Unveiling and deploying durability of late blight resistance in potato

from natural stacking to cisgenic stacking

Kwang Ryong Jo

Thesis committee

Promotors

Prof. dr. R.G.F. Visser
Professor of Plant Breeding
Wageningen University

Prof. dr. ir. E. Jacobsen
Professor of Plant Breeding (Genetical Variation and Reproduction)
Wageningen University

Co-promotor

Dr. J.H. Vossen
Researcher, Wageningen UR Plant Breeding
Wageningen University & Research Centre

Other members

Prof. dr. ir. P.C. Struik, Wageningen University
Dr. J.M. de Haas, HZPC, Metslawier
Prof. dr. ir. B.P.H.J. Thomma, Wageningen University
Dr. ir. G.J.T. Kessel, Wageningen University

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Unveiling and deploying durability of late blight resistance in potato

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Kwang Ryong Jo

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Chapter 1

General introduction

Parts are taken from the submitted book chapter: **Vossen JH**, **Jo KR**, **Vosman B (2012)** Mining the genus *Solanum* for increasing disease resistance

General introduction

Potato as a food crop

After its first introduction to Europe in the sixteenth century, the potato (*Solanum tuberosum*) has been growing in almost all countries of the world and became the most important non-cereal food crop. In 2010, with a total world production of over 324 Mt, it ranks as the world's third most important crop consumed by people after rice (696 M tonnes) and wheat (654 M tonnes). Also, potato ranks after maize (840 M tonnes) that is mainly used as feed or for bioethanol production (FAOSTAT 2012, Updated: 07 August 2012, http://faostat.fao.org). The importance of potato as food is strongly increasing in developing countries and its cultivation as a main crop or double crop or intercrop is widely encouraged because the potato produces a higher yield, has better food quality, can be harvested earlier and can grow in harsher climate conditions than any other major crop. The potato provides more nutritious diets for people as well as energy-rich starch. The potato contains significant amounts of high quality protein, high levels of vitamin C, and various minerals (Kärenlampi and White 2009). There have been many debates on the origin of cultivated potatoes and now it is reconsidered to be domesticated at 3800 m above sea level of the Andes between Peru and Bolivia between 7000–10,000 years ago (Spooner et al. 2005; Navarre et al. 2009). Although potato has a full potential as a "strategic food" to ensure food security and it is a very accommodating and adaptable plant that will produce well even without ideal soil and growing conditions (www.potato 2008. org), potato plants are vulnerable to a number of pests and diseases. Among a wide range of biotic constraints in potato cultivation, late blight, caused by the oomycete pathogen *Phytophthora infestans,* is the most devastating disease and therefore the first priority to control. Once the onset of disease symptoms is recognized, it spreads rapidly and can destroy a whole crop within a few weeks. *P. infestans* grows well in cool and wet climate conditions and has a hemibiotrophic life cycle which comprises a sexual or asexual reproduction cycle (Fry 2008). Sexual reproduction that occurs between two mating types, A1 and A2, leads to sexual recombination and consequent increased genetic diversity of the *P. infestans* population and thereby makes managing potato late blight increasingly difficult. The seed tubers, dumps, volunteers and some weed hosts that are infected serve as *P. infestans* inoculum sources and spores released from infected plants can migrate long distances by the wind. There are several practical methods to protect potato plants from late blight. The main protection for commercial potato crops can be achieved by disease free seed potatoes that are

produced by tissue culture and hydroponic based mass multiplication systems (Corrêa et al. 2009). Late blight can also be managed via rotation, the adjusted planting date, soil treatment, fungicide applications during growing season and proper harvesting and storage practices (http://extension.umass.edu). Although disease free seed potatoes or fungicides are effective measures for potato crop protection, financial and environmental costs for the production or development are considerable. Assuming 16% losses due to late blight at a global potato production of 324 Mt and € 330/hectare of control efforts (including chemicals costs and average 15 times of applications costs), the accumulated costs are estimated at more than € 5.2 billion/year worldwide (Haverkort et al. 2009). Besides these economic losses, frequent fungicide applications are harmful to both humans and environment. Furthermore, the capacity of the pathogen to develop resistance to modern fungicides (Goodwin et al. 1996; Grünwald et al. 2001) necessitates the development of durably resistant varieties. The emergence of fungicide resistant *P. infestans* genotypes like blue13 (13_A2) and green33 (33_A2) has been reported that exhibit resistance to phenylamides (Cooke et al. 2007; Cooke et al. 2012) and fluazinam (Dr G. Kessel, personal communication), respectively. Therefore, the use of resistant varieties is the most effective and environmentally friendly approach to the management of late blight and there is an increasing necessity to develop potatoes that genetically encode late blight resistance.

Late blight resistance resources

The family of Solanaceae is of high economic importance and is composed of more than 3,000 species which include important crop and model plants such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*) and eggplant (*Solanum melongena*) (Knapp 2002), but also wild species occurring in very different habitats (Spooner and Hijmans 2001). About 15,000 wild potato accessions are being maintained in large collections worldwide and the establishment of core and mini collections enables an effective use of the existing variation in gene banks while maintaining the variability, as has been proposed before (Frankel and Brown 1984; Hoekstra 2009). Plant breeders try to improve varieties by introducing new alleles, resulting in higher yields and better quality or resistance characteristics. Identifying new, promising alleles is not an easy task. In the post-genomics era, mining of a crop's (wild) gene pool for novel and superior alleles for agronomically important traits is becoming more and more feasible. Genebanks all over the world contain huge untapped resources of distinct alleles that may have potential application in crop

breeding programs. The genome sequence of potato (Potato Genome Sequencing Consortium 2011) and tomato (The Tomato Genome Consortium 2012) will facilitate mining for novel alleles or paralogs of resistance (*R*) genes. These may be found in the largely untapped resources of crossable species within the genus *Solanum* allowing their exploitation in breeding programs. Also, insight into sequence diversity at the *R* gene loci in wild *Solanum* species with different resistance response against economically important diseases will result in a better understanding of the mechanism of *R* gene functionality and evolution but can also help to identify new alleles or paralogs with different race specificities, and develop allele-specific diagnostic markers for marker assisted breeding. The Mexican hexaploid wild *Solanum* species, *Solanum demissum*, has been the most widely used source of resistance to *P. infestans* since the early part of last century when the first crosses between *S. tuberosum* and *S. demissum* were carried out (Umaerus et al. 1983). Breeding activities have mainly focused on the high resistance conferred by the dominant major *R* genes because it was easy to transfer and follow. Presently, many cultivars with *S. demissum* in their pedigree are available (Umaerus et al. 1983; Swiezynski et al. 1997). The potato differential set that initially included clonal selections made individually by W. Black (Scotland) and C. Mastenbroek (the Netherlands), and by W.R. Mills and L.C. Peterson (USA) and that afterwards was amended (Black et al. 1953), comprises 11 clones originating from *S. demissum* for the detection of late blight resistance factors (Trognitz and Trognitz 2007). The differential set collected by Mastenbroek (1952) is referred to as the Dutch or Mastenbroek differential set: Ma*R1* to Ma*R11*. Ma*R1* to Ma*R4* were developed by Mastenbroek and the other *R* gene differentials are identical to the Scottish differential set developed by Black (Huang et al. 2005). In the past, the *S. demissum R* genes from Ma*R1*, Ma*R2*, Ma*R3*, Ma*R4* and Ma*R10* were overcome rapidly (Wastie 1991), but *S. demissum* is still considered a valuable source for resistance (Niederhauser and Mills 1953; Colon et al. 1995). Especially, the Ma*R8* and *MaR9* have been reported to show broad spectrum resistance both under laboratory and under field conditions (Fry and Goodwin 1997; Swiezynski et al. 2000; Haynes et al. 2002; Bisognin et al. 2002; Zhang and Kim 2007).

Potato - *P. infestans* pathosystem

The oomycete plant pathogen *Phytophthora infestans* secretes a diverse repertoire of effector proteins that modulate host innate immunity and enable parasitic infection (Birch et al. 2006; Dodds and Rathjen 2010; Bozkurt et al. 2012). Plants monitor the presence of effectors using receptors encoded by disease resistance (*R*) genes, which

can activate plant immunity in response to pathogens and restrict their growth (Figure 1).

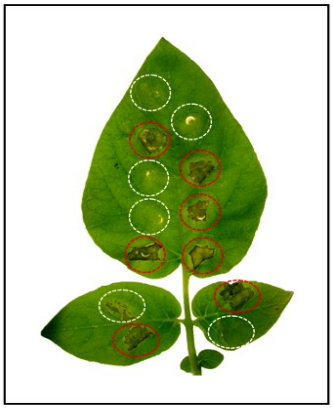

Figure 1. Effector induced hypersensitive response (HR) in *Solanum tuberosum* genotype Ma*R8.* Available effectors from *P. infestans* were applied using agroinfiltration in leaves of the resistant plant Ma*R8*. The dotted circles surround the infiltrated leaf area. The red dotted circles surround the effectors that elicited HR. These effectors are selected for validation of *R-Avr* interactions by additional genetic analysis.

Understanding the mechanisms by which effectors perturb plant processes and modulate immunity is central to the study of plant-microbe interactions.

Resistance (R) genes in potato

More than 100 *R* genes which confer resistance to a diversity of pathogens including bacteria, fungi, oomycetes, viruses, insects and nematodes have been identified and/or cloned from various plants, by a wide variety of methods including map-based cloning, transposon tagging, and similarity based DNA library screening (Sanchez et al. 2006; Vleeshouwers et al. 2011a). *R* genes often encode receptors for pathogen derived ligands and they are classified based on the combination of different domains (e.g. CC=coiled coil, TIR=toll interleukin receptor, Protein Kinase, NB=nucleotide binding, Lec (lectin), and LRRs=leucine rich repeats). Five classes can be identified, transmembrane proteins with extracellular LRRs (receptor like proteins, RLPs), transmembrane proteins with extracellular LRRs and intracellular protein kinase (receptor like kinases, RLKs), transmembrane proteins with extracellular "lectine like" domain and intracellular protein kinase (lectin receptor kinases, LecRKs), and intracellular NB-LRR proteins which can be divided in CC-NB-LRR and TIR-NB-LRR (Dubery et al. 2012). The NB-LRR class is the most abundant and has been extensively studied (Hulbert et al. 2001). Although NB-LRR genes are assumed to cause pathogen race specific (or vertical) resistance, it has also been suggested that members of the NB-LRR gene family are candidates for quantitative trait loci (QTL) that are responsible for horizontal resistance (Rietman et al. 2012; Sanz et al. 2012;

Gebhardt and Valkonen 2001). Most characterized plant NB-LRR genes are physically clustered in the plant genome. The homologous sequences in such a cluster are referred to as paralogs (Gebhardt and Valkonen 2001) and paralogs can confer resistance to different isolates of the same pathogen (Dodds et al. 2001; Li et al. 2011; Lokossou 2010) or to different pathogens (Dodds et al. 2001; van der Vossen et al. 2000). Some paralogs may also be considered as molecular fossils of evolution, whose activity is unclear or even absent, e.g., many pseudogenes have been found. In most *R* gene clusters the number of paralogs is very high and often an allelic relationship is hard to determine (Kuang et al. 2004). However, as the genome structure between species in the Solanaceae family is highly conserved positional conservation of *R* gene clusters (synteny) is observed across Solanaceous species (Grube et al. 2000; Park et al. 2009; Figure 2). Therefore, even when relatively unknown genetic sources are used, it is likely that the genes conferring resistance are linked to syntenic clusters of *R* genes known from well-studied species like potato and tomato. An overview of mapped and cloned *R* genes from Solanaceae is given in Figure 2. During the 20th century, breeding for late blight resistance started with the introgression of major dominant *R* genes from the Mexican wild species *Solanum demissum.* Eleven *S. demissum R* genes have been supposed to be available (Black et al. 1953; Malcolmson and Black 1966; Malcolmson 1969; Bradshaw et al. 2006). However, resistance obtained by introgression of *S. demissum R* genes, *R1, R2, R3,* and *R10* was overcome rapidly in European potato varieties (Malcolmson 1969; Wastie 1991). This motivated to focus on the introgression of additional late blight *R* genes for stacking in order to increase durability. Efforts to identify broad-spectrum resistance derived from other wild *Solanum* species beside *S. demissum* have revealed a wealth of major resistance to *P. infestans* (*Rpi)* genes (Hawkes 1990; Jansky 2000; Hoekstra 2009; Vleeshouwers et al. 2011a). Especially during the last decades many new *Rpi* genes have been identified, mapped and cloned. Besides the *R* genes from *S. demissum, Rpi* genes from different species have only been scarcely introgressed into potatoes (Ewing et al. 2001; Haverkort et al. 2008). Eight late blight *R* genes from *S. demissum* have been mapped: *R1* on chromosome V (Leonards-Schippers et al. 1992), *R2* on chromosome IV (Li et al. 1998), *R3a, R3b, R4, R6* and *R7* on chromosome XI (El-Kharbotly et al. 1996; Huang et al. 2005; Verzaux 2010) and *R8* on chromosome IX (Jo et al. 2011, **Chapter 2**). Very recently, two closely linked genes *R9a* and *R9b* were identified from the Ma*R9* differential plant and mapped near the *R8* locus on chromosome IX (**Chapter 4**). Besides, *Rpi* genes from other wild potato species have been identified and mapped. So far, over 20 functional late blight *R* genes have been cloned and all belong to the NB-LRR class.

