycles were as follows: 29 cycles of 94 °C for 1 min, primer annealing temperature for 2 min, 72 °C for 90s, followed by a final extension step of 72 °C for 5 min. Amplification products were denatured by the addition of an equal amount of stop solution (95 % formamide containing bromophenol blue and xylene cyanol) and heated to 98 °C for 10 min. Two to five microlitres of the reaction were run on 6 % denaturing polyacrylamide gels containing 6 M urea at 100 W for 2-4 hours. Gels were dried and exposed to X-ray film as for AFLP reactions.

Conventional PCRs were done in□□□□µl reaction volumes containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM each dNTP, 0.4 µM of each primer, 0.5 U *Taq* polymerase and 10-100 ng template DNA. Thermal cycling conditions typically consisted of an initial denaturation step of 94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, primer annealing temperature (Table 3) for 30 s, 72 °C for 1 min per kb of amplified product followed by a final extension step of 72 °C for 10 min. For sequencing, primers and dNTPs were removed from PCR products by incubation with 0.1 U μl^{-1} Exonuclease I and 0.1 U μl^{-1} SAP at 37 °C for 30 min followed by incubation at 80 °C for 20 min to denature the enzymes. Long rang PCR reactions were done using Phusion High Fidelity DNA polymerase (Finnzymes Oy, Finland) according to the manufacturer's protocol.

Construction of a S. venturii BAC library containing Rpi-vnt1.1 and Rpi-vnt1.2

The *S. venturii* plant K39 which was used to construct the BAC library is a transheterozygote carrying both *Rpi-vnt1.1*, originally from the resistant parent plant A618 (accession CGN18108), and *Rpi-vnt1.2* from the resistant plant A624 (accession CGN18279).

Plant material was grown on Murashige and Skoog (MS) medium without sucrose *in vitro* and young leaf tissue was harvested and stored at -80 °C. Twenty grams of frozen leaf tissue was used to prepare DNA plugs containing high-molecular-weight (HMW) DNA using a method slightly modified from Liu and Whittier (1994) and Chalhoub et al. (2004). The DNA plugs were prepared in 0.7 % inCert agarose (Biozym, Oldendorf, Germany), washed in lysis buffer

solution (1 % sodium lauryl sarcosine, 0.2 mg/ml proteinase K and 3.8 mg/ml sodium diethyldithiocarbamate dissolved in 0.5 M EDTA, pH 8.5) and stored at 4 °C in 0.5 M EDTA until required. The stored plugs were soaked in TE buffer, chopped into small pieces and partially digested with 5 units of *Hind*III for 1 hour to generate DNA fragments of a size range 50-300 kb.

Triple size selection was used to improve the size and uniformity of the inserts as described in Chalhoub et al. (2004). The first size selection was performed on 1 % Seakem LE agarose (Biozym, Oldendorf, Germany) using clamped homogeneous electric field (CHEF) pulsed field gel electrophoresis (Bio-Rad, Hercules, USA) at 1-40 seconds, 120°, 16 hours and 200 V in 0.25x TBE buffer directly followed by the second size selection in the same gel at 4-5 seconds, 120°, 6 hours and 180 V in the same buffer. Agarose gel slices containing partially digested DNA between 100 and 200 Kb were excised and divided into two. For the third size selection, the excised gel slices were separately run on 1 % Sea Plaque GTG Low-melting point agarose (Biozym, Oldendorf, Germany) at 3-4.5 seconds, 120°, 14 hours and 180 V. Size-selected DNA fragments were excised from the gel and stored at 4 °C in 0.5 M EDTA (pH 8). DNA was recovered in 40 µl 1x TAE buffer by electro-elution using a Bio-Rad Electro-elution system (Bio-Rad, Hercules, USA).

The total eluted DNA was ligated in a 100 µl reaction with 10 ng pIndigoBAC-5 vector (EpiCentre Biotechnologies, Madison, USA) and 800 U T4 DNA ligase (New England Biolabs, Ipswich, USA). The ligation was dialysed against 0.5 x TE buffer for 3 hours using Millipore membrane (Millipore, Billerica, USA). Three microliters of dialysed ligation was used to transform DH10□ electrocompetent cells (Invitrogen, Paisley, UK) by electroporation. The BAC library was picked into 274 384-well microtiter plates (Genetix Ltd., Dorset, UK).

Transformation of S. tuberosum cv. Desiree with Rpi-vnt1.1

A 4.3kb fragment carrying the *Rpi-vnt1.1* promoter, open reading frame (ORF) and terminator was amplified by PCR using the primers vnt1long-F and vnt1long-R (Table 3) from the BAC clone K39_266I9. This fragment was cloned into pGEM-T Easy (Promega) and sequenced to confirm no mistakes had been introduced during PCR. The resulting plasmid was digested with *Eco*RI and the fragment containing the original gene cloned into the *Eco*RI site of pBin19. The resulting plasmid was named pSLJ21152. Plasmid pSLJ21152 was introduced into *Agrobacterium tumefaciens* strain AGL1.

Transformation of potato cultivar Desiree was carried out as described in Kumar et al. (1996). Transformation of tomato cv. Moneymaker was carried out as described in (Fillatti et al. 1987). Transgenic plants capable of growth on kanamycin were weaned out of tissue culture into sterile peat blocks before being transplanted to the glasshouse. PCR using primers vnt1ORF-F and vnt1ORF-R, which amplify the full-length *Rpi-vnt1.1* ORF (Table 3) was used to determine whether the kanamycin-resistant plants also harboured the *Rpi-vnt1.1* transgene.

ACKNOWLEDGEMENTS

The authors thank Sara Perkins for growing and maintaining plants, Matthew Smoker and Jodie Pike for generating transgenic plants and Robin Buell for sequencing BAC clones. SF was supported by grant BB/C007522/1 from the UK Biotechnology and Biological Sciences Research Council. We are indebted to Barbara Baker for funding from the National Science Foundation Plant Genome res. Program (grant DBI-0218166) which supported GB and the costs of the BAC sequencing. The Jones laboratory is funded by the Gatsby Charitable Foundation.

CHAPTER 4

The potato late blight resistance alleles *Rpi-vnt1.1, Rpi-vnt1.2* and *Rpi-vnt1.3* from *S. venturii* are not widely spread across *Solanum* section *Petota* and have evolved most probably by Illegitimate recombination

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Keywords

Late blight, Potato, Allele mining, Resistance gene

ABSTRACT

The *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* alleles located on chromosome *9* of the potato wild species *Solanum venturii* belong to the Coiled Coil Nucleotide Binding Site Leucine Rich Repeat (CC-NBS-LRR) class of plant *R*-genes. A collection containing 200 wild *Solanum* species with 5 genotypes each was screened. *Rpi-vnt1* alleles were found in *S. venturii*, as expected, *S. mochiquense* and *S. weberbaueri*. *Rpi-vnt1*-like homologs sharing between 80%-97% homology were cloned from 13 wild species belonging to the *Tuberosa* serie 16. Subsequent alignment of *Rpi-vnt1*-like homolog OPL753-8 with *Rpi-vnt1* alleles revealed the presence of illegitimate recombination (IR) signatures suggesting that two successive deletion events might have occurred in the CC domain. Meanwhile, the analysis of a Neighbor Joining tree, based on AFLP marker data (Jacobs 2008) from all the accessions carrying *Rpi-vnt1* alleles or *Rpi-vnt1*-like homologs, showed that *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* alleles were monophyletic. Signatures of illegitimate recombination and the monophyletic grouping of *Rpi-vnt1* alleles relative to potato accessions suggested how *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* may have evolved. A first IR might have occurred between *OPL753-8*-like homologs to generate *Rpi-vnt1.2* which might have served as template for a second IR generating *Rpi-vnt1.1*. Subsequently *Rpi-vnt1.3* diverged from *Rpi-vnt1.2* by one non synonymous nucleotide substitution. A novel late blight resistance gene that was named *Rpi-vnt2*, was identified in *S. venturii*. *Rpi-vnt2* confers resistance to the only virulent strain to *Rpi-vnt1*.

INTRODUCTION

Late blight disease, caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases on potato. Resistance to *Phytophthora infestans*, initially detected in *Solanum demissum*, was considered as the main resistance source (Black and Gallegly 1957; Malcolmson and Black 1966) as at least 11 specificities (*R1-R11*) have been identified. A comprehensive survey of wild tuber bearing *Solanum* species revealed that the genus *Solanum* harbors a wealth of late blight resistance sources. Several *R*-genes for late blight resistance have been cloned: *R1* (chromosome *5*) and *R3a* (chromosome *11*) from *S. demissum* (Ballvora et al. 2002; Huang et al. 2005), *Rpi-blb1, Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* on chromosomes *8, 6* and *4*, respectively (Naess et al. 2000; Van der Vossen et al. 2003, 2005; Lokossou et al. 2009), *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* from *S. venturii* on chromosome *9* (Foster et al. 2009; Pel et al. 2009), functional homologs of *Rpi-blb1*: *Rpi-sto1* and *Rpi-pta1* (Vleeshouwers et al. 2008), *R2*, *R2*-like and *Rpi-abpt* on chromosome *4* (Lokossou et al. 2009). All these resistance genes belong to the CC-NBS-LRR or Lz-NBS-LRR subfamily.

The NBS-LRR gene family is the most abundant in plant species. It was estimated that at least 200 different NBS-LRR genes are present in Arabidopsis representing up to 1% of its genome (Meyers et al. 1999). Characterization of 149 *R* loci from the *Columbia* ecotype of *Arabidopsis*, showed that NBS-LRR were often organized as clusters or singletons (Meyers et al. 2003). The NBS-LRR gene family has been subdivided into two subfamilies based on motifs located in the N terminal. One subfamily codes for a TIR (Toll-Interleukin receptor-like region) sharing homology to the *Drosophila Toll* and mammalian *Interleukin-1* receptors (Qureshi et al. 1999). The second subfamily codes for a coiled-coils (CC) structure near the N terminal which is sometimes in the form of a leucine zipper (Lz) (Baker et al. 1997; Lupas 1996; Pan et al. 2001; Pan et al. 2000).

Plants must evolve novel *R*-gene specificity in order to counteract the high rate of evolution of the so called effectors from the pathogen side. Genetic recombination between alleles or related sequences contributes to genetic variation among *R*-gene clusters. Recombination events by equal crossing over leads to domain swaps in the protein whereas unequal crossing over changes the number of genes present in the cluster (Leister 2004). Modification of the length of the open reading frame appears to be also a major contributor to *R*-gene diversification. Dixon and co-workers (1998) reported on deletion/expansion events involving individual LRR repeats in the *Cf-5* gene. Wicker and Yahiaoui (2007) demonstrated that such deletion/expansion was due to illegitimate recombination (IR) occurring between stretches of

1-10 base pairs. This asymmetric pairing followed by sequence exchange can result in either duplications or deletions. Such events generate IR signature or short repeat motifs that serve as template for recombination. Tracking IR signature can be used to study *R*-gene lineage.

Recently, the *Solanum* section *Petota* was subject to reclassification based on AFLP markers and cpDNA including 1000 accessions (Jacobs et al. 2008). A classification in 16 groups, based on genetic similarities, was achieved explaining more variation than the old species classification. Furthermore, mis- and over-classification was identified leading to change the nomenclature of accessions carrying *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* alleles (Foster et al. 2009; Pel et al 2009). Within section *Petota*, hybridization was shown to have played a significant role in the origin of certain taxa (Jacobs 2008). Along with hybridization, polyploidization is also regarded as a major event to generate new taxa (Hawkes 1990).

Allele mining studies enable to identify wild species or cultivars, with the same or different alleles of a given *R*-gene. From a breeding standpoint, the main aim to carry out such a survey, is to avoid redundant breeding efforts and select for the most crossable wild species harboring the *R*-gene of interest. However, distantly related wild species like *S. bulbocastanum* cannot be hybridized directly with *S. tuberosum* cultivars. For instance, Hermen et al. (1973) used *S. acaule* (2n=4x) and *S. phureja* (2n=2x) as bridging species. Wang et al. (2008) studied the frequency of *Rpi-Blb1* or RB within 47 species representing 13 series. Highly conserved *Rpi-Blb1* homologues were indentified in *S. stoloniferum* (*Rpi-sto1*), *S. papita* (*Rpi-pta1*) and *S. polytrichon* (*Rpi-plt1*) which are tetraploid species that belong to the serie 18 *Longipadicellata* (Hawkes 1990) and can be crossed with *S. tuberosum*. This offers the possibility to transfer late blight resistance genes into cultivars by introgression. From a more scientific standpoint, allele mining leads to identification of functional and non-functional homologs revealing the allelic diversity of a specific *R*-gene and thus helping to understand *R*-gene evolution. Based on the high homology between *Rpi-blb1*, *Rpi-sto1*, *Rpi-pta1* and *Rpi-plt1*, a similar cluster organization and previous cytogenetic studies (Hawkes 1990), *S. bulbocastanum* may be the wild ancestor of *S. stoloniferum* (Wang et al. 2008).

Here we report on the allele mining study of the potato late blight resistance allele *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* within *Petota* section. This study aimed to determine whether any other wild species carried *Rpi-vnt1* alleles, the allelic frequency and diversity of the former three resistance genes and whether *S. venturii* had some other *R* loci for late blight resistance. Subsequent cloning of *Rpi-vnt1*-like homologs is expected to give insights on the evolution of *Rpi-vnt1* alleles.

RESULTS

Screening of wild species

The wild potato species collection within the Center for BioSystems Genomics (CBSG), containing one thousand accessions which are each represented by five genotypes, was screened using two specific primer pair (A and B). Both primer pairs allowed amplification of PCR products from *Rpi-vnt1* alleles. Subsequent alignment of *Rpi-vnt1* alleles with $Tm2^2$ gene showed that the start codon of *Rpi-vnt1* alleles was 100bp upstream the one of $Tm2^2$ gene (Pel et al., 2009). The primer pair A, located in the 5' UTR of $Tm2^2$ gene was expected to be more stringent for $Tm2^2$-like homologs than the primer B designed on the conserved start codon of $Tm2^2$ gene.

Upon PCR using primer pair B, accessions from 14 wild species (*S. weberbaueri*, *S. mochiquense*, *S. venturii*, *S. orophilum*, *S. stenotomum*, *S. okadae*, *S. neorossii*, *S. raphanifolium*, *S. multidissectum*, *S. vernei*, *S. phureja*, *S. sucrense*, S. *microdontum gigantophyllum*, *S. tarijense* and *S. oplocense*) allowed the amplification of a PCR product with the expected size of 2.6kb. Only 8 accession from the previous set allowed the amplification of a PCR product with the expected size of 2.7kb using primer pair A (*S. weberbaueri*, *S. mochiquense*, S. *venturii*, *S. orophilum*, *S. stenotomum*, *S. multidissectum*, S. *microdontum gigantophyllum*, *S. tarijense* and *S. oplocense*). After cloning and sequencing, these accessions could be divided into two groups.

The first group (1) contained accessions from *S. venturii* and *S. weberbaueri* carrying either *Rpi-vnt1.1*, *Rpi-vnt1.2* or *Rpi-vnt1.3*. 10 out of 14, 1 out of 4 and 1 out of 4 accessions of *S. venturii*, *S. weberbaueri* and *S. mochiquense* respectively were found to carry one of the three *Rpi-vnt1* alleles. As shown in Table 1, *Rpi-vnt1.1* was found in 5 accessions (CGN18108, CPC7129, CGN 18109 GLKS32794 and CGN22703), *Rpi-vnt1.2* in 1 accession (CGN18129) and *Rpi-vnt1.3* in 4 accessions (CGN18000, CGN17998, CGN17999 and 961508). Interestingly, a fourth allele was detected in the accessions CGN22703, GLKS32794 and CGN17999. This allele shared 99.9% homology with *Rpi-vnt1.1* and contained an insertion of 4 nucleotides (TTCC) at position 436bp leading to a frame shift. This non-functional allele based on predicted translation, named "TTCC", was present in combination with either *Rpi-vnt1.1* or *Rpi-vnt1.3*. One late blight resistant genotype (969-2) from CGN22703 was found to

carry only the non-functional TTCC allele. This result suggested the presence of a different late blight resistance gene(s) than *Rpi-vnt1* alleles.

Specie	accession CGN	accession BGRC	accession CPC	accession GLKS	Taxonomic cluster[1]	CBSG phenotype (90128 strain)	CBSG genotype	*Rpi-vnt1* alleles	Homology with *Rpi-vnt1.1* (%, DNA level)	Homology with *Tm2*^{2} (%, DNA level)	Homology with Rpi-vnt1.1 (%, protein level)	Homology with Tm2^2 (%, protein level)	Primer pairs	Genbank number
S. venturii	18108				3	R	367-1	*Rpi-vnt1.1*	100	81.7	100	73.1	A + B	FJ423044
			7129		3	R	283-1	*Rpi-vnt1.1*	100	81.7	100	73.1		
	18109				3	R	366-1	*Rpi-vnt1.1*	100	82.7	100	73.1		
				32794	3	R	250-3	*Rpi-vnt1.1*	100	83.7	100	73.1		
	22703				3	R	969-3	*Rpi-vnt1.1*	100	84.7	100	73.1		
	22703				3	R	969-5	*Rpi-vnt1.1*	100	81.7	100	73.1		
	18279$				nd	R	741-1	*Rpi-vnt1.2*	98.3	80.5	98.2	72		FJ423045
	18279$				nd	R	741-2	*Rpi-vnt1.2*	98.3	80.5	98.2	72		
	18000				3	R	365-1	*Rpi-vnt1.3*	98.3	80.5	98.2	72		FJ423046
	961508				3	R	896-2/-3/-4	*Rpi-vnt1.3*	98.3	80.5	98.2	72		
	17998				3	R	368-1	*Rpi-vnt1.3*	98.3	80.5	98.2	72		
				32794	3	R	250-2	*Rpi-vnt1.1 - ttcc allele*	100/99.9	81.7/84.6	100/n.a.	73.1/n.a.		FJ423044 GU386358
	22703				3	R	969-1	*Rpi-vnt1.1 - ttcc allele*	100/99.9	81.7/84.6	100/n.a.	73.1/n.a.		
	22703				3	R	969-4	*Rpi-vnt1.1 - ttcc allele*	100/99.9	81.7/84.6	100/n.a.	73.1/n.a.		
	17999				3	R	740-1	*Rpi-vnt1.3 - ttcc allele*	100/99.9	81.7/84.6	100/n.a.	72/n.a.		
	22703				3	R	969-2	*ttcc* allele	99.9	84.6	-	-		GU386358
S. weberbaueri				159.1	5	R	253-2 / -4	*Rpi-vnt1.1*	100	81.7	100	73.1	A + B	FJ423044
			6032		5	R	300-2 /-3	-	86.3	85.3	74	67	B	GU338341
	18262				10	R	933-1	-	95	84.4	88.6	75.6	B	GU338343
	18262				10	S	933-2	-	90.2	84.2	-	-	B	GU338344
S. mochiquense				2319$	nd	R	186-1	*Rpi-vnt1.1*	100	81.7	100	73.1	A + B	FJ423044
S. okadae	22709				15	QR	745-1	-	95.2	84.6	88.8	75.5	B	GU338334
		27158			15	QR	742-2	-	89.2	85.2	74.7	73.3		GU338333
	20599				15	QR	970-3	-	86	85	70.9	72.7		GU338335
	20599				15	QR	970-4	-	89.3	85.3	74.9	73.4		GU338336
S. neorossii	18280				16	S	735-1	-	94.8	84.2	-	-	B	GU338331
					16	QR	735-4		91.6	84.2	-	-		GU338332
S. orophilum				5301	10	R	196-1	-	95.0	79.0	-	-	A + B	GU338319
				5301	10	S	196-2	-	92.6	79.1	-	-		GU338320
S. stenotonum^2	18161				-	S	829-2	-	92.4	79.1	-	-	A + B	GU338321
	18161				-	R	829-3	-	92.9	79.5	-	-		GU338323
S. raphanifolium				644	1	R	209-1	-	95.3	84.3	-	-	B	GU338338
S. oplocense	21352				16	R	750-4	-	92.9	79.5	88.4	75.4	A + B	GU338317
	21319				16	R	753-1	-	92.9	79.5	88.4	75.4		GU338318
S. multidissectum	17685				10	S	726-2	-	92.0	78.6	-	-	A + B	GU338313
	17685				10	R	727-4_1	-	92.6	79.3	-	-	A + B	GU338314
	17685				10	R	727-4_2	-	95.7	80.0	-	-	A + B	GU338315
	17840				10	S	731-2	-	91.8	84.4	-	-	B	GU338329
	17840				10	R	731-3	-	92.4	79.0	-	-	A + B	GU338316
	21344				10	S	722-1	-	92.1	84.8	-	-	B	GU338328
S. phureja^2			7909		-	RQ	772-4	-	92.8	84.5	-	-	B	GU338337
S. tarijense	18198				14	R	855-5	-	94.9	80.1	-	-	A + B	GU338325
	18107				14	R	868-1	-	94.8	80.0	-	-		GU338326
S. sucrense	20631				16	R	845-2	-	80.7	84.4	-	-	B	GU338339
S. microdontum gigantophyllum	18083				4	R	362-4	-	97	80.5	88.4	75.3	B	GU338312
S. vernei	18111				9	R	898-2	-	92.1	84.1	-	-	B	GU338327

Table 1. List of accessions of wild potato species in which *Rpi-vnt1* alleles or *Rpi-vnt1*-like homologs were identified using two specific primer pairs. Both primer pairs (A and B) contained the same reverse primer designed on the stop codon of *Rpi-vnt1* alleles combined with two different forward primers: one designed on the start codon of *Rpi-vnt1* alleles (primer pair A) and the other one on the conserved start codon of *Tm2*[2] gene (primer pair B). Accessions highlighted in bold were initially classified as *S. okadae* or *S. neorossii* and recently reclassified as *S. venturii* (Jacobs et al., 2008).

[1] = Based on Jacobs et al., 2008

[2] = not included in the phylogenetic study (Jacobs et al., 2008)

[$] = Accession removed from phylogenetic analysis, due to conflicting positions in dendrogram (Jacobs et al., 2008)

nd = non determined

Differences at the nucleic level can discriminate between *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3* and the non functional allele (Figure 1). *Rpi-vnt1.2* and *Rpi-vnt1.3* have an insertion of 42 nucleotides (nt) at the position 36bp. Within this insertion, *Rpi-vnt1.2* carries a thymidine (T) whereas *Rpi-vnt1.3* has a cytosine (C) at position 74bp. This SNP translates a phenylalanine (F) or a serine (S) for *Rpi-vnt1.2* and *Rpi-vnt1.3* respectively. In the LRR domain, three SNPs are present at 1646bp, 1912bp and 2202bp in two combinations: A/T/G or T/C/A for *Rpi-vnt1.1* or *Rpi-vnt1.2*/*1.3* respectively. Only the SNPs at the positions 1646 and 2202 bp generate different amino acids (a.a) which have similar chemical characteristics. As previously mentioned, the non functional TTCC allele has an insertion of four nucleotides, cctt, at position 436bp and a guanosine (G) at position 188.

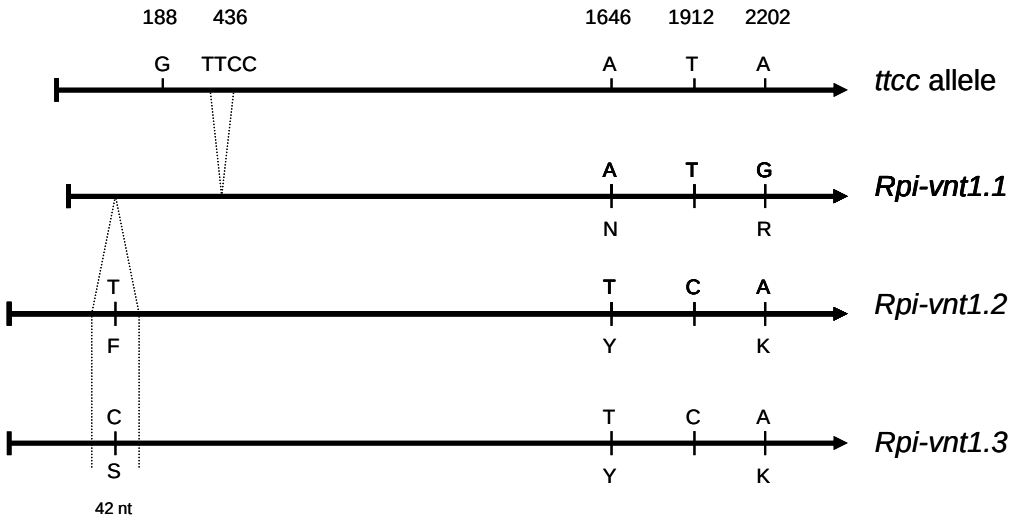


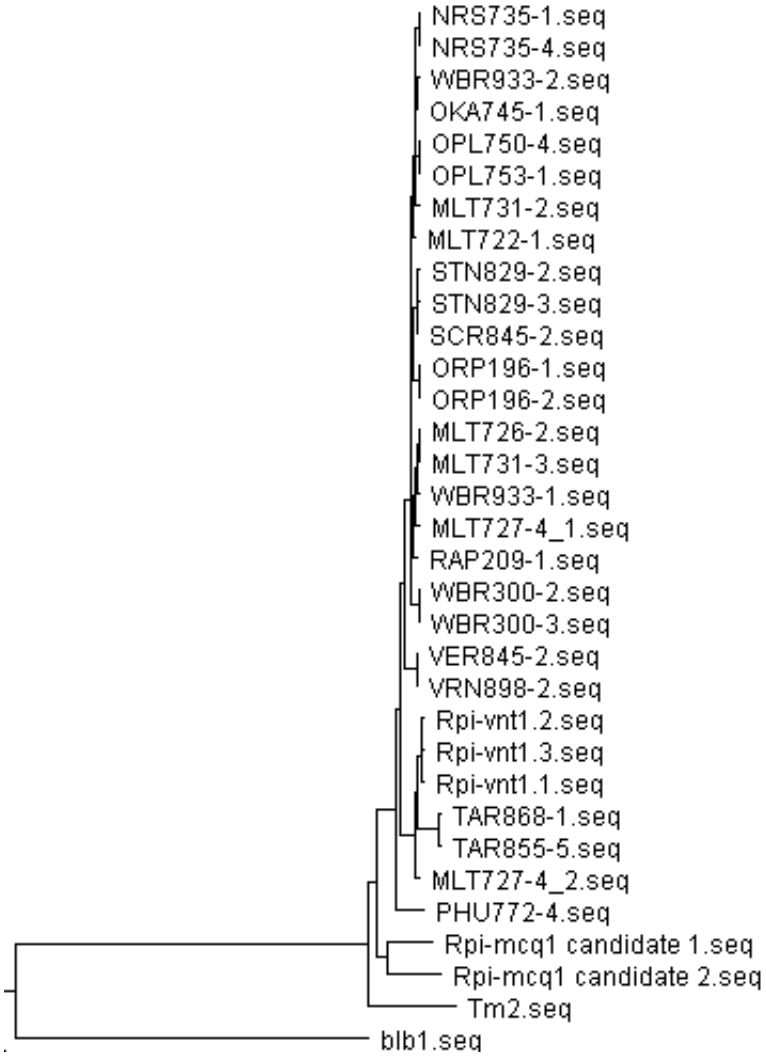
Figure 1. Scheme highlighting the SNPs and amino acids differences between the non functional allele *ttcc*, *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*. SNPs are located on each gene and amino acid differences under each gene. The non functional *ttcc* allele has a frame shit due to the insertion of four nucleotides (TTCC). *Rpi-vnt1.2* and *Rpi-vnt1.3* have an insertion of 42 nucleotide in the 5'end. A non synonymous SNP in this insertion allows to discriminate them.

The second (2) group contained accessions, from *S. weberbaueri*, *S. orophilum*, *S. stenotomum*, *S. okadae*, *S. neorossii*, *S. raphanifolium*, *S. multidissectum*, *S. vernei*, *S. phureja*, *S. sucrense*, S. *microdontum gigantophyllum*, *S. tarijense* and *S. oplocense*, carrying *Rpi-vnt1*-like homologs sharing between 80.7-97% homology (Table 1). Subsequent alignment using Clustal W showed that all the *Rpi-vnt1*-like sequences and *Rpi-vnt1* alleles clustered together whereas *Tm2*^{2} and *Rpi-mcq1* candidates 1 and 2 (Patent WO2009013468) belonged to a different clade (Figure 2a). This result demonstrated that the primer pairs A and B were specific to *Rpi-vnt1 R*-gene cluster. *Rpi-vnt1*-like homologs detected using the primer pair A were identical to those detected using the primer B within accessions from *S.*

weberbaueri, S. *venturii*, *S. orophilum*, *S. stenotomum*, *S. multidissectum*, S. *microdontum gigantophyllum*, *S. tarijense* and *S. oplocense*.

After sequence analysis, open reading frames (ORFs) with an homology between 70.9 and 88.8% at the protein level were found in 6 accessions from *S. microdontum gigantophyllum*, S. *weberbaueri*, *S. oplocense*, *S. okadae* and *S. neorossii*. Interestingly, only proteins from *S. weberbaueri* (WBR933-1) and *S. oplocense* (OPL753-8/OPL750-4, identical proteins) had the same LRR repeats determined in the Rpi-vnt1 proteins (Pel et al. 2009).

2a-



2b-

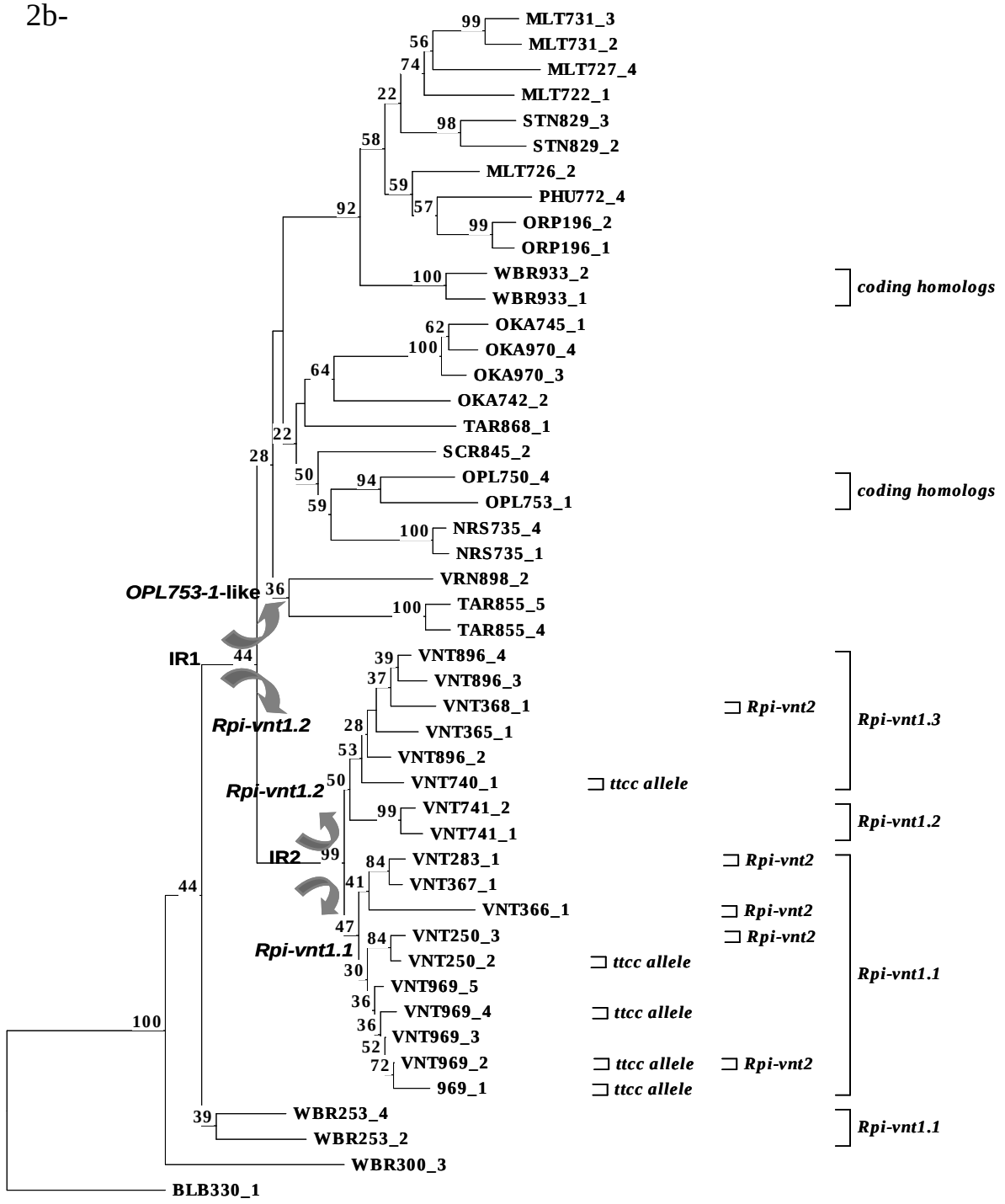


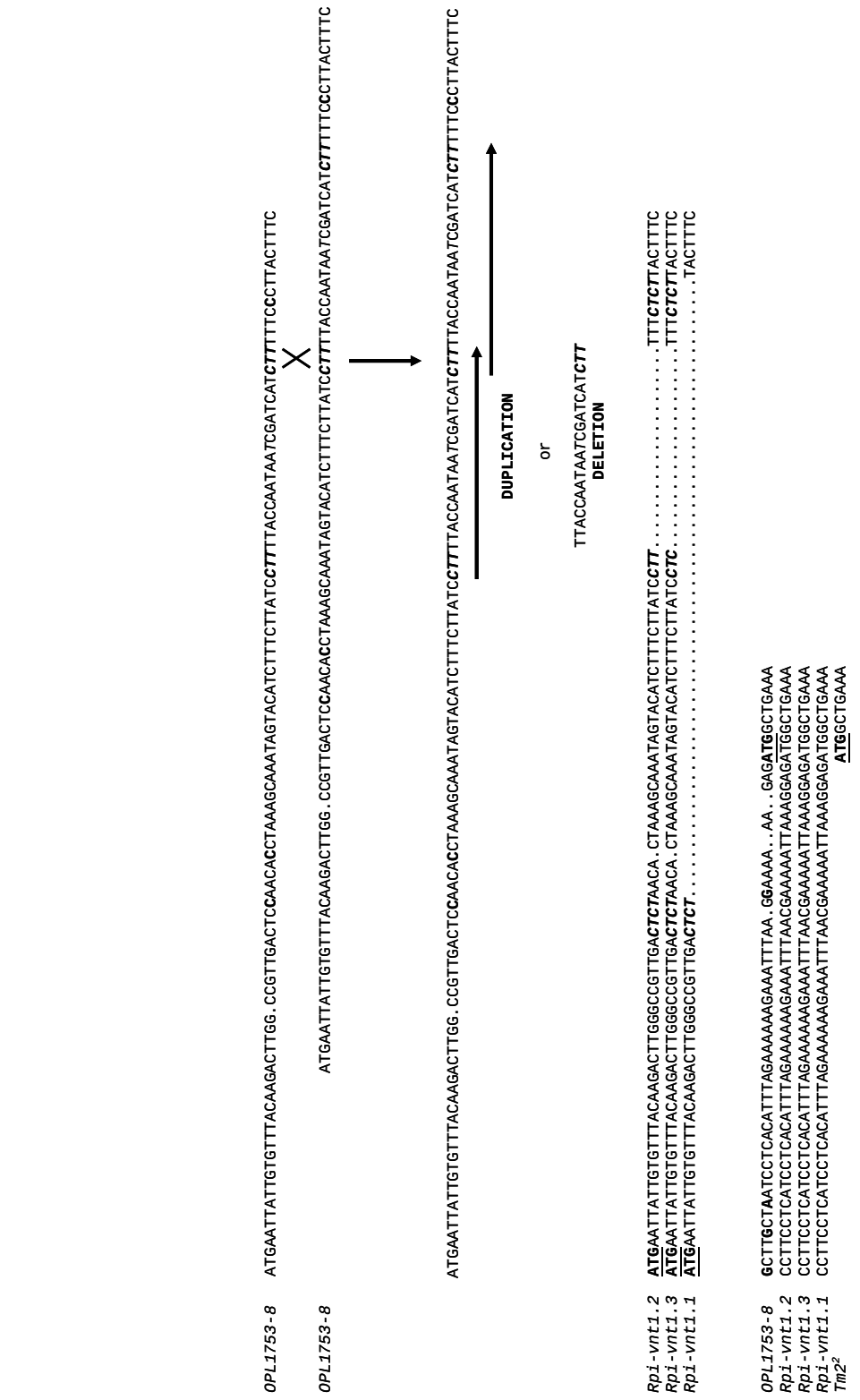
Figure 2. a- Tree based on a Clustal W alignment of *Rpi-vnt1* alleles, *Rpi-vnt1*-like homologs, *Tm2*^{2} and *Rpi-mcq1* candidates 1 and 2 using *Rpi-blb1* as an outer. The letter codes OKA, NRS, WBR, SCR, STN, ORP, MLT, RAP, VRN, GIG, TAR and PHU stand for *S. okadae*, *S. neorossii*, *S. weberbaueri*, *S. sucrense*, *S. stenotomum*, *S. orophilum*, *S. multidissectum*, *S. raphanifolium*, *S. vernei*, *S. microdontum gigantophyllum*, *S. tarijense* and *S. phureja* respectively. **b-** Taxonomic Neighbour Joining tree, based on AFLP data generated by Jacobs et al., 2008, of the genotypes carrying *Rpi-vnt1*-like homologs, *Rpi-vnt1* alleles and *Rpi-vnt2* gene. The presence of *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3*, *Rpi-vnt2* and *Rpi-vnt1*-like coding homologs is indicated. A first illegitimate recombination (IR1) might have occurred between *OPL753-8*-like homologs generating *Rpi-vnt1.2*. *Rpi-vnt1.1* and *Rpi-vnt1.3* might have evolved from *Rpi-vnt1.2* by a second IR (IR2) and one non synonymous nucleotide substitution respectively.

R-gene evolution

Both OPL753-8 and WRB933-1 had their start codon at the same position as the *Tm2²* gene. However, only OPL753-8 allowed a PCR amplification with both primer pairs A and B providing sequence information of its 5'UTR. Subsequent alignment of *Rpi-vnt1* alleles with *S. oplocense* (OPL753-8) nucleic sequence revealed that a deletion or an insertion occurred in the 5'end upstream the position of the *Tm2²* gene start codon (Figure 3). As previously described, *Rpi-vnt1.2* and *Rpi-vnt1.3* have an insertion of 42 nucleotide (nt) at the position 44 which was not present in *Rpi-vnt1.1*. In the same region, OPL753-8 had an extra insertion of 21 nt. Close examination of this region revealed traces of illegitimate recombination (IR) signatures. According to Wicker et al. (2007), IR seems to occur between short 1-8 bp stretches of homology leading to a duplication or deletion event. Blast analysis of the inserted or deleted sequence showed no homology with the rest of the sequences. This result suggested that a deletion might have occurred rather than a duplication event.

We could hypothesize that a first illegitimate recombination might have occurred between *Rpi-vnt1*-like homologs highly similar to OPL753-8 generating *Rpi-vnt1.2*. IR signatures could be identified as CTT in *OPL753-8* and *Rpi-vnt1.2* genes (Figure 3). The only SNP found between *Rpi-vnt1.2* and *Rpi-vnt1.3* was precisely located in one of the IR signature at position 75bp with sequence CTC instead of CTT. This SNP suggested that *Rpi-vnt1.2* might have evolved prior to *Rpi-vnt1.3*. A second IR might have occurred between *Rpi-vnt1.2* or *Rpi-vnt1.3* genes to generate *Rpi-vnt1.1*. Stretches of 4nt (CTCT) were identified as IR signatures at positions 32 and 78nt (Figure 3).

Figure 3. Scheme, adapted from Wicker et al., 2007, showing the illegitimate recombination mechanism from which *Rpi-vnt1* alleles might have evolved. *OPL1753-8* was chosen as template to illustrate the mechanism. A first IR might have occurred between CTT homologous sites (italic bold) generating *Rpi-vnt1.2* and *Rpi-vnt1.3*. Then a second IR might have occurred between CTCT homologous sites (italic bold) generating *Rpi-vnt1.1*. SNPs between *OPL1753-8* and *Rpi-vnt1* alleles are in bold. The start codon of each gene is underlined and bold.



Interestingly, the first putative IR previously described with CCT as IR signature motif might also have occurred in *Rpi-vnt1*-like homologs. For instance, some of them had a deletion of 21nt (TAR855-5, TAR868-1 and GIG362-4) at the same position as *Rpi-vnt1.2* and *Rpi-vnt1.3* (supplementary data S1). Moreover, two other putative IR leading to a deletion of 35nt and 116nt with GGTG and TTG motifs as IR signatures were identified in *Rpi-vn1*-like homologs from *S. stenotomum* and *S. weberbaueri* respectively (supplementary data S1).

A Neighbor Joining (NJ) tree of accessions carrying the functional alleles and *Rpi-vnt1*-like homologs (Figure 2b), based on AFLP data (Jacobs et al., 2008), showed that *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* were partially monophyletic. This lack of monophyletic grouping was due to one *S. weberbaueri* accession (WBR253-2/4) and *S. mochiquense* accession (186-1) carrying the *Rpi-vnt1.1* gene. Surprisingly, WBR253-2/4 did not cluster with the other *S. weberbaueri* accessions in the study of Jacobs et al. (2008). The *S. mochiquense* accession (186-1) was not included due to conflicting positions in dendrogram. Jacobs et al. (2008) removed it also from the phylogenetic. A second NJ tree was constructed using several resistant and susceptible genotypes per accession of the wild species carrying *Rpi-vnt1* alleles or *Rpi-vnt1*-like homologs (supplementary data S2). WBR253-2 clustered in the same clade as *S. venturii* whereas WBR253-4 was found with some other *S. weberbaueri* accessions further away. This observation suggested that indeed, WBR253-2/4 might be an interspecific hybrid between *S. weberbaueri* and *S. venturii*. Therefore *Rpi-vnt1* alleles were most probably monophyletic within *S. venturii* accessions. Moreover they seemed to be fixed for the accession CGN18109.

Another late blight resistance locus in S. venturii: *Rpi-vnt2*

As previously mentioned, a resistant genotype (969-2) from CGN22703 was found to carry only the non functional allele TTCC. The genotype 969-2 was further phenotyped using nine different *Phytophthora infestans* isolates to determine its resistance spectrum (Table 2). Although a more narrow resistance spectrum than *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* was determined, the genotype 969-2, showed to be resistant to EC1 which is the only *P. infestans* strain able to overcome *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*. All the accessions of *S. venturii* previously checked for the presence of *Rpi-vnt1* alleles were challenged with EC1. The figure 2b shows the accessions carrying resistance to EC1 strain. The population 7756 containing 95 F1 individuals was phenotyped with EC1 to check the segregation of the resistance. A ratio

1:1 (47 resistant, 7 questionable and 41 susceptible) was observed. This result show that the resistance to EC1 is conferred by a single dominant *R*-gene and that was named *Rpi-vnt2*.

Species	Accession	Genotype	IPO C	H30P04	90128	89148-09	NL00228	VK98014	USA618	Katshaar	EC1
S. venturii	18008	367-1	R	R	R	R	R	R	R	R	S
	22703	969.2	R	R	R	R	S	S	S	S	R
		969.4	R	R	R	R	R	R	R	R	S
S. neorossii	18280	735.1	S	S	S	S	S	S	S	S	S
		735.4	S	S	S	S	S	S	S	S	S
S. okadae	?	742.2	S	S	S	S	S	S	S	S	S
	20599	970.4	S	R	R	R	S	R	S	S	S
cv. Bintje	/	/	S	S	S	S	S	S	S	S	S
cv. Desiree::Rpi-vnt1.1	/	/	R	R	R	R	R	R	R	R	S

Table 2. Phenotyping results of genotypes which were further investigated for late blight resistance. The genotype (367-1, CGN18008) from which *Rpi-vnt1.1* was originally cloned and a primary transformant of *Rpi-vnt1.1* in cv. Desiree background were used as resistant controls and cv. Bintje as susceptible control. R: resistant. S: susceptible.

Late blight resistance in S. okadae and S. neorossii

Previous taxonomic work clustered accessions from *S. okadae*, *S. neorossii* and *S. venturii* in three different groups (Hawkes 1990). Based on cpPDNA and AFLP marker analysis to construct a backbone phylogeny, Jacobs et al (2008) generated also three distinct clades for *S. okadae*, *S. neorossii* and *S. venturii* but identified misclassification of accessions between the three wild species. The CGN accessions 18108, 18109, 17998, 17999, 22703 and 18269 initially classified as *S. okadae* and the CGN accession 18000 from *S. neorossii* were shown to belong to the clade of *S. venturii*. Therefore they were reclassified as *S. venturii* (Table 1). The remaining CGN accessions, 962076, 962078, 18157, 22709, 18129, 20599 and 17599, 18051, CPC6047, 18280 which were not clustered in the *S. venturii* clade were shown to be true *S. okadae* and *S. neorossii* accessions respectively (Jacobs 2008). As previously mentioned true accessions of *S. okadae* and *S. neorossii* allowed the amplification of a PCR product. Following cloning and sequencing, only *Rpi-vnt1*-like homologs sharing between 87.5 and 96.4 homology with *Rpi-vnt1* alleles were found (Table1). Seedlings from the genotypes harboring these *Rpi-vnt1*-like homologs (735-1, 735-4, 742-2 and 970-4) were further phenotyped using a set of 9 *Phytophthora infestans* isolates in DLA. The original resistant genotype from which *Rpi-vnt1.1* was cloned and a primary transformant of *Rpi-vnt1.1* in cv. *Desiree* background, were used as resistant controls and cv. *Bintje* as susceptible control. Table 2 shows the resistance spectrum for each genotype tested. As expected, both resistant controls showed to be resistant to all the isolates tested except to EC1. Cv. *Bintje* had a high level of sporulation for all the isolates. The genotype 970-4 was resistant to four isolates out of nine (H30P04, 90128, 89148-09 and VK98014) whereas the genotypes 742-2

(*S. okadae*) and 735-1/4 (both from *S. neorossii*) were fully susceptible to the nine isolates used. These phenotyping results suggested that the detected *Rpi-vnt1*-like homologs in the genotypes 742-2 and 735-1/4 were not involved in late blight resistance.

In order to characterize the resistance observed in the genotypes 970-4, a cross was made between 970-4 (CGN20599 from *S. okadae*) and 735-2 (susceptible genotype from *S. neorossii*). The segregation of the resistance was analyzed by DLA using three isolates (90128, H30P04 and VK98014). A ratio of 1:1 (resistant : susceptible) was determined using 90128 whereas H30P04 showed a 1:3 ratio. Surprisingly, this population was fully susceptible to VK98014. Most of the resistant individuals showed big necrosis spots of 3-10 mm without sporulation. These results suggest that the resistance observed in the genotype 970-4 could be due to the pyramiding of several resistance QTLs which was undone in the segregating population. No correlation could be determined between resistant individuals to different isolates. Therefore, no single strong dominant resistance genes could be identified in the genotype 970-4.

DISCUSSION

The screening of a large collection of potato wild species, using specific primer pairs to *Rpi-vnt1* alleles, showed that only two other wild species, *S. weberbaueri* and *S. mochiquense*, carried one of the three functional alleles, *Rpi-vnt1.1*. Many *Rpi-vnt1*-like homologs, sharing between 80.7-97% homology with *Rpi-vnt1* alleles, were present in 13 wild species. According to Jacobs et al (2008), these 13 wild species belong to different phylogenetic clades. *S. oplocense* and *S. sucrense* clustered in clade 16, *S. orophilum*, *S. weberbaueri* and *S. multidissectum* in clade 10, *S. raphanifolium* in clade 1, *S. vernei* in clade 9, S. *microdontum gigantophyllum* in clade 4, *S. okadae* in clade 15, *S. neorossii* in clade 16 and *S. tarijense* in clade 14. Three wild species, *S. stenotomum* and *S. phureja* were not included in the study of Jacobs et al. 2008. According to Hawkes (1990), these 13 wild species belong to the serie 16 named *Tuberosa* either in the wild or cultivated species subsection. This result suggested that *Rpi-vnt1* alleles were not widely spread across *Solanum* section *Petota* and might have more recently evolved in comparison to other ancient *R*-genes like *Rpi-blb1* (Van der Vossen et al. 2003; Wang et al. 2008).

The use of two primers pairs, based on the start codons of *Rpi-vnt1* alleles (primer A) and *Tm2*^{2} gene (primer B), enabled to detect full length and truncated *Rpi-vnt1*-like homologs respectively. Most of the accessions allowed a PCR product with both primers A and B but few of them did not, suggesting that their 5'UTRs diversified preventing the primer A from annealing. The only ORFs found had their start codon at the same position has *Tm2*^{2} gene suggesting that ORF starting 100bp upstream like *Rpi-vnt1* alleles may be rather unique. Subsequent alignment of OPL753-8 (*Rpi-vnt1*-like homolog from *S. oplocense*) with *Rpi-vnt1* alleles gave insight onto the plausible evolution of *Rpi-vnt1* alleles. In their 5'UTRs, putative illegitimate recombination (IR) signatures (Wicker et al., 2007) were identified in the CC domain of *Rpi-vnt1* alleles suggesting that they might have evolved through two successive IR events leading to deletions. These deletions changed the position of the start codon approximately 100bp upstream the conserved *Tm2*^{2} gene start codon. The extension of the ORF may be considered as a major diversification event as the coiled coil (CC) domain of *Rpi-vnt1* alleles evolved four alpha helices with the first one located upstream the conserved *Tm2*^{2} gene start codon. This first alpha helix is required to trigger HR (Pel et al. 2009). Wicker and co-workers demonstrated that IR was almost only involved in size variation of the LRR domain of *Pm3* genes following duplication events in wheat. IR might also have taken place in the CC domain and influenced the position of the start codon as shown in this study.

More *Rpi-vnt1*-like homologs had the same deletions in their 5'UTRs or even larger deletion in their LRR domains. It suggested that illegitimate recombination could be a significant mechanism involved in *Rpi-vnt1*-like homolog diversification.

A neighbor Joining (NJ) tree based on AFLP data (Jacobs et al., 2008) from all the *S. venturii* accessions showed that *Rpi-vnt1.2* and *Rpi-vnt1.3* were monophyletic whereas *Rpi-vnt1.1* lacked monophyletic grouping. This was due to an accession from *S. weberbaueri* carrying *Rpi-vnt1.1* which did not cluster in the same clade as *S. venturii* accession carrying *Rpi-vnt1.1*. As it did not cluster either with the other *S. weberbaueri* accessions, we hypothesized that it might be an interspecific hybrid between *S. weberbaueri* and *S. venturii* from which *Rpi-vnt1.1* was introgressed. Signatures of illegitimate recombination and the monophyletic grouping of *Rpi-vnt1* alleles relative to potato accessions suggested how *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* could have evolved. Indeed, a first IR (IR1) might have occurred to generate *Rpi-vnt1.2* which might have served as template for a second IR (IR2) generating *Rpi-vnt1.1*. Subsequently *Rpi-vnt1.3* diverged from *Rpi-vnt1.2* by one non synonymous nucleotide substitution.

The *Rpi-vnt1*-like homologs OPL753-8 encoding the same LRR repeats as *Rpi-vnt1* alleles was considered the most plausible template for IR event. In contrast, this hypothesis was in opposition with the place of OPL753-8 accession in the dendrogram. Indeed OPL753-8 accession did not cluster together with *S. venturii* accessions. This observation might suggest that the most plausible ancestor of *Rpi-vnt1* alleles diverged so much that none of the primers used in this study would produce a PCR product. Then OPL753-8 might be considered a plausible candidate to illustrate IR event but not a plausible accession ancestor.

Kuang et al. (2004) studied haplotype diversity at the RGC2 cluster in cultivated lettuce and wild relatives. They clearly showed two types of RGC2 genes. Type I genes evolve quickly and have frequent sequence exchange due to chimeric structures. Type II genes are more conserved and appear to have a very low recombination frequency. *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*, had 3 SNPs in the LRR domain. Only two SNPs were not synonymous but generated amino acids with the similar chemical characteristics. This observation together with the same resistance spectrum of *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* suggested that their LRR domains might be under purifying selection as described by Kuang et al. (2004) for the type II genes. Then *Rpi-vnt1* alleles may belong to the type II gene.

The allele mining study of *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* within accessions of *S. venturii*, revealed the presence of another resistant locus which was named *Rpi-vnt2*.

The finding that a particular wild species evolved several functional *Rpi* genes was observed in *S. demissum* carrying eleven *R*-genes (*R1*-*R11*) and in *S. bulbocastanum*, in which three *R*-genes, *Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3*, were cloned from the chromosomes *8*, *6* and *4* respectively (Van der Vossen et al.2003, 2005; Lokossou et al. 2009). *Rpi-vnt2* was not found to be monophyletic like *Rpi-vnt1* alleles. Upon selection pressure, accessions carrying a broad resistance gene, for instance *Rpi-vnt1* alleles, might have been selected to the detriment of *Rpi-vnt2*, conferring a more narrow resistance spectrum. It would be quite interesting in proceeding with the cloning and allele mining study of *Rpi-vnt2* to unveil late blight resistance evolution in *S. venturii*.

As previously mentioned, in the phylogeny study based on cpDNA (Jacobs 2008), some accessions of the wild species *S. okadae* and *S. neorossii* were found to be misclassified and thus reclassified as *S. venturii*. All the accessions reclassified as *S. venturii* carried either *Rpi-vnt1.1*, *Rpi-vnt1.2* or *Rpi-vnt1.3*. Accessions confirmed as *S. okadae* or *S. neorossii* did not harbor *Rpi-vnt1* alleles nor strong resistance to late blight. The allele mining study and further phenotyping strengthened the reclassification of some accessions.

Chromosome *9* of tomato and potato seem to be a hot spot for resistance because several *R*-genes, conferring resistance to a broad range of pathogens are located on this chromosome (Gebhardt and Valkonen 2001). For instance, the long arm of chromosome *9* of potato harbours resistance genes to three different viruses, PVY, PVX and PVM (Marczewski et al. 2006; Sato et al. 2006; Tommiska et al. 1998) and QTLs conferring resistance to *Globodera pallida* and *Erwinia carotovora* (Gebhardt and Valkonen 2001; Rouppe van der Voort et al. 2000). In the same region, there are at least two *Tm2*-like clusters (*Rpi-vnt1.1*/*1.2*/*1.3* and *Rpi-mcq1* genes) involved in late blight resistance located on chromosome *9*. To what extent *Tm-2*-like genes from potato are involved in quantitative/qualitative resistance against different pathogens than *P. infestans* is unknown. A broad allele mining analysis of *Tm-2*-like genes (*Tm-2*^{2}, *Rpi-vnt1.1*/*1.2*/*1.3* and *Rpi-mcq1* gene) would provide information on *Tm-2*-like genes diversity and insight into *R*-gene evolution on the long arm of chromosome *9* of potato.

MATERIALS AND METHODS

Plant materials

We used the same plant material from genus *Solanum* section *Petota* as described in Jacobs et al. (2008). In total, 196 different taxa were screened. At least 5 accessions from each available species and 5 individual plants per accession were used.

Following screening with *P. infestans*, resistant genotype oka970-4 from accession CGN20599 was used to make an inter-specific mapping population. The mapping population 3066 was generated by crossing [oka970-4 (resistant, CGN20599) x nrs735-2(susceptible, CGN18280)]. The mapping population 7756 generated by crossing [(vnt283-1 (resistant, CGN22703) x vnt368-6 (susceptible, CGN18109)] was used to map resistance to EC1 strain in *S. venturii*.

PCR

Two primer pairs (A and B) containing the same reverse primer designed on the stop codon of *Rpi-vnt1* alleles (stop-vnt1-rev: ttatagtacctgtgatattctcaac) was combined with two different forward primers: one designed on the start codon of *Rpi-vnt1* alleles (start-vnt1-for: atgaattattgtgtttacaagacttg, from pair A) and the other one on the start codon of *Tm-2*^{2} gene (start-tm2-for: atggctgaaattcttctcacagc, from pair B). *Rpi-vnt1* alleles and *Tm-2*^{2} gene share a homology of 75% at the nucleic level (Pel et al. 2009; Foster et al. 2009; Lanfermeijer et al. 2003). The gene encoding the elongation factor 1-alpha (*ef1α*, AB061263) was used as positive control (*ef1α* Forward: attggaaacggatatgctcca and *ef1α* reverse: tccttacctgaacgcctgtca). PCR using specific primer pairs A, B and *ef1α* with *Pfu* Turbo polymerase in 50µl reaction-mixture was prepared containing 50ng of gDNA, 1µl of the forward primer (10µM), 1µl of the reverse primer (10µM), 0.8µl dNTPs (5mM each), 5µl 10X buffer, 1µl of *Pfu* Turbo (Invitrogen). The following PCR program was used: 94°C for 3 mins, 94°C for 30 sec, 55°C for 30 sec, 72°C for 2 mins, 72°C for 5 mins during 29 cycles. Completed PCR reaction were run in 1% agarose gel.

Sequencing

Cloned fragments, in pGEM®-T Easy Vector from Promega, or PCR products generated with *Pfu* Turbo polymerase (Invitrogen) were sequenced as follows: 10µl sequencing reaction mixtures were made using 5µl of PCR product or 5ng of plasmid, 3µl of buffer, 1µl of DETT (Amersham) and 1µl of forward or reverse primer. The PCR program used was 25 cycles of

94ºC for 20sec, 50ºC for 15sec, 60ºC for 1min. The sequences were generated on ABI 3730XL sequencers.

Disease assay

Detached leaf assays (DLA) on accessions were carried out as described by Vleeshouwers (Vleeshouwers et al. 1999). Leaves were inoculated with 10µl droplets of inoculum ($5x10^4$ zoospores/ml) on the abaxial side and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16h/8h day/night. At 6 days post inoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant.

Trees construction

Taxonomic NJ trees were constructed according to Nei and Li (1979) using the software Treeconw (version 1.3b) based on AFLP marker data (Jacobs et al., 2008). Nucleic sequences were aligned with Megalign using Clustal W algorithm (DNAstar version 6).

Figure S1. Alignment of *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3*, *Tm2*^{2} and *Rpi-vnt1*-like homologs amplified with both primer pairs (A and B). a- 5'region depicting the same deletion as *Rpi-vnt1.2* and *Rpi-vnt1.3*. The same putative IR signatures (motif CTT) are shown in bold. b- Region of the LRR domain depicting a deletions of 115nt with putative IR signatures (motif TTG). c- End of the LRR domain depicting a deletion of 35nt with putative IR signatures (motif GGTG).

a-

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OPL753-8      ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGACTCCAACACCTAAAGCAAATAGTACATCTTTCTTATCCTTTACCAATAATCGATCATCTTTTCCCTTACTTTCGC
STN829-2      ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGACTCCAACACCTAAAGCAAATAGTGCATCTTTCCTATCC-TTTACCAATAATTGATCATCTTTTCTCTTACTTTCCC
STN829-3      ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGACTCCAACACCTAAAGCAAATAGTGCATCTTTCCTATCC-TTTACCAATAATTGATCATCTTTTCTCTTACTTTCCC
MLT724-4_2    ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGAATCCAACACCTAAAGCAAATAGTACATCTTTCTTATCCTTTTACCAATAATTGTTCATCTTTTCTCTTACTTTCCC
MLT726-2      ATGAATTATTGTGTTTACAAGACTTTG-CCGTTGAATCCAACACCTAAAGCAAATAGTACATCTTTCTTATCCTTTTACCAATAATTGTTCATCTTTTCTCTTACTTTCCC
MLT727-4_1    ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGACTCCAACACCTAAAGCAAATAGTGCATCTCTCTTATCCTTTTACCAATAATCGATCATCTTTTCCCTTACTTTCCC
MLT731-2      ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGAATCCAACACCTAAAGCAAATAGTACATCTTTCTTATCCTTTTACCAATAATTGTTCATCTTTTCTCTTACTTTCCC
ORP196-1      ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGACTCCAACACCTAAAGCAAATAGTGCTTCTTTCTTATCCTTTTACCAATAATTGATCATCTTTTCTCTTACTTTCCC
ORP196-2      ATGAATTATTGTGTTTACAAGACTTGG-CCGTTGACTCCAACACCTAAAGCAAATAGTGCTTCTTTCTTATCCTTTTACCAATAATTGATCATCTTTTCTCTTACTTTCCC
TAR855-5      ATGAATTATTGTGTTTACAAGACTTGGGCCGTTGACTCCAACAC-TAAAGCAAATAGTACATCTTTCTTATCCTTT----------TTTCTCTTACTTTCCC
TAR868-1      ATGAATTATTGTGTTTACAAGACTTGGGCCGTTGACTCCAACAC-TAAAGCAAATAGTACATCTTTCTTATCCTTT----------TTTCTCTTACTTTCCC
GIG362-4      ATGAATTATTGTGTTTACAAGACTTGGGCTGTTGACTCCAACA-CTAAAGCAAATAGTACATCTTTCTTATCCTTT----------TTTCTCTTACTTTCCC
Rpi-vnt1.2    ATGAATTATTGTGTTTACAAGACTTGGGCCGTTGACTCTAACA-CTAAAGCAAATAGTACATCTTTCTTATCCTTT----------TTTCTCTTACTTTCCC
Rpi-vnt1.3    ATGAATTATTGTGTTTACAAGACTTGGGCCGTTGACTCTAACA-CTAAAGCAAATAGTACATCTTTCTTATCCTC----------TTTCTCTTACTTTCCC
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b-

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Rpi-vnt1.2 GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGGTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
Rpi-vnt1.3 GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGGTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
STN829-2   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
STN829-3   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
GIG362-4   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACAAGAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
TAR855-5   GCGTTAGAGGTTCTGAAGCTCCGATTTTTCAAGAACACAAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCAAGTTGGGTTTGCTTTGTTTCTCAACAGTGCTCTTGAACATTGAAGCAT
TAR868-1   GCGTTAGAGGTTCTGAAGCTCCGATTTTTCAAGAACACAAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCAAGTTGGGTTTGCTTTGTTTCTCAACAGTGCTCTTGAACATTGAAGCAT
MLT726-2   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCGCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTGTTGAACATTGAAGCAT
MLT727-4_1 GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGAAAATAAATTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
MLT727-4_2 GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCGCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTGTTGAACATTGAAGCAT
MLT731-3   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCGCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTGTTGAACATTGAAGCAT
OPL7553-8  GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTCTTGAACATTGAAGCAT
WBR933-1   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAACTTGTCGTCGCATCCAAATATTGTCGAGTTGGGTTTGTTTGGTTTCTCAGCAATGCTGTTGAACATTGAAGCAT
WBR933-2   GCGTTGGAGGTTCTGAAGCTCAGATTTTTCAAGAACACGAGTGAGCAAATAAATTTGTCGTCCCATCCAAATATTGTCGAGTTGGGTTTGTTTGG-TTCTCAGCAATGCTCTTGAACATTGAAGCAT
WBR300-1   GCGTTG----------AAGCAT
WBR300-2   GCGTTG----------AAGCAT
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c-

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OPL753-8    GCTTAGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
Rpi-vnt1.2  GCTTTGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
Rpi-vnt1.3  GCTTTGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
ORP196-1    GCTTAGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCTGCAA
ORP196-2    GCTTAGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCTGCAA
GIG362-4    GCTTAGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
MLT726-2    GCTTAGGTGCAGCCATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
MLT727-4_1  GCTTAGGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
MLT727-4_2  GCTTAGGTGCAGCCATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
MLT731-3    GCTTAGGTGCAGCCATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
WBR933-1    GCTTAGGTGCAGCCATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
WBR933-1    GCTTAGGTGCAGACATGGTGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
WBR300-1    GCTTATGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
WBR300-2    GCTTATGTGCAGACATGATGCAGAAAAAATGGATCTCTCTGGTGATAGCTTTCCGCAA
STN829-2    GCTTAGGTG----------ATAGCTTTCCGCAA
STN829-3    GCTTAGGTG----------ATAGCTTTCCGCAA
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Figure S2. Neighbour Joining tree, based on AFLP data generated by Jacobs et al., 2008, of all accessions from species in which *Rpi-vnt1*-like homologs or *Rpi-vnt1* alleles were detected.