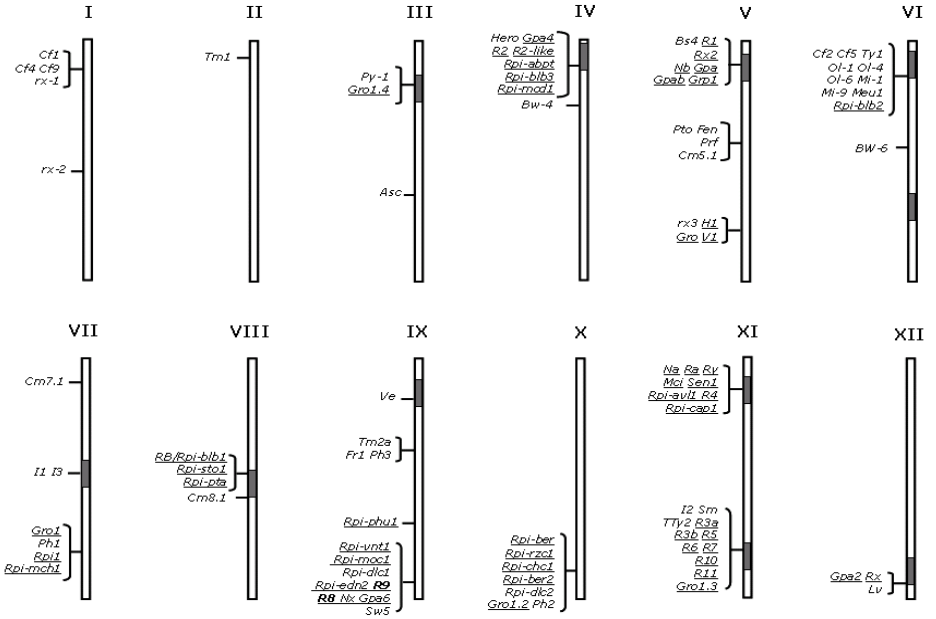

Figure 2. **Genetic locations of disease resistance traits in Solanaceae.** Twelve linkage groups are shown and the position of *R* genes is indicated. The *R* genes for potato are underlined and those for other species, mainly tomato, are not underlined. Map segments having QTL for resistance to *Phytophthora infestans* in potato are shown in black colour. *R8* and *R9* mapped on chromosome IX (**Chapter 2, 4**).

These include five *Solanum demissum* genes, namely *R1* (Ballvora et al. 2002)*, R2* (Lokossou et al. 2009)*, R3a* (Huang et al. 2005)*, R3b* (Li et al. 2011) and *R9a* (**Chapter 5**), and *Rpi* genes derived from other *Solanum* species, e.g., *RB/Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005; Lokossou et al. 2009), *Rpi-sto1* from *S. stoloniferum* (Vleeshouwers et al. 2008), *Rpi-vnt1* from *S. venturii* (Pel et al. 2009; Foster et al. 2009), *Rpi-mcq1* from *S. mochiquense* (Jones et al. 2010), and *Rpi-chc1* from *S. chacoense* (Vossen et al. 2012).

Avirulence (Avr) genes in P. infestans

In 2009, the sequence of the *P. infestans* genome of ~240Mb size has been published (Haas et al. 2009). The genome of *P. infestans* revealed large complex families of effector genes encoding secreted proteins involving pathogenesis which fall into two broad categories of apoplastic effectors and cytoplasmic effectors (Dodds and Rathjen

2010). The former accumulate in the plant intercellular space (apoplast) and include secreted hydrolytic enzymes such as proteases, lipases and glycosylases, enzyme inhibitors to protect against host defence enzymes, and necrotizing toxins. The latter are translocated directly into the plant cell by specialized infection structures known as haustoria (Whisson et al. 2007). 563 RXLR and 196 Crinkler (CRN) cytoplasmic effectors have been revealed by annotation of the *P. infestans* genome (Haas et al. 2009). The domain structure of *P. infestans* AVR proteins shows a typical modular structure with a N-terminal (signal peptide) domain, RXLR motif (Arg-X-Leu-Arg, where X indicates any amino acid), and C-terminal effector domain that often contains conserved amino acids residues (W, Y, and L) and tandem repeats (Oliva et al. 2010; Schomack et al. 2010; Win et al. 2012). The N-terminal domain plays a role in secretion and host translocation whereas the variable C-terminal domain carries the effector biochemical activity. Like RXLRs, CRNs are modular proteins. CRNs are defined by a highly conserved N-terminal, 50-amino-acid LFLAK domain and an adjacent diversified DWL domain followed by a diverse C-terminal domain (Haas et al. 2009). The *Avr* genes reside in the gene sparse regions with bigger distance to their neighboring genes (Haas et al. 2009) and represent the highly variable peripheral genome. Boutemy et al. (2011) reported the crystal structures of the effector domains from two oomycete RXLR effectors and proposed that the core α-helical fold (termed the 'WY-domain') provides both a degree of molecular stability and plasticity for effector virulence activities. To date, a catalog of more than eight *Rpi* and *Avr* gene pairs for the potato-*P. infestans* pathosystem is available, including *R1/Avr1* (Ballvora et al. 2002; Tyler 2009), *R2/Avr2* (Lokossou 2010; Champouret 2010), *R3a/Avr3a* (Huang et al. 2005; Armstrong et al. 2005), *R3b/Avr3b* (Li et al. 2011; Rietman 2011), *R4/Avr4* (van Poppel et al. 2008), *Rpi-blb1/Avrblb1* (Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008), *Rpi-blb2/Avrblb2* (van der Vossen et al. 2005; Oh et al. 2009), and *Rpi-vnt1/Avrvnt1* (Foster et al. 2009; Pel 2010), which have proven valuable in e.g., dissecting resistance in genetically modified (Zhu et al. 2012) and classical breeding material (Rietman et al. 2012).

Molecular interaction mechanisms

Forty years after the statement of Flor's "gene-for-gene" concept involving both plant resistance genes and pathogen a-virulence factors (Flor 1971), it has become apparent that plant *R* genes encode components of the plant immune system that confer the capacity to recognize and respond to specific pathogens (Dodds and Rathjen 2010). Here we summarize the integrated molecular picture of the plant-

pathogen interactions, i.e., potato-*P. infestans* interactions (Jones and Dangl 2006; Birch et al. 2006; Dodds and Rathjen 2010; Figure 3).

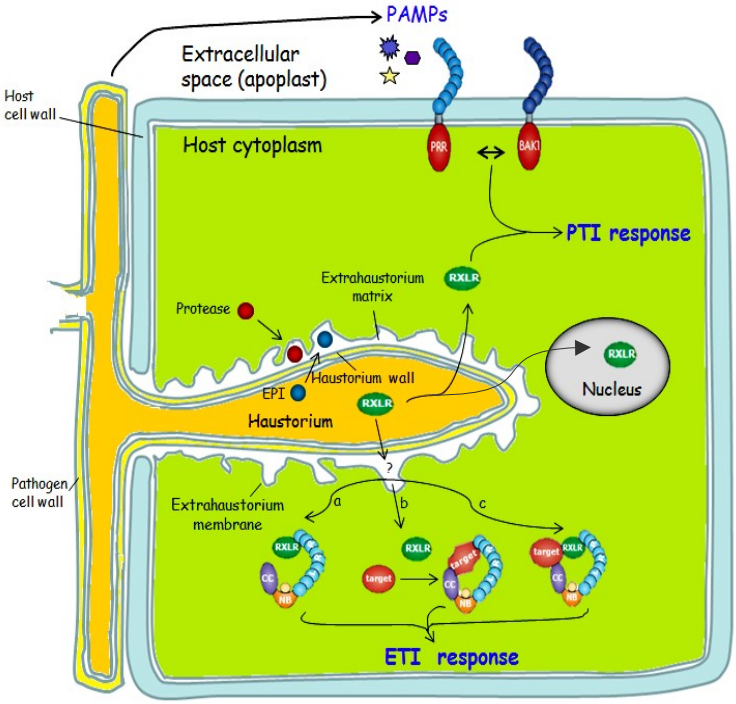

Figure 3. **The principles of potato-*P. infestans* interactions.** (see text, modified from Dodds and Rathjen 2010)

P. infestans extends its hyphae into the extracellular spaces of plant tissues. Specialized feeding structures known as haustoria, are formed that penetrate the host cell walls but not the plasma membrane. Molecules deriving from the pathogen (cell wall) are released into the extracellular spaces (pathogen associated molecular patterns = PAMPs) and recognized by pattern recognition receptors (PRRs) on the cell surface that successively elicit PAMP-triggered immunity (PTI) (Boller et al. 2009). PRRs consist of two classes, transmembrane receptor kinases that couple an extracellular domain (LRR) and an intracellular domain with protein kinase or other unknown activity and transmembrane receptor-like proteins that lack any apparent internal signalling domain (Zipfel 2008; Dodds and Rathjen 2010). Many PRRs interact

with BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1) or related proteins that have a LRR receptor kinase organisation, to initiate the PTI signalling pathway. The leucine-rich repeat receptor-like kinase (LRR-RLK) BAK1/SERK3 that has been described in *Arabidopsis thaliana* and *N. benthamiana* is a major modulator of PAMP-triggered immunity (PTI) (Shan et al. 2008). Recently, Chaparro-Garcia et al. (2010) demonstrated that *N. benthamiana* receptor-like kinase SERK3/BAK1 (NbSERK3) significantly contributes to resistance to *P. infestans* and regulates the immune responses triggered by the *P. infestans* PAMP protein INF1. Not only PAMPs are recognised by transmembrane receptors, also extracellular AVRs of pathogens whose niche is restricted to the intercellular space (Bozkurt et al. 2012). *P. infestans* also encodes extracellular protease inhibitor (EPI) proteins that target extracellular host-defense-associated proteases. So far no *Rpi* genes have been identified that can recognize these extracellular effectors. On the other hand intracellular detection of *Pi* effectors by Rpi proteins is very common. Oomycetes deliver effectors from haustoria or other intracellular structures into the host cell by an unknown mechanism. These intracellular effectors often act to suppress PTI. However, many are recognized by intracellular NB-LRR receptors, and thereby induce effector triggered immunity (ETI). NB-LRR receptors can recognize pathogen effectors either directly or by indirect mechanisms (Dodds and Rathjen 2010; Figure 3). Direct recognition (a) triggers immune signalling by physically binding of the effector to the receptor. In the guard and decoy models (b), the effector modifies an accessory protein, which may be its virulence target (guardee) or a structural mimic of such a target (decoy). Successively, the modified accessory protein is recognized by the receptor. In the bait model (c) the interaction of an effector with an accessory protein is directly recognized by the receptor.

Approaches for mapping and cloning of *R* genes

NBS profiling

Many plant *R* genes are a member of a multigene cluster composed of multiple copies with high sequence similarity (Song et al. 2003). The NB region of NB-LRR *R* genes and their analogs (RGAs) contains highly conserved common motifs like the P-loop, the kinase 2, kinase 3a and GLPL motifs (Meyers et al. 2003). These conserved motifs have been targeted successfully for PCR amplification using two degenerate primers. Successive sequence analysis has identified (parts of) NBS regions from various plant species (Pflieger et al. 1999; Zhang and Gassmann 2007). NBS profiling uses only one primer in conserved motifs and efficiently tags NBS-LRR type of *R* genes and their

analogs (van der Linden et al. 2004). The technique involves three different steps. (1) Digestion of genomic DNA with a restriction enzyme and ligation of adaptors to compatible restriction ends. (2) PCR amplification of NBS containing fragments using a NBS primer and an adaptor primer. (3) Separation of amplified fragments by polyacrylamide gel electrophoresis. The technique produces a multilocus profile of the genome. NBS profiling can easily be adapted to target other conserved gene families, which is referred to as motif-directed profiling (van der Linden et al. 2004). Also NBS profiling can be adapted to target particular *R* gene clusters. *R* genes from the same cluster usually have similarities in their sequences not shared with other *R* genes (McDowell and Simon 2006; Meyers et al. 2005), allowing the design of specific primers for a particular *R* gene cluster. NBS profiling could therefore also be adapted to reach high marker saturation in *R* gene clusters of interest (Verzaux et al. 2011; Jo et al. 2011, **Chapter 2; Chapter 4**). This technique is referred to as cluster directed profiling (CDP). Mapping of *R* genes is strongly facilitated by allele mining through NBS profiling. Typical examples of *R* gene mapping using a NBS profiling approach are provided by Pel et al. (2009) and Jacobs et al. (2010).