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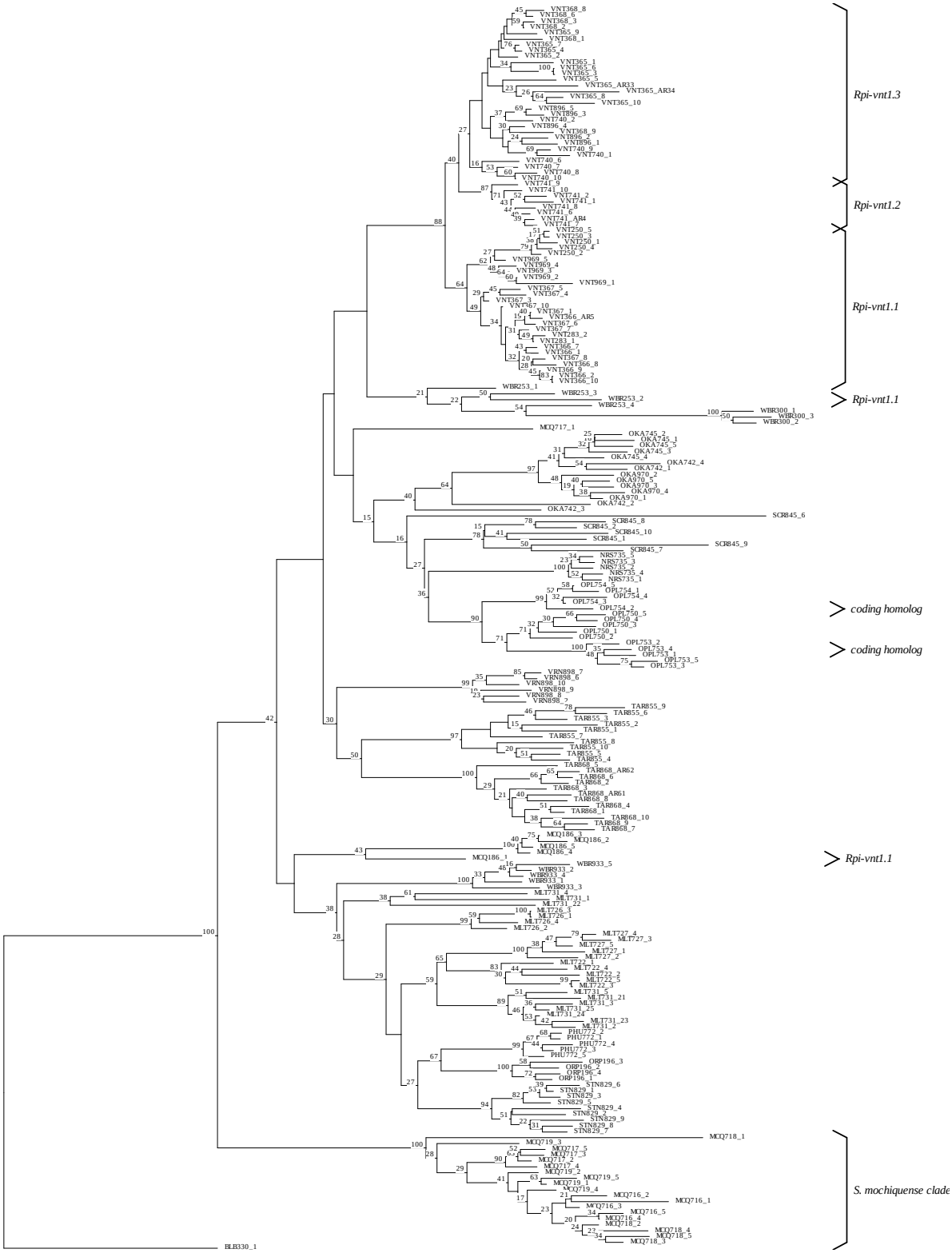
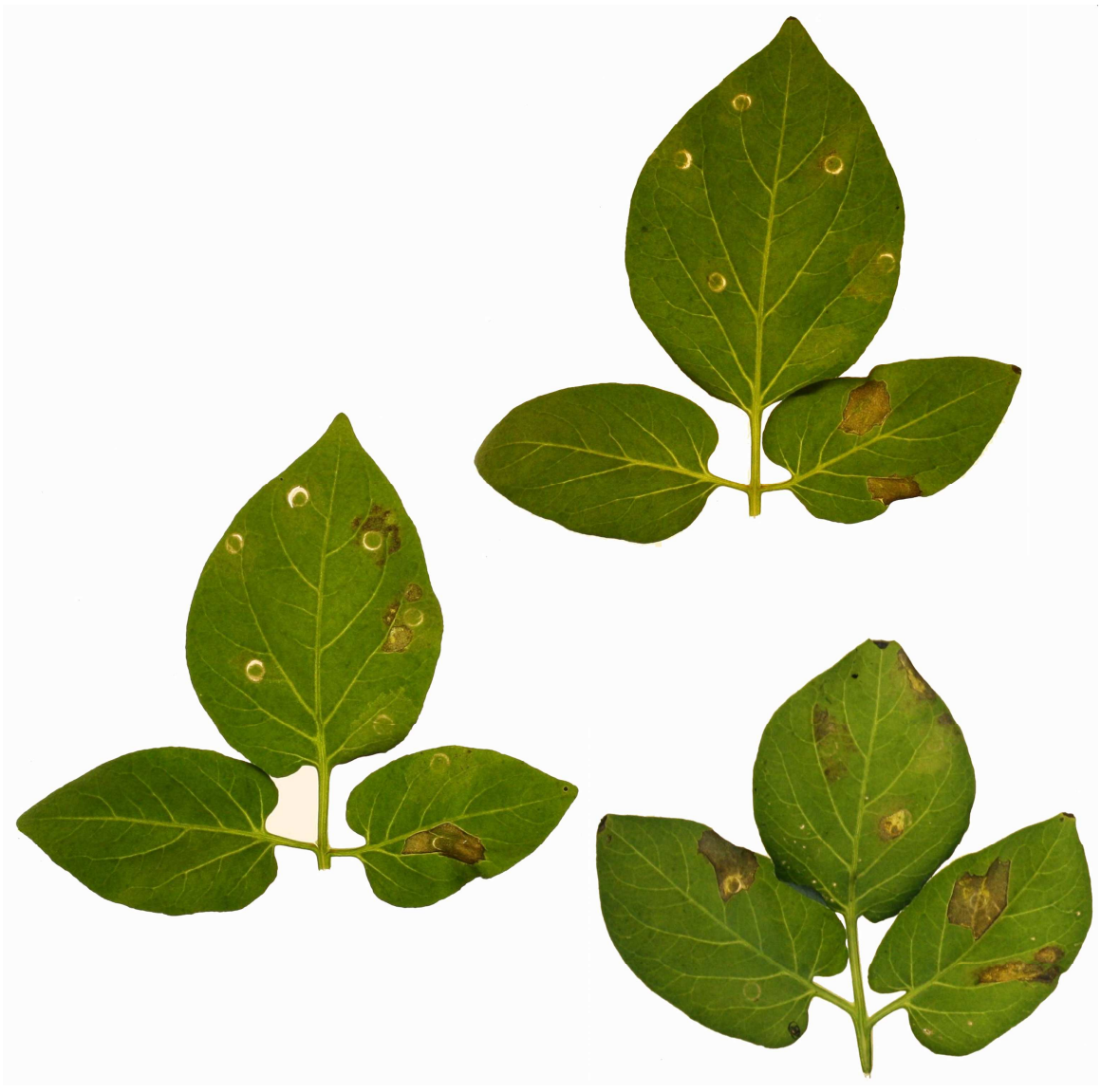


ACKNOWLEDGMENTS

The presented work was financially supported by the FP6 programme BioExploit Food-CT-2005-513959.

CHAPTER 5

The newly identified *Avr-vnt1* is silenced in a *Phytophthora infestans* strain that is virulent on plants expressing *Rpi-vnt1.1, Rpi-vnt1.2* and *Rpi-vnt1.3*



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Transient expression of *Avr-vnt1* gene in wild type potato leaves expressing *Rpi-vnt1.1* gene

The newly identified *Avr-vnt1* is silenced in a *Phytophthora infestans* strain that is virulent on plants expressing *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*

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ABSTRACT

Late blight caused by *Phytophthora infestans* is the most damaging disease on potato. Resistance to late blight is conditioned by resistance (*R*) gene recognizing effector proteins secreted by *P. infestans* and translocated into the host cell. Identification of *Avr-vnt1*, the cognate avirulence gene of *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1*.3, was achieved by an efficient and high throughput functional screening of a genome-wide set of expressed RXLR effectors of *P. infestans* in resistant wild potato species. Genomic studies showed that *Avr-vnt1* was located in a gene-sparse region at a single locus in the reference genome T30-4. Four *Avr-vnt1* alleles, sharing a sequence identity between 97.7-99.7% and 95.4-100% at the nucleic and protein levels respectively, were mined from 9 *P. infestans* isolates. One of the four alleles had an early stop codon leading to a truncated protein and the three others encoded full length proteins of 153 amino acids. Occurrence of specific variants in *P. infestans* strains was not related to avirulence phenotypes on *Rpi-vnt1* plants. At the transcriptional level however, no *Avr-vnt1* mRNA was detected in the virulent strain EC1. Therefore, we conclude that the silencing of *Avr-vnt1* led to virulence on *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* plants.

INTRODUCTION

The plant pathogen *Phytophthora infestans* is the causal agent of late blight, one of the most devastating diseases on the potato crop. *P. infestans* belongs to the Oomycetes, which form a diverse group of fungus-like eukaryotic microorganisms. Since the notorious Irish famine in 1840, a consequence of potato late blight, breeders searched for resistance sources within cultivars and wild potato species. In the 1950s, promising monogenic resistance genes to *P. infestans* (*Rpi*, *R1-R11*) were identified in the Mexican species *S. demissum*, but a few years after their introgression, they were overcome by *P. infestans* (Black and Gallegly 1957; Malcolmson and Black 1966). By now, several *Rpi*-genes are cloned and characterized. Two of them, *R1* and *R3a* were found in *S. demissum* on chromosome *5* and *11* (Ballvora et al. 2002; Huang et al. 2004, 2005). From another Mexican species, *S. bulbocastanum Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* were cloned and mapped on chromosome *8, 6* and *4*, respectively (Van der Vossen et al. 2003, 2005; Lokossou et al. 2009; Song et al. 2003). In the meanwhile, a comprehensive survey of resistance to *P. infestans* revealed a wealth of resistance genes in wild *Solanum* species section *Petota*, also from South American origin. From *S. venturii*, three alleles of *Rpi-vnt1* were recently cloned, i.e. *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* (Foster et al. 2009; Pel et al. 2009). *Rpi-vnt1* alleles were shown to be *Tm2*^{2}-like, sharing 75% homology at the nucleic acid level, and mapped on chromosome *9*. Compared to Rpi-vnt1.1, Rpi-vnt1.2 and Rpi-vnt1.3 harbor a 14 amino acids (a.a.) indel in the N-terminal region of the protein and two different a.a. in the LRR domain. One synonymous SNP in the N terminal can distinguish between *Rpi-vnt1.2* and *Rpi-vnt1.3*. So far, despite these differences, *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* genes exhibit the same resistance spectrum, and *P. infestans* EC1 is the only strain found to overcome *Rpi-vnt1* (Pel et al. 2009).

All cloned Rpi genes belong to the CC-NB-LRR family. Resistance (*R*) genes can be grouped in three main classes: Nucleotide Binding Site Leucine Rich Repeat (NBS-LRR), LRR Receptor Like Kinase (LRR-RLK) and LRR Receptor Like Protein (LRR-RLP). The NBS-LRR class is among the most abundant in all plant species (Meyers et al. 2003, 1999). *R*-gene based resistance to any plant pathogen was conceptualized to make a model known as gene-for-gene interaction (Flor 1971). When matching Avirulence *(Avr)* and Resistance *(R)* proteins are present in the pathogen and the plant respectively, a resistance response is triggered resulting in a hyper-sensitive response (HR) causing necrosis and cell death at the infection site (Dangl et al. 1996). If one of these components is missing the plant-pathogen interaction will not result in a cell death response.

Recently, various *Avr* genes from *P. infestans* were identified following different cloning strategies. For instance, *Avr3a* was cloned by association genetics and was shown to have an avirulence activity by transient co-expression with *R3a* in a heterologous *Nicotiana benthamiana* system (Armstrong 2005). The identification of *Avr1* and *Avr2* was achieved by classical map-based cloning in *P. infestans* (Francine Govers, personal communication; Morales et al., unpublished results; Lokossou et al. 2009). *Avr4* was identified using a cDNA-AFLP based strategy (Guo et al. 2006) prior to the cloning of its corresponding *R*-gene (*R4*) (Van Poppel et al. 2008). *Avr-blb1* and *Avr-blb2* (Oh et al. 2009; Vleeshouwers et al. 2008) were identified using an effector-genomics (effectoromics) approach, namely activity screens with a large collection of candidate effectors. Characterization of identified *Avr* genes showed how *P. infestans* evolved to avoid recognition using various mechanisms such as deletion, frame shift and non synonymous point mutations. For instance, *Avr1* is absent in the virulent strains on potato genotypes carrying the *R1* genes (Francine Govers, personal communication). The 'virulent' allele of *Avr4* has a frame shift leading to a truncated protein which is no longer recognized by the *R4* gene (Van Poppel et al. 2008). Non-synonymous point mutations affecting *R*-gene based recognition were identified between *Avr3a*/*avr3a*, *Avr-blb1*/*avr-blb1* and *Avr-blb2*/*avr-blb2* (Armstrong 2005; Bos et al. 2009; Oh et al. 2009). There are no reports of down-regulation of *Avr* genes at the transcriptional level in *P. infestans* thus far. However, recent studies showed that transcriptional differences of *Avr1a* and *Avr3a* among *P. sojae* strains cause changes in virulence (Dong et al. 2009; Qutob et al. 2009). Moreover, loss of *Avr1b-1* transcription has been reported in strains that are virulent on soybean plants carrying *Rps1b* (Shan et al. 2004). Loss of elicitin transcription in a virulent isolate of *P. parasitica* led to virulence on tobacco plants (Kamoun et al. 1993). Changes observed in the avirulence phenotypes of some *Phytophthora* strains could be explained by epigenetic changes. Abu-El Samen et al. and Rutherford et al. (2003 and 1985 respectively) observed changes from avirulence to virulence and vice versa within two generations of single zoospore lines of *P. sojae* and *P. infestans*, respectively.

A common feature of all identified *Avr* genes in Oomycetes is that they contain a N-terminal RXLR motif (Rehmany et al. 2005; Tyler et al. 2006). This latter motif was shown to be involved in the translocation of the effectors into host cells (Birch et al. 2006; Dou et al. 2008; Whisson et al. 2007). The genomes of *P. sojae* and *P. ramorum* revealed that the RXLR effectors family were abundant and evolved rapidly generating over 370 members for each species (Jiang et al. 2008). In *P. infestans*, the genome sequence revealed that RXLR effectors genes are located in Gene Sparse Regions (GSRs), where they can be subject to

diversification via duplication, recombination or point mutation (Haas et al. 2009). Known Avr genes of *P. infestans* are all RXLR effectors that are induced at 2 days post inoculation, a stage that is critical to establish infection. This suggests that RXLR genes that are up regulated during the early phases of in planta growth represent good *Avr* gene candidates.

In this study, we apply an effectoromics strategy to identify *Avr*-vnt1. We composed a genome-wide set of expressed RXLR effectors from the recently available *P. infestans* genome sequence and transiently express this large set of candidate effectors in the resistant wild potato and monitor for occurrence of cell death. We identified one RXLR effector as *Avr-vnt1* whose interaction with *Rpi-vnt1* alleles (*Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*) results in a hypersensitive response (HR). Study of its occurrence within *P. infestans* strains showed that *Avr-vnt1* is silenced in the virulent strain EC1.

RESULTS

Identification of Avr-vnt1

To construct a library of, candidate *Avr* genes, we selected over 200 *P. infestans* putative RXLR effectors predicted from strain T30-4, which was used for genome sequencing and annotation (Haas et al., 2009). RXLR cDNA's strongly upregultated at 2-3 days post inoculation (dpi) of potato leaves were among the effectors selected for cloning. In T30-4, 79 effectors were shown to be up regulated (Haas et al., 2009) at 2dpi, however with less stringent criteria are applied, the set of up-regulated effectors is expanded to ~130. In addition, RXLR effectors showing expression polymorphism with the aggressive isolate 3928A (Blue 13) (Sophien Kamoun, personal communication) were added to the set. Since both T30-4 and 3928A are avirulent on plants carrying *Rpi-vnt1.1* (Pel et al. 2009, and unpublished data), the corresponding *Avr* gene is expected to be present in this selection of effectors.

Effector sequences were obtained from the *Phytophthora infestans* database (Broad.mit.edu; http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html) and their sequences were trimmed of signal peptides. Afterwards sequences were synthesized and cloned into the *Agrobacterium tumefaciens* binary expression vector pMDC32.

To identify *Avr-vnt1*, three leaves of 3 plants from *S. venturii* were infiltrated with a subset of 150 effectors and scored for necrosis 2-4 days post inoculation (dpi), an empty pMDC32 vector was included as negative control and the R3a-Av3a co-infiltration (Mix 1:1) was included as positive control. The majority of putative effectors did not show any responses but one effector, PITG_16294, was showing severe cell death responses at 2 days post infiltration. Also infiltration of *R3a*/*Avr3a* resulted in a cell death response whereas infiltration of empty pMDC32 alone did not give any response as expected.

Figure 1 shows that a cell death response was observed in infiltrated spots with PITG_16294 specifically in vnt283-1 (numerical values are presented in Table S1). An independent repeated experiment confirmed that PITG_16294 specifically induced cell death in vnt283-1. This indicates that PITG_16294 is a strong candidate for *Avr-vnt1*.

To check whether cell death response to the infiltrated effector co-segregates with the resistant phenotype conferred by *Rpi-vnt1.1*, 97 F1 offspring plants of the population 7756, which segregates for *Rpi-vnt1.1* gene, were infiltrated with PITG_16294. Figure 1 and Table S2 show that 46 out of 97 F1 infiltrated individuals were found to give cell death response co-

segregating with the *Rpi-vnt1.1* gene in a 1:1 ratio at the phenotypic level. As positive controls, *R3a*/*Avr3a* and *Rpi-vnt1.1*/PITG_16294 were also infiltrated. The latter one served as positive control for the F1 progeny plants lacking the *Rpi-vnt1.1* gene. Therefore, cell death response is expected to be present in resistant and susceptible F1 progeny when *Rpi-vnt1.1*/PITG_16294 combination is co-infiltrated and only in resistant F1 progeny when PITG_16294 is infiltrated alone. Upon infiltration of *Rpi-vnt1.1* and *Avr3a* alone, no cell death response was observed. Surprisingly, the positive control *R3a*/*Avr3a* did not perform well as little cell death responses were observed. In contrast, almost all the spots co-infiltrated with *Rpi-vnt1.1*/PITG_16294 generated clear cell death responses. Interestingly, resistant F1 offsprings infiltrated with PITG_16294 depicted a cell death response at 2 dpi whereas susceptible F1 individuals co-infiltrated with *Rpi-vnt1.1*/PITG_16294 showed a cell death response at 3-4 dpi. The 97 F1 offsprings were subject to PCR analysis to check for the presence of *Rpi-vnt1.1* using specific primers. All the resistant offsprings showing cell death response upon PITG_16294 infiltration generated the expected PCR product which was absent in the susceptible parent and offsprings. From these data we concluded that PITG_16294 is *PiAvr-vnt1*.

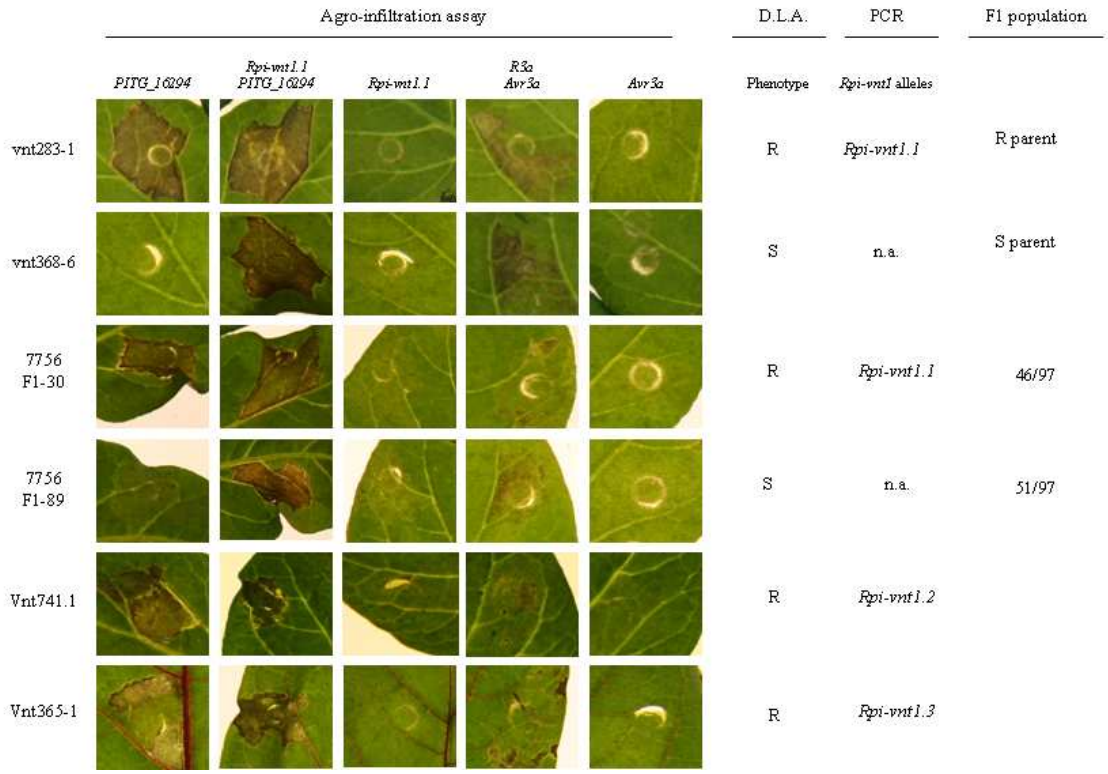


Figure 1. Transient expression of PITG_16294 in the wild potato species *S. venturii* (vnt). PITG_16294 was identified to cause a hypersensitive response (HR) when infiltrated in the resistant (R) genotype vnt283-1 carrying *Rpi-vnt1.1*. The F1 population (vnt283-1 x vnt368-6 susceptible, S) containing 97 individuals was infiltrated with PITG_16294 alone and *Rpi-vnt1.1*/ PITG_16294. *R3a*/*Avr3a*, *Rpi-vnt1.1*/ PITG_16294 and *Rpi-vnt1.1*, *Avr3a* were used as positive and negative controls respectively. The F1 individuals -30 and -89 are representative of the phenotypes observed on resistant and susceptible F1 progenies, respectively. Upon PITG_16294 infiltration 46 out of 97 were found to give an HR co-segregating with the *Rpi-vnt1.1* gene in a 1:1 ratio at the phenotypic and molecular level. "n.a." stands for no *Rpi-vnt1* alleles present. Infiltration of PITG_16294 on the genotypes vnt741-1 and vnt365-1 carrying *Rpi-vnt1.2* and *Rpi-vnt1.3*, respectively, triggered a HR showing that PITG_16294 is recognized by the three *Rpi-vnt1* alleles. Numerical values are presented in Table S1.

Avr-vnt1 characterization

Blastn analysis of PITG_16294 on the genome of *P. infestans* (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans) gave two hits, namely PITG_22547 and PITG_18880, which are both annotated as RXLR-EER effectors. PITG_16294 and PITG_22547 have only two synonymous SNP differences (position 321 and 327bp) whereas PITG_18880 shares only 73.8% identity at the nucleotide level. PITG_16294, PITG_22547 and PITG_18880 form a small family annotated as Tribe RXLR fam96 by Haas et al. (2009) and are located in the supercontigs 1.45 (154223-154684), 1.3922 (1355-1777) and 1.86 (103029-103493) respectively (Figure 2). To determine whether PITG_16294 and PITG_22547 are two haplotypes that were not collapsed during the genome assembly, their 5' regions were aligned. As PITG_22547 lacks sequence information in its 3'end, no 3' UTR was available. Only 1354bp upstream the start codon of PITG_22547 was aligned because of the short length of the super contig 3922. Interestingly, the 5' UTR regions of PITG_16294 and PITG_22547 showed 99.4% homology. In contrast PITG_18880 shares 50% with the 5' UTR regions of PITG_16294 and PITG_22547. This result strongly suggests that PITG_16294 and PITG_22547 are haplotypes. Therefore *Avr-vnt1* is likely to be located to a single heterozygous locus in the reference strain T30-4.

Microarray studies of RXLR-EER effector expression during infection showed that PITG_16294 and PITG_22547 were induced at 2dpi whereas PITG_18880 was not induced (Figure 2).

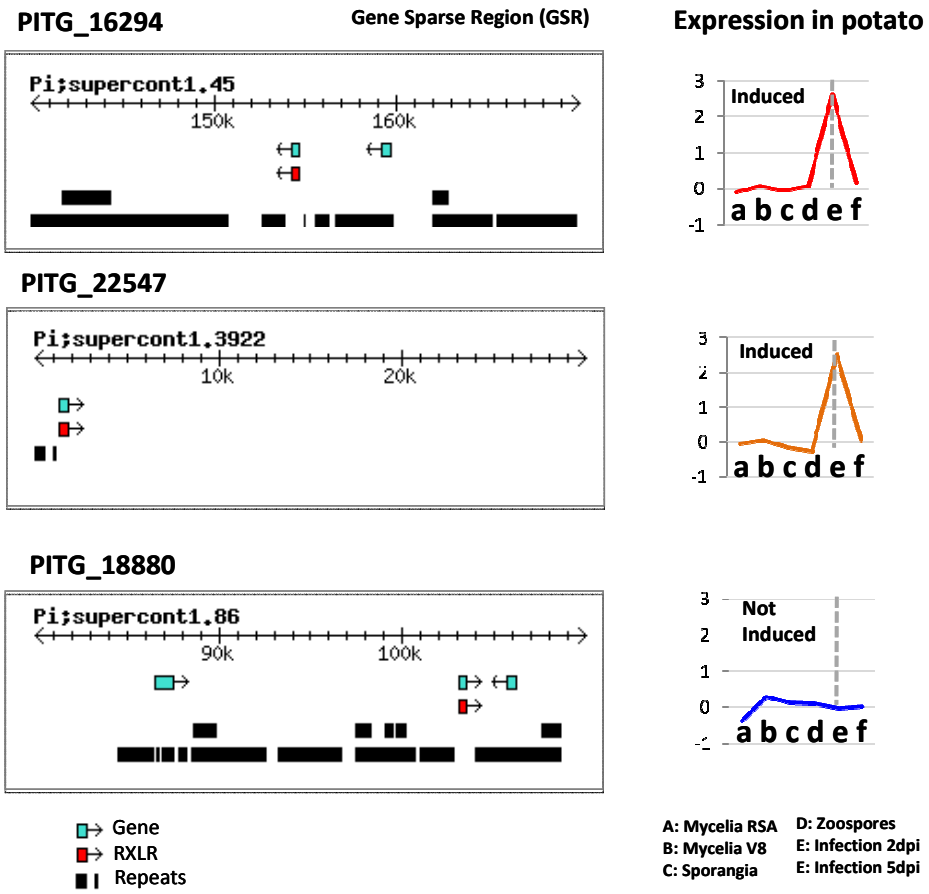


Figure 2. Genome environment and gene expression of three *Avr-vnt1* candidate RXLR genes A). PITG_16294, PITG-22547 and PITG_18880 genes belong to the RXLR family 96 and are locate to a gene sparse region (GSR) in the *P. infestans* T30-4 genome. B). There is differential induction in the effector family 96. PITG_16294 and PITG_22547, almost identical paralogs, are induced at 2 days post infection whereas PITG_18880 is not.

The 5'-terminal structure of *Avr-vnt1* was determined by comparing the genomic sequence with cDNA fragments generated by 5' rapid amplification of cDNA ends (RACE) from strain 90128. RACE identified 5' cDNA fragments comprising 5' untranslated region of 74bp. The conserved sequence surrounding the oomycete transcriptional start site GCTCATTYBNNNWTTY (Kamoun 2003) was partially identified in the genomic sequence of PITG_16294 and PITG_22547 and retrieved from the 5' RACE fragments. The first five nucleotides, GCTCA were found at position -66bp. The translational start codon of *Avr-vnt1* (ACCATG) matches with the consensus sequence indentified by (Kamoun 2003). The open reading frames of *Avr-vnt1* encode predicted peptides of 153 amino acids with a N-terminal region comprising a predicted signal peptide (SP) within the first 23 amino acids (a.a.) and a typical RXLR-EER motif between 48 and 63 a.a. (Figures 2 and S1). Subsequent analysis of the protein sequences of PITG_16294, PITG_22547 and PITG_18880 did not reveal any conserved motifs previously characterized such as the domains W, L or Y at the C-terminal region (Jiang et al. 2008) suggesting that *Avr-vnt1* belong to a distinct class of RXLR

effectors. The protein sequence of *Avr-vnt1* was further analyzed to determine secondary structure. In total 4 α helices ranging from 5 to 20 a.a. are predicted in the C-terminal region of Avr-vnt1 whereas PITG_18880 depict 6 putative α helices (Figure S1, http://www.sbg.bio.ic.ac.uk/phyre). It suggests that Avr-vnt1 and PITG_18880 have different conformation and may have different virulence targets.

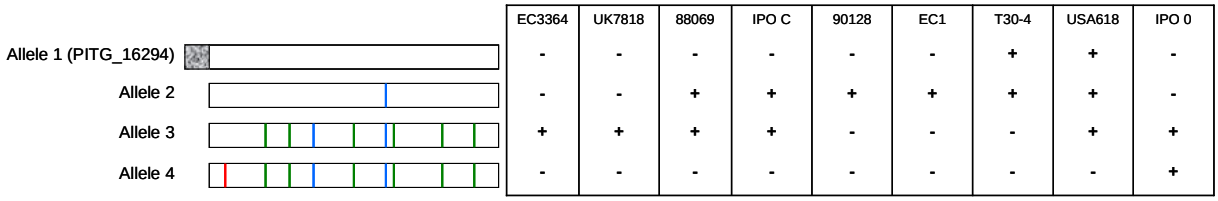
An expressed ortholog of Avr-vnt1 in P. mirabilis, PmAvr-vnt1, is under positive selection

By comparative genomics and transcriptomics, we identified an ortholog of *P. infestans Avr-vnt1* in the closely related species *P. mirabilis* (Sophien Kamoun personal communication. *PmAvr-vnt1* encodes a protein of 135 amino acids that exhibit a distinct C-terminal region with 13 polymorphic amino acids (Figure S2). Signatures of selection in the C-terminus domain of RXLR effectors genes at the intraspecific level have been reported by Win et al. (2007). In this study we estimated the signatures of selection at the interspecific level by comparing orthologs RXLR effector genes. We detected a high ratio of non-synonymous to synonymous substitutions **dN**/**dS** of 2.43 at the C-terminus domain, suggesting *Avr-vnt1* is under positive selection in *P. mirabilis*.

Avr-vnt1 allele mining

In order to determine whether *Avr-vnt1* has more than the two alleles present in the strain T30-4, genomic DNA from 9 *P. infestans* strains was PCR amplified with primers designed after the signal peptide and on the stop codon. All the strains generated the expected PCR product of 393bp. Subsequent cloning and sequencing revealed the presence of four alleles (Figure 3). PITG_16294, now named allele1, was only retrieved in T30-4, the donor of the genome sequence. PITG_22547 was not found back. However, the second allele retrieved from T30-4 contains the synonymous SNP at position 321 but not the synonymous SNPs at position 327. A third allele depicts 6 non synonymous SNPs sharing 95.4% homology at the protein level with the alleles 1 and 2. Then the fourth allele has a stop codon due to a SNP at position 112bp leading to a truncated protein. This allele 4 was only found in strain IPO-0, in combination with allele 3. In this set of tested *P. infestans* strains, allele 2 and 3 were found the most abundantly present, whereas allele 1 and 4 are relatively rare. Generally, the *P. infestans* strains carry one or two alleles, possibly reflecting the diploid state, however strain USA618 is carrying three different alleles of *Avr-vnt1*. Perhaps USA618 is triploid for

the *Avr-vnt1* locus; as triploidy in *P. infestans* has been reported (Carter et al. 1999). Surprisingly, the only strain overcoming *Rpi-vnt1* alleles, EC1, has the fully coding allele 2. Therefore EC1 cannot be distinguished from avirulent isolate 90128 for genetic variation of *Avr-vnt1* alleles.



	EC3364	UK7818	88069	IPO C	90128	EC1	T30-4	USA618	IPO 0
Allele 1 (PITG_16294)	-	-	-	-	-	-	+	+	-
Allele 2	-	-	+	+	+	+	+	+	-
Allele 3	+	+	+	+	-	-	-	+	+
Allele 4	-	-	-	-	-	-	-	-	+

Figure 3. *Avr-vnt1* alleles identified in *P. infestans* strains: EC3364, UK7818, 88069, IPO C, 90128, EC1, T30-4, USA618 and IPO-0. *Avr-vnt1* was PCR amplified with a primer pair designed after the signal peptide (grey box) and in the stop codon. Four alleles were identified carrying synonymous SNP (blue bar), non synonymous SNP (green bar) and a stop codon (red bar). The presence or absence of each allele in studied *P. infestans* strains is represented by a "**+**" or "**–**", respectively.

Avr-vnt1 is transcriptionally silenced in the Rpi-vnt1-virulent strain EC1

To test whether allele 2 is expressed during infection, leaves from *cv. Bintje* were inoculated with zoospore suspensions of EC1 or 90128 and sampled at 2, 3, 4 and 5 days post inoculation. Qualitative RT-PCR showed that *PiAvr-vnt1* allele 2 was expressed in 90128 at all time points with a peak in expression at day 2, in line with microarray data (Figure 2), and which is typical for *P. infestans Avr* genes. Interestingly, expression of *Avr-vnt1* was completely silenced in EC1 (Figure 4).

Subsequent sequence analysis of the promoter region of *Avr-vnt1* from 90128, H30P04, USA618, UK7818, 88069, IPO-0, IPO-C, EC3364 and EC1 strains did not reveal any polymorphism (deletion/insertion or SNPs) that are discriminating between avirulent and virulent strains (Table S3). This result suggests that the loss of expression of *Avr-vnt1* in EC1 may be due to a transcriptional repressor specific to EC1 or may have an epigenetic nature.

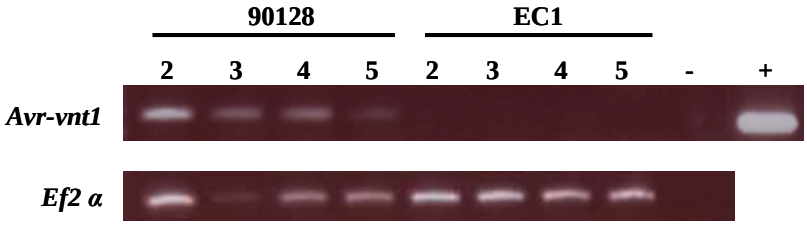


Figure 4. Qualitative expression analysis of *PiAvr-vnt1* was conducted on the strains 90128 (avirulent) and EC1 (virulent) both carrying allele 2. Challenged leaves from cv. *Bintje* were sampled at 2, 3, 4 and 5 days post inoculation (dpi). The elongation factor 2-alpha (*Ef2 α*) was used as endogenous control. A single *E. coli* colony carrying *PiAvr-vnt1* in the *pMDC32* vector was used as positive control.