QTL mapping

Plant pathogen resistance, at the phenotypic level, often does not behave as a single *R* gene but as a quantitative trait that is controlled by multiple genetic and environmental factors (Trognitz et al. 2002; Bai et al. 2003). Understanding the molecular basis for quantitative traits will facilitate diagnosis and the combination of superior alleles in crop improvement programs. The possible approaches to mapping genes that underlie quantitative traits fall broadly into two categories: candidate gene studies, which use either association or resequencing approaches, and linkage studies, which include both QTL mapping and genome-wide association studies (GWAS). Linkage disequilibrium (LD) mapping, or association analysis based on candidate genes is also considered as an allele mining approach (Malosetti et al. 2007). Some cases of close linkage between an *R* gene and quantitative trait loci (QTL) for pathogen resistance supports the hypothesis that qualitative and quantitative resistance have a similar molecular basis (Leonards-Schippers et al. 1994), thereby suggesting that genes showing sequence similarity to *R* genes are candidates for being factors underlying quantitative resistance (Rickert et al. 2003; Rietman 2011). Candidate genes participating in the control of the quantitative resistance to pathogens involve in the disease response network, which include *R* genes that recognize the pathogen and trigger the resistance response, genes involving signal transduction pathways and the

large group of pathogenesis related (PR) genes which are expressed in response to pathogen attack and are involved in the execution phase of the defence response (reviewed by Gebhardt and Valkonen 2001). The genetic dissection of complex plant traits in QTLs first became possible with the advent of DNA-based markers (Osborn et al. 1987). The first genes and their allelic variants underlying plant QTLs have been identified by positional cloning (reviewed in Salvi and Tuberosa 2005).

Next generation sequencing

Currently, most genome and transcriptome sequencing projects, which used Sanger sequencing methodology in the past, are being replaced by next generation sequencing (NGS) technologies. These NGS technologies are able to generate data inexpensively and at a rate that is several orders of magnitude faster than that of traditional technologies (reviewed in Ercolano et al. 2012). At present there are several next generation sequencers on the market. Most of these systems have different underlying biochemistries but all of these technologies sequence populations of PCR-amplified DNA molecules (Voelkerding et al. 2009). The versions, which sequence single molecules, are the exceptions. The amount of sequence data and the length of the reads are increasing with the continued development of the technology. Now resequencing and *de novo* sequencing of transcriptomes and genomes is becoming more and more accessible for individual labs (Varshney et al. 2009). This will lead to the discovery of novel useful variation which has been limiting the application of sequence-based selection in plants in the pre-NGS era (Henry 2011). The availability of large numbers of genetic markers can facilitate linkage mapping and whole genome scanning (WGS)-based association genetics are of practical use for MAS in marker-deficient crops (Varshney et al. 2009). Resequencing of several genomes (Cao et al. 2011) followed by the comparison of all candidate *R* genes is now feasible in *Arabidopsis* (Guo et al. 2011). Soon this type of analysis will also be applied for crops and their wild relatives. Resequencing of parts of the genome with duplicated sequences, like *R* gene clusters, will remain a challenge, especially in heterozygous species like potato (Potato Genome Sequencing Consortium 2012). Single molecule sequencing will offer great opportunities for this research field (Koren et al. 2012).

Cloning of R genes

Several late blight *R* genes have been cloned from potato wild relatives using allele- and paralog mining (Lokossou et al. 2009; Vleeshouwers et al. 2011a; Rietman 2011). Sometimes there is no clear distinction between allele- and paralog mining because of

the high similarity among genes. An example of true allele mining was shown by Vleeshouwers et al. (2008) who isolated functional alleles of *Rpi-blb1,* which include *Rpi-sto1* from *S. stoloniferum* and *Rpi-pta1* from *S. papita*. The entire genes were isolated by long range PCR using primers up and downstream of the coding regions. Specificity of the cloned genes was shown with different *P. infestans* isolates and with effector IpiO-1 and -2, which is recognized by *Rpi-blb1*, *Rpi-sto1* and *Rpi-pta1*. An allelic relationship between the three genes was also shown using marker (CT88) segregation studies (Wang et al. 2008). Sequence analyses showed that the putative functional homologs *Rpi-sto1* and *Rpi-pta1* are nearly identical to *Rpi-blb1*, with only 3 and 5 non-synonymous nucleotide substitutions inside the coding sequence, respectively. A slightly different example of allele mining was provided by Lokossou et al. (2009), who described the map based cloning and functional characterization of *Rpi-blb3* and *Rpi-abpt,* which are allelic variants *R2* and *R2*-like. An allele mining strategy was employed using a start stop codon approach. In this study a major technological improvement was made. The Gateway™ technology was used to clone the entire amplified coding sequences in a destination vector under the control of the *Rpi-blb3* promotor and terminator. Such efficient cloning strategies of candidate alleles were combined with transient complementation assays in *Nicotiana benthamiana* and allowed for the rapid cloning and identification of *R2* and *R2*-like alleles. Champouret (2010) used a similar technical approach to mine for *R3a* and *R2* alleles. A start stop codon approach was pursued and the *R3a* screen revealed alleles with identical activity in distantly related species. This is considered as true allele mining. Also the *R2* screen revealed many genes with identical activity, however, also a few genes were identified which had slightly different recognition specificities, suggesting that not only alleles but also paralogs were mined. This is an example where allele mining and paralog mining are overlapping (Champouret 2010). Paralog mining strategies can be pursued in order to facilitate map based cloning of novel *R* gene variants. An example of successful paralog mining came available using a *R2* mining approach applied on *S. microdontum*. This resulted in the isolation of *Rpi-mcd1* which is functionally distinct from *R2* since the *Avr2* gene was not recognised (Lokossou 2010). Another example is the cloning of the *Rpi-vnt1.1* gene (Pel et al. 2009). NBS profiling revealed a fragment that was co-segregating with resistance in a F1 population. The sequence of this NBS profiling band was similar to a known *R* gene (*Tm-2²*). PCR amplification of *Tm-2²* homologs identified the functional *Rpi-vnt1.1* gene. The mined allele had a different genetic position on the same chromosome as the *Tm-2²* gene. Also the biological activity was different and therefore this study followed a typical paralog mining approach. This study also illustrates a risk associated with paralog mining in multigene

families (Pel et al. 2009). Using the start stop codon primer pair derived from *Tm-2*^{2} only a part of the coding sequence was identified and a N-terminal extension, specific for the *Rpi-vnt1* alleles were overlooked. The entire coding sequence of the *Rpi-vnt1.1* allele was found after sequence analysis of a BAC clone derived from the genomic locus (Foster et al. 2009). *R* gene mediated resistance is often referred to as qualitative resistance in contrast to quantitative disease resistance which is often partial and is conferred by multiple genes. Mapping of quantitative trait loci (QTLs) is a labor- and time-consuming process which requires the generation and analysis of large experimental mapping populations. An alternative to positional cloning of QTLs may be the allele mining approach, which is based on the knowledge of a genes' function in controlling a characteristic of interest on the one hand, and genetic co-localization of a functional candidate gene with QTL of interest on the other (Pflieger et al. 2001; Faino et al. 2012). However, in this approach substantial *a priori* knowledge is required. DNA variation for genes fulfilling these criteria has been examined in natural populations of accessions related by descent for associations with positive or negative characteristic values (Li et al. 2005; Gonzalez-Martinez et al. 2007). Finding such associations indicates that DNA variations either at the candidate locus itself or at a physically linked locus is causal for the phenotypic variation, but defined prove for the involvement of the gene is still circumstantial.

Cisgenesis

Crop breeding is a process for the introduction of a few or many requirements into the resulting new varieties. With the development of technologies, the paradigm in breeding is changing and various biotechnological aspects can be taken into account. The tetrasomic inheritance and highly heterozygous nature of potato complicate the incorporation of new traits from wild species into an existing cultivar that needs to improve only one or two main characteristics. Although molecular marker-assisted backcrossing opens more precise and quicker ways to select individuals with traits or genes of interests, the unique favourable composition of alleles in the original variety can not be recovered in the end product and also a drawback of linkage drag remains. Genetic engineering, also termed genetic modification (GM), allows more delicate and directional introduction of traits or genes into the original varieties with little or potentially no interference for the host genome. Therefore, when one aims at improving existing varieties with a promising agronomic performance but lacking, e.g., resistance to specific disease, it can give an efficient strategy. Since the introduction of genetically modified (GM) crops in the mid-1990s, there has been a rapid adoption of

the technology and in 2011, the global acreage of genetically modified (GM) crops reached 160 million hectares (Que et al. 2010; James 2011). In terms of traits or genes of interest to be introduced, gene stacking is current trends for GM crops. The genes to be introduced have complementary effects, i.e., for insect or disease resistance, reducing the chance for resistance to be overcome. In addition crop management contributes to durability, as examplified by the case of *Bacillus thuringensis* (BT) toxin mediated insect resistance; a refuge area prevents the emergence and buildup of resistant biotic constraints. Simultaneous or consecutive delivery of multiple genes, potentially for multiple traits, in one stretch of recombinant DNA into the host plant, is a preferred option for genetic improvements of existing varieties. The introduced multiple genes segregating as one "haploblock" makes trait introgression and line conversion much simpler (Halpin 2005; Que et al. 2010). Although GM technology has a tremendous potential to crop improvement, concerns and scepticism on GM crops have been persisting with respect to consumers' preferences and environmental risks. Taking these concerns into account, a cisgenesis concept was introduced that combines the advantage of genetic modification involving speed, accuracy and sophistication of trait improvement with the introduction of natural genes from the conventional breeding pool. The natural gene transfer process of *Agrobacterium* mediated transformation is used to exclusively introduce natural genes from the plant species itself or from crossable plant species (Jacobsen and Schouten 2008). The term cisgene has a very restricted meaning compared to transgene in existing transgenic organisms. Therefore cisgenesis does not contaminate the host species' gene pool with genes from completely unrelated species, e.g., viruses, bacteria, animals and non-crossable plants. Also, it has advantages of no linkage drag and no interference in the host genome as described above. Now the availability of multiple broad spectrum late blight *R* genes that have been cloned from wild *Solanum* species enables to exploit cisgenic stacking to produce durable resistant potatoes within a short time frame of breeding (Haverkort et al. 2009). The implementation of cisgenesis concepts carries the anticipation that crops developed according to these guidelines eventually can be deregulated from the GMO safety regulations (Schouten et al. 2006; Jacobsen and Schouten 2008; Rommens 2010). At present, the very high cost associated with regulatory approval, in particular in the EU area, is a major impediment to the introduction of genetically modified crops. As a consequence, many genetically modified crops and traits are currently not included in breeding programmes although they may be considered useful by the general public (Holme et al. 2011). Up to now, two cisgenic plants have been reported which include a cisgenic apple plant resistant to scab (Vanblaere et al. 2011) and a cisgenic barley

with enhanced phytase activity (Holme et al. 2011). Very recently, the European Food Safety Authority GMO Panel states that cisgenic plants have a risk level similar to conventionally bred plants (EFSA 2012).

Scope of the thesis

Knowledge about late blight *R* genes and their function sheds light on how to develop broad spectrum and durable resistant potatoes. In this study, genetic components involving durable late blight resistance from some durable resistant potato materials are mapped and cloned. In the context of pathogen perception by the *R* genes, *Avr* genes are identified and investigated that match the *R* genes of study. Also, the introduction of a combination of broad spectrum *R* genes into potato varieties is pursued by cisgenic transformation approaches. Implications for the deployment of cisgenic varieties in potato breeding are discussed.

In **Chapter 2**, we studied *R8*-mediated resistance from the differential set plant Ma*R8* that has been long known to be highly resistant. We exploited NBS profiling and comparative genomics tools such as an anchored scaffold approach to locate the *R8* gene in a new locus on chromosome IX rather than the previously suggested position on chromosome XI.

In **Chapter 3**, we found a new R-Avr pair, R8/AVR8 by integrating effectoromics into genetic characterization. Also, it was described that the gene responsible for quantitative resistance in cv. Sarpo Mira is *R8* and abundance of *R8* resistance in both centres of diversity, Central and South America, was found through functional germplasm screens with Avr8, suggesting its ancient origin and effective potential durability.

In **Chapter 4**, the *R9*-mediated resistance from Ma*R9* could also be mapped near the *R8* locus on chromosome IX, and not on chromosome XI as was reported earlier. Furthermore, two closely linked genes were found to confer the *R9* resistance in our study using different approaches. This also gives new results and a first report for the exact chromosomal position of *R9.*

In **Chapter 5**, we cloned the *R9a* gene, one of two closely linked genes, and found new insights that *R9a* has an overlapping recognition spectrum with the broad spectrum resistance gene *Rpi-blb2.* Here, we pursued to perform map-based cloning

without the need to perform high resolution mapping and indeed led to successful isolation of *R9a* that confers broad spectrum resistance to late blight.

In **Chapter 6**, we investigated cisgenic transformation procedure in combination with transgenic approaches and obtained valuable information on the development of late blight resistant potatoes by cisgenic stacking of *R* genes in susceptible as well as resistant varieties. This is important for deploying *R* genes in agriculture and might lead to the production of durable late blight resistant commercial varieties that remain resistant even under heavy disease pressure.

The general discussion (**Chapter 7**) summarizes and discusses the conceptual ideas and ways for achieving durability, and issues of *R* gene deployment concerning the cisgenic approach.

Chapter 2

Mapping of the *S. demissum* late blight resistance gene *R8* to a new locus on chromosome IX

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Mapping of the *S. demissum* late blight resistance gene *R8* to a new locus on chromosome IX

Kwang Ryong Jo[1,2,3], Marjon Arens[1], Tok-Yong Kim[3], Maarten A. Jongsma[4], Richard G. F. Visser[1], Evert Jacobsen[1], Jack H. Vossen[1]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, P.O. Box 16, 6700 AA, Wageningen, The Netherlands, [2] Graduate School Experimental Plant Sciences, The Netherlands, [3] Research Institute of Agrobiology, Academy of Agricultural Sciences, Pyongyang, DPRK, [4] Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands

Abstract

The use of resistant varieties is an important tool in the management of late blight, which threatens potato production worldwide. Clone Ma*R8* from the Mastenbroek differential set has strong resistance to *Phytophthora infestans*, the causal agent of late blight. The F1 progeny of a cross between the susceptible cultivar Concurrent and Ma*R8* were assessed for late blight resistance in field trials inoculated with an incompatible *P. infestans* isolate. A 1:1 segregation of resistance and susceptibility was observed, indicating that the resistance gene referred to as *R8*, is present in simplex in the tetraploid Ma*R8* clone. NBS profiling and successive marker sequence comparison to the potato and tomato genome draft sequences, suggested that the *R8* gene is located on the long arm of chromosome IX and not on the short arm of chromosome XI as was suggested previously. Analysis of SSR, CAPS and SCAR markers confirmed that *R8* was on the distal end of the long arm of chromosome IX. *R* gene cluster directed profiling markers $CDP^{Sw5}4$ and $CDP^{Sw5}5$ flanked the *R8* gene at the distal end (1 cM). $CDP^{Tm2}1$-1, $CDP^{Tm2}1$-2 and $CDP^{Tm2}2$ flanked the *R8* gene on the proximal side (2 cM). An additional co-segregating marker ($CDP^{Hero}3$) was found, which will be useful for marker assisted breeding and map based cloning of *R8*.

Introduction

Phytophthora infestans, causing late blight in potato, is one of the most devastating pathogens and threatens food production worldwide. The use of resistant varieties is considered to be the most sustainable approach for the management of late blight. Today, commercial potato crops are mainly protected by the use of disease-free seeds and frequent fungicide application (Fry 2007; Struik and Wiersema 1999). However, considerable financial and environmental costs are incurred for fungicides and their

application (Vleeshouwers et al. 2011a). Production of disease-free seeds causes considerable additional costs for the refined infrastructure and regular operation of the facilities. Furthermore, the capacity of the pathogen to develop resistance to modern fungicides (Goodwin et al. 1996; Grünwald et al. 2001) necessitates the development of durably resistant varieties. Therefore, breeders have been extremely interested in creating resistant cultivars ever since the first late blight epidemic in Europe in the 1840s that caused the Irish potato famine. Breeding activities at the beginning of the twentieth century have mainly focused on dominant resistance genes, as the complete resistance they conferred was easy to follow and promised a fast and effective way to protect the crop against late blight. Dominant resistance genes were initially identified in the Mexican species *Solanum demissum* and introgressed by crossing and backcrossing into cultivated potato. Eleven potato resistance (*R*) gene differentials from *S. demissum* have been identified (Black et al. 1953; Malcolmson and Black 1966). The *S. demissum R* genes from Ma*R1*, -*R2*, -*R3*, -*R4* and -*R10* have been introgressed into potato cultivars. However, their durability proved to be a problem due to the rapid appearance of compatible races of the pathogen after market introduction (Wastie 1991). In recent years, other wild species of the genus *Solanum* are also being considered as possible sources of resistance in addition to *S. demissum*. Introgression of these genes into cultivars sometimes requires interspecific bridge crosses (Hermsen and Ramanna 1973). This approach resulted in the introgression of *Rpi-blb2* from *S. bulbocastanum* into the cultivars Toluca (NL 2006) and Bionica (NL 2008) (Haverkort et al. 2009). In the last two decades, the chromosomal positions of many *R* genes from *S. demissum* have been determined. Eight *R* genes have been mapped; *R1* on chromosome V (Leonards-Schippers et al. 1992), *R2* on chromosome IV (Li et al. 1998), *R3a* and *R3b* (Huang et al. 2004), *R6* and *R7* (El-Kharbotly et al. 1996), and *R10* and *R11* (Bradshaw et al. 2006) on chromosome XI. *R5, R8* and *R9* have been suggested to be allelic variants of *R3*, located on chromosome XI (Huang 2005). Furthermore, four *R* genes from *S. demissum* have been cloned, *R1* (Ballvora et al. 2002), *R2* (Lokossou et al. 2009), *R3a* (Huang et al. 2005) and *R3b* (Li et al. 2011). In the Mexican species *S. bulbocastanum*, three *R* genes were identified and cloned, the alleles *RB* (Helgeson et al. 1998; Naess et al. 2000; Song et al. 2003) and *Rpi-blb1* (van der Vossen et al. 2003) on chromosome VIII, *Rpi-blb2* on chromosome VI (van der Vossen et al. 2005) and *Rpi-blb3* on chromosome IV (Park et al. 2005). In the wild species *S. pinnatisectum* a dominant *R* gene, *Rpi1*, was mapped by Kuhl et al. (2001) to chromosome VII. On chromosome IX genes from *S. mochiquense* (*Rpi-moc1*; Smilde et al. 2005), *S. phureja* (*Rpi-phu1*; Sliwka et al. 2006), *S. venturii* (*Rpi-vnt1*; Foster et al. 2009; Pel et al. 2009), *S. dulcamara* (*Rpi-dlc1*; Golas et al. 2010)

and *S. caripense* (Trognitz and Trognitz 2004; Nakitandweet al. 2007) were mapped. In *S. berthaultii* three genes, *Rpi-ber* (Rauscher et al. 2006), *Rpi-ber1* and *Rpi-ber2* (Park et al. 2009), were mapped to the long arm of chromosome X. Besides these resistance genes, there are additional sources of resistance from a wide range of species, which have not been located in the genome yet. *S. microdontum*, *S. paucissectum* and *S. stoloniferum* are considered as important resistance sources (Sandbrink et al. 2000; Villamon et al. 2005; Wang et al. 2008). Despite the rapid breakdown of *R1, R2, R3, R4* and *R10* in the past, *S. demissum* is still considered a valuable source for both race-specific and race-non-specific resistance (Niederhauser and Mills 1953; Colon et al. 1995). Differentials Ma*R8* and Ma*R9* were shown to be durably resistant to several *P. infestans* isolates (Haynes et al. 2002), however, *R8* and *R9* have never been used in breeding (Huang 2005). Haynes et al. (2002) evaluated 22 potato clones including seven late blight differentials for late blight resistance in seven US locations in 1997. The authors found that the area under disease progress curve (AUDPC) of Ma*R8* was very low. Evaluation of the reaction of potato differentials to over 5,000 *P. infestans* isolates, collected in various parts of the world, showed that the resistances of differentials Ma*R5*, Ma*R8* and Ma*R9* were most durable (Swiezynski et al. 2000). Also, *P. infestans* isolates derived from clonal lineage US8, the most common and aggressive genotype of *P. infestans* present in the US (Fry and Goodwin 1997) overcame all known *R* gene differentials except Ma*R8* and Ma*R9*, both in detached leaf assay and in field trials (Bisognin et al. 2002). The apparent durability of the *R* gene in Ma*R8* led us to study the molecular basis of the resistance in this plant. Here, we describe the use of dedicated molecular marker techniques [NBS- and cluster directed profiling (CDP)] and genetic analysis of the resistance to *P. infestans* isolate IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) in Ma*R8*, and provide evidence that this resistance is located on the distal end of the long arm of chromosome IX.

Materials and methods

Plant material and mapping population

Ma*R8*, corresponding to 2424a(5) and PI 303149 (Black et al. 1953; Malcolmson and Black 1966), and cultivar Concurrent were maintained and *in vitro* multiplied in the laboratory of Plant Breeding. Ma*R8*, as resistant female parent, and the susceptible cultivar Concurrent were crossed to generate a F1 mapping population in the summer of 2008 (population code 3020). Seeds were sown under sterile conditions and 100 plants were maintained in *in vitro* culture.

Phytophthora infestans isolates and disease testing

Phytophthora infestans isolate IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) was kindly provided by Prof. Francine Govers (Laboratory of Phytopathology, Wageningen University). IPO-C was used in detached leaf assays as described by Vleeshouwers et al. (1999) but also to inoculate field trials. In 2009 and 2010, respectively, four and two *in vitro* plants per genotype from population 3020 were planted in the beginning of June. Spreader rows and the border rows consisted of the susceptible potato cultivars Bintje and Nicola, which served to support a local late blight epidemic. In the beginning of July, the trial fields were inoculated. For the inoculum production, a large number of detached leaves of potato cultivar Bintje were inoculated with isolate IPO-C. After 6 days, spores were washed off to prepare a spore suspension in large containers. Zoospore release was induced by incubating the containers at 10°C. After the release of the zoospores, the inoculum was adjusted to a concentration of 5×10^4 zoospores/ml. At nightfall, the zoospore suspension was sprayed on the potato field using a tractor using a spraying arm. After 2 weeks severe late blight symptoms were observed in susceptible plants and a clear segregation of resistance and susceptibility was observed in F1 population 3020. Scoring was performed in a qualitative way (resistant or susceptible).

DNA isolation and marker analysis

Genomic DNA was isolated as described by Fulton et al. (1995). Young leaf tissue was collected for DNA isolation according to the CTAB protocol with the Retsch machine (RETSCH Inc., Hannover, Germany). Primers used for marker analysis are listed in Table 1. PCR reactions were performed using DreamTaq™ polymerase (Fermentas) in a standard PCR program (start: 94°C for 30 s; amplification: 35 cycles of 94°C for 30 s, 55°C for 30 s; 72°C for 1 min; termination: 72°C for 2 min). NBS profiling was performed as described by van der Linden et al. (2004), with minor modifications. The restriction enzyme digestion of genomic DNA and the ligation of adapters were made in one incubation step. Restriction enzymes *Mse*I, *Hae*III and *Rsa*I were used for restriction ligation reactions and NBS primers NBS1, NBS2, NBS3, NBS5a6 and NBS9 in combination with the adaptor primer were used for the successive PCR reactions. Primers with corresponding names and sequences have been described previously (van der Linden et al. 2004; Wang et al. 2008; Mantovani et al. 2006; Brugmans et al. 2008). Totally, 15 primer enzyme combinations were used for NBS profiling. For *R* gene CDP, *R2* and *Tm-2²* primers (R2LF1, R2LF2, R2LF3, R2LF4, R2LR2, R2LR3, R2LR4, Tm1F, Tm1R, Tm3F, Tm3R, Tm6F, Tm15F, Tm15R, Tm19F, Tm19R, Mcq19F,

Mcq21R and Mcq23F) were used as described by Verzaux (2010). HotStarTaq™ polymerase (QIAGEN) was used in the first PCR and DreamTaq™ polymerase (Fermentas) in a second PCR. For designing *Hero*-CDP-primers, *Hero*-like sequences available from NCBI (http://www.ncbi.nlm.nih.gov/) and *S. phureja* DM1-3 516R44 (CIP801092) whole genome assembly scaffold sv3 available from the Potato Genome Sequencing Consortium (PGSC; http://www.potatogenome.net) were collected and aligned. Primers were designed on cluster specific conserved domains encoding CC and LRR. A total of six *Hero*-CDP degenerate primers were designed and one produced a marker that was linked to *R8* (Table 1). For *Sw-5*-CDP seven specific primers described by Dianese et al. (2010) were used. Like in NBS profiling, the CDP primers were used in combination with a labeled adaptor primer (fluorescent dye IRD700) to enable visualization on a denaturing polyacrylamide gel using a NEN_IR2 DNA analyser (LI-COR_Biosciences, Lincoln, NE, USA). NBS profiling was carried out first on a set of 10 resistant and 10 susceptible F1 plants, including the parents. If in this first round polymorphic bands between the parents and co-segregation of these bands with resistance in the F1 plants were found, a second round of NBS profiling was carried out on genomic DNA of the remaining F1 progeny. If multiple markers are found with one primer/enzyme combination, numbers behind the dash are consecutive numbers ordered from low to high molecular weight produced by the same primer enzyme combination. For example, marker CDP^{Tm2}1-1 and CDP^{Tm2}1-2 were produced using primer/enzyme combination Tm15R/*Mse*I. In order to screen for cleaved amplified polymorphic sequences (CAPS), PCR was done using primers listed in Table 1 and successively the PCR products were digested using the restriction enzymes listed in Table 1. 5 µl of PCR product were added to a 15 µl of restriction enzyme digestion according to the manufacturers' instructions.