Avr-vnt1recognizes the three Rpi-vnt1 alleles

In order to check whether PITG_16294 can also trigger HR with the other two alleles of *Rpi-vnt1*, a set of resistant and susceptible genotypes from *S. venturii*, *S. neorossii*, *S. mochiquense* and *S. weberbaueri*, previously characterized by Pel et al. (Submitted), was infiltrated. The outcome of the infiltrations is presented in Figure 1 and Table 1. The three *Rpi-vnt1* alleles (*Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*) are recognized by PITG_16294 and show a clear hypersensitive response. Therefore we conclude that PITG_16294 is the cognate *Avr-vnt1* for all three *Rpi-vnt1* alleles. Upon co-infiltration of *Rpi-vnt1.1* and *Avr-vnt1*, relatively low HR percentages were observed for some genotypes, such as vnt969-2, vnt365-1, vnt365-4 and nrs735-2. These genotypes might not be reluctant to transient agroinfiltration with the strain AGL1. Moreover, also with the *R3a*/*Avr3a* positive control, low percentages of cell death were observed.

Truncated versions of Rpi-vnt1.2 and Rpi-vnt1.3 proteins by having an indel of 33 amino acids in their N-terminal region do not confer late blight resistance. To study whether the entire CC domain of Rpi-vnt1 is required to trigger cell death with *Avr-vnt1*, transformants in cv. Desiree background carrying full-length or truncated *Rpi-vnt1.1* and *Rpi-vnt1.3* were infiltrated with *Avr-vnt1*. As expected, infiltrated genomic fragment of 4.3kb from a BAC clone carrying *Rpi-vnt1.1* and full-length transformants with *Avr-vnt1* showed HR but not the truncated version of *Rpi-vnt1.1* and *Rpi-vnt1.3*. All transformants in cv. Desiree background show a high level of HR (75-100%) and 63-96% to the positive controls of *Rpi-vnt1.1* / *Avr-vnt1* and R3a/Avr3a respectively. To *R3a*, *Avr3a* and *pmdc32* vector controls, a background was observed upon infiltration of alone for the transformants tested, which could be explained by the shape of the transformants presenting sometimes yellowish or curled leaves. In summary, the results of this experiment demonstrate that the entire CC domain is critical to trigger HR.

	Infiltrated genotypes	*Rpi-vnt1 alleles*	*Rpi-vnt1 Avr-vnt1*	*Avr-vnt1*	*Rpi-vnt1*	*R3a*	*Avr3a*	*R3a Avr3a*	*pmdc32*
Wild species	vnt366-1	1.1	92	100	0	0	0	29	0
	vnt367-1	1.1	100	100	1	0	0	33	0
	vnt969-4	1.1	100	100	4	0	13	83	0
	vnt969-1	n.a.	85	4	6	2	0	29	0
	vnt969-2	n.a.	42	0	2	0	4	36	0
	vnt367-4	n.a.	100	0	0	0	0	67	0
	vnt250-2	1.1	73	88	0	0	0	11	0
	wbr253-1	1.1	100	100	50	17	0	8	17
	mcq186-1	1.1	100	100	0	0	0	63	6
	mcq186-2	n.a.	75	25	0	0	0	75	0
	mcq186-4	n.a.	65	0	0	0	0	43	0
	vnt741-1	1.2	92	92	21	13	13	56	0
	vnt896-4	1.3	81	100	0	0	0	38	0
	vnt896-2	1.3	100	100	4	0	0	65	0
	vnt365-1	1.3	17	63	0	0	0	15	4
	vnt365-4	n.a.	42	92	0	0	0	13	2
	nrs735-2	n.a.	48	0	0	0	0	54	0
Transformants in cv. Desiree background	BAC clone Rpi-vnt1.1_1	1.1	100	50	0	54	56	63	38
	BAC clone Rpi-vnt1.1_3	1.1	96	100	0	23	19	75	2
	BAC clone Rpi-vnt1.1_4	1.1	100	100	6	50	29	88	8
	full length Rpi-vnt1.1_15*	1.1	100	71	17	4	17	81	8
	full length Rpi-vnt1.1_16*	1.1	85	71	13	46	17	65	8
	full length Rpi-vnt1.3_7*	1.3	75	25	23	33	13	75	8
	full length Rpi-vnt1.3_8*	1.3	100	50	0	50	44	85	40
	truncated Rpi-vnt1.1_2*	n.a.	83	13	0	29	13	96	42
	truncated Rpi-vnt1.1_4*	n.a.	100	2	0	21	6	67	0
	truncated Rpi-vnt1.3_7*	n.a.	100	25	13	52	21	85	27
	truncated Rpi-vnt1.3_8*	n.a.	100	21	0	35	29	96	8
	Desiree	n.a.	79	35	8	54	58	67	19

*: regulatory elements of *Rpi-blb3* gene (Lokossou et al. 2009)

n.a.: no functional *Rpi-vnt1* allele

Table 1. Scores of infiltrated genotypes from *S. venturii* (vnt), *S. weberbaueri* (wbr), *S. mochiquense* (mcq), *S. neorossii* (nrs) and genetic transformants in cv Desiree background. For each condition a percentage of hypersensitive response (HR) was calculated and shaded in dark grey (80-100%), grey (60-79%), pale grey (59-30%) or white (0-29%). Co infiltration with *Rpi-vnt1*/*Avr-vnt1* and *R3a*/*Avr3a* served as positive control. Genotypes from which *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* were originally cloned are presented in bold (Pel et al. 2009; Foster et al. 2009).

DISCUSSION

An efficient and high throughput functional screening of a genome-wide set of expressed RXLR effectors of *P. infestans* in resistant wild potato species has led to the identification of *Avr-vnt1*, the cognate avirulence gene of *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*. Genomic studies showed that *Avr-vnt1* is located in a Gene-Sparse Region at a single locus in the reference genome T30-4. Four *Avr-vnt1* alleles, sharing a homology between 97.7-99.7% and 95.4-100% at the nucleic and protein levels respectively, were mined from 10 *P. infestans* isolates. One of the four alleles had an early stop codon leading to a truncated protein and the three others encoded full length proteins of 153 amino acids. At the genomic DNA level, virulent and avirulent strains could not be discriminated. At the transcriptional level however, evidently no mRNA was detected in the virulent strain EC1, and we conclude that silencing of *Avr-vnt1* led to virulence on *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpivnt1.3* plants.

Silencing of *Avr* genes has thus far not been reported for *P. infestans*, but there are some reports from other Oomycetes and other plant pathogens. Loss or variation of effector gene transcription has been reported as a mechanism to gain virulence in *P. sojae Avr1-b-1*, *Avr1a* and *Avr3a* (Shan et al, 2004, Qutob et al, 2009, Dong et al 2009). A virulent *P. sojae* strain showed loss of *Avr1b*-1 expression and was able to escape recognition by *Rps1b* in soybean (Shan et al. 2004). Upon genetic complementation, a second locus, *Avr1b-2*, was found to be involved in the accumulation of *Avr1b-1* mRNA. The fact that the 5'UTR and promoter region of *Avr-vnt1* have few SNPs which seem not to be correlated with avirulence properties suggests that a silencing mechanism such as post transcriptional gene silencing (PTGS) may be involved. PTGS occurs after transcription by degrading double stranded RNA (dsRNA). Small RNAs (19–25 nucleotide-long) hybridizing to their target creating double stranded RNA (dsRNA) which is going to be processed towards degradation by several enzymes such as Dicer enzyme and RISCs complex (Voinnet 2008). Several studies reported on the role of PTGS in plant-microbe interactions (reviewed by Voinnet 2008) from the plant or pathogen side to activate or suppress plant defense respectively. The next step would be to determine whether the silencing of *Avr-vnt1* in EC1 strain is due to PTGS. The most straightforward approach would be to carry out a differential screen of small RNA libraries from avirulent and virulent strains. The role and significance of PTGS in *P. infestans* remain to be investigated.

The efficiency of the identification of *Avr-vnt1* effector in *P. infestans* resides in the availability of sequence and expression data for a repertoire of 563 predicted RXLR effectors. We also used the described RXLR families to characterize two other *Avr-vnt1* variants that

clustered with PITG_16294 in the same family (RXLRfam96) (Haas et al. 2009). *P. infestans* RXLR effector candidates were screened based on their expression patterns as described by Haas et al 2009. *PiAvr* genes are typically up-regulated at the early stage of infection in potato (2-3 days post inoculation, dpi). At this phase they are expected to play a major role in reprogramming plant cells, such as Avr3a which is involved in the suppression of PAMP related cell death (Bos, 2006), and therefore we postulate that selecting for highly expressed RxRL effectors significantly enriches the effector collection for *Avr* genes.

Another aspect that rendered the effector genomics approach successful is attributed to the choice of the functional expression testing system in *Solanum* plants and *P. infestans* effectors. In contrast to former strategies, undertaking classical and laborious map-based cloning or cDNA-AFLP marker development (Van der Lee et al. 2001; Armstrong 2005; Van Poppel et al. 2009), high throughput screening of candidate *Avr* genes in wild potato species provides broader perspectives. Although the model plant *Nicotiana benthamiana* is most commonly used for *in planta* functional *R*-/*Avr*- expression systems, in our case, infiltration of *N. benthamiana* leaves with *Rpi-vnt1.1* gene under its native regulatory elements resulted in a patchy and yellowish phenotype (data not shown). To avoid background upon co-infiltration of *Rpi-vnt1.1* and candidate effectors, we chose to infiltrate a wild resistant *Solanum* genotype naturally expressing *Rpi-vnt1.1*. Our observations showed that depending on the *R*-gene used, *N. benthamiana* is not always the most suitable background for *R*-/*Avr*- interaction studies and also wild *Solanum* plants can serve for this goal. Instead of focusing on one particular *Avr*/*Rpi*- gene interaction, high throughput screening of candidate *Avr* genes in wild potato species enables to study multiple interactions within a given late blight resistant genotype. For example, it was shown that *R3a* does not solely interact with *Avr3a,* but also with distinct homologues from *Phytophthora sojae* (Win et al. 2007). More recently, *R2* was shown to be recognized by a variety of RXLR effectors with share sequence homology (Rietman and Champouret in preparation).

Little is known about the intrinsic function of Avirulence proteins and how they can affect plant innate immunity, although insights are gaining. For example, (Bos 2006) showed that Avr3a could suppress program cell death (PCD) triggered by the INF1 elicitin protein characterized as a microbe-associated molecular pattern (MAMP) (Nürnberger et al. 2004). A similar inhibition has been recreated by its homologue in *P. sojae, Avr1b,* which is able to block BAX-induced cell death (Dou 2008). Moreover, another homologue in *P. sojae*, *Avh1b* is able to induce R3a-dependent cell death (Win et al., 2007). We stress that for agricultural exploitation of *Rpi-vnt1,* a thorough understanding of its virulence function is of high priority.

Therefore, the extensive study of the repertoire of *P. infestans* effectors focused on their intrinsic functions and genomic dynamics will lead to better understand the *P. infestans*-Potato interaction.

Materials and Methods

Plant material

All the genotypes from wild species used in this study are listed in Table S4. The intraspecific population 7756 (*S. venturii*) was made by crossing the resistant genotype 283-1 (CPC 7129) with the susceptible genotype 368-6 (CGN 17998).

Disease assays

Detached leaf assays (DLA) on the *Solanum* species were carried out as described by Vleeshouwers *et al*. (1999). Leaves were inoculated with 10µl droplets of inoculum ($5x10^4$ zoospores/ml) on the under side and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16h/8h day/night. At 6 days post inoculation, leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant. The *P. infestans* strains used are listed in Table S5.

Agroinfiltration

Agro-infiltration of RXLR effectors was carried out on wild potato species by infiltrating the lower side of plant of four weeks old. Agro-infiltration was prepared according to Van der Hoorn (2000) using *Agrobacterium tumefaciens* cultures with an OD_{600} of 0.3. Infiltrated leaves were scored four days post Agro-infiltration.

Gateway® cloning of RXLR effector into a binary expression vector

A set of 150 RXLR effectors up regulated at 2-3 days post inoculation was synthesized at Genscript. Each effector was obtained in PUC57 vector (http://www.genscript.com/vector/SD1176-pUC57_plasmid_DNA.html) and lyophilised. Once resuspended with water, a Gateway® LR cloning was performed according to http://botserv1.uzh.ch/home/grossnik/curtisvector/index_2.htmlhttp://www.untergasser.de/lab/protocols/lr_classic_gateway_reaction_ii_v1_0.htm). DH5α competent cells (Invitrogen) were transformed by heat shock with 5µl of the LR reaction mixture. Cells were selected on LB medium containing 50mg/ml of Kanamycine. Colonies were checked by PCR for the presence of the correct inserts. Positive colonies were grown overnight in LB medium supplemented with 50mg/ml of Kanamycine to extract the final expression vector. The final expression vector was transferred to *Agrobacterium tumefaciens* strain AGL01 through

electropration. Colonies were selected on LB medium supplemented with 50ug/ml of Kanamycine and 75 ug/ml chloramfenicol overnight at 30ºC.

5' Rapid Amplification of cDNA Ends

The GeneRacer™ Kit from Invitrogen was used according to the manufacturer's instructions to determine the 5' terminal structure of *Avr-vnt1* from the strains 90128, PIC 99189, IPO-0 and T30-4. A gene specific primer (GSP1-5race Table x) was designed to perform a nested PCR using cDNA template from relevant parental genotypes and transgenic plants and High Fidelity Platinum® *Taq* DNA Polymerase (HT Biotechnology Ltd). PCR products were cloned into the pGEM®-T Easy Vector (Promega) and sequenced.

RNA extraction

For RNA extraction, leaf tissue samples (challenged and unchallenged) were collected, immediately frozen in liquid nitrogen, and then kept at -80ºC. Total RNA was extracted using the RNeasy plant mini kit (Qiagen, Valencia, CA) 1.5µg of total RNA for each sample was treated with RNase-free DNase (Invitrogen) in a volume reaction of 15µl (1.5µl reaction buffer, 1.5µl DNase enzyme, RNase free water).

RT-PCR

Qualitative RT-PCR was performed with two steps. The first strand cDNA was synthesized using 1µg of total RNA (Biorad). Qualitative RT-PCR with Taq-polymerase in a 20µl reaction-mixture was prepared with 1µl of cDNA, 1µl of the forward primer (10µM), 1µl of the reverse primer (10µM), 1µl dNTPs (5mM each), 2µl 10X buffer, 5 units of Taq-polymerase (Perkin Elmer). The following PCR program was used: 94°C for 3 mins, 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, during 29 cycles, 72°C for 5 mins.

Sequencing

Cloned fragments in pGEM®-T Easy Vector from Promega were sequenced as follows: 10µl sequencing reaction mixtures were made using 5µl of PCR product or 5ng of plasmid, 3µl of buffer, 1µl of DETT (Amersham) and 1µl of forward or reverse primer. The PCR program used was 25 cycles of 94ºC for 20sec, 50ºC for 15sec, 60ºC for 1min. The sequences were generated on ABI 3730XL sequencers.

Primer pairs

All the primer pairs used in this study are listed in Table S5.

Positive Selection

To calculate the signatures of selection, we produced a phylip alignment in amino acid of the C-terminus domain in CodonAlign version 2.0. We estimated the ratio of non-synonymous vs. synonymous substitutions dN/dS from the phylip alignment using the yn00 algorithm of PAML version 3.14 (Yang and Nielsen 2000).

Figure S1. Alignment of *Avr-vnt1* alleles: PITG_16294 (allele 1), alleles 2, 3, 4, PITG_22547 and PITG_18880. Predicted signal peptide is presented in italic, the conserved RXLR-EER motif in bold and predicted α helices underlined (http://www.sbg.bio.ic.ac.uk/phyre).

```
PITG_16294  allele 1    MRVYAAFVVALVSLLATNSAVLAVTTPTKLAKLTAPEFTDNENRQ-RRLLRKQEGAELGDEERTSTQNLGNSLMRVFSKE
            allele 2    ----------------------.....................-.................................
            allele 3    ----------------------.....................-...................I......M.......
            allele 4    ----------------------.....................-..............------------------
PITG_22547              .............................................-.................................
PITG_18880              ...L.T..M.M.L...AS....TA...D.....MSTDLIE.RQ.DGV.F..N......D....I.IK.I..F.K.I....

PITG_16294  allele 1    ATRKYYLDLFKRADFTANLPKLAKKGGPDRLNDALKKLRKAGISEEKFAELKGAAAKYADDWYRIYGKLDPIRA
            allele 2    ................................................................................
            allele 3    ...............E...........N.................L.................N........
            allele 4    --------------------------------------------------------------------------------
PITG_22547              ..............................................................------------
PITG_18880              .RK.H..NK..Q..IES...N.....N......Q..K.T....QLK..ES..KG.SKE.F.KF.....V..
```

Figure S2. Alignment of an expressed ortholog of *Avr-vnt1* gene in *P. mirabilis*, *PmAvr-vnt1* (*Pm16294*) and *Avr-vnt1* (*Pi16294*). A high ratio of non-synonymous to synonymous (*) substitutions **dN**/**dS** of 2.43 at the C-terminus domain suggests that *Avr-vnt1* is under positive selection in *P. mirabilis*. Amino acids under positive selection are indicated with a double dot (**:**).

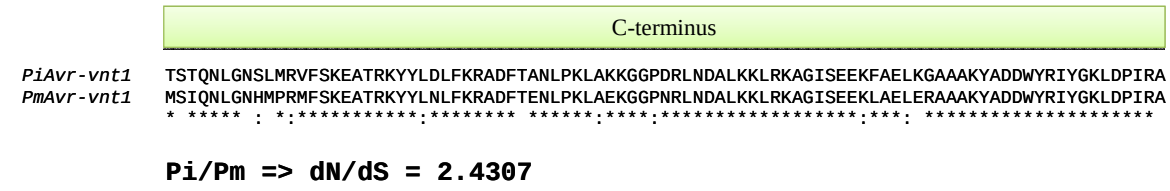


Table S1. Table showing PITG_16294 infiltration scores on the genotypes: vnt283-1, vnt368-6, 7756 F1 30, 7756 F1 89, vnt741-1 and vnt365-1. We infiltrated three leaves per plant. This experiment was done using two biological replicates. Co-infiltration *R3a*/*Avr3a* and *Rpi-vnt1.1*/PITG_16294 were used as positive control whereas infiltration of *Avr3a* and *Rpi-vnt1.1* alone served as negative controls. For each condition a percentage of cell death response was calculated. and shaded in dark grey (80-100%), grey (60-79%), pale grey (59-30%) or white (0-29%).

Genotype	*Rpi-vnt1* alleles	Agro-infiltration (4 dpi)				
		Rpi- vnt1.1 PITG_16294	*PITG_16294*	*Rpi- vnt1.1*	***Avr3a***	***R3a Avr3a***
vnt283-1	1.1	89	89	1.39	0	58.3
vnt368-6	n.a.	100	0	0	0	0
7756 F1 30	1.1	100	100	0	0	4.17
7757 F1 89	n.a.	100	0	0	0	8.33
vnt741.1	1.2	92	92	13	13	56
vnt365.1	1.3	17	63	0	0	15

n.a.: no functional *Rpi-vnt1* allele

Table S2. Detached leaf assay (D.L.A.)and *Avr-vnt1* infiltration scores on the population 7756 (resistant vnt283-1 x susceptible vnt368-6). D.L.A. was performed twice using the strains PIC 99183 and EC1 (avirulent and virulent on *Rpi-vnt1.1* respectively). The EC1 resistant phenotype segregating with a 1:1 ratio is due to the presence of *Rpi-vnt2* (Pel et al. 2010 submitted). Only the F1 plant 34 showed a phenotypic conflict (*). Three leaves from each F1 individual were infiltrated. For each condition a percentage of hypersensitive response (HR) was calculated. Co infiltration with *Rpi-vnt1*/*Avr-vnt1* and *R3a*/*Avr3a* served as positive control. The population 7756 was PCR amplified to check the presence of *Rpi-vnt1.1* using *ef1α* gene as PCR control for genomic DNA quality.

F1 individuals POP 7756	PCR amplification		D.L.A. (6 dpi)		Agro-infiltration (4 dpi)					*Rpi gene*
	Rpi-vnt1.1	*ef1a*	EC1	PIC99183	*Rpi- vnt1.1 PiAvrvnt1*	*PiAvrvnt1*	*Rpi-vnt1.1*	Avr3a	*R3a Avr3a*	
29	-	+	R	S	100	0	0	0	8.33	…
96	-	+	R	S	100	4	0	0	4.17	…
19	-	+	R	S	100	0	0	0	0.00	…
32	-	+	R	S	100	0	0	0	0.00	…
33	-	+	R	S	100	0	0	0	8.33	…
38	-	-	R	S	100	67	67	50	54.17	…
45	-	+	R	S	100	0	0	0	0.00	…
50	-	+	R	S	100	0	0	0	0.00	…
56	-	+	R	S	71	0	0	0	0.00	…
57	-	+	R	S	100	0	58	0	33.33	…
59	-	+	R	S	75	0	0	0	4.17	…
64	-	+	R	S	100	33	33	4	66.67	…
88	-	+	R	S	75	4	0	0	1.67	…
89	-	+	R	S	100	0	0	0	8.33	…
99	-	+	R	S	75	0	0	0	0.00	…
9	-	+	R	S	100	0	0	0	0.00	…
24	-	+	R	S	100	0	0	0	4.17	…
51	-	+	R	S	100	17	0	0	29.17	…
54	-	(+)	R	S	83	0	0	0	0.00	…
60	-	+	R	S	75	0	0	0	4.17	…
84	-	+	R	S	100	0	0	8	8.33	…
4	-	+	R	S	42	4	4	0	8.33	…
16	-	+	R	S	100	0	0	0	0.00	…
35	-	+	R	S	100	8	0	0	4.17	…
46	-	-	R	S	42	0	0	0	0.00	…
62	-	(+)	R	S	100	0	0	0	41.67	…
78	-	+	R	S	100	0	0	0	4.17	…
90	-	+	R	S	83	0	0	0	0.00	…
93	-	+	R	S	75	0	0	0	0.00	…
91	-	+	R	S	83	0	0	0	0.00	…
70	-	+	S	S	71	0	0	0	4.17	…
21	-	+	S	S	75	0	0	0	0.00	…
18	-	+	S	S	100	0	0	4	0.00	…
20	-	+	S	S	67	0	4	0	0.00	…
23	-	+	S	S	100	0	0	0	4.17	…
25	-	+	S	S	100	0	0	0	50.00	…
26	-	+	S	S	100	0	4	0	8.33	…
34*	-	+	S	S	100	0	4	0	0.00	…
39	-	+	S	S	71	0	33	42	33.33	…
40	-	+	S	S	100	0	0	0	0.00	…
43	-	+	S	S	83	0	0	0	0.00	…

58	-	+	S	S	100	4	0	0	33.33	…
63	-	+	S	S	67	0	0	0	0.00	…
66	-	+	S	S	100	0	0	0	0.00	…
68	-	+	S	S	100	0	8	8	8.33	…
69	-	+	S	S	29	0	0	0	4.17	…
75	-	+	S	S	75	0	0	0	0.00	…
80	-	+	S	S	100	0	0	0	0.00	…
81	-	+	S	S	100	0	0	0	0.00	…
92	-	+	S	S	100	0	0	0	4.17	…
98	-	+	S	S	100	0	0	0	0.00	…
37	+	+	S	R	100	100	0	0	8.33	*Rpi-vnt1.1*
48	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
1	+	+	R	R	100	100	0	0	8.33	*Rpi-vnt1.1*
22	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
36	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
42	+	+	R	R	100	100	25	0	8.33	*Rpi-vnt1.1*
65	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
71	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
86	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
100	+	+	R	R	100	100	0	0	4.17	*Rpi-vnt1.1*
2	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
7	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
8	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
41	+	+	R	R	100	100	0	0	4.17	*Rpi-vnt1.1*
85	+	+	R	R	100	100	0	0	4.17	*Rpi-vnt1.1*
12	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
13	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
27	(+)	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
67	+	+	R	R	100	100	0	0	12.50	*Rpi-vnt1.1*
11	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
47	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
6	+	+	R	R	100	100	21	8	12.50	*Rpi-vnt1.1*
95	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
61	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
83	+	+	R	R	100	100	0	0	4.17	*Rpi-vnt1.1*
17	+	+	R	R	100	100	0	0	0.00	*Rpi-vnt1.1*
53	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
82	+	+	S	R	100	100	0	0	8.33	*Rpi-vnt1.1*
30	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
31	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
77	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
87	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
3	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
5	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
10	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
14	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
15	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
28	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
44	+	+	S	R	100	100	0	0	0.00	*Rpi-vnt1.1*
49	+	+	S	R	100	100	75	42	41.67	*Rpi-vnt1.1*
52	+	+	S	R	75	100	0	0	0.00	*Rpi-vnt1.1*
72	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
73	+	+	S	R	54	100	0	0	0.00	*Rpi-vnt1.1*
74	+	+	S	R	100	100	0	0	8.33	*Rpi-vnt1.1*

76	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
79	+	+	S	R	100	100	0	0	4.17	*Rpi-vnt1.1*
283-1	+	+	R	R	100	100	0	0	0	*Rpi-vnt1.1*
368-6	-	+	S	S	100	0	0	0	0	…

*: conflicting phenotype

(+): faint PCR product

R: resistant

S: susceptible

Table S3. Polymorphisms identified within the 1.3 kb promoter region of Avr-vnt1 cloned from 88069, EC1, EC3364, H30P04, IPO C, T30-4 and USA618 strains. A binary (0-1) system was used to encode SNPs. Only non redundant promoter sequences from a given strain are presented. “ins” and “del” stand for insertion and deletion of few base pairs respectively.

Clones	Polymorphisms																													
	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1	0-1
	A-G	G-A	C-T	T-A	C-T	T-C	T-A	ins-del	C-T	A-C	T-C	A-T	del -G	A-T	T-G	C-T	T-A	G-T	G-A	A-C	G-T	C-A	G-C	T-G	T-A	C-T	A-T	G-del	A-G	G-T	C-T
88069_10	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
88069_9	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
EC1_23	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
EC1_24	-	-	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
EC3364_14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0
EC3364_15	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
H30P04_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	1
H30P04_2	-	-	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	1	0	0	0	0	0	0	0	1
H30P04_3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
H30P04_4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	0
IPO C_5	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0
IPO C_6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1
IPO C_7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
T-30-4_1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T-30-4_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	1	1	0	0	0	0	0	0	0	0	0	0	1
USA618_31	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
USA618_33	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
USA618_34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	1	1	0

Table S4. List of the plant material (genotypes and accessions) used in this study.

Solanum Species	Genotype*	CGN Accession	CPC Accession	GLKS Accession
S. weberbaueri	wbr253-1	…	…	159.1
S. mochiquense	mcq186-1	…	…	2319
	mcq186-2	…	…	2319
	mcq186-4	…	…	2319
S. venturii	vnt367-1	18108	…	…
	vnt367-4	18108	…	…
	vnt365-1	18000	…	…
	vnt365-4	18000	…	…
	vnt969-1	22703	…	…
	vnt969-2	22703	…	…
	vnt969-4	22703	…	…
	vnt250-2	…	…	32794
	vnt741-1	18279	…	…
	vnt896-2	961508	…	…
	vnt896-4	961508	…	…
	vnt366-1	18109	…	…
	vnt366-8	18109	…	…
	vnt283-1	…	7129	…
	vnt368-6	17998	…	…
S. neorossii	nrs735-2	18280	…	…

* Genotype number from the Centre for BioSystems Genomics (CBSG) (Jacobs 2008).

Table S5. List of the *P. infestans* isolates used in disease assays and allele mining of *Avr-vnt1*.

Isolate ID	Country of Origin	Obtained from	Isolation year	Virulence profile
90128	Geldrop, The Netherlands	Govers, Phytopathology WUR	1990	1.3.4.7.(8)
IPO-C	Belgium	Kessel, PRI, WUR	1982	1.2.3.4.6.7.10.11
PIC 99189	Metepec, Mexico	Kessel, PRI, WUR	1999	1.2.5.7.8.10.11
USA618	Toluca Valley, Mexico	Fry, Cornell, USA	n.d.	1.2.3.6.7.10.11
88069	The Netherlands	Govers, Phytopathology WUR	1988	1.3.4.7
EC3364	Ecuador	Govers, Phytopathology WUR	2004	not available
T30-4*	The Netherlands	Govers, Phytopathology WUR	no date	7
UK7818	United Kingdom	Govers, Phytopathology WUR	1978	not available
PIC 99183	Metepec, Mexico	Kessel, PRI, WUR	1999	1.3.4.5.7.8.10.11
EC1	Ecuador	Birch, SCRI, Scotland	n.d.	1.3.4.7.10.11
IPO-0	The Netherlands	Kessel, PRI, WUR	n.d.	0

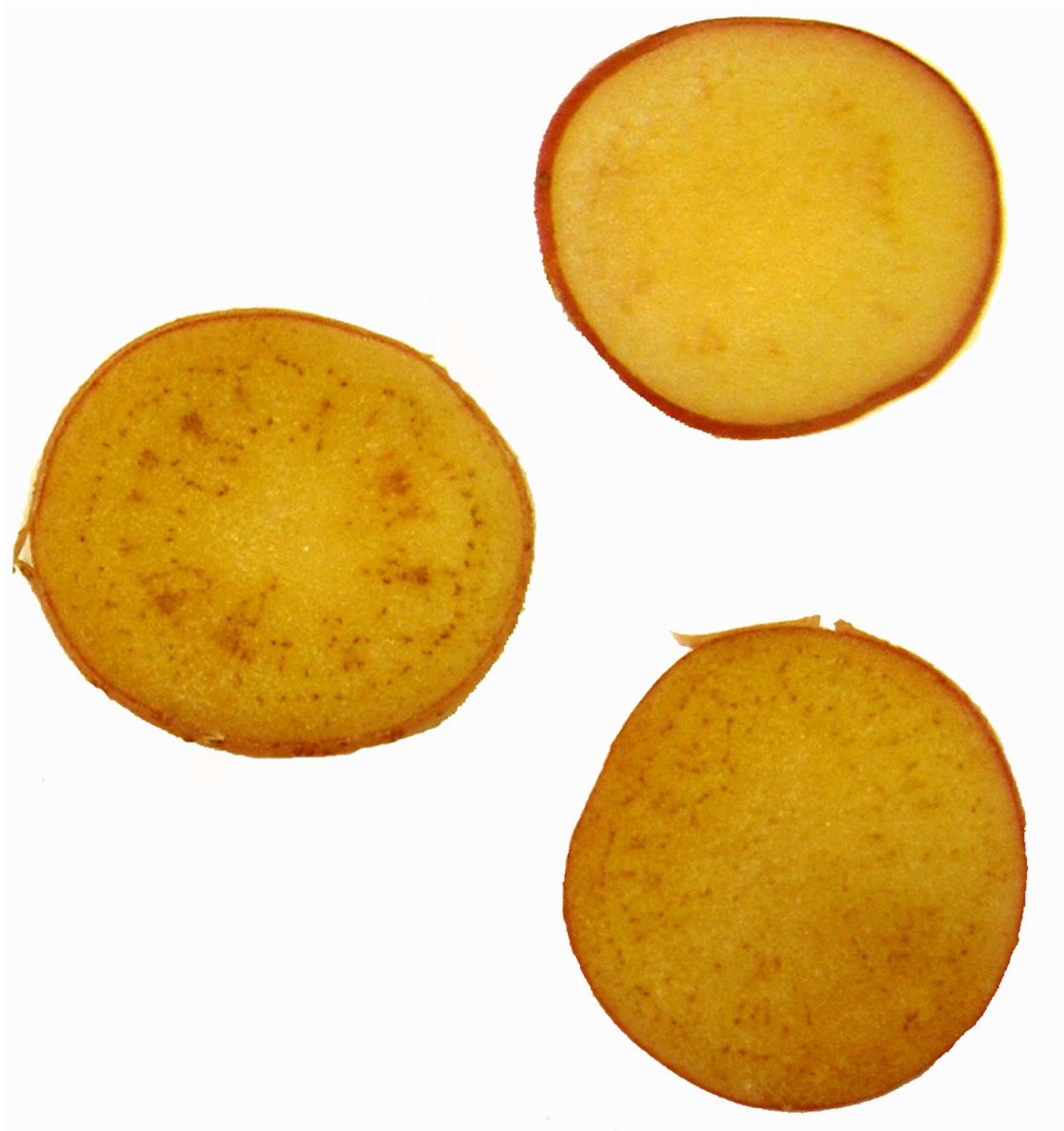
* Laboratory progeny

Table S6. List of primers used in this study.

Gene	Primer orientation	Primer sequence	Annealing temperature(ºC)	Genbank accession
PiAvr-vnt1 -SP	Forward	gtaacgaccccgaccaagtt	60	not available
	Reverse	tcaagctctaataggatcaagc		
PiAvr-vnt1 promoter	Forward	ctactgcatgtagaacaacatc	55	not available
	Reverse	caagcgacgctgacgattc		
PiAvr-vnt1 Q-PCR	Forward	cgaagttgacggctcctg	60	not available
	Reverse	ggctcgcttgaacaaatcc		
ef2 α	Forward	gtcattgccacctacttc	60	Torto et al. 2002
	Reverse	catcatcttgtcctcagc		

CHAPTER 6

Expression levels of Resistance/Avirulence gene matching pair are involved in tuber blight resistance in *Solanum tuberosum*



Pel, M. A., Hutten R. C. B., Jacobsen E.,. Van der Vossen E. A. G., Govers F., Visser R. G. F. and Van Eck H. J.