Isolation and sequence analysis of NBS fragments

Fragments were excised as described in the Odyssey manual for band extraction (Westburg, The Netherlands) and re-amplified with the specific profiling primer and the adapter primer. PCR products were checked on agarose gels and purified with QIAquick PCR purification spin columns (QIAGEN Benelux, The Netherlands). Fragments were cloned into the pGEM-T Easy vector (Promega, USA) prior to sequencing with M13 primers. Sequencing was carried out with the BigDye Terminator kit and an ABI 3700 automated sequencer from Applied Biosystems (USA). Blast analysis of the sequences was performed using the websites from NCBI, PGCS and SGN (http://blast.ncbi.nlm.nih.gov/; http://potatogenomics. plantbiology.msu.edu/;

http://solgenomics. net). ClustalX (Jeanmougin et al. 1998) was used to align sequences.

Map construction

Co-segregating, simplex-inherited NBS and CDP markers from the tetraploid female parent (Ma*R8*) were scored as dominant markers (Wu et al. 1992). The marker order was determined by TetraploidMap (Hackett and Luo 2003; http://www.bioss.ac.uk). The map distance was calculated based on the frequency of the recombination between markers. Publicly available potato and tomato genetic maps from the SH x RH population (Van Os et al. 2006), SGN (http://sgn.cornell.edu/cview/map.pl?map_id = 9&show_offsets= 1&show_ruler = 1) and GABI (http://www.gabipd.org/database/) databases were included for comparison of marker positions and synteny.

Results

Segregation of resistance in the mapping population

F1 progeny and the parental clones Ma*R8* and cv. Concurrent were screened for resistance against *P. infestans* isolate IPO-C.

Table 1. Markers used for mapping of *R8* in the F1 population Ma*R8* x Concurrent

Marker name	Primer name	Primer sequence (5′- 3′) *	Polymorphism	Reference
NBS5a6H	NBS5a6	YYTKRTHGTMITKGATGAYRTITGG	*Hae*III	van der Linden et al. 2004
NBS1M	NBS1	GCIARWGTWGTYTTICCYRAICC	*Mse*I	
CDP^{Tm2}1-1	Tm15R	GTAACAAGTCATGTATGCGAC	*Mse*I	Verzaux 2010
CDP^{Tm2}1-2	Tm15R	GTAACAAGTCATGTATGCGAC	*Mse*I	Verzaux 2010
CDP^{Tm2}2	Tm19F	GCCAAATAGTATTGTCAAGCTC	*Mse*I	Verzaux 2010
CDP^{Hero}3	Hero4064F	RRAGATTCAGCCATKGARATTAAGAAA	*Hae*III	This study
CDP^{Sw5}4	Sw55F	AGTCTCCAAACATTCCTGCTTCTC	*Mse*I	Dianese et al. 2010
CDP^{Sw5}5	Sw55F	AGTCTCCAAACATTCCTGCTTCTC	*Hae*III	Dianese et al. 2010
Stm1021	STM1021 F	GGAGTCAAAGTTTGCTCACATC	SSR	Collins et al. 1999
	STM1021 R	CACCCTCAACCCCCATATC		
TG328	TG328F	TGAATGGACTGGTGATCTGC	SCAR	This study
	TG328R	TTGGAAAGAATTGGCTTTTGA		
184-81	184-81F	CCACCGTATGCTCCGCCGTC	CAPS, *RsaI*	This study
	184-81R	GTTCCACTTAGCCTTGTCTTGCTCA		
General primers for profiling				
*Mse*I adaptor	Mse-ad-top	CCCGAAAGTATAGATCCCAT		
	Mse-ad-bottom	TAATGGGATCTATACTT		
Blunt adaptor	B-ad-top	ACTCGATTCTCAACCCGAAAGTATAGATCCCA		van der Linden et al. 2004
	B-ad-bottom	TGGGATCTATACTT		
Adaptor primer		ACTCGATTCTCAACCCGAAAG		

F; forward primer, R; reverse primer. * All markers were produced using a standard PCR program (see ''Materials and methods'')

The detached leaf assay with leaves from greenhouse grown plants turned out not to be suitable for the F1 population. In contrast to the mother plant Ma*R8*, the F1 plants showed no clear resistance. Initial screens indicated some variation in resistance; however, these findings were not reproducible for most of the individuals. In contrast, highly reproducible results were obtained in two field trials performed in Wageningen, The Netherlands, in the summer of 2009 and 2010. Ma*R8* plants remained devoid of late blight symptoms, while cv. Concurrent was completely infected within 2 weeks after inoculation. Among 100 F1 genotypes screened, 52 were resistant, 46 were susceptible and 2 showed intermediate phenotypes. This demonstrates that the resistance in Ma*R8* is inherited as a dominant simplex allele (χ^2 = 0.54, P >0.05) at a single locus. The corresponding gene is referred to as *R8* hereafter.

Identification of R8 flanking markers

In order to identify markers linked to *R8*, we used NBS profiling since this technique can also give an indication about the *R* gene family of the targeted gene. Initially, NBS profiling experiments were carried out using combinations of the NBS5a6 primer and three enzymes (*Hae*III, *Rsa*I and *Mse*I) on both parents and 10 resistant and 10 susceptible F1 individuals from the mapping population. Marker NBS5a6H was linked to the resistance phenotype and was found at a frequency of one recombinant in twenty F1 plants. Subsequently, an additional set of NBS primers (NBS1, NBS2, NBS3 and NBS9) was used which resulted in the identification of an additional marker, NBS1M showing linkage to the resistance but without recombinants in twenty F1 plants. The NBS5a6H and NBS1M markers were tested on the complete F1 progeny. 22 additional recombinants were found between NBS5a6H and *R8*, and three recombinants were identified between NBS1M and *R8*. These recombinants were not overlapping resulting in 26 recombinants between NBS1M and NBS5a6. This showed that the two NBS profiling markers flank the *R8* gene (Figure 1).

Localization of R8 flanking markers in the genome

The NBS5a6H (361 bp) and NBS1M (301 bp) fragments were cut out of the gel and sequenced (genbank accession numbers: JF317286 and JF317287 respectively). In potato scaffold PGSC0003DMS000000483, a 93% identity match was found for the

NBS5a6H sequence. PGSC0003DMS000000483 could be located to chromosome IX using genetic and physical maps of tomato (Figure 1). NBS1M showed 97% identity to potato scaffold PGSC0003DM S000001347.

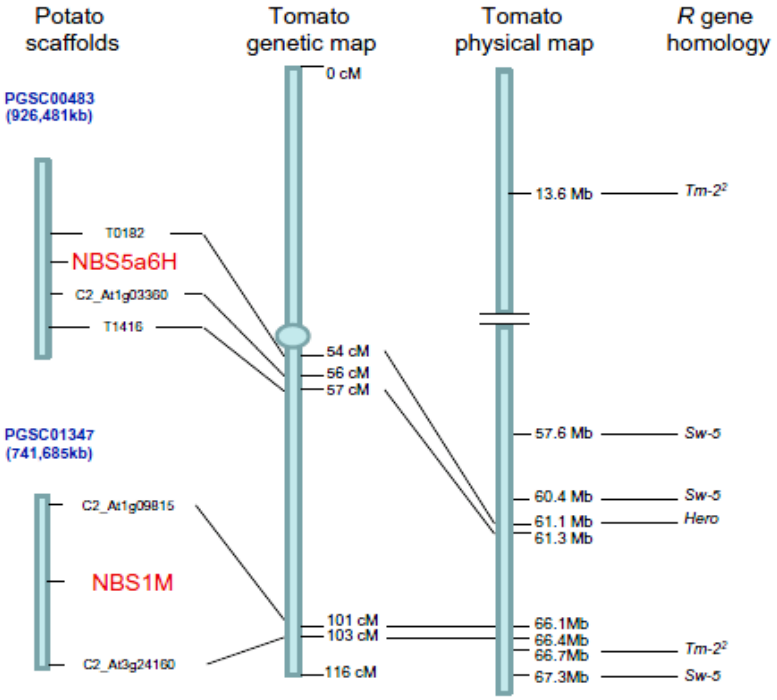

Figure 1. **Positions of NBS profiling markers and *R* gene homologs on chromosome IX.** Markers in large font indicate the NBS profiling markers that were linked to *R8*. Potato genome sequences, the tomato genome sequences and the marker sequence database from the SGN were searched using the NBS5a6H and NBS1M by BLAST analysis. The bars on the left indicate *S. phureja* scaffolds (PGSC v3). In the middle are the tomato EXPEN 2000 genetic map and the tomato SL2.40 Ch9 physical map (SGN). On the right positions of tomato genome sequences with homology to *R* genes are shown. Horizontal and diagonal lines indicate corresponding marker positions in the different maps.

This scaffold could be linked to the telomeric region at the long arm of chromosome IX using markers C2_At1g09815 and C2_At3g24160 (Figure 1). The proposed inversions between potato and tomato on chromosome IX (Tanksley et al. 1992) did not affect the positioning of the *R8* flanking markers. For marker NBS1M, there was no similarity to sequences with known function. The sequence of marker NBS5a6H, however, showed 90% identity to the tomato *Hero* gene (Ernst et al. 2002), which is located on

chromosome IV. Apparently, *Hero*-like genes are not only present on chromosome IV but are located in other genomic regions as well (Figure 1).

Localisation of R8 on chromosome IX

In order to verify that *R8* and its flanking markers were on chromosome IX, more closely linked markers near the *R8* gene were required. Therefore, *R* gene CDP was performed. Two *R* gene clusters known to locate on chromosome IV (*R2* and *Hero*), and two clusters known to locate on chromosome IX (*Tm-2²* and *Sw-5*) were targeted for *R* gene-CDP. Using *R2*-CDP no bands linked to the resistance were found among 24 primer/enzyme combinations (data not shown). Three linked markers, CDP^{Tm2}1-1 (240 bp), CDP^{Tm2}1-2 (345 bp) and CDP^{Tm2}2 (120 bp), were identified using *Tm-2²* primers out of 36 primer/enzyme combinations. CDP^{Tm2}1-1 and CDP^{Tm2}1-2 were identified using the same primer enzyme combination (Tm15R/*Mse*I). All *Tm-2²*-CDP markers are at 2 cM distance (proximal) from *R8* (Figure 2). Two markers, CDP^{Sw5}4 (277 bp) and CDP^{Sw5}5 (165 bp), were identified using *Sw-5*-CDP. Both markers were located at 1 cM to the opposite side (distal) of the *R8* gene as CDP^{Tm2}1-1, CDP^{Tm2}1-2 and CDP^{Tm2}2 (Figure 2). Interestingly, one fully co-segregating marker, CDP^{Hero}3 (500 bp; Figure 3), was found using *Hero*-CDP out of 18 primer/enzyme combinations. All CDP markers were excised from the gel and subjected to sequence analysis. CDP^{Tm2}1-1 and CDP^{Tm2}2 indeed showed similarity to *Tm-2²*. CDP^{Sw5}4 and CDP^{Sw5}5 were confirmed to be similar to *Sw-5*, a *S. lycopersicon* tospovirus resistance gene (Brommonschenkel and Tanksley 1997; Spassova et al. 2001). Unfortunately, the sequences of CDP^{Tm2}1-2 and CDP3 remained unresolved due to technical reasons. The relative positions of the *Tm-2²* and *Sw-5* homologous markers in the *R8* map are in agreement with relative positions of *Rpi-moc1*, which is homologous to *Tm-2²* (Foster et al. 2009) and *Sw-5*, as inferred from publically available genetic maps of chromosome IX (Figure 2). In addition, the draft sequence of the complete tomato chromosome IX shows that *Tm-2²* and *Sw-5* like sequences are located close to each other near the telomere (Figure 1). To further confirm the map position of *R8* and the newly identified profiling markers on chromosome IX, known markers (GP101, S2g3, TG591A, GP41, CT220, T0521, S1d11, S1d5-a, T1065, TG328, TG424, and St_At3g23400) from the SGN and GABI databases on the long arm of chromosome IX were selected and tested for linked polymorphisms after digestion with 24 selected restriction enzymes. Only TG328 did display an informative SCAR type polymorphism. A segregation of 87 presence to 12 absence genotypes was found which fits a 5:1 ratio (χ^2 = 0.23, p >0.05), indicating that the TG328 marker allele is present in duplex in Ma*R8*. Also three SSR markers (Stm1010,

Stm1021, Stm0017) (Milbourne et al. 1998; Collins et al. 1999) were screened and one SSR marker, Stm1021, present in RH9 BIN65 of the SH x RH map located at 9 cM proximal to *R8*. Since no other useful polymorphisms could be found in known genetic markers in this region, we mined for potential polymorphic regions in the potato genome covering this region. TG328 located to SH9 BIN77 of the SH x RH map, was linked to *Rpi-moc1* in the GABI map, and located 2 cM proximal relative to *R8* (Figure 2).

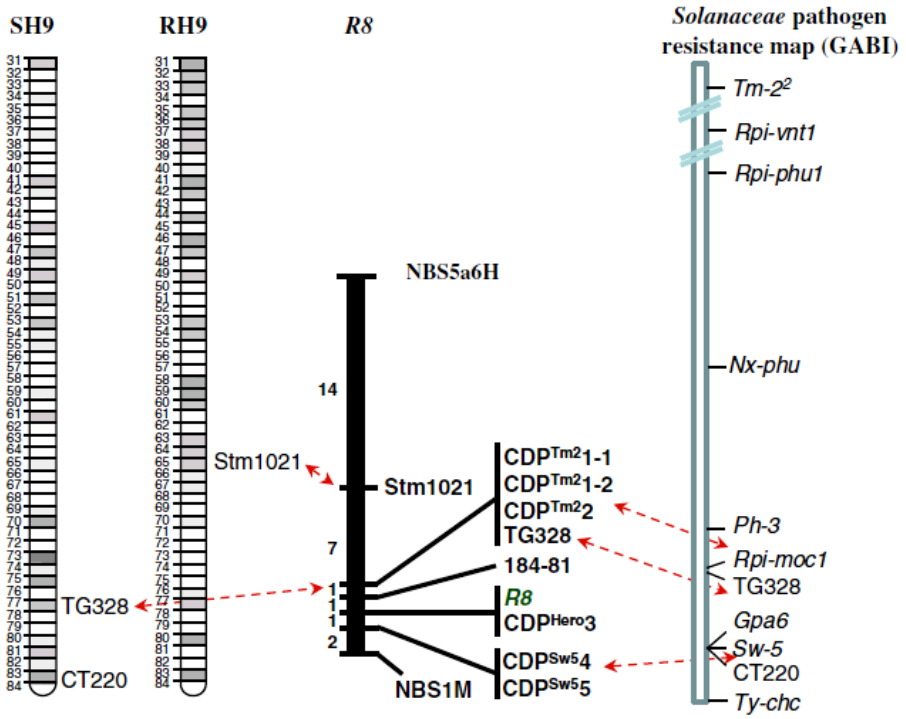

Figure 2. **Comparison of different genetic maps of chromosome IX.** From left to right the potato SHxRH map (Van Os et al. 2006), the *R8* map produced in this study and the combined Solanaceae pathogen resistance map as extracted from the GABI website (May 20th, 2011). Only the long arms of chromosome IX are shown. The dotted arrows indicate relative positions of studied markers shared between the different maps.

Scaffold PGSC0003DM S000000184 which contained the flanking markers TG328, CDP^{Tm2}1-1 and CDP^{Tm2}2, was aligned to the tomato genome and several polymorphic regions were identified. PCR screens within these regions eventually identified additional polymorphic marker (184-81), which located 1 cM proximal to *R8* (Figure 2).

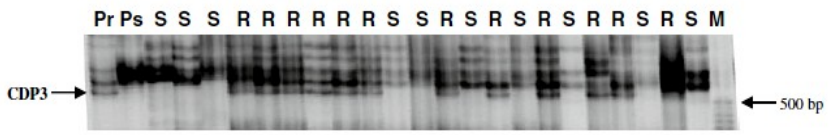

Figure 3. **Morphology of marker CDP^{Hero}3.** A part of a LI-COR gel containing co-segregating marker CDP^{Hero}3 which was obtained with Hero4064F1/*Hae*III primer/enzyme combination. Pr, Ps, R, S and M indicate the resistant parent, susceptible parent, resistant and susceptible F1 genotypes, and molecular weight marker, respectively. The arrow on the left points at the CDP^{Hero}3 band and the right arrow points at molecular weight marker.

Discussion

In this study, we report the genetic mapping of the *R8* late blight resistance gene from the differential clone Ma*R8* to the distal end of chromosome IX. Previously, it was suggested that *R8* was a *R3* family member located on chromosome XI (Huang et al. 2005). This suggestion was generally accepted in literature. However, from this study it is now clear that this is not the case. Rather, *R8* is located distal to Stm1021 and TG328, present in RH9 BIN65 and SH9 BIN77, respectively, of the SH x RH map (Figure 2, Van Os et al. 2006), and proximal to *Sw-5*. Also *Rpi-edn2*, a gene from *S.x edinense* conferring resistance to IPO-C (Verzaux 2010), was located distal (~15 cM) to Stm1021. Interestingly, Stm1021 was found to locate in between two known *R* gene clusters on chromosome IX, distal to the cluster containing *Rpi-vnt1* (Foster et al. 2009; Pel et al. 2009) and proximal to the cluster containing *Rpi-moc1* (Smilde et al. 2005), which is probably the same as *Rpi-mcq1* (Foster et al. 2009). Both *Rpi-vnt1* and *Rpi-mcq1* were described to be homologous to *Tm-2^2*, a tomato gene conferring resistance to Tomato Mosaic Virus (Lanfermeijer et al. 2003). This gene is located near the centromere on the long arm of chromosome IX. We conclude that *Tm-2^2* like sequences are dispersed over the long arm of chromosome IX and are concentrated in at least three different clusters. Besides the *Rpi-vnt1, Rpi-mcq1, Rpi-edn2* and *R8*, the *Rpi-phu1* gene also was mapped to the long arm of chromosome IX (Sliwka et al. 2006), although more proximal than *R8*, as inferred from map comparisons. The multitude of late blight resistance genes in this genomic region raises the question whether they could represent different alleles of the same gene or whether they are indeed different genes. Mining for *R* gene homologs in this region has revealed several potential *R* gene clusters, suggesting that each cluster could potentially harbor

different late blight resistance genes. Primer enzyme combination Tm19F and *Mse*I, used to produce CDP^{Tm2}2, was previously shown to link to *Rpi-edn2* at 6 cM distance (Verzaux 2010). CDP^{Tm2}2, however, produced a marker band of a different size. Further research is required to show whether *R8* and *Rpi-edn2* are allelic variants or different genes. Although we show that *Sw-5, Tm-2^2* or *Hero* sequences are present in this part of chromosome IX (Figure 2), we cannot yet clarify to which family the *R8* gene belongs. In this study, we mapped eight profiling markers, of which six were *R* gene CDP markers. CDP is a refinement of the motif-directed profiling (MDP) marker technology (van der Linden et al. 2004). However, it can easily be adapted to target other conserved gene families. For instance, it was adapted for Prx-Profiling (Peroxidase Profiling) in barley to map peroxidase genes and correlate them with QTL map positions for resistance (González et al. 2010). *R* genes from the same cluster usually have similarities in their sequences not shared with other *R* genes (McDowell and Simon 2006; Meyers et al. 2005). So it is possible to design specific primers for a particular *R* gene cluster. In this study, we have adapted the MDP technology to achieve marker saturation in an *R* gene cluster of interest, referred to as CDP. We show that comparative genomics tools can be used to predict chromosomal positions of the sequenced profiling markers. Besides the virtue of homology-based marker landing, an important pitfall is illustrated in this study. Highly similar sequences may sometimes be found in different clusters, as was shown for fragment NBS5a6H. For this fragment, high similarity was found with a sequence of tomato *Hero* gene that had previously been mapped to tomato chromosome IV (Ernst et al. 2002). The putative map position on chromosome IV for this marker, inferred from the high sequence similarity to Hero, was incorrect. The availability of the sequence assembly of the entire chromosomes from tomato has allowed us to identify multiple gene clusters on the long arm of chromosome IX. These clusters have high levels of homology ([90% identity] to *Sw-5* and *Tm-2^2* respectively which are physically separated by millions of base pairs. This finding may provide indications as to how *R* gene clusters evolve. Several studies have indicated the role of unequal crossing over, resulting in local duplications leading to rapid evolution of *R* gene clusters (Leister 2004; Kuang et al. 2004; McDowell and Simon 2006). Duplication over long distances, as observed in this study, would suggest an excision and subsequent insertion mechanism. This could be associated with the excision and insertion of retrotransposons, which are present in many *R* gene clusters. An excision insertion hypothesis for duplication is supported by the finding of a *Hero*-like gene on chromosome IX. This duplication cannot be a result from intra-chromosomal rearrangements such as unequal crossing over. Finally, we would like to emphasize that differential clone Ma*R8* is durably resistant to many *P.*

infestans isolates (Haynes et al. 2002), but it was not extensively used in breeding (Huang 2005). Localization of *R8* is a first important step for introgression breeding and for molecular cloning of this gene. The combination of several of the *R* genes from *S. demissum* and other wild potatoes using cisgenic modification or pyramiding breeding strategies offer good ways to protect the plant against late blight (Jacobsen and Schouten 2007; Zhu et al. 2011). In this perspective, marker $CDP^{Hero}3$ and its closely flanking markers are suitable for tagging *R8* introgressions in breeding material and to distinguish *R8* from other *R* genes in stacking approaches. Furthermore, the identified markers will be instrumental for the map based cloning of the *R8* gene.

Acknowledgments

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Chapter 3

The avirulence factor AVR8 for the Mexican *S. demissum* late blight resistance gene *R8* is recognized by cv. Sarpo Mira and resistant wild *Solanum* species from South America

The avirulence factor AVR8 for the Mexican *S. demissum* late blight resistance gene *R8* is recognized by cv. Sarpo Mira and resistant wild *Solanum* species from South America

Kwang Ryong Jo[1,2,3], Hendrik Rietman[1], Gerard Bijsterbosch[1], Liliana M. Cano[4], Tok-Yong Kim[3], Evert Jacobsen[1], Richard G. F. Visser[1], Sophien Kamoun[4], Jack H. Vossen[1] and Vivianne G. A. A. Vleeshouwers[1]

[1] Wageningen UR Plant Breeding, P.O. Box 386, 6700 AJ Wageningen, The Netherlands, [2] Graduate School Experimental Plant Sciences, The Netherlands, [3] Research Institute of Agrobiology, Academy of Agricultural Sciences, Pyongyang, DPRK, [4] Sainsbury Laboratory, Norwich NR4 7UH, United Kingdom

Summary

Recognition-dependent disease resistance is increasingly important for developing late blight resistant potatoes. The introgressed resistance (*R*) genes from *Solanum demissum* are mostly quickly defeated but there are a few exceptions. *R8* seems to confer a more durable resistance. We used an effectoromics approach to study *R8* resistance based on the elicitation of cell death in the Ma*R8* differential potato by agroinfiltration. Using a collection of *in planta*-induced RXLR effectors of the potato late blight pathogen *Phytophthora infestans*, our screen revealed a *R8* specific cell death to one RXLR effector; PITG_07558. The response to this effector co-segregated with the *R8*-mediated resistance to *P. infestans* isolate IPO-C in a F1 population and therefore this gene was designated as *Avr8*. Interestingly, *Avr8* has been recently reported as *AvrSmira2,* which was found to associate with field resistance in cultivar Sarpo Mira with *Rpi-Smira2*. To investigate the correlation between R8 and Rpi-Smira2, we crossed the resistant Sarpo Mira with the susceptible clone RH89-039-16 and performed genetic mapping studies in the segregating population. Indeed we found that *Rpi-Smira2* localized in the *R8* locus. The notion that field resistance in Sarpo Mira is caused by *R8* is supported by the fact that also *R8*-mediated resistance was only observed under field conditions and matched similar levels of delay in the onset of *P. infestans* symptoms. To search for the geographical and phylogenetic origin of *R8* in the *Solanum* gene pool, we carried out a functional screen for AVR8 responsiveness in 98 wild genotypes (72 accessions of 40 species) of *Solanum* section *Petota*. From this screen, we identified twelve AVR8 responding *Solanum* accessions originating both from Central and South America. Durability, origin and perspectives in potato resistance breeding of *R8* and AVR8 recognition specificity are discussed.