Transient expression of *Avr-2* gene in tuber tissue from a stable transformant expressing *Rpi-blb3* gene

Expression levels of Resistance/Avirulence gene matching pair are involved in tuber blight resistance in *Solanum tuberosum*

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ABSTRACT

Little is known about the genetic mechanisms governing tuber late blight resistance. Previous studies focused on analyzing the correlation between foliar and tuber resistance in wild species and cultivars at the phenotypic level. Using a transgenic approach four *R* genes, *Rpi-blb3*, *R3a*, *R1* and *Rpi-vnt1.1* were studied in the cultivar (cv) Desiree background for tuber resistance. *R1* is known to confer both foliage and tuber resistance and *R3a* is foliage specific (Oberhagemann et al. 1999; Park et al. 2005). Here we demonstrate that co-expression of the matching *R*-/*Avr*- genes in tuber slices can trigger a Hypersensitive Response (HR). Phenotypic and molecular analysis of three sets of transformants for *Rpi-blb3*, *R3a* and *Rpi-vnt1.1*, and transcriptional analysis of the corresponding effector genes (*Avr2*, *Avr3a* and *Avr-vnt1* respectively) during leaf and tuber infection showed that the expression level of a given *R*-gene in combination with the expression level of the matching effector gene determines tuber late blight resistance. This indicates that an optimal stoichiometry between R- and Avr-proteins is necessary to efficiently trigger a resistance response in the tuber. These results demonstrate that foliar and tuber late blight resistance are controlled by the same genes.

INTRODUCTION

The potato plant, *Solanum tuberosum*, belonging to the *Solanaceae* family, was introduced in Europe by Spanish colonialists in the 16th century. Since that time, it spread around the globe and became one of the most important crops in the world. Its economic value resides in its tubers used for human consumption and starch derived products. A potato tuber is formed from an underground stem called stolon. It is comprised of different types of tissues. The potato skin or periderm is made up of three types of cells: the phellem (cork), the phellogen (cork cambium) and the phelloderm. The periderm forms a barrier at the surface of the tuber that protects it from infection and dehydration (Lulai 2002).

Late blight disease caused by the oomycete *Phytophthora infestans*, is the most damaging disease for the potato crop. *P. infestans* is able to infect all parts of the plant including leaves, stems, inflorescences and tubers. Although most research focuses on late blight foliar resistance, few studies were conducted to investigate tuber resistance. For instance, vascular regions of potato stems and tubers were found to be significantly more resistant to *P. infestans* than the other organs of the plant (Kassim et al., 1976). In the late nineties, Pathak (1987) observed spots in tubers which were similar in appearance to the hypersensitive response (HR), located in the outermost 20-30 cell layers of the cortex. More than one cell was involved in this type of necrosis. He also hypothesized that tuber resistance could be attributed to three major components of the tuber: the periderm, the cortex cell layers and the medulla.

Previously others who studied the inheritance of tuber late blight resistance tried to determine which genetic mechanisms control foliar and tuber blight resistance and what the overlap is. All these studies were conducted on tubers obtained from cultivars, progenitors clones or wild species. A first group of studies pointed at the correlation between foliage and tuber resistance (Wastie et al. 1987). Moreover, Stewart et al. (1992, 1994) showed that indeed foliage and tuber resistance were correlated in certain cultivars like *Stirling*. Quantitative tuber blight resistance was identified by Bradshaw et al. (2006) on chromosome *4* of a dihaploid potato clone (*Solanum tuberosum subsp tuberosum*). A second group of studies have reported on distinct genetic mechanisms controlling foliar and tuber blight resistance (Flier et al. 2001; Liu and Halterman 2009; Nyankanga et al. 2008). Interestingly, Park et al. (2005) showed that differences observed between cultivars for tuber blight resistance may be due to the intrinsic specificity of a given resistance (*R*) gene. *R3a* and *Rpi-abpt* are shown to be foliage specific only whereas other *R*-genes such as *R1* and *Rpi-phu1*, are foliage and tuber

specific (Park et al. 2005; Sliwka et al. 2006). Millett and Bradeen (San Diego 2005) investigated the specificity of the *RB* gene, also known as *Rpi-blb1* (Van der Vossen et al. 2003), to see whether it could confer tuber resistance or not. The *RB* gene was found to be constitutively expressed in leaf and tuber but conferred only foliar resistance. To date, no *R*-genes are known which confer only tuber resistance.

So far, expression of late blight *R* genes was only analyzed in leaf tissue and not in tubers. Recently, high transcript levels of the *RB* gene were demonstrated to correspond to an enhanced resistance level in leaf tissue (Bradeen et al. 2009; Kramer et al. 2009). Brugmans et al. (2008) performed NBS profiling using cDNA from leaf, stem and root showing different *R*-gene expression patterns between tissues and between individuals within a particular tissue.

Several expression studies on *P. infestans* genes were conducted during leaf infection (Birch and Whisson 2001; Tian et al. 2006). Genome wide analysis in various tissues, also in infected leaf tissue were reported by Judelson et al. (2008) and Haas et al. (2009). The latter gives a special focus on genes encoding a RXLR motif. It is a common feature of all identified *Avr* genes in Oomycetes located in their N-termini (Rehmany et al. 2005; Tyler et al. 2006). This latter motif was shown to be involved in the translocation of the effectors into host cells (Birch et al. 2006; Dou et al. 2008; Whisson et al. 2007). The genomes of *P. sojae* and *P. ramorum* revealed that the RXLR effectors family were abundant and evolved rapidly generating over 370 members for each species (Jiang et al. 2008). In *P. infestans*, the genome sequence revealed that RXLR effectors genes are located in Gene Sparse Regions (GSRs), where they can be subject to diversification via duplication, recombination or point mutation (Haas et al. 2009). Known Avr genes of *P. infestans* are all RXLR effectors that are induced at 2 days post inoculation, a stage that is critical to establish infection. Knowing the different cellular environments between leaf and tuber, *P. infestans* genes may depict a different expression pattern in leaf and tuber tissues.

R-gene based resistance to any plant pathogen was conceptualized to make a model known as gene-for-gene interaction (Flor 1971). When matching Avirulence *(Avr)* and Resistance *(R)* proteins are present in the pathogen and the plant respectively, a resistance response is triggered resulting in a hyper-sensitive response (HR) causing necrosis and cell death at the infection site (Dangl et al. 1996). If one of these components is missing the plant-pathogen interaction will not result in a cell death response. *P. infestans Avr*-genes expression may be influenced by the type of plant tissues. All the *Avr*'s and *R*'s that are studied here belong to the RXLR and NBS-LRR families respectively.

To better understand the mechanisms underlying tuber resistance three hypotheses were tested. (**1**) The first one was to determine whether a hypersensitive response (HR) could be triggered, as soon as a pair of matching *R-Avr* genes was stably and transiently expressed in tuber tissue, respectively. To do so an *Agrobacterium tumefaciens* Transient Assay (ATTA) in transgenic tubers carrying either *R1, R3a, Rpi-blb3* or *Rpi-vnt1.1* (Ballvora et al. 2002; Huang et al. 2005; Lokossou et al. 2009; van der Vossen Edwin 2003) was developed. Presence of true HR would show that all the components required to trigger HR are present in tuber as well as in leaf. (**2**) The second hypothesis was to check whether the *R*-gene expression level in tuber tissue was decisive for late blight tuber resistance. A panel of transformants for *Rpi-blb3, R3a* and *Rpi-vnt1.1* was made to monitor *R*-gene expression level and determine whether their expression levels in tuber tissue are correlated with late blight resistance and susceptibility. (**3**) The third hypothesis was to determine whether the pathogen's *Avr*-gene expression level in infected tuber tissue was decisive for late blight tuber resistance.
We here report that the same genes control foliar and tuber resistance. Moreover the expression ratio of a given *R*-gene/effector pair is determinant for tuber late blight resistance**.**

RESULTS

Transient expression of an R-gene/Avr-gene in potato

Primary transformants carrying *R1, Rpi-blb3, R3a* or *Rpi-vnt1.1* in cv. *Desiree* background, showing clear foliar and tuber resistance and expressing the transgene in tuber tissue were chosen to perform ATTAs in tuber. In an attempt to trigger a Hypersensitive Response (HR) in tubers as the result of the co-expression of corresponding pairs of *R*-gene/*Avr*-gene, tubers from transgenic plants carrying *R1, Blb3, R3a* or *Rpi-vnt1.1* were infiltrated with the corresponding Avirulence (*Avr*) gene (*Avr1, Avr2, Avr3a* and *Avr-vnt1* respectively) (Francine Govers personal communication; Lokossou et al. 2009; Armstrong et al. 2005; Chapter 5). After three days post infiltration, necrosis was observed for all the *R*-gene/*Avr* gene pairs tested (Figure 1). *R1* tuber slices showed dense necrosis all over the surface of the infiltrated slices whereas necrosis observed for tuber slices containing *Rpi-blb3, R3a* and *Rpi-vnt1.1* were less dense. Tuber slices of cv. *Desiree* were used as negative control. They did not show any necrosis, neither due to the *Agrobacterium tumefaciens* strain (*AGL1*) used for transient expression nor due to the expression of an effector of *P. infestans* alone. Figure 1 shows 10 times magnified microscopic observation of tuber slices from cv. Desiree::*R1* infiltrated with

either *Avr1* or *AGL1*. Here we showed that *R*-genes were able to recognize their matching effectors in tuber tissue resulting in necrosis formation. In order to confirm that the observed necrosis is a hypersensitive response (HR), the expression pattern of the *HSR203J* gene, known as a molecular marker of a HR (Pontier et al. 2001) was studied in infiltrated tuber slices. As shown in Figure 2, the expression of the *HSR203J* gene was 10, 12, 10.2 and 4 fold expression up-regulated in untransformed cv. Desiree slices once infiltrated with *Agrobacterium tumefaciens* carrying a construct expressing *Avr1, Avr2, Avr3a* or *Avr-vnt1,* respectively. *HSR203J* gene was even more up regulated in transgenic tuber slices expressing the matching *R*-gene (*R1, Rpi-blb3, R3a* and *Rpi-vnt1.1*) showing 17, 19, 18 and 7.5 fold expression once infiltrated with *Agrobacterium tumefaciens* carrying a construct expressing *Avr1, Avr2, Avr3a* and *Avr-vnt1,* respectively. These results confirm that the co-expression of matching *R*-/*Avr*- genes in tuber slices triggers true HR.

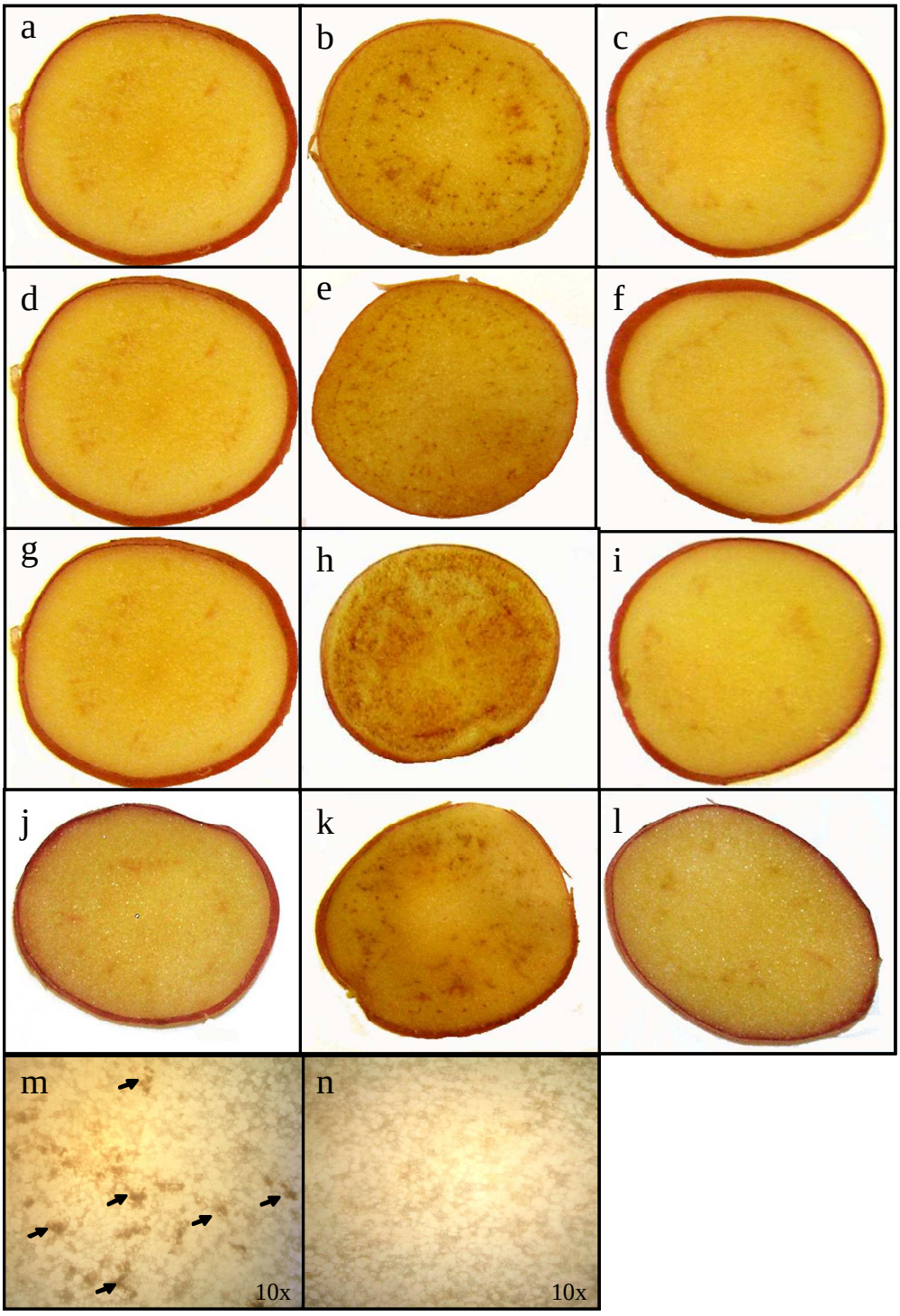


Figure 1. *Agrobacterium tumefaciens* Transient Assays (ATTA). **a**, **d**, **g**, **j** cv. Desiree tuber slices incubated with empty AGL01 strain. **b** and **c**- *Rpi-blb3* transgenic tuber in cv. Desiree background and wild type (WT) cv. Desiree, respectively, both incubated with *Avr2*. **e** and **f**- *R3a* transgenic tuber in cv. Desiree background and WT cv. Desiree, respectively, both incubated with *Avr3a*. **h** and **i**- *R1* transgenic tuber in cv. Desiree background and WT cv. Desiree, respectively, both incubated with *Avr1*. **k** and **l**- *Rpi-vnt1.1* transgenic tuber in cv. Desiree background and WT cv. Desiree, respectively, both incubated with *Avr-vnt1*. **m** and **n**- microscopic observation of tuber slices from cv. Desiree::*R1* and wild type cv. Desiree transiently transformed with *Avr1* respectively. Black arrows indicate necrosis observed when *Avr1* is transiently expressed in cv. Desiree::*R1* tubers.

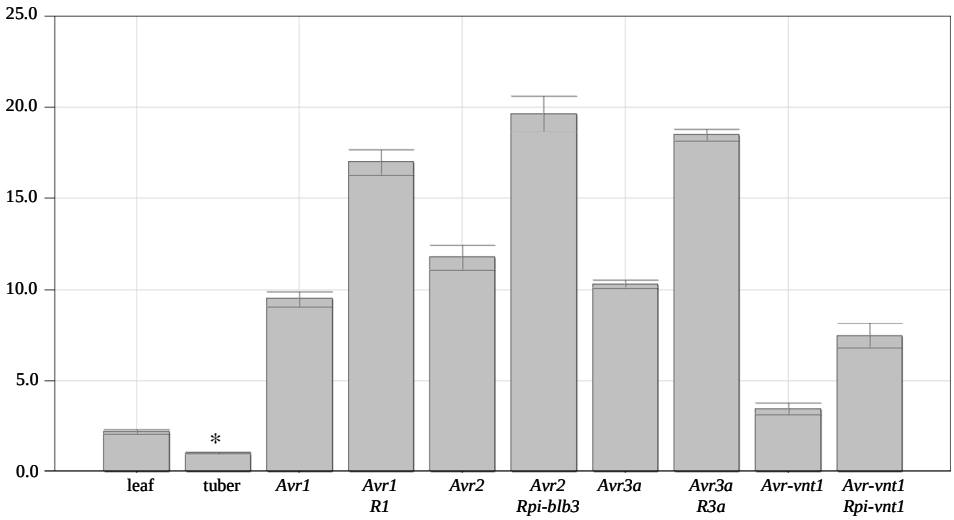


Figure 2. a, Relative expression level of the *HSR203j* gene in tuber slices from the transformants *R1*, A03-15 (carrying *Rpi-blb3*), A04-15 (carrying *R3a*) and Vnt1-3 (carrying *Rpi-vnt1.1*) infiltrated with *Avr1*, *Avr2*, *Avr3a* and *Avr-vnt1* respectively was studied by QRT-PCR. Tuber slices from untransformed cv. Desiree separately infiltrated with *Agrobacterium tumefaciens* expressing each effector gene were used as control. Tissues were sampled 2 days post infiltration. Expression levels were normalized with *ef1α* gene. An arbitrary value of 1 was given to the expression level of *HSR203j* gene in non treated tuber tissue (*). **b**, As *Avr-vnt1* was recently identified, the expression of the *HSR203j* gene was studied in a different experiment. This could explain why the expression levels for all the conditions are lower than those obtained for *R1*, *Rpi-blb3* and *R3a* genes. Both experiments included five tuber slices from two independent experiments for studied condition. A standard deviation of 5% was applied to the graphs.

Resistance signaling pathway(s)

To be fully functional R proteins need protein chaperones such as *RAR1*, *Sgt1* and *HSP90.2* for stabilization and correct folding (Shirasu 2009; Shirasu and Schulze 2003). Upon effector recognition, signal transduction is critical to trigger HR. In Arabidopsis *ESD1* and *NDR1* genes were reported to be determinant nodes in defense pathways (Aarts and Metz 1998). To check whether defense pathways in tuber tissues may be a limiting step for tuber resistance, we analyzed the expression of the potato homologs (*st*: *S. tuberosum*) *stRAR1*, *stSgt1*, *stESD1* and *stNDR1* (Pajerowska 2005) and the gene a*tHSP90*.2 (*Arabidopsis*) in uninfected leaves and tubers from cv. Desiree and in leaves and tubers challenged with *P. infestans* strain 90128 (Figure 3). The gene *stEf1α* was used as an endogenous control to standardize expression levels of *stRAR1*, *stSgt1*, *stESD1*, *stNDR1* and *atHSP90.2* genes. In order to better visualize differences between studied tissues, an arbitrary value of 1 was given to *stRAR1*, *stSgt1*, *stESD1*, *stNDR1* and *atHSP90.2* genes expression levels in tuber tissue. In unchallenged tissue, *stRAR1* gene was found to be expressed at the same level in leaf and tuber (1.1 fold expression) whereas *stSgt1b* and *HSP90.2* genes had lower expression levels in leaves as

compared to tubers (0.8 and 0.5 fold expression change, respectively). *stRAR1* gene did not show down regulation in tuber. Under unchallenged condition, *stESD1* and *stNDR1* genes were higher expressed in leaves than in tubers (2 and 4 fold expression compared to the expression level of 1 in the tuber respectively). However, upon infection, *stEDS1* and *stNDR1* were much higher expressed in challenged tuber tissue (on average 20 and 30 fold expression, respectively) than in challenged leaf tissue (on average 4 and 10 fold expression, respectively) (Figure 4). These results suggest that signal transduction initiated by *EDS1*- and *NDR1*-dependent R proteins may not be affected in tuber tissue challenged with *P. infestans*. These results suggest that tuber blight susceptibility is not caused by a lack of R protein functionality or reduced signal transduction.

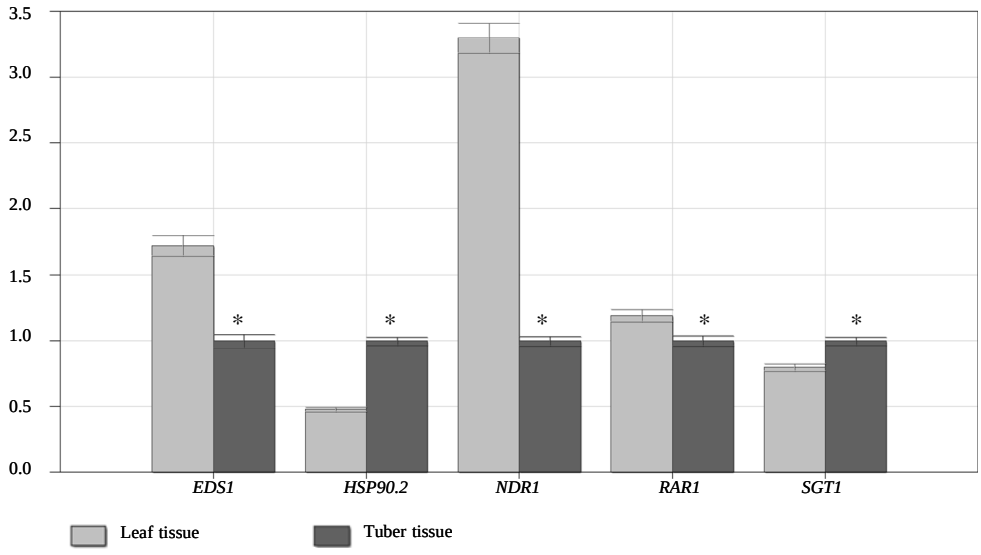


Figure 3. Relative expression levels of *stRAR1*, *stStg1b*, *stEDS1*, *stNRD1* and *HSP90.2* genes in non challenged leaf (at 4 and 5 weeks in the greenhouse) and non challenged tuber (at 0 week after tuber harvest of cv. Desiree) were studied by QRT-PCR. Expression levels were normalized with *ef1α* gene. An arbitrary value of 1 was given to the tuber samples (*).A standard deviation of 5% was applied to the graphs.

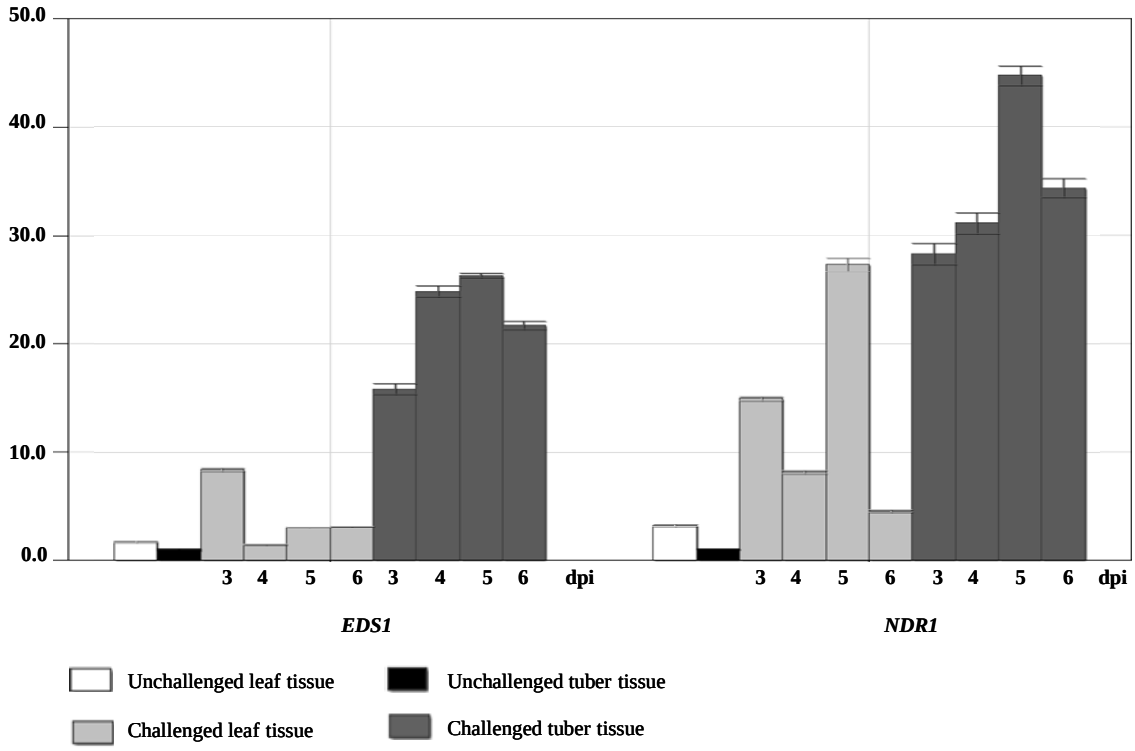


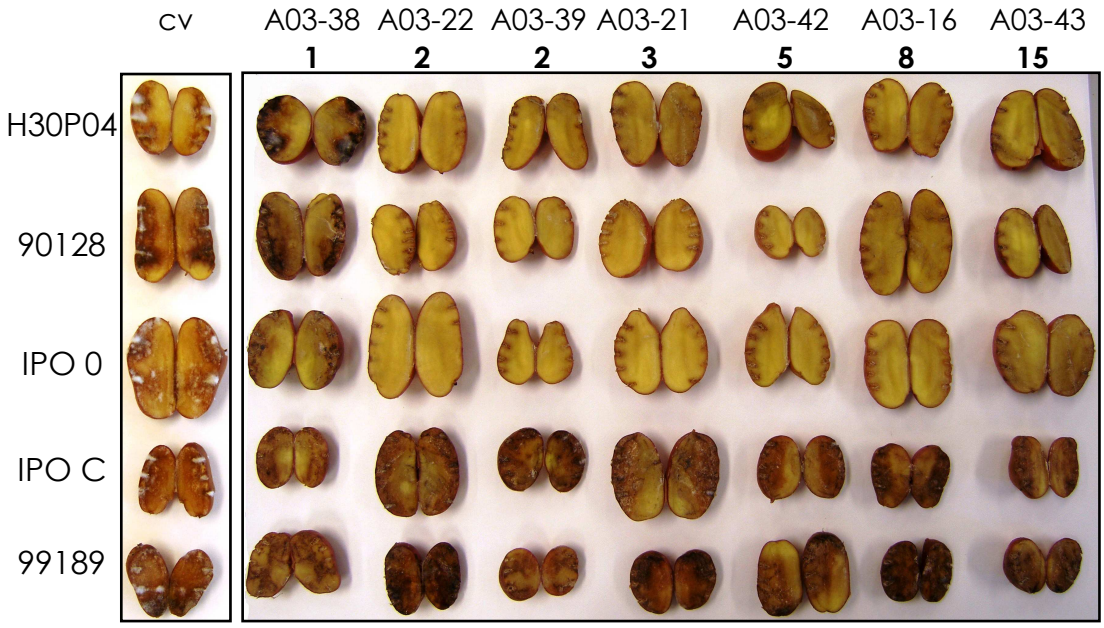
Figure 4. Relative expression levels of *stEDS1* and *stNRD1* genes in challenged leaf and tuber with the strain 90128 (at 3, 4, 5 and 6 days post inoculation, dpi, of cv. Desiree) were studied by QRT-PCR. Expression levels were normalized with *ef1α* gene. An arbitrary value of 1 was given to the unchallenged tuber samples (*).A standard deviation of 5% was applied to the graphs.

R-gene expression in leaf and tuber tissues

Compiling previous result showing that a true HR can be triggered in tuber tissue, we test the second hypothesis investigating whether the tissue specific expression level of a given *R*-gene may determine tuber blight resistance.

To make a diverse set of transformants carrying each different transgene copy numbers, 20 primary transformants carrying *Rpi-blb3*, *R3a* or *Rpi-vnt1.1* genes driven by their native promoter sequence were generated. In these transformants the copy number of the transgene was determined and their resistance/susceptibility phenotype upon inoculation with five *P. infestans* strains was analyzed. For the *R*-genes, *Rpi-blb3*, *R3a* or *Rpi-vnt1.1*, a set of transformants was selected based on a clear foliar resistant phenotype and insertion number ranging from 1 up to 12. For *R1* only one primary transformant was used. This transgenic line, which was kindly provided by Dr. Christiane Gebhardt carries a single copy insertion of

the transgene (Ballvora et al. 2002). Tubers from each diverse set were challenged with five *P. infestans* strains (H30P04, 90128, IPO-0, IPO-C and PIC99189; Table S1) and phenotyped (Figure 5). Interestingly, transformants of *Rpi-blb3* carrying more than one insertion (A03-22, -39, -21-, 42, -16 and -43) display the same resistance spectrum in leaf and tuber. The transformant A03-38 which carries one insertion was susceptible to the five strains tested. Transformants of *R3a* showed two different resistance spectra when challenged with avirulent isolates such as IPO-0 and PIC99189. For instance, all transformants were resistant to PIC99189 whereas those carrying less than three insertions (A03-22 and -3) were susceptible to IPO-0. Regarding transgenic plants carrying Rpi-vnt1.1, challenged tubers showed different phenotypes when compared to each other for a given isolate. The single *R1* primary transformant showed to be tuber blight resistant to IPO-0 but susceptible to an avirulent strain: H30P04. This is in contradiction with the foliar resistance spectrum of the *R1* gene.



A

Transformant	A03-38	A03-22	A03-39	A03-21	A03-42	A03-16	A03-43
Copy Number	1	2	2	3	5	8	15
H30P04	S	R	R	R	R	R	R
90128	S	R	R	R	R	R	R
IPO 0	S	R	R	R	R	R	R
IPO C	S	S	S	S	S	S	S
99189	S	S	S	S	S	S	S

B

Transformant	Vnt1-1	Vnt1-3	Vnt1-4	Vnt1-6	Vnt1-7	Vnt1-8	Vnt1-9
Copy Number	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
H30P04	S	R	R	R	R	R	R
90128	S	R	S	R	R	S	S
IPO 0	S	S	S	R	S	S	S
IPO C	S	R	S	Q	R	R	S
99189	S	R	S	R	R	R	R

C

Transformant	A04-22	A04-3	A04-21	A04-15	A04-30	A04-2
Copy Number	1	2	3 or 4	6	8	12
H30P04	S	S	S	S	S	S
90128	S	S	S	S	S	S
IPO 0	S	QS	R	R	R	R
IPO C	S	S	S	S	S	S
99189	R	R	R	R	R	R

Figure 5. Tuber blight resistance assay: transgenic tubers in cv Desiree background carrying *Rpi-blb3* (**A**), *R3a* (**B**) or *Rpi-vnt1.1* (**C**) were challenged with the strains H30P04, 90128, IPO-0, IPO-C and PIC 99189. The way of scoring tuber blight resistance and susceptibility is illustrated by challenged wounded transgenic tubers carrying *Rpi-blb3* gene. Tuber with brownish to black lesions were scored as susceptible whereas intact tuber as resistant. Three tables show the phenotypic results for the three *R*-gene panels. Insert numbers of the transgene are shown in bold black under the name of each transformant. Challenged tubers were cut at 6 days post inoculation and incubated for 3 more days in the dark. Tubers from cv. Desiree were used as a susceptible control.

The differences observed in the resistance spectrum of a given *R*-gene can be explained at the molecular level. Therefore the expression level of each studied *R* gene was determined for all the transformants in leaf and tuber tissues (Figures 6 and S1). Comparison of *R*-gene

expression level between leaf and tuber tissue showed that *R*-gene expression level was higher in leaf than in tuber for all the transformants tested except for the single *R1* transformant and some of *Rpi-vnt1.1* transformants.

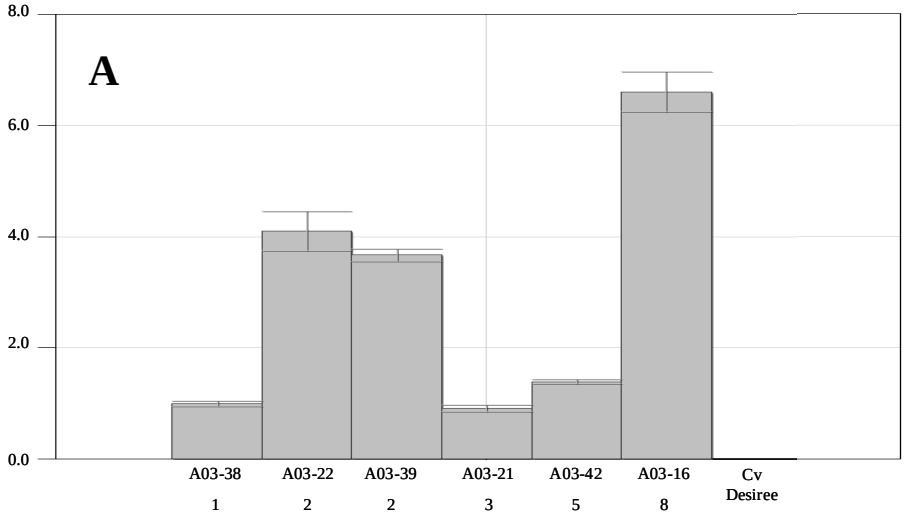
The transformants carrying one (A03-38) and five (A03-42) insertions of *Rpi-blb3* had the lowest expression level of *Rpi-blb3* gene. Interestingly A04-38 and A03-42 were clearly scored as susceptible and resistant respectively when challenged with a strain that is avirulent on *Rpi-blb3* plants (Figure 6). We hypothesized that A03-42 may express *Rpi-blb3* just above the required threshold to trigger an effective resistance response. The detection sensitivity of the quantitative RT-PCR might not be sufficient to separate these two transformants at the molecular level.

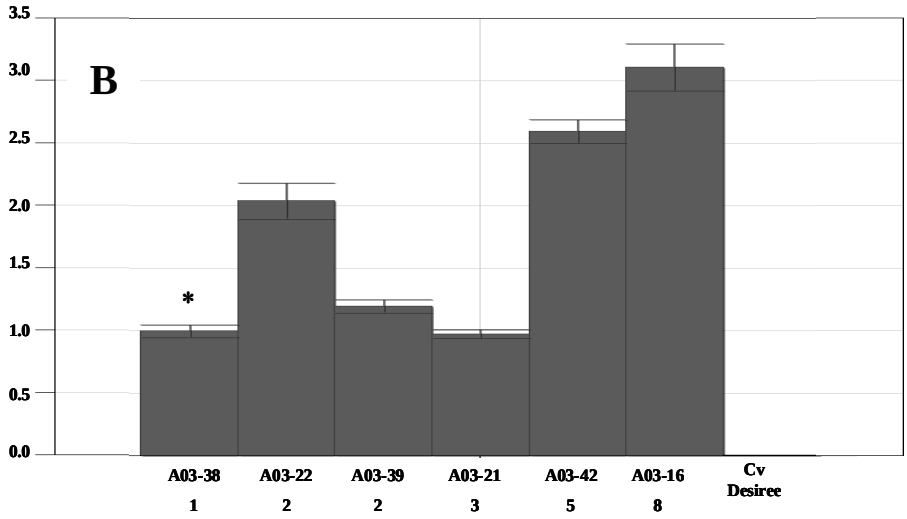
In the case of *R3a* transformants, transformant A04-22 which was scored as susceptible in tuber tissue to all the strains tested, had the lowest *R3a* gene expression level and carried only one transgene. The transformants A04-3 and A04-21 had a similar expression level but A04-3 and A04-21 were found to expanding lesions scored as quantitative resistant and fully resistant to IPO-0, respectively. Here again, it was not possible to distinguish between the transformants A04-3 and A04-21 at the molecular level although two different phenotypes were observed. As observed with *Rpi-blb3* transformants, Q-RT-PCR might not be sensitive enough to discriminate, at the molecular level, between the transformants A04-3 and A04-21. It suggests that a minimal *R3a* gene expression level threshold is necessary to confer tuber resistance.

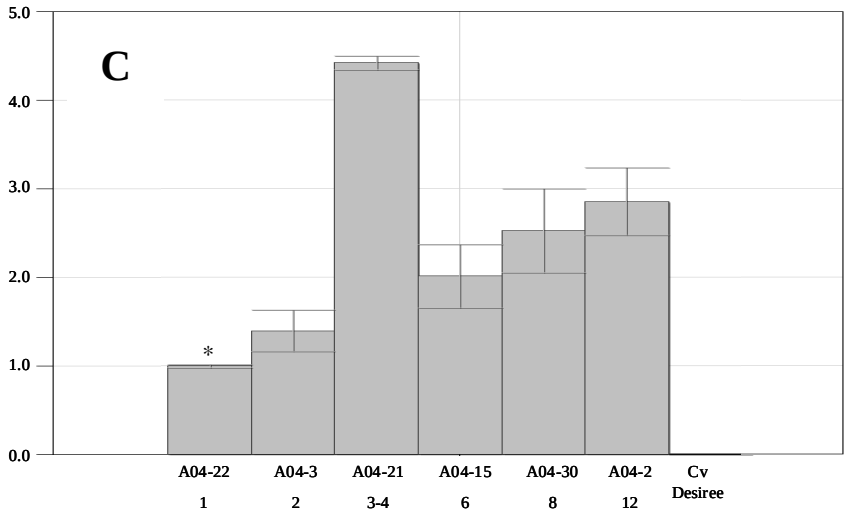
Two out of seven transformants carrying *Rpi-vnt1.1* gene had a higher expression in tuber than in leaf (SF3 and SF7). Interestingly four different resistance spectra were observed when challenged with five avirulent strains (H30P04, 90128, IPO-0, IPO-C and PIC 99189). Surprisingly, the transformant Vnt1-6 which is resistant to the five *P. infestans* strains tested, has a lower expression level than Vnt1-3 and -7 scored as resistant to four strains. The specific primer used to monitor the expression of *Rpi-vnt1.1* were difficult to design. As previously mentioned *Rpi-vnt1.1* share a homology of 75% with *Tm2*^{2} gene and located in one of the three *Tm2*-like R-gene clusters of the chromosome *9* (Pel et al. 2009). The abundance of *Tm2*-like homologs renders difficult to design specific primers to *Rpi-vnt1.1* reaching the requirements of quantitative expression analysis such as G/C content and absence of secondary structure in primers. Upon the testing of 12 primer pairs, only one primer pair gave *Rpi-vnt1.1* specific PCR product. This primer pair was not designed following the requirements for quantitative expression analysis. The non optimal quality of the primer pair

design might explain the lack of correlation between the phenotype and *Rpi-vnt1.1* gene expression level.

Compilation of phenotypic and molecular data suggest that *Rpi-blb3* and *R3a* must reach a certain expression threshold to trigger resistance in tuber tissue. Variation of the tuber resistance spectrum of *R3a* might be due to the expression level of the corresponding effector. It suggests that the ratio between *R*- and *Avr*- genes expression may determine tuber late blight resistance.



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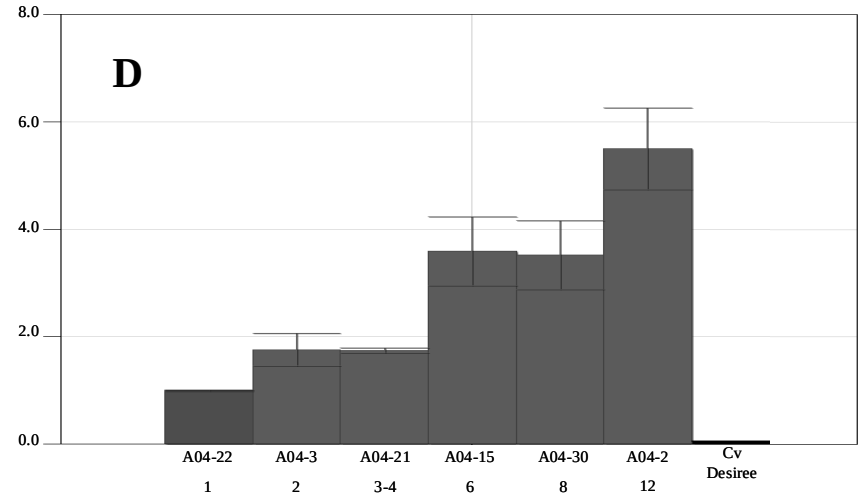
Cv
Desiree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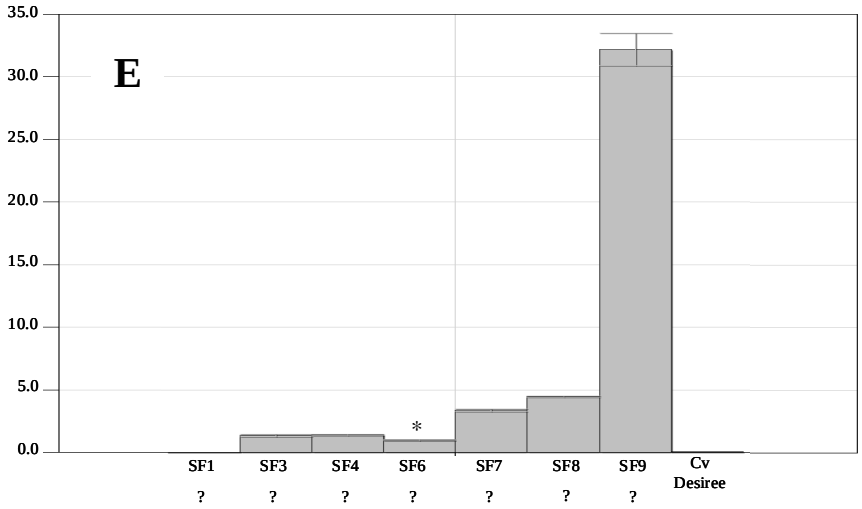
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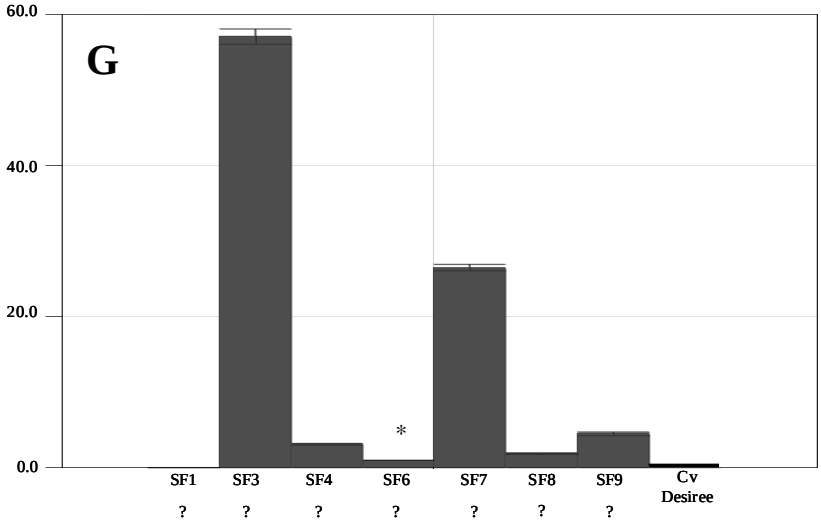


Figure 6. Relative expression levels of *Rpi-blb3* (**A** and **B**), *R3a* (**C** and **D**) and *Rpi-vnt1.1* (**E** and **F**) transformants in non challenged leaf (4 and 5 weeks old in the greenhouse, pale grey on the graph) and tuber (at 0 week old after tuber harvest, dark grey on the graph), respectively, were studied by QRT-PCR. The transformant A03-43 (15 insertions) had a very high expression level, that is why it was not included in the graph. The transformant Vnt1-1 might be an escape as no signal was detected. Expression levels were normalized with *ef1α* gene. An arbitrary value of 1 was given to the transformants A03-38, A04-22 and Vnt1-6 (*) to better compare the change in gene expression between leaf or tuber tissue. Comparison of *R*-gene expression levels between leaf and tuber tissue is presented in Figure S1. A and B- relative. A standard deviation of 5% was applied to the graphs.

Effector gene expression in leaf and tuber tissues

During infection process, *P. infestans* may have to adapt its physiology depending on the type of plant tissue by up-regulating or repressing certain sets of genes. We can speculate that expression of genes encoding effectors might show different expression patterns between leaf and tuber tissues. Changes in the expression pattern of effectors might influence the interaction between a given effector, its virulence target and the corresponding *R*-gene. Therefore the expression of *Avr1*, *Avr2*, *Avr3a* and *Avr-vnt1* was studied in leaves and tubers from cv. *Desiree* challenged with four *P. infestans* strains (90128, IPO-0, PIC 99189 and H30P04). Infected tissues were sampled at 3, 4, 5 and 6 dpi. *Avr1*, *Avr2*, *Avr3a* and *Avr-vnt1* were qualitatively expressed at all time points in the leaves and tubers (data not shown). Upon cloning and sequencing of the RT-PCR products, it was confirmed that the recessive alleles or closely related homologs of the *Avr*-genes, *avr1*, *avr2* and *avr-vnt1*, did not bias the RT-PCR signal. Transcripts of *Avr3a* and *avr3a* could not be amplified separately, because RT-PCR products of both alleles were found. Quantitative RT-PCR showed that *Avr1*, *Avr2*, *Avr3a* and *Avr-vnt1* have different expression patterns in leaf and tuber (Figure 7).

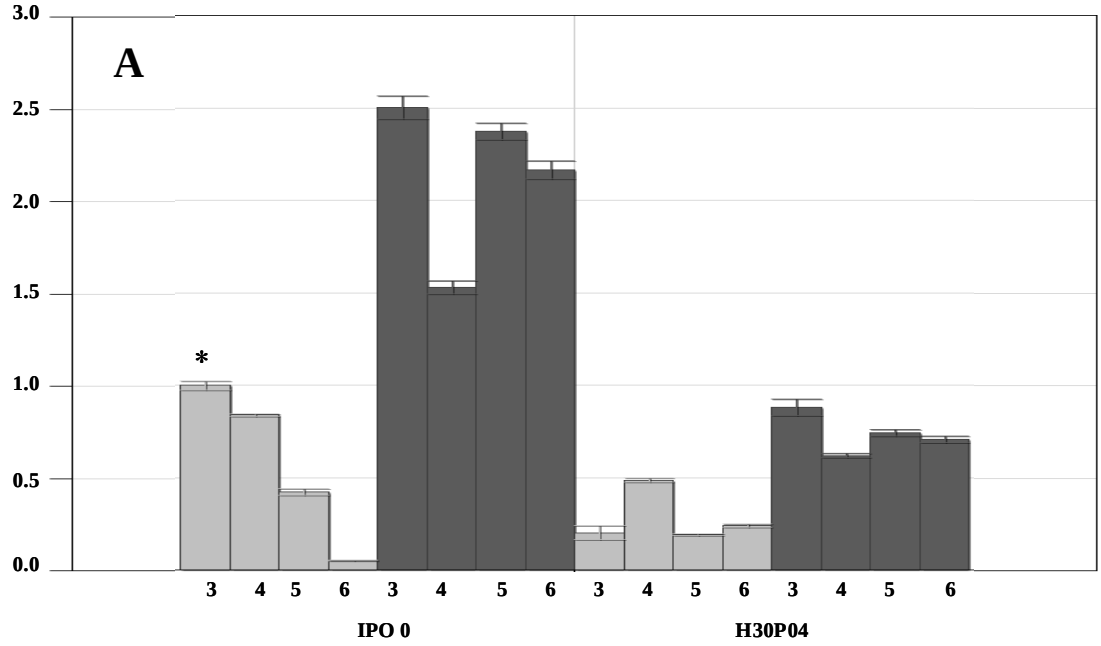
Avr1 expression level was studied in the strains IPO-0 and H30P04 (Figure 7a). Overall, *Avr1* was higher expressed in IPO-0 than in H30P04 when comparing strains and higher expressed in tuber than in leaf when comparing tissues. Changes in expression patterns between IPO-0 and H30P04 do not affect foliar resistance as the *R1* transformant was fully resistant to both strains. However, H30P04 showed a compatible interaction on tuber carrying *R1*. During tuber infection *Avr1* is on average 3.0 fold more expressed in IPO-0 as compared to H30P04. This result suggests that the expression level of *Avr1* affects the resistance response conferred by the *R1* gene in tuber.

Avr2 expression level was studied in 90128, IPO-0 and H30P04 strains (Figure 7b). The same expression pattern was observed for the strain 90128 in leaf and tuber tissues. In IPO-0 and H30P04 *Avr2* expression is higher in tuber than in leaf (4.8 and 1.5 fold expression at 3 days post inoculation, dpi, respectively). Variations in the expression pattern of *Avr2* do not influence the resistance response as all the transformants carrying at least 2 insertions were fully resistant to these three strains (Figure 6).

Avr3a expression was studied in IPO-0 and PIC 99189 strains (Figure 7c). Overall, *Avr3a* was higher expressed in tuber than in leaf. However, both strains showed different expression patterns for *Avr3a* in tuber tissue. For instance, in PIC 99189 *Avr3a* expression gradually increased during infection, whereas in IPO-0 *Avr3a* expression was high in early stages of infection but decreased afterwards. At 3 dpi *Avr3a* in IPO-0 was 6.0 fold higher than in PIC99189. As previously shown, different resistance spectra were observed for *R3a* transformants when challenged with PIC99189 and IPO-0 (Figure 6). They were all resistant to PIC99189 whereas transformants carrying less than 3 insertions were susceptible to IPO-0. Apparently, variation in the expression pattern of *Avr3a* affects *R3a* mediated resistance for a given transformant.

Avr-vnt1 expression was studied in 90128, IPO-0, H30P04 and 99189 strains (Figure 7d). *Avr-vnt1* expression level in the strains IPO-0, H30P04 and 99189 was higher in tuber than in leaf whereas the strain 90128 had the same level of expression in both tissues. Due to the lack of correlation between the phenotype and the expression level of *Rpi-vnt1.1*, it was not possible to determine whether the expression level of *Avr-vnt1* plays a role in *Rpi-vnt1* mediated resistance.

Expression analysis of genes encoding avirulence factors shows that the expression level of *Avr2* gene influences *Rpi-blb3* mediated resistance whereas the expression level of *Avr3a* affects *R3a* mediated resistance.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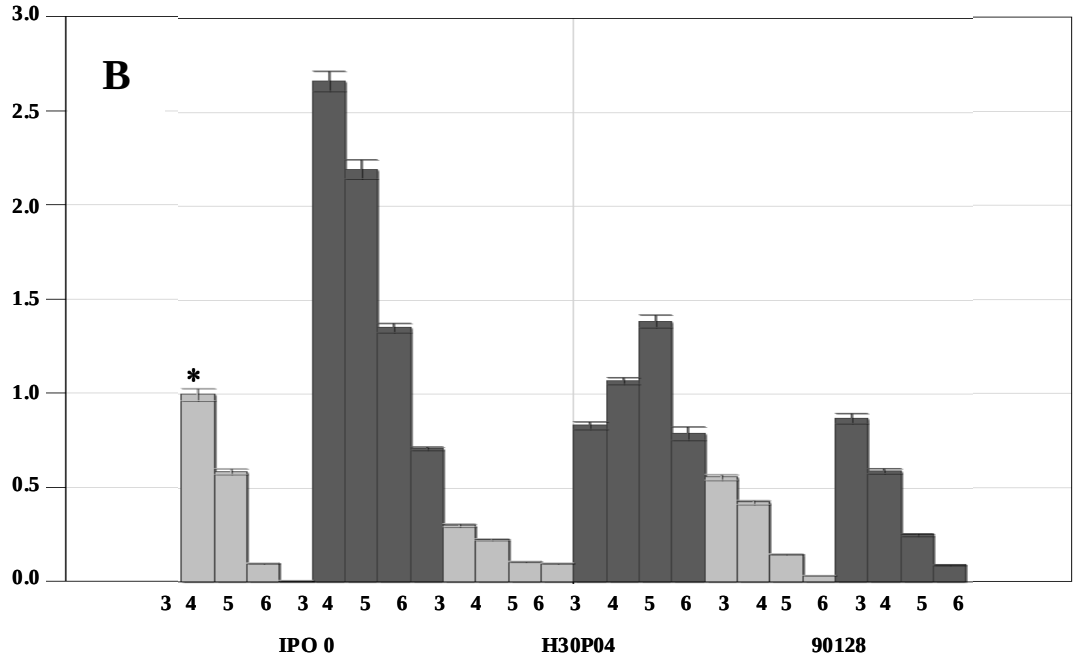
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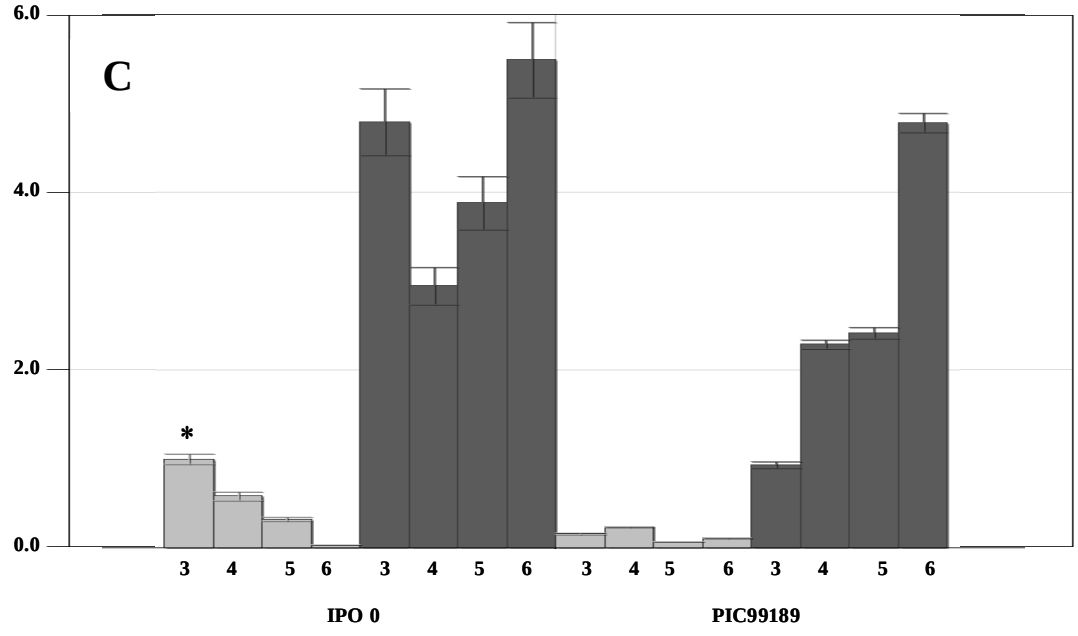
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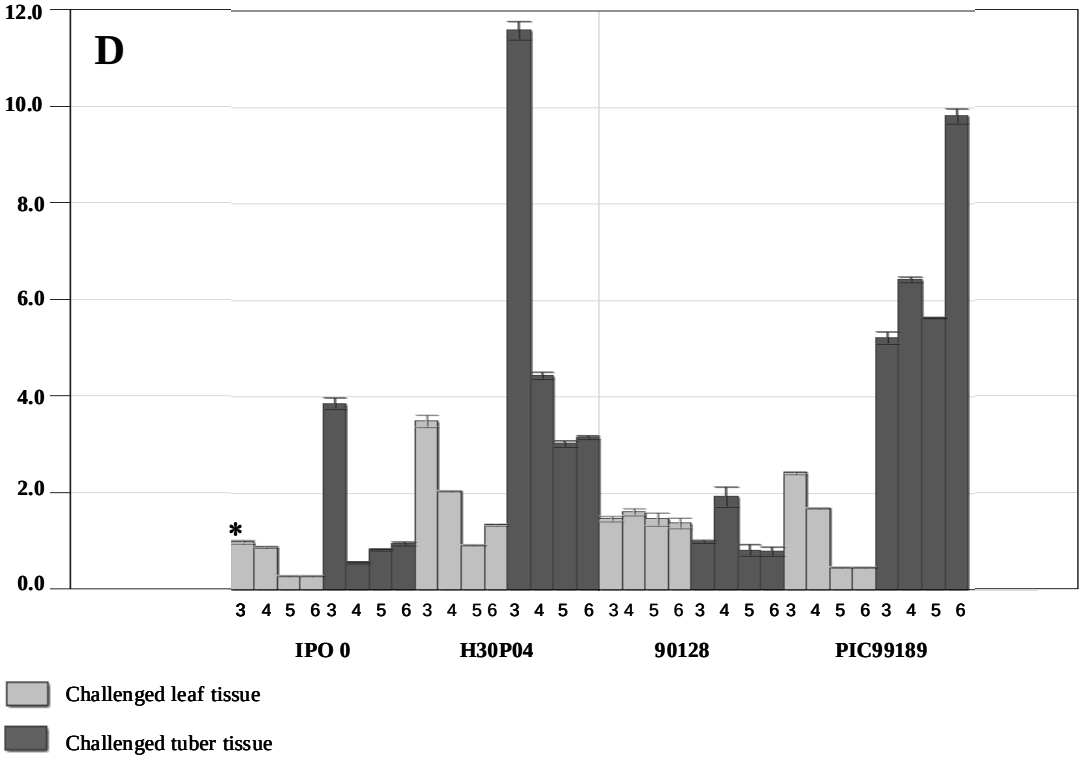


Figure 7. Relative expression levels of *Avr1* (**A**), *Avr2* (**B**), *Avr3a* (**C**) and *Avr-vnt1* (**D**) in challenged leaf and tuber from cv. Desiree at 3, 4, 5 and 6 dpi were studied by QRT-PCR. Expression levels were normalized with *ef2* α gene. An arbitrary value of 1 was given to the leaf samples at 3 dpi from IPO-0 strain (*).A standard deviation of 5% was applied to the graphs.

DISCUSSION

For many years, tuber blight resistance was investigated at the phenotypic level in cultivars or progenitor clones. Subsequent cloning of *Rpi* and *Avirulence* genes combined with a transgenic approach allows for the first time to study tuber late resistance at the molecular level.

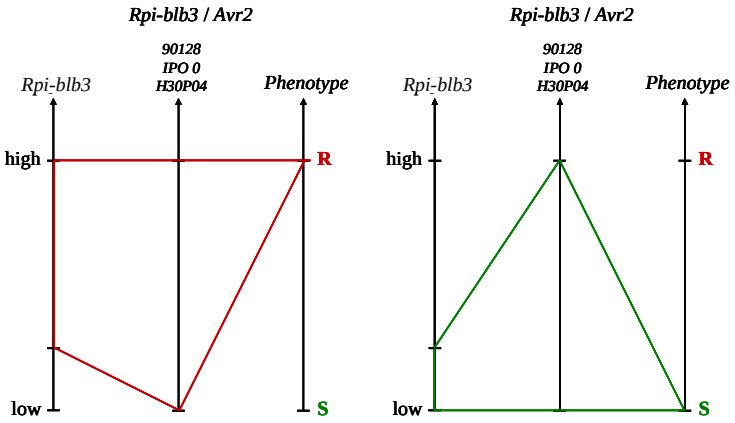
Although Pathak et al. (1987) observed necrosis in tuber tissue, the presence of hypersensitive response (HR) in tuber tissue was never clearly demonstrated. We showed that upon transient expression of *Avr*-genes in tubers carrying the matching *R*-genes HR could be triggered. Aarts et al. (1998) identified two resistance pathways defined by R protein structure types (TIR-NB-LRR and CC-NB-LRR): *EDS1*- and *NDR1*-dependent respectively. *EDS1* and its partner *PAD4* constitute a regulatory platform that is essential for basal resistance. They were found to be important activators of salicylic acid (SA) signaling and also mediate antagonism between the jasmonic acid (JA) and ethylene (ET) defense response pathways (Wiermer et al. 2005). *NDR1* was identified by mutational analyses in *Arabidopsis* and found to be required for *R* gene-mediated resistance with *RPS2*, *RPS5*, and *RPM1* (Aarts and Metz 1998). Tornero et al. (2002) studied *RPP7* resistance pathways by knocking down *NDR1* gene. They identified three phenotypic classes: HR, trailing necrosis and free hyphae (Tornero et al. 2002). The molecular characterization of the observed necrosis in blight resistant tuber showed that true HR was triggered. It suggests that the functionality of defense pathways is not affected in tuber tissues. Beyond *EDS*- and *NDR1*- pathways, a recent study on signaling pathways of *ddnd1* and *dnd2* mutants in Arabidopsis suggested the existence of new defense pathways (Genger et al. 2008) which remain to be explored.

A study on the relationship between *Bremia lactucae* (downy mildew) and *Lactuca sativa* (lettuce) reported on gene-dosage effects to understand host-parasite specificity (Crute and Norwood 1986). Later on, gene dosage effects were found to be involved in pathogen resistance across various crops. For instance, (Sacco et al. 2001) demonstrated that a higher dose of *Lr3* genes in wheat was correlated with a higher resistance level. Loutre et al. (2009) studied the *Lr10*-mediated leaf rust resistance in tetraploid and hexaploid wheat showing that two CC-NB-LRR genes (*Lr10* and *RGA2*) are required to confer resistance. The ratio of the expression of both *R*-genes was hypothesized to be critical for correct functioning.

Gene dosage effects have been studied only at the *R*-gene side and no study has been reported on the effects of the ratio of gene expression between *R*-/*Avr*- genes. Here we showed for the *Rpi-blb3*/*Avr2* gene pair that the expression level of the *Rpi* gene determines resistance. For

the *R3a*/*Avr3a* gene pair, the ratio of gene expression between *R*-/*Avr*- genes determines tuber resistance. Due to the lack of transformants carrying *R1* gene, the characterization of *R1* mediated resistance in tuber tissue could not be done. The phenotypic outcomes of different *Rpi*-/*Avr*- gene expression ratio's studied here are illustrated in the Figure 8. Although tuber blight resistance was observed for the *Rpi-vnt1.1* transformants, no ratio between *Rpi-vnt1.1* and *Avr-vnt1* expression levels could be determined. These different types of *Rpi*-/*Avr*- genes interaction may reflect the activation of different defense pathways whose activation may rely on different mechanisms.

A


Rpi-blb3 / Avr2
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H30P04
Rpi-blb3
Phenotype
high
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R
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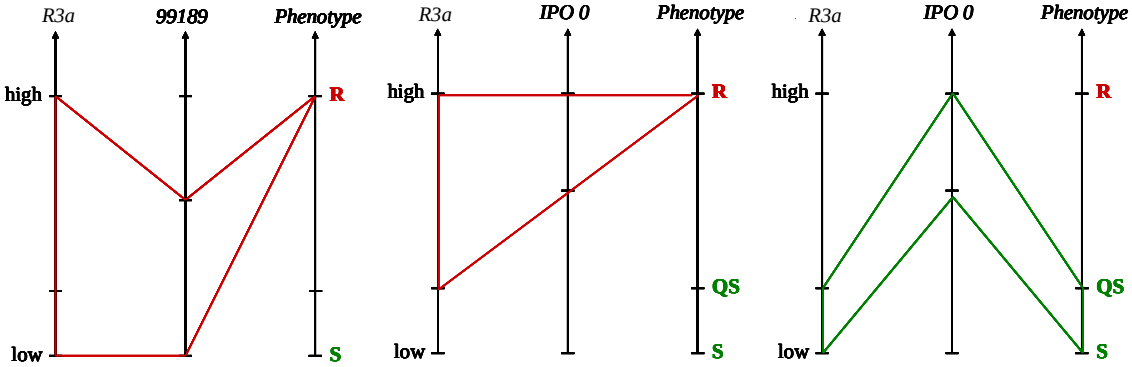
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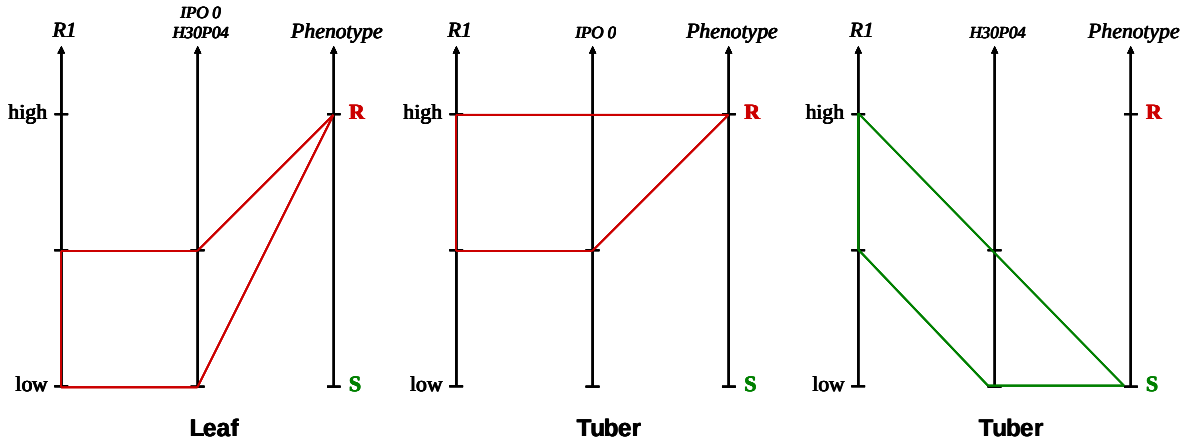
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R1
H30P04
Phenotype
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low
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S
Tuber

Figure 8. Scheme representing *R-*/*Avr*- genes expression thresholds determining tuber late blight resistance and susceptibility. Red and green lines stand for resistance and susceptible phenotypes respectively. **A**- *Rpi-blb3*/*Avr2* interaction in tuber tissue with the strains 90128, IPO-0 and H30P04. *Rpi-blb3* gene must reach a certain expression threshold in order to trigger resistance. This required expression threshold is not affected by variation of the expression of *Avr2* gene. There *Rpi-blb3*-mediated resistance is *R*-gene expression dependent. **B**- *R3a*/*Avr3a* interaction in tuber tissue is also *R*-gene expression dependent. However variation of the expression of *Avr3a* affect the required *R3a* expression threshold. Therefore *R3a*-mediated resistance is *R*- and *Avr*-expression genes dependent. **C**- *R1*/*Avr1* was partially characterized as only one transformant was obtained. Nevertheless, it seems that *R1*-mediated resistance might be affected by variation of the expression of *Avr1* in tuber tissue.