Introduction

Although the potato (*Solanum tuberosum*) is the world's third most important food crop after rice and wheat, it still continues to severely suffer from late blight, a devastating disease caused by *Phytophthora infestans*. *P. infestans* is a specialized pathogen, causing disease in the foliage and tubers of potato (Fry and Goodwin, 1997). To control late blight during the season, frequent fungicide applications are required, which are harmful to both humans and environment (Deahl et al. 1993; Goodwin et al. 1994; Grünwald et al. 2001). The coexistence of two mating types (A1 and A2) in many parts of the world (Fry et al. 1993; Goodwin et al. 1994) leads to sexual recombination and consequent increased genetic diversity of the *P. infestans* population makes managing potato late blight increasingly difficult. Late blight disease management is also affected by the emergence of fungicide-resistant *P. infestans* genotypes (Cooke et al. 2007; Cooke et al. 2012). Therefore there is an increased necessity to develop potatoes that possess durable late blight resistance. Breeding for late blight resistance in the beginning of the twentieth century concentrated on using major dominant resistance (*R*) genes from the Mexican wild species *Solanum demissum.* Introgressing these *R* genes into *S. tuberosum* led to a differential set of eleven breeding lines Ma*R1*–Ma*R11* (Black et al. 1953; Malcolmson and Black 1966; Malcolmson 1969; Bradshaw et al. 2006). However, resistance conferred by *R1, R2, R3,* and *R10* was rapidly overcome in European potato varieties (Malcolmson 1969; Wastie 1991). This was problematic and highlighted the use of introgression of additional resistance (*Rpi*) genes for stacking in order to increase durability to the late blight disease. Avirulence (AVR) proteins to oomycete pathogens that have been identified thus far all belong to the RXLR class of effector proteins (Vleeshouwers et al. 2011a). The *Avr* genes encode modular, secreted proteins with a RXLR motif for translocation into the host cell, followed by diverse, rapidly evolving C-terminal effector domains (Win et al. 2007). Typically, *Avr* genes of *P. infestans* meet three criteria, i.e., they function inside the host cell, they reside in gene-sparse (GSRs), repeat-rich regions that are thought to contribute to genome plasticity in the genome and they are highly up-regulated during the early biotrophic phase of infection in potato (Haas et al. 2009; Raffaele et al. 2010; Cooke et al. 2012). The emerging field of effector biology has led to application of effector-driven breeding, in which effectors are exploited for improving the use and deployment of disease resistance (Vleeshouwers et al. 2011a). Up to now, more than eight *Avr* genes for the potato-*P.infestans* pathosystem have been discovered. *Avr1,* which is perceived by the first cloned late blight resistance gene *R1,* has been isolated using positional cloning

(Ballvora et al. 2002; Tyler 2009). It locates in a repeat-rich region of the *P. infestans* genome and is highly expressed during the biotrophic phase of potato infection. *Avr2*, as a member of a highly diverse family of 18 RXLR effectors, was identified by a combination of map-based cloning, transient expression *in planta*, pathogen transformation and DNA sequence variation across diverse isolates (Gilroy et al. 2011). The family member *PexRD11* was detected by effectoromics screens and other family members by sequence similarity searches of the *Pi* genome (Rietman 2011; Vleeshouwers et al. 2011a). AVR2 family members are recognized by R2 family members (Lokossou et al. 2009). Functional allele mining revealed specific response to PEXRD11 in *S.x edinense* and *S. hjertingii* and two, two, and four copies of functional *R2* homologs were present in *S.x edinense, S. schenckii* and *S. hjertingii,* respectively (Champouret 2010). *Avr3a* was cloned by an association genetics approach and has two alleles, *Avr3a*^{KI} and *Avr3a*^{EM} in *P. infestans* populations (Armstrong et al. 2005). The avirulent allele *AVR3a*^{KI} is recognized by *R3a* (Huang et al. 2005). *R4* was not cloned yet but the corresponding effector *Avr4* that encodes a RXLR-dEER effector has been identified by a map-based cloning approach (van Poppel et al. 2008). *Avrblb1* that was identified by effectoromics screens is identical to the *in planta*-induced gene *ipiO,* and interacts with *Rpi-blb1* (Song et al. 2003; van der Vossen et al. 2003; Vleeshouwers et al. 2008). *Avrblb2* was detected by allele mining and functional screening of candidate RXLR effector genes (van der Vossen et al. 2005; Oh et al. 2009) and interacts with *Rpi-blb2*. Similar to *Avr2*, *Avrblb1* and *Avrblb2* occur in multiple copies in the *P. infestans* genome and are highly diverse: *Avrblb1* has up to four paralogous copies per genotype, and *Avrblb2* seven paralogs (Champouret 2010; Oh et al. 2009). *Avrvnt1,* the avirulence gene that interacts with *Rpi-vnt1*, was identified by effectoromics and three homologous RXLR effectors in the T30-4 genome were detected, with four variants among *P. infestans* isolates (Foster et al. 2009; Pel 2010). Recently, the genetic basis has been elucidated from potatoes that have retained a considerable level of resistance in the field, such as differential set plants Ma*R8* and Ma*R9* and cultivar Sarpo Mira (Jo et al. 2011; Kim et al. 2012; Rietman et al. 2012). Functional studies with effectors have shown that cultivar Sarpo Mira contains a pyramid of *R* genes, namely *R3a, R3b, R4, Rpi-Smira1* and *Rpi-Smira2* (Rietman et al. 2012). The differential Ma*R9* contains *R1, Rpi-abpt1, R3a, R3b, R4, R8,* and *R9* (Kim et al. 2012), and Ma*R8* contains *R8*, as well as *R3a*, *R3b*, and *R4* (Kim et al. 2012). Interestingly, *R8* was only detected under field conditions, not in detached leaf assays (Jo et al. 2011; Kim et al. 2012). Also the major determinant for late blight resistance in Sarpo Mira, *Rpi-Smira2,* was only detected in field trials and was associated with responses to the RXLR effector *AvrSmira2* (Rietman et al. 2012).

The recent work of Orłowska et al. (2012) showed that foliar resistance to late blight in Sarpo Mira depends on the integrity of the plant and is similar to our findings with Ma*R8* and Sarpo Mira material. In this study, we exploited an effectoromics approach to identify the *Avr8* gene matching the major resistance gene, *R8*, in differential clone Ma*R8* (Jo et al. 2011). Interestingly, *Avr8* turns out to be identical to *AvrSmira2*. Using marker analysis in F1 populations, we found that *Avr8/AvrSmira2* responsiveness and the quantitative resistance conferred by *Rpi-Smira2* in Sarpo Mira (Rietman et al. 2012) mapped at the *R8* locus, suggesting that *R8* and *Rpi-Smira2* are identical or functional homologs. A functional allele-mining with AVR8 in an extensive set of wild *Solanum* from Central and South America led to the discovery of various wild *Solanum* that may contain *R8* functional homologs. The use of this germplasm for breeding and potential for obtaining durable resistance is discussed.

Materials and methods

- Plant material

The potato differential plant Ma*R8*, also known as 2424a(5), was used as female parent in a cross with the susceptible cultivar Concurrent to generate a F1 mapping population 3020. Cultivar Sarpo Mira was crossed with the susceptible clone RH89-039-16, a donor of the potato genome sequence, to produce F1 population 3079. A hundred or thirty of genotypes of populations 3020 or 3079, respectively, and their parents, were clonally maintained *in vitro* culture containing Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose at 20°C. The wild *Solanum* plant materials which were maintained *in vitro* at Wageningen UR Plant Breeding, were used for effector screening and functional allele mining. Information on species, origin, collection site, GPS coordinates and other genbank code are accessible on the Sol*R*gene database.

- *Phytophthora infestans* isolate and blight resistance tests

Phytophthora infestans isolate IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) was used in field trials. Field trials for the mapping populations were done in 2009, 2010 and 2011 in Wageningen, The Netherlands, as described in previous studies (Jo et al. 2011; Rietman et al. 2012). Field trials for germplasm accessions were performed in the growing seasons of the years 2005 and 2007 in Wageningen, The Netherlands (Vleeshouwers et al. 2011b). Disease assessments were made in four replicates per genotype by estimating the percentage of leaf area covered with late blight lesions at multiple time points after inoculation. From these readings, the AUDPC was calculated

(Fry 1978) and the AUDPC values were transformed to 1 (susceptible)–9 (resistant) scale.

- Screening of *in planta*-induced RXLR effectors

A genome-wide collection of RXLR effectors was selected from the *P. infestans* genome sequence based on presence of a predicted signal peptide, a RXLR motif, and an elevated gene expression at 6 hours post inoculation (hpi) to 3 days post inoculation (dpi) (Haas et al. 2009). Using Gateway™ technology, effectors were subcloned into pK7WG2 and transformed into *Agrobacterium tumefaciens* strain AGL1, pSoup, and pVirG cells by electroporation. Agroinfiltration was performed as previously described (Rietman et al. 2012). Briefly, *A. tumefaciens* strains from frozen glycerol stocks were grown overnight at 28°C in 3 ml of LB medium supplemented with appropriate antibiotics. The next day these cultures were used to inoculate 15 ml of YEB medium (5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g yeast extract, 2 ml 1 M $MgSO_4$ in 1 L of milli-Q water) supplemented with antibiotics, 10 µl/L of 200 mM acetosyringone and 1000 µl/L of 1M MES. On the third day, the cells were harvested and resuspended to a final OD_{600} of 0.3 in MMA (20 g sucrose, 5 g MS salts and 1.95 g MES in 1 liter of distilled water, adjusted to pH5.6 with KOH supplemented with 1 ml/L of 200 mM acetosyringone). Leaves of plants were infiltrated with this suspension. Two leaves per plant and three replicate plants of 4 to 5 weeks old were infiltrated with the following constructs: effectors, *R3a* (Huang et al. 2005) and *Avr3a* (Armstrong et al. 2005) as the positive control and empty pK7WG2 (Karimi et al. 2002) as the negative control. Responses were scored 3 to 4 days after infiltration.

- DNA isolation and marker analysis

Total genomic DNA was isolated from young leaves as described by Fulton et al. (1995). The Retsch homogenizer (RETSCH Inc., Hannover, Germany) was used to grind young plant materials frozen in liquid nitrogen. For mapping *Avr8*-responsiveness in F1 populations markers described by Jo et al. (2011) were used (Table 2). For the identification of markers associated with the recognition of AVR8 in various *Solanum* genotypes, a modification of the NBS profiling protocol of van der Linden et al. (2004) was carried out as described in Jo et al. (2011). The concentrations of genomic DNA for all samples were adjusted to 300 ng/µl prior to profiling experiments. PCR reactions for markers 184-81 and Stm1021 were performed using the primers in Table 2 and DreamTaq™ polymerase (Fermentas) in a simple PCR program (94°C for 60 s followed by 30 cycles of 94°C for 30 s, 58°C for 60 s, 72°C for

90s and a final extension time of 5 min at 72°C). The polymorphism for marker 184-81 was detected by digesting the PCR product with the restriction enzyme listed in Table 2 and 1% agarose gel electrophoresis. Marker Stm1021 is a simple sequence repeat marker. Polymorphisms were detected using polyacrylamide gel electrophoresis. Fragments were prepared using a labelled forward primer (fluorescent dye IRD800) to enable visualization on a denaturing polyacrylamide gel using a NEN® IR2 DNA analyser (LI-COR® Biosciences, Lincoln, NE, USA).

- Map construction

The marker order was determined by TetraploidMap (Hackett and Luo 2003; http://www.bioss.ac.uk/knowledge/tetraploidmap/). The map distance was calculated based on the frequency of the recombination between markers.

Results

In planta-induced RXLR effector screening reveals candidate genes for Avr8

Ma*R8* was functionally profiled for response to a collection of 234 predicted RXLR effectors selected from the *Phytophthora infestans* genome sequence described by Haas et al. (2009). Responses to effectors were quantitatively scored for the level of cell death, ranging from 0% (no symptoms) to 100% (confluent cell death in all replicates) four days after agroinfiltration. Out of the 234 tested effectors, 13 effectors triggered more than 30% of cell death in the Ma*R8* plant (Table 1). Among those 13 effectors, four effectors, i.e., *Avr3a, Avr3b, Avr4* and *AvrSmira2* (PITG_07558), were described previously to confer avirulence activity in Sarpo Mira (Rietman 2011).

Response to AVR8 is co-segregating with R8 specific resistance

To investigate which of the identified effectors had an avirulence function towards *R8*, we adopted a genetic approach. Ma*R8* was crossed with the susceptible cultivar Concurrent and 100 F1 genotypes (population 3020) were assessed for resistance to *P. infestans* isolate IPO-C in detached leaf tests and in replicate field trials (Jo et al. 2011). This isolate is virulent on potatoes carrying *R3a*, *R3b*, and *R4*, and therefore, those *R* genes are expected not to interfere with the *R8* phenotype. The population 3020 showed a clear segregation of 1:1 ratio for resistance and susceptibility in field trials but not in laboratory tests (data not shown). Subsequently, we tested this population for response to the effectors that were recognized in Ma*R8* (Table 1).