Breeding for late blight resistance is an important goal in potato breeding programs. Breeding for foliar resistance has received most attention because assessment of foliar resistance is more straightforward than tuber resistance regarding disease assays. All the studies investigating tuber blight resistance, were conducted on cultivars or wild species. Here we studied tuber resistance in the same genetic background using a transgenic approach to monitor the expression level of *Rpi*- and *Avr*- genes. We showed that any *R*-gene could confer tuber resistance as soon as the required *Rpi*-/*Avr*- expression ratio is reached. This ratio can be achieved by higher copy numbers of the transgene driven by its native promoter or by replacing the native promoter with a stronger promoter. The observation of lack of correlation between foliar and tuber blight resistance in cultivars and wild species from previous studies (Flier et al. 2001; Liu and Halterman 2009; Nyankanga et al. 2008) can be explained by a low expression of *R*-gene native promoter in tuber tissue. In contrast, *R*-genes defined as foliage and tuber specific (Park et al. 2005) had sufficiently high expression levels in tubers to trigger an effective resistance response.

Differences in expression levels between tissues and *R*-genes, highlight the uniqueness of each *R*-gene promoter. Analysis of a set of transformants with transgene copy numbers ranging from 1 to 12 for *Rpi-blb3*, *R3a* and *Rpi-vnt1.1*, showed no correlation between the number of transgene insertions and the expression level of a given *R*-gene. Variation of transgene expression in plants can be explained by different factors such as RNA silencing, position effects at the insertion site, somaclonal variation and flanking regulatory sequences (Butaye et al. 2005; Matzke and Matzke 1998).

Tubers have an efficient physical barrier based on a thick periderm preventing a pathogen from penetrating. For instance, *P. infestans* is only able to infect tubers via wounds or tuber eyes once they are out of the ground. Therefore wild potato developed efficient defense in leaf which is the most exposed tissue to *P. infestans* in natural condition without human intervention. Natural selection may have favored strong *R*-gene promoters in leaf undermining tuber tissue specificity.

Transcriptional analysis of *Avr1*, *Avr2*, *Avr3a* and *Avr-vnt1* during leaf and tuber infection, revealed that tissues from the plant host may influence the expression patterns of genes encoding in *P. infestans*. Judelson et al. (2009) studied the metabolic adaptation of *P. infestans* during growth on leaves, tuber and artificial media. Interestingly, genes involved in gluconeogenesis and amino acids catabolism were up regulated in tuber tissue when compared to other tissues. This result may be a consequence of the high abundance of glucose and free amino acids in tuber tissues. To what extent, leaf and tuber tissues, used as substrate, may affect the transcriptional regulation of *P. infestans* effector genes is yet to be investigated at a large scale. Microarray study of the whole transcriptome of *P. infestans* would certainly highlight gene families or physiological pathways which are up or down regulated in leaf or tuber tissues. Beside transcriptional analysis, cytological, pathogenicity and virulence studies would contribute to better characterize tuber infection by *P. infestans*.

MATERIALS AND METHODS

Plant materials

Primary transformants in cv Desiree genetic background carrying different number of insertion events for *Rpi-blb3* and *R3a* genes were obtained from Lokossou et al. (2009). The transformant carrying *R1* in cv Desiree genetic background was kindly provided by Dr. Christian Gebhardt (Max Planck Institute-Cologne; Ballvora et al. 2002). Those expressing *Rpi-vnt1.1* in cv Desiree genetic background were previously described by Pel et al. (2009).

Disease assays

Detached leaf assay

Detached leaf assays (DLA) were carried out as described by Vleeshouwers *et al*. (1999). Leaves were inoculated with 10 µl droplets of inoculum ($5x10^4$ zoospores/ml) on the abaxial surface and incubated at 15°C for 6 days in a climate chamber with a photoperiod of 16h/8h day/night. At 6 days post inoculation (dpi), leaves showing sporulation were scored as susceptible whereas leaves showing no symptoms or necrotic lesions were scored as resistant. The *P. infestans* strains used were IPO-0, 90128, H30P04, PIC 99189, IPO-C (Table S1).

Wounded tuber assay

Tubers were first cleaned with 5% sodium hypochlorite for 5 mins and then rinsed three times with tap water. With the tip of a pipette, five aligned holes of 5 mm in depth were made for each tuber. 10 µl of inoculum ($5x10^4$ zoospores/ml) was applied to the wound. Inoculated tubers were kept for 6 days at 15ºC with 100% humidity in a dark climate chamber. At 6 dpi, tubers were cut and incubated for 3 more days.

Transient expression of genes encoding RXLR effector in tuber tissue

Agrobacterium tumefaciens Transient Assay (ATTA) were performed on tuber slices from transgenic and wild type cv. Desiree. Tuber slices of 2-3 min thick were incubated for 15mins with a solution of *A. tumefaciens* (*AGL01* strain) carrying an *Avr* gene (*Avr1*, *PiAvr2*, *Avr3a* or *PiAvr-vnt1* cloned in pGRAB vector) or empty strain. Agro-infection of tuber slices was carried out according to the method of (Van der Hoorn et al. 2000) developed on leaf tissue, using a OD_{600} of 0.1. Slices were phenotyped 2-4 days post infection. This assay was repeated three times.

RNA extraction

For RNA extraction, tissue samples (leaf and tuber) were collected, immediately frozen in liquid nitrogen, and then kept at -80ºC. Total RNA was extracted from leaf tissue using the RNeasy plant mini kit (Qiagen, Valencia, CA) and from tuber using TRIZOL reagent (Invitrogen), and mRNA was purified with a messenger RNA kit (Qiagen). 1.5 µg of total RNA for each sample was treated with RNase-free DNase (Invitrogen) in a volume of 15 µl (1.5 µl reaction buffer, 1.5 µl DNase enzyme, RNase free water).

RT-PCR / QRT-PCR

Qualitative and quantitative RT-PCR were performed with two steps. The first strand cDNA was synthesized using 1 µg of total RNA (Biorad). Qualitative RT-PCR with Taq-polymerase in a 20 µl reaction-mixture was prepared with 1 µl of cDNA, 1µl of the forward primer (10 µM), 1 µl of the reverse primer (10 µM), 1 µl dNTPs(5 mM each), 2 µl 10X buffer, 5 units of Taq-polymerase (Perkin Elmer). The following PCR program was used: 94°C for 3 mins, 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, during 29 cycles, 72°C for 5 mins. Quantitative RT-PCR mix using SYBR green super mix (Biorad real time PCR kit) was performed with 20 times diluted cDNA. The following PCR program was used: 95°C for 3 mins, 95°C for 15 sec and 60°C for 45 sec during 40 cycles, 95°C for 1 min, 65°C for 1 min and 65°C for 10 sec during 61 cycles (melting curve analysis). Amplifications of *ef2*α and ef1α genes (Table S2) were used as constitutive controls for *P. infestans* and cv Desiree respectively (Torto et al., 2002 and Nakane et al. 2003).

Primers

All primer sequences are listed in the Table S2. Primer pairs used in Q-PCR were designed with *Beacon Designer* 7.01 software.

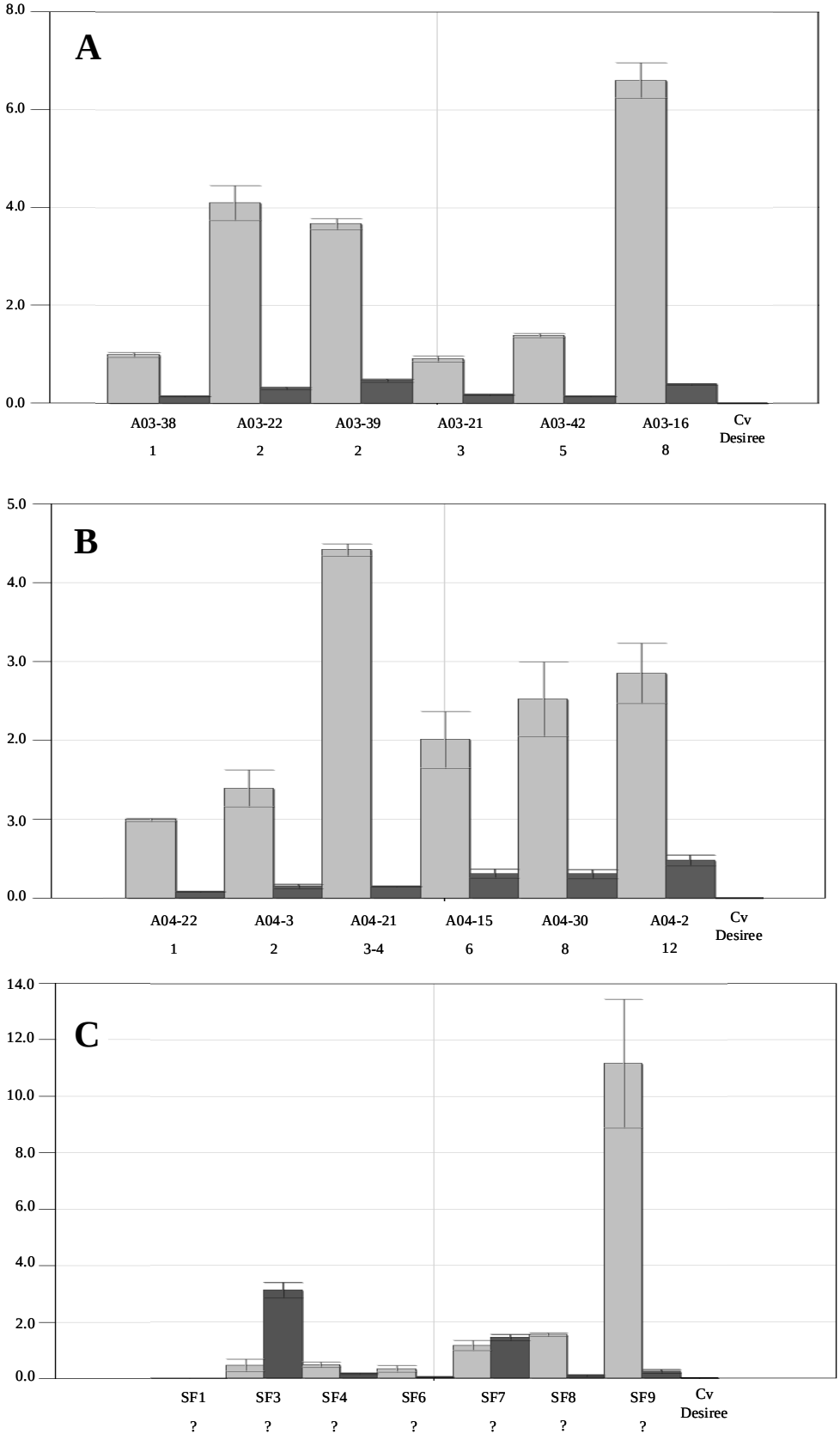
Sequencing

Cloned fragments in pGEM®-T Easy Vector from Promega, or PCR products generated with Taq-polymerase (Perkin Elmer) were sequenced as follows: 10 µl sequencing reaction mixtures were made using 5 µl of PCR product or 5 ng of plasmid, 3 µl of buffer, 1µl of DETT (Amersham) and 1µl of forward or reverse primer. The PCR program used was 25 cycles of 94ºC for 20 sec, 50ºC for 15 sec, 60ºC for 1min. The sequences were generated on ABI 3730XL sequencers.

Defense related genes

Pajerowska et al. (2005) identified in *Solanum tuberosum* (st) potato homologs of well known and characterized defense related genes from Arabidopsis: *stRAR1*, *stSgt1*, , *stESD1* and *stNDR1*. The Genbank accession numbers of these potato homologs and the primer pairs used to study their expression are shown in Table S2.

Figure S1. Relative expression levels of *Rpi-blb3* (**A**), *R3a* (**B**), *Rpi-vnt1.1* (**C**) and *R1* (**D**) transformants in non challenged leaf (4-5 weeks old in the greenhouse) and tuber (at 0 week after tuber harvest). Expression levels were normalized with *ef1α* gene.



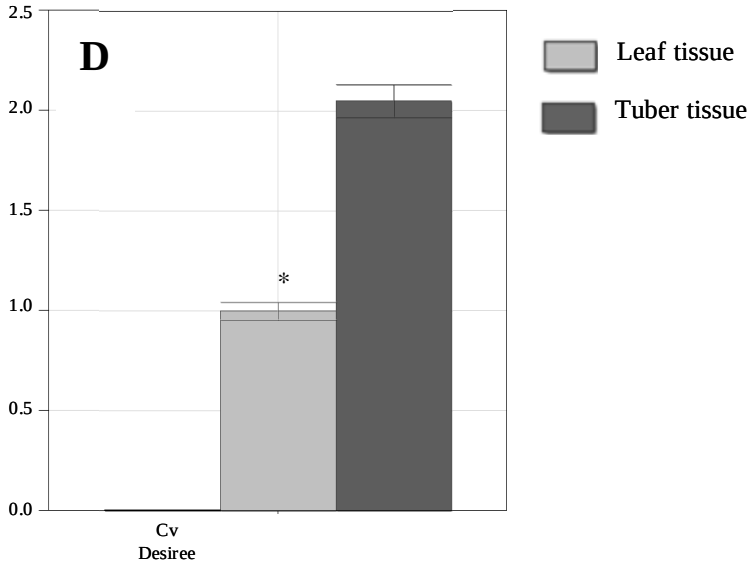

D
2.5
2.0
1.5
1.0
0.5
0.0
*
Cv
Desiree
Leaf tissue
Tuber tissue

Table S1. List of *P. infestans* strains used in disease assays.

Table S1. List of *P. infestans* strains used in disease assays				
Isolate ID	**Country of Origin**	**Obtained from**	**Isolation year**	**Virulence profile**
90128	Geldrop, The Netherlands	Govers, Phytopathology WUR	1990	1.3.4.7.(8)
H30P04*	The Netherlands	Govers, Phytopathology WUR	n.d.	7
IPO-C	Belgium	Kessel, PRI, WUR	1982	1.2.3.4.6.7.10.11
PIC 99189	Metepec, Mexico	Kessel, PRI, WUR	1999	1.2.5.7.8.10.11
IPO-0	The Netherlands	Kessel, PRI, WUR	n.d.	0

*: Laboratory progeny

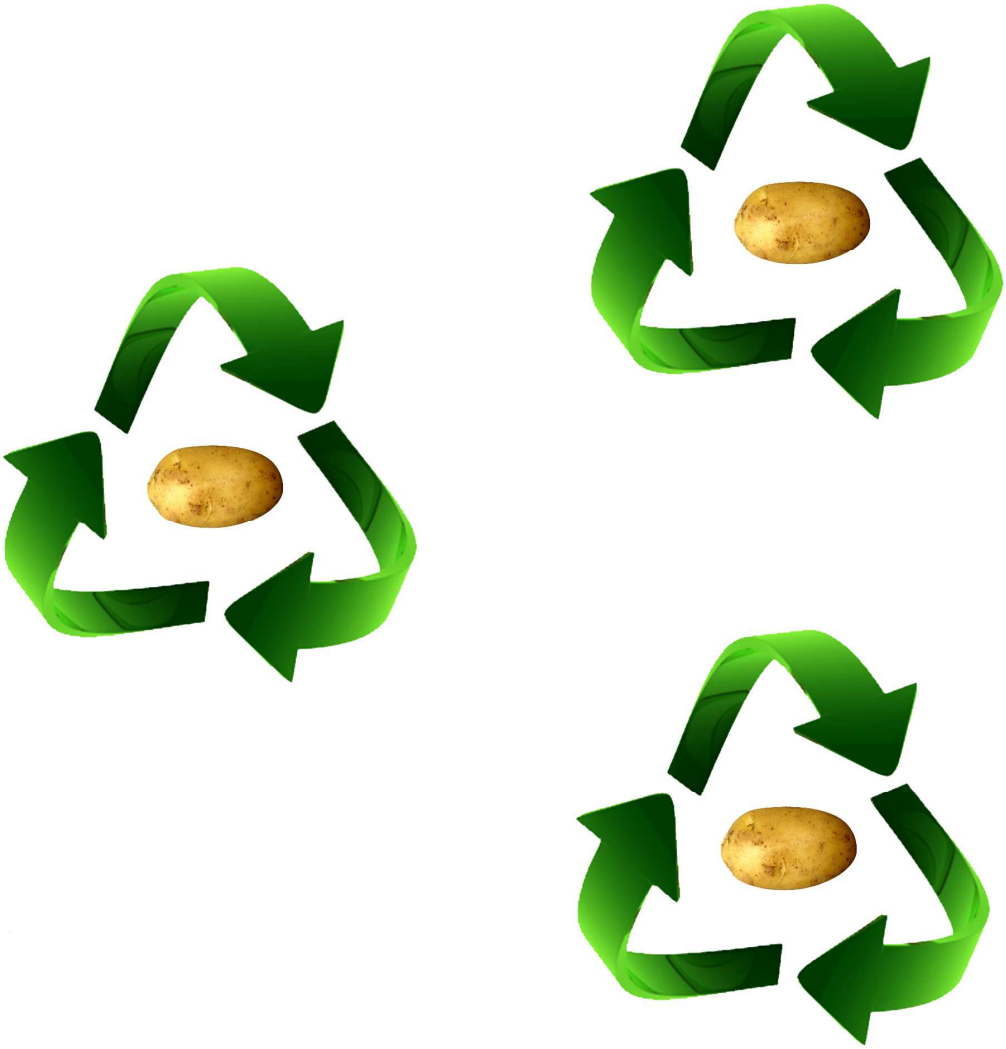
n.d.: not determined

Table S2. List of primers used to performed QRT-PCR analysis in leaf and tuber tissues.

Gene	Primer orientation	Primer sequence	Annealing temperature(ºC)	Genbank accession
R1	Forward	gggatcagattaccaaacct	60	AF447489
	Reverse	tagtgaggatatgtcacgagtg	…	…
R3a	Forward	ccggagtggaagcaatggg	60	AY849382
	Reverse	cttcaaggtagtgggcagtatgctt	…	…
Rpi-blb3	Forward	tgtcgctgaaagagatagacc	60	FJ536346
	Reverse	caccttttgccattggtttag	…	…
Rpi-vnt1.1	Forward	atgaattattgtgtttacaagacttg	58	FJ423044
	Reverse	cagccatctcctttaatttttc	…	…
ef1 α	Forward	attggaaacggatatgctcca	58-60	AB061263
	Reverse	tccttacctgaacgcctgtca	…	…
Avr1	Forward	aagaccgacgagttcaag	60	n.a.
	Reverse	tgatcctccacttaacagc	…	…
Avr3a	Forward	atgtggctgcgttgacggaga	60	AJ893356
	Reverse	tgagccccaggtgcatcaggta	…	…
Avr2	Forward	atgcgtctcgcctacatttt	60	n.a.
	Reverse	aatcctctctcttcctcgatc	…	…
Avr-vnt1	Forward	cgaagttgacggctcctg	60	n.a.
	Reverse	ggctcgcttgaacaaatcc	…	…
ef2 α	Forward	gtcattgccacctacttc	60	Torto et al. 2002
	Reverse	catcatcttgtcctcagc	…	…
stRAR1	Forward	atgatggaacgaagcagtg	58	AY615275
	Reverse	ttggtagcagatggtgttg	…	…
stStg1	Forward	tacgatgtgatgtctacc	58	AY679160
	Reverse	ttgaggaaggataactgg	…	…
stHSP90.2	Forward	ccgacaagactaacaacac	58	NM_124985
	Reverse	gtcagcaaccaagtaagc	…	…
stEDS1	Forward	tctggtgtctggaatgttg	58	AY679160
	Reverse	aagaggagcaagcatcatc	…	…
stNDR1	Forward	gctctctttatctggctaagtc	58	AY615280
	Reverse	gattggatcttgtggtgttgg	…	…
HSR203J	Forward	gtaatgatagttcggttgataagc	60	AB200918
	Reverse	ggaagacggagacaataatagc	…	…

CHAPTER 7

General Discussion



GENERAL DISCUSSION

This thesis presents a study conducted in the frame work of the EU project FP6 program BioExploit Food-CT-2005-513959 named BioExploit. The study starts with wild species germplasm, in particular *Solanum venturii* as a source of resistance (*R*)genes. These *R*-genes have been mapped, cloned, characterized and tested in transgenic plants. The study of *Rpi-vnt1* alleles illustrates the different aspects of cloning of *R*pi-*vnt1* alleles and an allele mining study unveiled the possible evolution of this *R*-gene. It also reports on the identification of its matching effector gene, *Avr-vnt1* and on a much lesser studied aspect of late blight resistance which is tuber late blight resistance of *R*pi-*vnt1* but also of the *Rpi-blb3, R3a* and *R1* genes.

Resistance to *Phytophthora infestans* (*Rpi*) *genes in Potato*

To date, eight *Rpi*-gene clusters have been identified and characterized (chromosome *4*: *R2*-like cluster, chromosome *5*: *R1* cluster, chromosome *6*: *Rpi-blb2* cluster (or *Mi* cluster), chromosome *8*: *Rpi-blb1* cluster, chromosome *9*: *Rpi-vnt1* and *Rpi-mcq1* clusters, chromosome *10*: *Rpi-ber1* and chromosome *11*: *R3a/b* cluster. In the past decade, several late blight *R*-genes were cloned following a classical map based cloning approach (*Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3*, *R3a*, *R1*) (Van der Vossen et al. 2003, 2005; Lokossou et al. 2009; Huang et al. 2004; Ballvora et al. 2002). The cloning of *Rpi-vnt1* alleles was achieved by combining a PCR based strategy using *Tm2a* homologous primers and a classical map cloning approach (**Chapters 2 and 3**). *Rpi-vnt1* alleles were mapped in the same cluster as the $Tm2^2$ resistance gene conferring resistance to Tomato Mosaic Virus (ToMV) using NBS profiling (Van der Linden et al. 2004) and bulk segregant analysis (BSA). RGA markers linked to *Rpi-vnt1* alleles shared 75% homology at the DNA level with $Tm2^2$ gene. This result highlights the fact that genomic information from characterized *R*-gene clusters conferring resistance to any disease, can be efficiently used to localize new *R*-genes. However anchored markers linked to the *R*-gene of interest are needed to confirm localization. The genomic information generated by the Potato Genome Sequencing project will help in the localization and BAC walking steps. The DNA fingerprinting technique specifically targeting *R*-genes, NBS profiling, can easily be customized to target a specific *R*-gene cluster. Therefore markers within a given *R*-gene cluster can be generated easily for genetic mapping. In the case of *Rpi-vnt1* alleles, long range PCR primers were designed to clone and sequence all the *Tm2*-like homologs. Upon

sequence analysis and cosegregation, good candidate genes, fully coding, were chosen for complementation analysis.

Classical map based cloning and NBS-homology based mining are two complementary strategies providing robust physical mapping and candidate genes respectively. The potato genome contains *Rpi*-gene (Resistance to *Phytophthora infestans*) clusters on almost all the chromosomes (*4*, *5*, *6*, *7*, *8*, *9*, *10* and *11*). Remarkably, not a single *R*-gene has been mapped on chromosome *1* and *2*, and no *Rpi*-gene on chromosome *12*. It seems that the identification of new *R*-gene clusters is reaching the end as new *R*-gene specificities are indentified in known clusters. For instance, a resistant accession of the wild species *S. huancabambense* carries an *R*-gene mapped on chromosome 9 (*Rpi-mcq1* cluster) showing a different resistance spectrum than *Rpi-mcq1* (unpublished results). In the near future, once *Rpi*-genes depicting new specificities are mapped, the cloning step is likely to use a straightforward candidate gene approach based on previous characterized *R*-genes from the same cluster.

So far, all the cloned resistance genes against *P. infestans* belong to the CC/Lz-NB-LRR family. A more exhaustive survey of TIR-NB-LRR genes may reveal the proportion of CC relative to TIR within the potato genome. In *Arabidopsis*, TIR-NB-LRR genes represent two-third of NB-LRR genes in *Arabidopsis*. Several TIR and CC *R*-genes from Arabidopsis (*RPP1*, *RPP2a*, *RPP5*, *RPS4* and *RPS2*, *RPS5*, *RPPM1*, *RPP13*, *RPP7*, *RPP8* genes respectively) have been characterized well, and confer resistance to fungal or bacterial pathogens such as *Peronospora parasitica* and *Pseudomonas syringae* (Holub 2001). In *Arabidopsis*, it is not apparent that a specific *R*-gene family evolved for a specific pathogen. Would this be the case for Potato? Were the first successful *R*-genes against *P. infestans* CC-NB-LRR? Positive selection on this latter could have diminished the chances for the TIR-NB-LRR family to evolve recognition of *P. infestans* effectors.

Dynamics of R-gene clusters

As mentioned in **Chapters 2** and **3**, *Rpi-vnt1* alleles share 75% homology at the nucleotide level with $Tm2^2$ gene conferring resistance to *Tomato Mosaic Virus* (*ToMV*). In potato the *Tm2*-like cluster is divided in three sub clusters: *Tm2*-like, *Rpi-vnt1* alleles and *Rpi-mcq1* gene. It is rather interesting to observe that potato may have evolved *R*-genes conferring resistance to *P. infestans* and ToMV from a common *R*-gene ancestor. This illustrates the potential of *R*-gene clusters to generate and to maintain a wealth of *R*-gene analogs (RGAs) that can evolve towards new pathogen recognition of effectors. Within the allele mining study

of *Rpi-vnt1* alleles, coding *Tm2*-like homologs were identified in the late blight susceptible accessions of *S. oplocense* (**Chapter 4**). Interestingly, these *Tm2*-like homologs had their start codon at the same position as $Tm2^2$ gene. In other words, the start codon from *S. oplocense* is located 100bp downstream of the start codon of *Rpi-vnt1* alleles. This first 100bp region encodes the first of the four alpha helices of the Coil Coiled (CC) domain of *Rpi-vnt1* alleles (**Chapter 2**). These coding *Tm2*-like homologs might be functional and confer resistance to other potato pathogens. (Van der Vossen et al. 2005) showed that a similar locus could carry *R*-genes conferring resistance to two completely different pathogens. The *Rpi-blb2* and the *Mi-1* genes conferring resistance to *P. infestans* in potato and nematodes in tomato, respectively, mapped on the chromosome *6*, share 82% homology at the protein level. Therefore it would be worthwhile investigating whether this vnt-allele from *S. oplocense* shows resistance to ToMV.

R-gene evolution involves several genetic mechanisms that are nicely reviewed (Leister 2004; Meyers et al. 2003, 2005) but the question of the fate of *R*-gene alleles remains open. Are the defeated *Rpi*-gene alleles going to be extinct in a host population because of its transient status, or are they becoming very rare until they are re-selected upon re-emergence of the corresponding avirulence gene? These two phenomena were defined as "transient polymorphism model" and "recycling polymorphism model" (also known as trench warfare) (Holub 2001; Stahl et al. 1999). Holub et al. (2001) stated that these two models were not mutually exclusive and combined them into a new model called "evolved recycling polymorphism model". Basically, this latter model proposed that for a given *R*-gene, the host population starts with transient cycles towards recycling alleles as they start diverging. The recycling step is only achieved with an extensive reservoir of diversified *R*-gene. The status of numerous *Tm2*-like homologs, observed in **Chapter 2**, would suggest that the $Tm2^2$ locus has achieved extensive diversification and is in a recycling step.

Novel strategies to deploy late blight resistance genes in potato breeding

Breeding is a laborious and time consuming process aiming to stack a specific combination of beneficial traits for a given crop use and environment. In the field of breeding for resistance, geographical disease occurrence must be considered in order to make the best adapted variety for a given region. Unfortunately, late blight occurs all over the world. Although *Rpi*-genes are available, certain *Rpi*-gene based strategies may have severe implications regarding the durability of the resistance. Indeed, in the late fifties, introgressed *Rpi*-genes from *S.*

demissum were easily overcome by *P. infestans* within a few years (Turkensteen 1993). Disillusion of horizontal resistance revived the interest for *R*-gene based resistance this last decade. Knowing that a single *Rpi*-gene can be overcome quickly, two strategies emerged to deploy simultaneously several *Rpi*-genes to reduce selection pressure on *P. infestans*: polyculture and stacking of *Rpi*-genes. The first strategy is a crop heterogeneity-based strategy. It could be achieved with a mixture of one cultivar carrying different *R*-genes A theoretical epidemiology study suggested that genetic heterogeneity provides greater disease suppression over large areas (Garrett and Mundt 1999). Research on disease control in rice, showed that crop heterogeneity was an efficient solution against the vulnerability of monocultured crops to disease (Zhu et al. 2000). A mixture of susceptible and resistant rice varieties had a greater yield, whereas blast incidence was significantly less severe than in monoculture. Polyculture of *R*-genes implies the introgression of single *R*-genes and stacking them to avoid stepping stones. A classical breeding method would be time consuming and would face linkage drag issues. The most efficient strategy to achieve *R*-gene polyculture within the same genetic background would be to use a transgenic approach. Linkage drag and breeding efforts are not the only arguments in favor of a transgenic approach to breed for late blight resistance in potato. One more argument is provided by the results of **Chapter 6**. The *R*-gene expression level determines tuber blight resistance. Improvement of tuber blight resistance is required to prevent infected tubers from being a disease vector. Transport of infected seed tubers may mix late blight isolates from different parts of the world creating new isolates via sexual reproduction. That is why, besides crop loss, more efforts are needed to achieve tuber late blight resistance. Meanwhile, new insights into tuber late blight resistance may help to breed for other potato tuber diseases. Within cultivars and wild species, one copy of a given *R*-gene is present and the expression level appeared to be insufficient to trigger tuber late blight resistance e.g. *R3a*, *Rpi-abpt* (Park et al. 2005b). Therefore, to reach the required *Rpi*-gene expression level in tuber tissue, a transgenic approach would be the most efficient solution. This can be achieved either by copy number or promoter strength.

Although a transgenic approach is attractive from a breeding point of view, it faces a lack of acceptance among western politicians and much opposition among consumer groups and environmental activists. To counteract political and ethical issues, the concept of cisgenesis recently emerged. However, recent results on tuber blight resistance (**Chapter 6**) show the limits of cisgenesis in potato. As mentioned earlier, copy number or promoter strength which implies several copies or a different promoter than the native promoter of the *R*-gene of interest. This is in contradiction with the requirements of cisgenesis.

Once a strategy to breed for late blight resistance is chosen, one must carefully decide which *Rpi*-genes to deploy. It has to be based on the complementarities of the resistance spectrum but also on the type of effectors recognized by the *R*-genes used. A multi-locus effector is more difficult to mutate than a single locus effector. To successfully breed for late blight resistance, the identification and characterization of *P. infestans* effectors are required. The identification and cloning of effector recognized by *Rpi*-genes became more efficient with *in planta* screening. Agro-infiltration of late blight resistant genotypes from wild species offers a high throughput platform to identify *Rpi*-/*Avr*- gene interactions. The identification and characterization of both components of a matching *Rpi*-/*Avr*- genes pair is critical to better understand and further investigate the p*otato*-*P. infestans* interaction. For instance, the cloning of *Avr*-*vnt1* (**Chapter 5**) opens the perspectives to identify its virulence target and to study its intrinsic function. A better understanding of the function and the "genomic dynamics" of secreted effectors may lead to identify the "*Achilles*'s heel" of *P. infestans* (Birch et al. 2008). Such knowledge could be used to engineer artificial *Rpi*-gene. For instance, subtle changes in the Rx1 protein in potato resulted in a broader resistance spectrum to *Potato virus X* and to the distantly related *Poplar mosaic virus* (Farnham and Baulcombe 2006). Studying *P. infestans* epidemiology in different regions of the world could provide useful information regarding which *Rpi*-gene must be deployed. Monitoring *P. infestans* directly in the field could lead to a better decision-support-system (DSS) to control late blight by using an efficient *Rpi*-gene combination and, if necessary, spraying at the right time of the year.

Besides single dominant *R*-gene based resistance, other leads such as recessive *R*-genes and non-host resistance to *P. infestans* may contribute to late blight resistance.

Dominant vs. recessive R-genes

Screening for late blight resistance within wild potato species is focused on single dominant *Rpi*-genes. That is why only dominant *Rpi*-genes have been mapped and/or cloned so far. One can wonder whether recessive *Rpi*-genes have evolved in potato, and if so, would it be worthwhile to proceed further into that direction to breed for late blight resistance. Recessive resistance genes were first studied in viral pathosystem in which almost half of all reported *R*-genes show recessive inheritance (Kang et al. 2005). Although most *R*-genes studied within bacterial and fungal pathosystems behave as dominant *R*-genes, few recessive *R*-genes were identified and cloned such as the *RRS1-R* gene conferring resistance to *Ralstonia*

solanacearum in *Arabidopsis thaliana* (Deslandes et al. 2002), the rice gene *xa5* and *xa13* conferring resistance to *Xanthomonas oryzae* (Chu et al. 2006; Iyer and McCouch 2004) and the barley gene *mlo* conferring resistance to powdery mildew (Buschges et al. 1997). Interestingly these recessive *R*-genes encode proteins which are not the well known and characterized NB-LRR R-proteins. For instance, *RRS1-R* has a WRKY transcription factor domain in its C terminus. *Xa5* gene encodes a *TFIIA*-gamma, a small subunit of the transcription factor *IIA* and *xa13*, is a membrane localized protein. A seven-transmembrane protein of unknown function is encoded by the *mlo* gene. Behind recessive resistance genes lays the concept of loss of compatible interaction between plant and pathogen to achieve resistance. The existence of plant genes required for susceptibility (*S*-genes) to certain pathogens was questioned by Eckardt (2002) and recently reviewed by Pavan et al. (2009). Basically a pathogen uses a host target to trigger infection. In the case of an *S*-gene, this host target can be activated to suppress innate plant defense or be essential for pathogen growth (Pavan et al. 2009). So far no recessive resistance genes or *S*-genes have been identified in potato. The challenge would be first to identify such genes and then exploit them in late blight resistance breeding along with dominant *R*-genes.

Non-host resistance to Phytophthora infestans

Non-host resistance is defined by the situation when all members of a plant species are resistant to all members of a given pathogen species (Heath 1991, Thordal-Christensen 2003). (Mysore and Ryu 2004) defined two types of non-host resistance. Type I Non-host resistance does not produce any visible symptoms, whereas the hypersensitive response (HR) is characteristic for type II Non-host resistance. (Peart et al. 2002) showed that SGT1 was required for host and Non-host resistance suggesting that signaling pathways may converge. Non-host resistance was hypothesized to involve recognition of general effectors, essential for the pathogen, or multiple effectors by an arsenal of ancient *R*-genes (Heath 2000; Huitema et al. 2003; Staskawicz et al. 1995). Breeding for late blight resistance focuses on exploiting host resistance against *P. infestans* by introgressing single dominant *R*-genes. Previous introgressions of *R*-genes from *S. demissum* have not been durable as *P. infestans* overcame quickly these *R*-genes (Turkensteen 1993). Studies of non-host resistance to *P. infestans* may unveil new prospects which might lead to engineer durable resistance. *Phytophthora* species have a high degree of specialization and can infect a limited range of species (Kamoun et al. 1999a). Therefore a large choice of plant species is available to study Non-host resistance to

P. infestans. Study of several plant-pathogen systems such as *Arabidopsis-Phytophthora* and *Nicotiana- Phytophthora* leads to a better understanding of the molecular mechanisms involved in non-host resistance to *Phytophthora* species. A typical hypersensitive response (HR) response classified as type II Non-host resistance (Huitema et al. 2003; Kamoun 2001; Vleeshouwers et al. 2000) is observed for the pathosystem *P. infestans-Arabidopsis thaliana*. So far only one *Phytophthora* species, *Phytophthora porri*, was reported to infect *Arabidopsis* (Roetschi et al. 2001). The non-host resistance observed in the pathogen system *Nicotiana-Phytophthora* results in diverse HR phenotypes intensities depending on the plant species. An extracellular protein of 10-kD, named INF1, inducing HR was extensively studied and hypothesized to function as an avirulence factor (Kamoun et al. 1998). *P. infestans* isolates lacking INF1 protein have been reported to infect *N. benthamiana* plants. However the same strains deficient for INF1 protein were unable to infect *N. tobacco* plants (Kamoun et al. 1999b; Kamoun et al. 1997). In an ongoing study genotypes from *S. microdontum* were identified which were responsive to INF1 resulting in HR production. The perspective of the cloning of the INF1 receptor from *S. microdontum* may lead to engineer broad spectrum resistance to *P. infestans.*

Potato diseases

Most emphasis is given to *P. infestans* causing late blight disease which is world wide the most devastating disease for potato. Therefore significant advances have been achieved in our understanding of the potato-*P. infestans* interaction. Nonetheless, several other potato pathogens causing fungal diseases (early blight, *Alternaria solani*; verticillium wilt), bacterial diseases (white mold, *Ralstonia solanacearum*; soft rotting, *Pectobacterium astrosepticum*; ring rot, *Clavibacter michiganensis*; phytoplasma), viral diseases (leaf roll and mosaic viruses), cyst nematodes (*Globodera* spp) and root-knot nematodes (*Meloidogyninae* spp) diseases are also a problem for potato growers (Den Nijs 2007; Elphinstone 2007; Fry 2007; Hamm 2007; Mugniéry 2007; Secor 2007; Valkonen 2007). Fungicides are often applied on the basis of decision-support-system (DSS) which use weather forecast and incorporate the physiological maturity of the crop. Regarding bacterial diseases, resistant cultivars and curative chemical control are lacking. Two European directive (EC Directives 93/85/EEC and 98/57/EC) require to test seed and ware potatoes to eradicate any contaminated material. In

contrast, *R*-genes were identified against potato virus and successfully used in cultivars such as the *Rx* gene (Maule et al. 2007) and *Ry-sto* (Song and Schwarzfischer 2008).

Although progress on understanding and controlling diseases on potato has been made, a lot more remains to be done to set up efficient disease control strategies excluding fungicides input, not only for *P. infestans* but also for other fungal diseases in potato.

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SUMMARY

Late blight (LB), caused by the oomycete *Phytophthora infestans*, is one of the most devastating diseases on potato. Resistance (*R*) genes from the wild species *Solanum demissum* have been used by breeders to generate late blight resistant cultivars, but resistance was soon overcome by the pathogen. A more recent screening of a large number of wild species has led to the identification of novel sources of resistance, many of which are currently being characterized. *R*-gene based resistance to any plant pathogen has been conceptualized according to a model known as gene-for-gene interaction. When matching avirulence (*Avr*) and resistance (*R*) proteins are produced by the pathogen and the plant respectively, a resistance response is triggered resulting in a hyper-sensitive response (HR) causing necrosis and cell death at the infection site. If one of these components is missing the plant-pathogen interaction is compatible and will result in completion of the life cycle of the pathogen. **This thesis** describes the cloning and the characterization of the resistant alleles *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* from *Solanum venturii* and their counterpart *Avr-vnt1* from *Phytophthora infestans*. The *Rpi-*/*Avr-* genes pair *Rpi-vnt1*/*Avr-vnt1* along with *R3a*/*Avr3a* and *Rpi-blb3*/*Avr2* have been used to study the genetic and molecular mechanisms behind tuber blight resistance.

The cloning of *Rpi-vnt1* alleles (*Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3*) was achieved by the combination of long range PCR (**Chapter 2**) and a classical map based cloning strategy (**Chapter 3**). The long range PCR made use of *Tm2* homologous PCR primers, upon identification of *Tm2* sequence homology in associated markers generated with an NBS-targeted fingerprinting technique. *Rpi-vnt1* alleles belong to the CC-NBS-LRR class of plant *R* genes and encode predicted peptides of 891 and 905 amino acids, respectively, which share 75% amino acid (a.a.) identity with the ToMV resistance protein Tm-2^2 from tomato. Compared to *Rpi-vnt1.1*, the allele *Rpi-vnt1.3* harbors a 14 amino acid insertion in the N-terminal region of the protein and two different amino acids in the LRR domain. Despite these differences, *Rpi-vnt1.1* and *Rpi-vnt1.3* genes have the same resistance spectrum.

An allele mining study of *Rpi-vnt1* alleles across *Solanum* section *Petota* showed that the three functional alleles were confined within *S. venturii* as only two accessions from the closely related species *S. weberbaueri* and *S. mochiquense* carried *Rpi-vnt1.1* (**Chapter 4**). Subsequent alignment of *Rpi-vnt1*-like homologs with *Rpi-vnt1* alleles revealed the presence of illegitimate recombination (IR) signatures suggesting that two successive deletion events might have occurred in the CC domain. Meanwhile, the construction of a Neighbor Joining

tree, based on AFLP data from all the accessions carrying *Rpi-vnt1* alleles or *Rpi-vnt1*-like homologs showed that *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* alleles belong to a monophyletic clade. Signatures of illegitimate recombination and the monophyletic grouping of *Rpi-vnt1* alleles suggested how *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* could have evolved. Extensive phenotyping with various *Phytophthora* isolates identified another *Rpi* gene in *S. venturii* named *Rpi-vnt2*, complementing the *Rpi-vnt1* allelic resistance spectrum. The genetic position of this second independent locus is not yet identified.

The identification of the matching avirulence factor from the pathogen, *Avr-vnt1*, was achieved by using an efficient and high throughput effector screen of resistant wild potato species (**Chapter 5**). *Avr-vnt1* encodes a typical RXLR-EER effector which expression is induced 2 days post inoculation. *Avr-vnt1* is located on a single locus in the reference strain T30-4. Among nine isolates, four alleles were identified. The virulent strain EC1 carries a functional coding sequence of *Avr-vnt1* but fails to express the gene.

In Chapter 6, the genetic and molecular mechanisms of tuber late blight have been investigated. Using transgenic cv. Desiree plants transformed with *Rpi-vnt1.1*, *R3a* or *Rpi-blb3* tuber blight resistance could be studied in an identical genetic background. First, we demonstrated that transient co-expression of the matching *Avr*- genes in these transgenic tuber slices trigger a hypersensitive responses (HR), showing that the presence and interaction of both proteins is sufficient to establish tuber blight resistance. Second, phenotypic and molecular analysis of a panel of transformants for *Rpi-vnt1.1*, *R3a* and *Rpi-blb3*, and transcriptional analysis of the corresponding effectors (*Avr-vnt1*, *Avr3a* and *Avr2* respectively) during leaf and tuber infection showed that the expression level of a given *R*-gene should equal or exceed the expression level of the matching effector in order to trigger an efficient resistance response in the tuber. Therefore, foliar and tuber late blight resistance are controlled by similar genetic mechanisms. The perceived lack of phenotypic correlation between foliage and tuber blight resistance is thus solely due to the tissue specific expression level of the *Rpi*-gene.

In the general discussion **(Chapter 7)**, results from the experimental chapters are discussed in a broader perspective.

SAMENVATTING

De aardappelziekte die veroorzaakt wordt door de waterschimmel *Phytophthora infestans,* is een van de meest destructieve plantenziekten. Een aangeboren weerstand tegen deze ziekte, gebaseerd op resistentiegenen (*R*-genen) die hun oorsprong hebben in de wilde botanische soort *Solanum demissum* is reeds door plantenveredelaars gebruikt om aardappelrassen te ontwikkelen die onvatbaar zijn. Helaas is deze resistentie na korte tijd al doorbroken, omdat de schimmel zich kon aanpassen aan deze resistante rassen. Recent is opnieuw gezocht naar resistentiegenen. Een grote collectie van wilde soorten is getoets en nieuwe bronnen voor resistentie worden momenteel gekarakteriseerd. Het type resistentie dat gebaseerd is op *R*-genen maakt deel uit van het theoretische model dat bekend is als de 'gen-voor-gen' hypothese. Dit model stelt dat een reactie optreedt, indien er corresponderende eiwitten van de avirulentie (*Avr*) and resistentie (*R*) genen gevormd worden door respectievelijk het pathogeen en de plant. Deze resistentie reactie die optreedt nadat de corresponderende eiwitten gelijktijdig aanwezig zijn, resulteert in een overgevoeligheidsreactie die bestaat uit het afsterven van weefsel door geprogrammeerde celdood op de plek van de infectie. Indien een van deze componenten ontbreekt is sprake van een compatibele interactie tussen de ziekteverwekker en de plant. De ziekteverwekker kan zich dan vermeerderen op de plant en zijn levenscyclus volbrengen. **Dit proefschrif** beschrijft het isoleren en het karakteriseren van verschillende allelen van een locus betrokken bij resistentie. De allelen *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* zijn afkomstig uit *Solanum venturii* en het corresponderende eiwit uit *Phytophthora infestans* is heet *Avr-vnt1*. De combinaties van *Rpi-*/*Avr-* genen zoals *Rpi-vnt1*/*Avr-vnt1* alsmede ook de combinaties *R3a*/*Avr3a* en *Rpi-blb3*/*Avr2* zijn gebruikt om de genetische en moleculaire mechanismen te bestuderen die betrokken zijn bij resistentie van knolweefsel.

Het isoleren van allelen van het *Rpi-vnt1* gen (*Rpi-vnt1.1*, *Rpi-vnt1.2* en *Rpi-vnt1.3*) is mogelijk gebleken door gecombineerd gebruik van zowel *long range* PCR (**Hoofdstuk 2**) als ook de conventionele werkwijze die uitgaat van een plaatsbepaling van het gezochte gen via genkartering (**Hoofdstuk 3**). De *long range* PCR werkwijze gebruikte PCR primers die homologie hadden met het *Tm2* resistentiegen. Dit reeds eerder geïsoleerde resistentiegen kwam in beeld toen DNA sequenties van gekoppelde NBS-specifieke merkers in een database vergeleken werden met bekende genen. De geisoleerde *Rpi-vnt1* allelen behoren tot de CC-NBS-LRR klasse van plant *R* genen, en coderen voor eiwitten met een lengte van 891 en 905 aminozuren, welke een hoge mate van gelijkenis vertonen met (75% homologie op basis van

aminozuren) met het eiwit van het resistentiegen *Tm-2*^{2} uit tomaat tegen het tomaten mozaiek virus (ToMV). Een vergelijking tussen de verschillende allelen *Rpi-vnt1.1*, *Rpi-vnt1.2* en *Rpi-vnt1.3* laat zien dat het tweede en derde allel een insertie bevat van 14 aminozuren in de N-terminale regio van het eiwit. Voorts verschillen de laatste twee allelen nog op twee verschillende aminozuurposities in het LRR domein. Niettegenstaande deze verschillen hebben *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* genen eenzelfde resistentiespectrum.

In **hoofdstuk 4** wordt een studie beschreven naar het opdelven van nog andere *Rpi-vnt1* allelen dwars door de hele *Solanum* sectie *Petota*. Hieruit bleek dat de drie functionele allelen slechts binnen *S. venturii* gevonden konden worden, en nog twee accessies van de nauw met *S. venturii* verwante wilde soorten *S. weberbaueri* and *S. mochiquense*. Nadat vervolgens ook deze *Rpi-vnt1*-achtige homologen uit deze andere soorten opgelijnd werden met de eerdere *Rpi-vnt1* allelen, werden aanwijzingen gevonden voor het optreden van onrechtmatige overkruising (*illegitimate recombination*; IR). De aangetroffen sporen die op IR duiden, veronderstellen dat twee keer opeenvolgend een deletie heeft plaatsgevonden in het CC domein van de gensequentie. Tegelijkertijd is ook een dendrogram geconstrueerd met het *Neighbor Joining* algoritme, gebruik makend van AFLP gegevens van alle accessies van wilde soorten die *Rpi-vnt1* allelen of *Rpi-vnt1*-achtige homologen bevatten. Uit de topologie van het dendrogram bleek dat *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* allelen tot een monofyletische groep behoren. De sporen van onrechtmatige overkruising, samen met de monofyletische positie van *Rpi-vnt1* allelen geven suggestieve aanwijzingen hoe *Rpi-vnt1.1*, *Rpi-vnt1.2* and *Rpi-vnt1.3* zouden kunnen zijn ontstaan. Uitgebreide toetsen met een reeks aan *Phytophthora* isolaten leidde ook nog tot de identificatie van een tweede *Rpi* gen in *S. venturii*, genaamd *Rpi-vnt2*, en een complementair resistentiespectrum vertoont ten opzichte van *Rpi-vnt1* allelen. De positie van dit locus op de genetische kaart van aardappelchromosomen is vooralsnog niet komen vast te staan.

De corresponderende avirulentie factor van het pathogeen, *Avr-vnt1*, kon ook achterhaald worden (**Hoofdstuk 5**). Hiertoe is een methode met hoge doorloopsnelheid gebruikt waarmee efficiënt resistente wilde aardappelsoorten getoetst kunnen worden. *Avr-vnt1* codeert voor een zo kenmerkend RXLR-EER effectoreiwit, waarvan de genexpressie op de tweede dag na infectie toeneemt. *Avr-vnt1* is gelegen op een enkelvoudige positie in de referentiestam T30-4. In negen andere stammen konden nog vier effectorallelen gevonden worden. De virulente stam EC1 bevat de functionele coderende DNA sequentie van *Avr-vnt1*, maar brengt dit gen echter niet tot expressie.

In **Hoofdstuk 6**, worden de genetische en moleculaire mechanismen van knolresistentie tegen de aardappelziekte onderzocht. Door gebruik te maken van transgene cv. Desiree planten die apart getransformeerd werden met *Rpi-vnt1.1*, *R3a* of *Rpi-blb3*, kon knolresistentie in dezelfde genetisch achtergrond worden onderzocht. In de eerste plaats kon aangetoond worden dat tijdelijke co-expressie van de corresponderende *Avr*- genen een overgevoeligheidsreactie (HR) oproepen in transgene knolschijfjes, hetgeen betekent dat de gelijktijdige aanwezigheid van beide eiwitten en hun interactie adequaat is om knolresistentie te bereiken. Ten tweede, met de fenotypische en moleculaire analyse van een groep transformanten met *Rpi-vnt1.1*, *R3a* of *Rpi-blb3*, en de analyse van het transcriptieniveau van de corresponderende effectors (respectievelijk *Avr-vnt1*, *Avr3a* en *Avr2*) gedurende de infectie van blad of knolweefsel, kon worden aangetoond dat het expressieniveau van een gegeven *R*-gen tenminste gelijk of hoger dient te zijn dan het expressieniveau van de corresponderende effector om een adequate resistentie reactie te bewerkstellingen. Derhalve wordt geconcludeerd dat resistentie in blad- of knolweefsel gereguleerd wordt door eenzelfde genetisch mechanisme. Het zo vaak gesuggereerde onbreken van een correlatie tussen blad- en knolresistentie is dus alleen het gevolg van verschillen in de weefselspecifieke expressieniveaus van betrokken *Rpi*-genen.

In the algemene discussie **(Hoofdstuk 7)**, worden de resultaten uit de experimentele hoofdstukken in een breder kader geplaatst.

RESUME

Le mildiou, engendré par *Phytophthora infestans,* est l'une des maladies les plus dévastatrices pour la pomme de terre. Des gènes de resistance provenant de l'espèce sauvage *Solanum demissum* ont été utilisés par les sélectionneurs afin de créer des cultivars de pomme de terre résistant au mildiou. Cependant, le pathogène *P. infestans* a rapidement cassé cette resistance. Une analyse récente d'un grand nombre d'espèces sauvage de pomme de terre a permis d'identifier de nouvelles sources de résistance qui sont pour la plus part en train d'être étudiées. Résistance á n'importe quel pathogène basée sur des gènes de résistance, a été conceptualisé en un model connu sous le nom de « Gène-pour-Gènes » intéraction. Lorsque les protéines d'avirulence (Avr) et de resistance (R) sont produites par le pathogène et la plante respectivement, une réaction de défense est déclenchée se traduisant par une réaction hypersensible (HR) caractérisée par une nécrose au site d'infection. Si l'une des deux protéines est manquante, l'intéraction entre la plante et le pathogène est qualifiée de compatible permettant au pathogène de poursuivre son cycle d'infection normalement. Cette thèse décrit le clonage et la caractérisation des allèles résistants *Rpi-vnt1.1, Rpi-vnt1.2* et *Rpi-vnt1.3* issus de *Solanum venturii* ainsi que le gène d'avirulence *Avr-vnt1* de *P. infestans*. Les paires de gènes de resistance et d'avirulence *Rpi-vnt1*/*Avr-vnt1* avec *R3a*/*Avr3a* et *Rpi-blb3*/*Avr2* ont été utilisées pour étudier les mécanismes génétiques et moléculaires de la resistance au mildiou dans les tubercules de la pomme de terre.

Le clonage des allèles résistants *Rpi-vnt1* (*Rpi-vnt1.1, Rpi-vnt1.2* et *Rpi-vnt1.3*) a été réalise en combinant la technique de « Long range PCR » (**Chapitre 2**) et une approche classique de clonage positionnel (**Chapitre 3**). L'identification de marqueurs moléculaires ayant une forte homologie avec le gène *Tm-2*^{2} a permis d'utiliser des amorces homologues au gène *Tm-2*^{2} pour la technique de « Long range PCR ». Les allèles *Rpi-vnt1* appartiennent á la famille des gènes de résistance CC-NB-LLR chez les plantes. Le gène *Rpi-vnt1.1* code pour une protéine de 891 acides amines tandis que les gènes *Rpi-vnt1.2* et *Rpi-vnt1.3* codent pour des protéines de 905 acides amines chacun. Ces trois protéines ont une homologie de 75% avec la protéine de résistance Tm-2^2 conférant la résistance au virus ToMV chez la tomate. Comparé á *Rpi-vnt1.1*, les protéines Rpi-vnt1.2 et Rpi-vnt1.3 ont une insertion de 14 acides amines dans le N terminus et deux acides aminés différents dans le LRR domaine. Malgré ces différences, les gènes *Rpi-vnt1.1*, *Rpi-vnt1.2* et *Rpi-vnt1.3* ont le même spectre de résistance.

L'étude des allèles de *Rpi-vnt1* á travers la section *Petota* des solanacée a montré que les trois allèles fonctionnelles *Rpi-vnt1.1, Rpi-vnt1.2* et *Rpi-vnt1.3* étaient confinés dans l'espèce *S.*

venturii. En effet, seulement deux accessions de pomme de terre provenant d'espèces proches telle que *S. weberbaueri* et *S. mochiquense* portent l'allèle *Rpi-vn1.1* (**Chapitre 4**). L'alignement de gènes homologues aux allèles *Rpi-vnt1* a révélé la présence de motifs caractéristiques de recombinaisons illégitimes. Cette observation suggère que deux délétions ont pu survenir successivement dans le domaine Coiled coil. La construction d'un arbre basé sur la technique « Neighbor Joining » utilisant des marqueurs moléculaires AFLP provenant des accessions de pomme de terre portant les allèles *Rpi-vnt1*, a montré que *Rpi-vnt1.1*, *Rpi-vnt1.2* et *Rpi-vnt1.3* appartiennent chacun á un clade monophylétique. La présence de motifs caractéristiques de recombinaisons illégitimes et le caractère monophylétique des accessions ayant l'une des trois allèles suggèrent comment *Rpi-vnt1.1*, *Rpi-vnt1.2* et *Rpi-vnt1.3* auraient pu émergés. De plus, un phénotypage exhaustif des accessions de l'espèce *S. venturii* avec différentes souches de *P. infestans* a identifié un autre *Rpi* gène dénommé *Rpi-vnt2*, complémentant le spectre de résistance des trois allèles de *Rpi-vnt1*. La position génétique de *Rpi-vnt2* n'a pas encore été déterminée.

L'identification du gène d'avirulence de *P. infestans*, *Avr-vnt1*, a été réalisée en utilisant une méthode efficace permettant de tester, directement sur des espèces de pomme de terre sauvages et résistantes á *P. infestans*, un grand nombre de gènes codant des effecteurs (**Chapitre 5**). Le gène d'avirulence *Avr-vnt1* code un effecteur ayant un motif caractéristique de *P. infestans*, le motif RXLR-EER, dont l'expression est induite deux jours après inoculation. *Avr-vnt1* est localisé á un simple locus dans la souche de référence T30-4. Parmi neuf souches, quatre allèles ont été identifiés. La souche virulente EC1 a un allèle fonctionnel de *Av-vnt1* mais non exprimé pendant l'infection.

Dans le **Chapitre 6**, les mécanismes génétiques et moléculaires de la résistance au mildiou dans les tubercules de pomme de terre a été étudiés. Le cultivar Désirée a été utilisé pour générer des plantes génétiquement modifiées avec les gènes *Rpi-vnt1.1*, *R3a* ou *Rpi-blb3* afin d'avoir le même background génétique lors de l'étude de la résistance dans les tubercules. Nous avons démontrés que l'expression transitoire des gènes d'avirulence *Avr-vnt1*, *Avr3a* et *Avr-blb2* dans les tubercules exprimant *Rpi-vnt1.1*, *R3a* ou *Rpi-blb3*, déclenchait une réaction hypersensible (HR), respectivement. L'analyse phénotypique et moléculaire des transformants portant *Rpi-vnt1.1*, *R3a* ou *Rpi-blb3* et l'analyse transcriptionelle des gènes d'avirulence *Avr-vnt1*, *Avr3a* et *Avr-blb2* pendant l'infection des feuilles et des tubercules, a montré que le niveau d'expression d'un gène de résistance donné devait être égal ou supérieur á celui du gène d'avirulence pour déclencher une réaction de résistance efficace dans les tubercules. En conclusion, la résistance au mildiou dans les feuilles et les tubercules est contrôlée par les

mêmes mécanismes génétiques. Le manque de corrélation entre la résistance dans les feuilles et les tubercules précédemment décrite, est donc due á une expression spécifique des gènes résistance dépendant du type de tissus.

Dans la discussion générale (**Chapitre 7**), les résultats des chapitres expérimentaux sont discutés et mis en perspectives.

ACKNOWLEDGEMENTS

I came from London to Wageningen four years ago. I have to admit that it was not as easy as I would have expected. A loss to overcome, a different culture to understand and new challenges towards a PhD degree. Here I am to acknowledge all the people who helped me to achieve this milestone in my life.

Richard Visser and Evert Jacobsen have been my promoters during these four years. I am grateful to Richard for his guidance and fruitful discussions on the structure of my PhD thesis. I really appreciated the talks with Evert who tried to make me think “out of the box”. This is sometimes difficult when focused on a PhD to think beyond your experiments. .

The first day at Plant Breeding, I was told that I would work with Edwin Van der Vossen who did not take part in my me recruitment. I have to say that I was rather confused and stressed. I will never know for sure whether he would have chosen me or not. I am very grateful to Edwin who ,during the first two years of my PhD, fulfilled completely his role of supervisor. I would like to thank him for teaching me how to initiate and conduct scientific research.

My deep and sincere acknowledgments to Herman van Eck who accepted to take over my supervision half way in my PhD. I really enjoyed his supervision “a la carte” giving me more freedom to take initiatives.

The last person who took part in my supervision is Vivianne Vleeshouwers regarding the effector part. It was really a pleasure to interact with her. I also would like to acknowledge Sophien Kamoun for his useful and efficient input in the effector chapter.

For four years, I really enjoyed working at Plant Breeding. The lab 1.162 under the supervision of Annelies had such a great atmosphere. Annelies, Marjon, Maarten, Karin, Anoma, Nicolas and Estelle are really fun to work with in the lab. I would like to give a special thanks to Marian, my bench mate. For four years, she managed to stand my mess! Beyond the lab 1.162, I enjoyed interacting with very helpful and friendly people such as Doret, Koen, Gert and Martijn. My acknowledgments goes also to DirkJan and Gerda who helped me efficiently with my plants and tuber material in the greenhouse.

I would like to acknowledge “the lunch and coffee break” group composed of Antoine, Anoma, Nicolas, Estelle, Jeroen, Benoit, Piere-Yves, Bjorn (NL) and Bjorn (BL) for the nice interaction. I will miss you all for sure.

Before coming to the Netherlands, some people contributed to make me what I am and where I stand today. Once upon a time, a sexy girl, seated in the first row of a amphitheatre, was eating a sweet in the shape of a crocodile during a lesson of organic chemistry at the University of Toulouse. Somehow we became friends thanks to Haribo sweets! First in Toulouse, then in Montpelier and finally in England, we have lived so many special moments together that I can surely consider Alexia as my sister of heart. Laure, as known as Laurette, is also special to me. Although we do not see each other so often, we always share great moments together usually in a restaurant. We share a common passion for eating.
During my stay in England, Jenny and Geoff taught me proper English via a game, the scrabble, which seems rather boring at the first glance but very funny if played with extravert people. One of the best way to explain the meaning of words is to mine their meaning, especially when you want to explain ”to burp”. I thank you both for the lovely time spent together in Cambridge and London. Another person who counted for me and still does is Gil or shall I say Marmita. He supported my choice towards a PhD and helped me with his wisdom. But above all, he remains deep in my heart as my first true love. Wish him the best, Shalom Marmita!

During my PhD in Wageningen, I met “Les biloutes du 50, biloute beko et biloute moussaka”. In the north of France “une biloute” is a close friend that you can rely on in any situations. In order of appearance, Anais and Antoine, you are my oxygen in the Netherlands. Dinners and parties at Lombardi 50 are so memorable. Above all you made me feel at home. I may surely say that you are more than friends for me; I shall say family. Then “biloute beko”, Laetitia, I was at high school with her. We lost contact and met each other on the battle field of the We day 2 years ago. What a small world! She has so much energy, she is just amazing. I will always remember the parties in Utrecht, Wageningen and now Toulouse. You are truly a friend of heart. The last “biloute” is Natasha. What a communicative person, always a love story to tell! I enjoy so much her spontaneity.

For two years, I have lived in a shared house located in an alternative student neighbourhood. It was rather challenging for someone who is not vegetarian (impossible as being French), in favour of genetic modified organisms and not so fond of organic products! But somehow I managed to get accepted in a house, Droef 65, thanks to my cooking skills. I spent a wonderful time there with eight different droef women over the two years: Ansa and Yvette (The Capoera Girls), Birthe (white wine addict), Isolde (Dear Blondy), Tatie (short but

tough), Berta (Super Girl), Kim (Scary Froggy) and Ulla (The Rabbit Girl). I already miss you all. You are more than welcome anytime in Utrecht for a fancy dinner.

I would have never imagined to settle down in The Netherlands after my PhD but someone made me feel at home. Roland as known as Monsieur chips. He is a great deal of “joie de vivre”. I can definitely say that he is the one for me. I know that I am not so flexible and sometimes grumpy but somehow he manages to handle that. I thank him for being there for me and supporting me. He is my life. I find fantastic that I can be part of his paternity. Of course this was only possible with two special people, Maike and Desiree who are also part of my life and family now.
In the Netherlands, I also found a second family of heart in which I am glad to belong. I could not imagine to meet more welcoming, sweeter and nicer people. Joke, Jan, tante Rietje, oom Kees (Maurice), tante Liesbeth, Arnold, Brigitte, Timon, Milan, Eric Vincent, Jean-Marie and Anita, I thank them all for accepting me in their family.

From my heart, I thank my parents without whom I will not stand here today. They did support and encourage me in the path that I have chosen. Above all, their understanding, tolerance and love made what I am today. I love you.

ABOUT THE AUTHOR

Mathieu A. Pel was born on January 1st, 1981, in Versailles, France. After obtaining his scientific Bacalaureat in 1999, he studied biology at the University of Toulouse III. He obtained his BsC specialized in plants science in 2003. Then, he obtained his master degree specialized in plant genetics and genomics at the University of Montpellier II. During his master he performed an internship in Cambridge (UK) within the laboratory of Biogemma UK. Once he completed his Master, he worked for the Medical Research Council in London as service provider for DNA arrays analysis. In 2006, he started his PhD at the University of Wageningen at the department of plant breeding under the supervision of Edwin van der Vossen and Herman van Eck. After finishing his PhD, he obtained a position as researcher in phytopathology in leafy vegetables in opened field at Enza Zaden (The Netherlands).

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: **Mathieu Pel**
Date: **21 May 2010**
Group: **Laboratory of Plant Breeding, Wageningen University**

1) Start-up phase	*date*
► **First presentation of your project**	
Late blight resistance genes: Rpi-oka1 and Rpi-nrs1	Mar 20, 2006
► **Writing or rewriting a project proposal**	
Mapping, Isolation and characterization of genes responsible for Late Blight Resistance in Potato	May 2006
► **Writing a review or book chapter**	
► **MSc courses**	
Plant-Microbe Interactions (PHP 30306)	2008
► **Laboratory use of isotopes**	
Radiation Hygiene, level 5 B	2007
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
EPS PhD Student Day, Wageningen University	Sep 19, 2006
EPS PhD Student Day, Wageningen University	Sep 13, 2007
EPS PhD Student Day, Leiden University	Feb 26, 2009
► **EPS Theme Symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 02, 2007
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 22, 2009
► **NWO Lunteren days and other National Platforms**	
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 03-04, 2006
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2007
NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
► **Seminars (series), workshops and symposia**	
EPS flying seminars(2)	2006
EPS flying seminars(2)	2007
EPS flying seminars(2)	2008
Seminars series(2)	2006
Seminars series(2)	2007
Seminars series(2)	2008
Seminars series(2)	2009
Plant Research Day (Plant Breeding)	Mar 2009
► **International symposia and congresses**	
Oomycete Molecular Genetics Network Wageningen	May 04-06, 2006
MPMI sorento 2007	Jul 21-25, 2007
SOL, Cologne 2008	Oct 12-16, 2008
MPMI Quebec 2009	Jul 19-23, 2009
► **Presentations**	
Poster: Oomycete Molecular Genetics Network Wageningen	May 04-06, 2006
Poster: Lunteren	Apr 02-03, 2007
Poster: MPMI 2007	Jul 21-25, 2007
Oral: NWO-ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
Poster: Summer School: 'On the Evolution of Plant Pathogen Interactions: from Principles to Practice'	Jun 18-20, 2008
Oral: SOL, Cologne 2008	Oct 12-16, 2008
Poster: The Graduateschool EPS 'Peer Review'	Jun 16. 2009
Poster: MPMI Quebec 2009	Jul 19-23, 2009
► **IAB interview**	Sep 2008
► **Excursions**	
Subtotal Scientific Exposure	*16.4 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Summer School 'Signaling in Plant Development and Plant Defence'	Jun 19-21, 2006
Summer School: 'On the Evolution of Plant Pathogen Interactions: from Principles to Practice'	Jun 18-20, 2008
► **Journal club**	
Literature discussion : SFPs	2006-2009
► **Individual research training**	
Subtotal In-Depth Studies	*4.8 credits**

4) Personal development	*date*
► **Skill training courses**	
WGS course 'Information Literacy' (including Endnote, basic and advanced)	2006
WGS course 'Career Perspectives'	2009
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*2.2 credits**
TOTAL NUMBER OF CREDIT POINTS*	**36.9**

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

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

This project was financially supported by the FP6 programme BioExploit Food-CT-2005-513959.

Cover picture and design by Mathieu Pel.

Printed by Wöhrmann Print Service, Zutphen, The Netherlands