Table 1. RXLR effectors that trigger cell death response on the differential plant Ma*R8*

Effector [a]		SignalP[b]				Expression in potato[c]		Response
Gene or construct ID	Gene annotation	HMM Prob.	NN mean NS score	RXLR	RXLR Tribe[d]	16 hpi	48 hpi	Ma*R8*(%)[e]
PITG_15039		0.993	0.928	RILV	1	0.83	1.12	100
PITG_22880		0.999	0.928	na	1	0.74	0.09	100
PITG_07558	*Avr8*	1.000	0.963	RSLR	2	1.11	0.24	95
PITG_04097		1.000	0.786	RSLR	5	1.40	1.28	75
PITG_18683		1.000	0.862	RSLR	5	-0.54	2.61	47
PITG_04169		0.995	0.918	RSLR	10	0.13	0.65	83
PITG_07387	*Avr4*	0.999	0.903	RFLR	52	0.03	2.00	100
PITG_10540		0.999	0.850	RFLR	57	-0.06	1.37	86
PITG_14371_KI[f]	*Avr3a* (KI)	na	na	RLLR	58	na	na	100
PITG_14374		0.992	na	RFLR	58	0.44	0.91	100
PITG_18215	*Avr3b*	0.999	0.857	RSLR	124	1.51	3.07	100
PITG_23129		0.956	0.452	RLLR	128	1.38	1.28	78
PITG_23131		0.983	0.831	RLLR	128	0.97	1.09	94
pMDC32	Neg control	-		-		na	na	0
R3a:Avr3a	Pos control	-		-		na	na	100

[a] Description of predicted RXLR effectors from the *P. infestans* genome reference strain T30-4. [b] Hidden Markov model (HMM) score and S-mean value predicted using SignalPv2.0. NN = neutral networks output, NS = S score (signal peptide score). [c] Gene induction on potato in T30-4 reference genome strain. *In planta*-gene induction was estimated relative to the expression levels in mycelium. Hours post inoculation (hpi). [d] RXLR Tribe ID as described by Haas et al. (2009). [e] Percentage of cell death response upon agroinfiltration, based on an average of quantitative scores in at least nine replicates. [f] AVR3aKI is the avirulent allele of AVR3a. na; not assessed.

In an initial screen, agroinfiltration was done on ten resistant and ten susceptible individuals of population 3020. Co-infiltration of *R3a/Avr3a* and the empty vector pK7WG2 were included as positive and negative controls, respectively. High levels of necrosis in the range of 30%-80% were observed in the negative control for five plants in the resistant set and six in the susceptible set. Also, the Concurrent parent displayed nonspecific response to agroinfiltration (Figure 1). To establish a subset of the population that does not show nonspecific cell death to the negative control, we tested an additional 23 individuals of population 3020 for their responses to the empty vector pK7WG2 and *R3a/Avr3a* (co)infiltration. Altogether, nine resistant and nine susceptible plants showed no cell death to the negative control but did show cell death to the positive control. We subjected this mini-population to an agroinfiltration experiment with the *Avr8* candidates in Table 1. The response to one effector PITG_07558 fully matched with the resistance to *P. infestans* isolate IPO-C in field trials (Figure 1). To further confirm these co-segregation results on a large set of the 3020 population, 56 additional genotypes of population 3020 were tested. Despite the occurrence of certain levels of cell death responses to negative controls in five plants,

for the remaining progeny clear distinction between PITG_07558-infiltrated spots (90-100%) and negative control spots (30-60%) was observed.

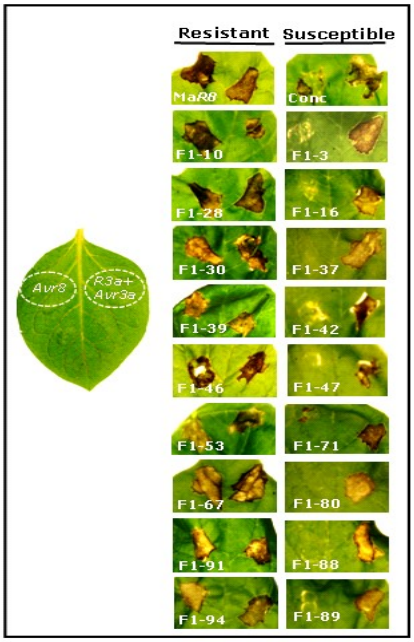


Figure 1. **Agroinfiltration assay for parents Ma*R8* and cv Concurrent and nine resistant and nine susceptible progeny.** *R-Avr* interactions were validated by co-segregation of responses to the effector AVR8 with resistance to *P. infestans* isolate IPO-C in F1 population (Ma*R8* x Concurrent). Avr8 was transiently expressed (left side) in Ma*R8*, Concurrent and F1 progeny plants by agroinfiltration. A 1:1 mixture of *R3a* and *Avr3a* was infiltrated as positive controls (right side).

All 34 plants that responded to PITG_07558 were resistant to *P. infestans* in the field, whereas all 31 plants that failed to respond to PITG_07558 were susceptible. Thus, response to PITG_07558 fully co-segregated with *R8* specific resistance to *P. infestans* and we designated PITG_07558 "*Avr8*". To test whether the response to *Avr8* co-segregates with the presence of a *R8* specific marker, we tested 65 genotypes which were used in agroinfiltration with marker CDP3 (Jo et al. 2011). All 34 resistant AVR8-responding plants contained CDP3, whereas all 31 susceptible non-responding plants did not. These data further confirm that the response to *Avr8* is associated with *R8* specific resistance.

Avr8 gene structure and expression

Avr8 (PITG_07558) is a single copy gene in the T30-4 reference genome located at supercontig 11 (Haas et al. 2009; Figure 2A). Indeed, the *P. infestans* transcripts database displayed only one expressed *Avr8* copy (http://www.broadinstitute.org). *Avr8* encodes a secreted protein with a typical RXLR effector of 244 amino acids and most likely an EER-like motif in its N-terminal domain and a WY-domain in its C-

terminal domain (Figure 2B). Similar to other *P. infestans Avr* genes, *Avr8* resides in a gene-sparse repeat-rich region (GSR) of the *P. infestans* genome (Figure 2A).

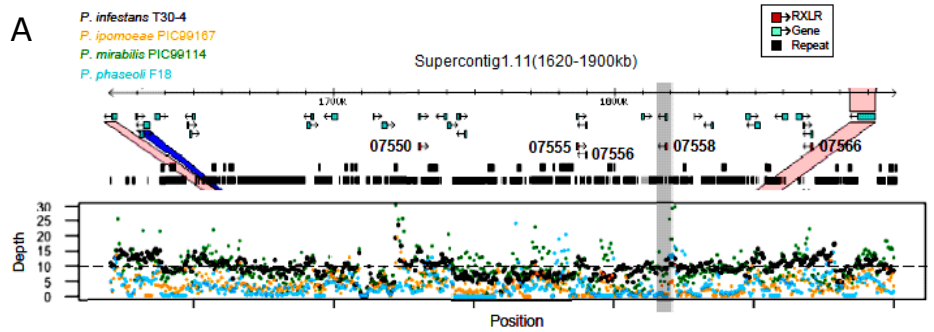


Figure 2. ***Avr8* effector features**. **A**. *Avr8*-containing genomic region. The 1620-1900kb of genomic region in supercontig 1.11 was shown. This region includes *AvrSmira1* (PITG_07550), PITG_07555, PITG_07556, PITG_07566. The *Avr8* gene is present in *P. infestans* and *P. mirabilis* but not in *P. ipomoeae* and *P. phaseoli*. **B**. The *Avr8* gene is a single copy gene and encodes a secreted protein with a signal peptide followed by a typical RXLR motif and most likely an EER-like motif at the N-terminal and a WY domain at the C-terminal effector region. **C**. A heat map showing expression levels of five RXLRs including the Avr8 gene from a time course infection on potato (6 to 120 hpi). Out of these RXLR effectors, PITG_07550, PITG_07555, PITG_07556, PITG_07566, Avr8 (PITG_07558) is the only one specifically induced at 6/16hpi. my; mycelia, sp; sporangia, zo;

zoospores. Gene induction is relative to mycelia as previously reported by Raffaele et al. (2010b). The gene expression values are from the reference genome strain T30-4 (Haas et al. 2009).

The *Avr8* gene is present in all thus far sequenced *P. infestans* isolates 90128, PIC99189, 06_3928A (13_A2), NL07434 and P17777 (US22) (Haas et al. 2009, Cooke et al. 2012 and unpublished), and no polymorphisms have been found thus far. Additional studies for genetic variation of *Avr8* are underway (data not shown). Sequencing of the closely related *P. mirabilis* showed that *Avr8* is also conserved in that species (Liliana Cano, Sophien Kamoun, unpublished), but not in other clade 1c *Phytophthora* species.

Rpi-Smira2 is localized on chromosome IX

In our previous studies, it was shown that the response to PITG_07558 correlates with field resistance that is conferred by the late blight resistance gene *Rpi-Smira2* in cv. Sarpo Mira (Rietman et al. 2012).

Table 2. Markers used for mapping of *Rpi-Smira2* in the F1 population (Sarpo Mira x RH)

Marker	Primer sequence (5'→3')	Marker type	Tm[a] (°C)	Product size (bp)	References
CDP3	Hero4064F: RRAGATTCAGCCATKGARATTAAGAAA	CDP/ *Hae*III	55	500bp	Jo et al. (2011)
184-81	184-81F: CCACCGTATGCTCCGCCGTC 184-81R: GTTCCACTTAGCCTTGTCTTGCTCA	CAPS/ *Rsa*I	58	480bp	Jo et al. (2011)
CDP4	Sw55F: AGTCTCCAAACATTCCTGCTTCTC	CDP/ *Mse*I	55	277bp	Jo et al. (2011)
Stm1021	Stm1021F: GGAGTCAAAGTTTGCTCACATC Stm1021R: CACCCTCAACCCCCATATC	SSR	58	210bp	Collins et al. (1999)

Sequences of adapter and adapter primer for CDP profiling

*Mse*I adapter	*Mse*-ad-top: CCCGAAAGTATAGATCCCAT *Mse*-ad-bottom: TAATGGGATCTATACTT	van der Linden et al. (2004)
Blunt adapter	B-ad-top: ACTCGATTCTCAACCCGAAAGTATAGATCCCA B-ad-bottom: TGGGATCTATACTT	
Adapter primer	ACTCGATTCTCAACCCGAAAG	

[a] annealing temperature

The fact that *Rpi-Smira2* interacts with the same effector as *R8* suggests that both genes localize on a similar position of chromosome IX. In order to prove this, the flanking markers to *R8*, 184-81 and CDP4 (Jo et al. 2011), were tested in Sarpo Mira x RH population (population 3079). In population 3079, flanking marker CDP4 located 1

cM distal to HR responses to AVR8 upon agroinfiltration (AVR8-HR) and the opposite flanking marker 184-81 2 cM proximal to AVR8-HR (Figure 3). This position is similar to position of the *R8* gene (Jo et al. 2011). For CDP3, a segregation of 22 presence to 4 absence in the tested genotypes was found which fitted a 5:1 ratio (χ^2= 0.87, >0.05). Some CDP3 positive genotypes were not responsive to AVR8 while none of the AVR8 responsive genotypes lacked the marker band.

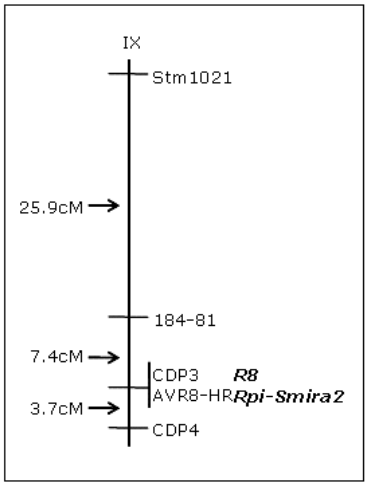


Figure 3. ***Rpi-Smira2* resides in *R8* locus on chromosome IX**. The quantitative resistance conferred by *Rpi-Smira2* in cv. Sarpo Mira based on AVR8 responsiveness (AVR8-HR) mapped between *R8* flanking markers 184-81 and CDP4 where *R8* was also localized (Jo et al. 2011). The map distances were calculated based on the frequency of the recombination between markers.

This suggested that CDP3 was present in duplex and that only one marker allele was linked to *Rpi-Smira2* which was present in simplex. Therefore we concluded that *Rpi-Smira2* resides in the *R8* locus on chromosome IX.

Functional profiling of wild Solanum species for response to AVR8

To determine the representation of *R8* functional homologs in wild *Solanum* species, 98 genotypes (72 accessions of 40 species) which are geographically and phylogenetically diverse were selected from wild *Solanum* section *Petota* germplasm (Vleeshouwers et al. 2011b). Wild *Solanum* genotypes that display resistance to *P. infestans* isolate IPO-C in detached leaf tests as well as in field trials were selected. The plants were functionally tested for cell death responses to AVR8 by agroinfiltration. Also, the potato differential set Ma*R1* to Ma*R11* were included in this

study. From these 109 genotypes, 60 genotypes showed nonspecific cell death responses to *Agrobacterium* or did not produce a response to the positive control construct and therefore could not be accurately grouped as responsive or nonresponsive. 35 genotypes that were well amenable to agroinfiltration did not show an *Avr8* response. Twelve genotypes from various wild *Solanum* accessions and two genotypes from the potato differential set displayed specific cell death in response to AVR8 (Table 3). These include genotypes from *S. demissum, S. tarijense, S. microdontum gigantophyllum, S. stoloniferum, S. schenkii* and two unclassified *Solanum* section *Petota* species. From the potato differential set Ma*R1* to Ma*R11,* as expected, Ma*R8* as well as Ma*R9* showed the response to AVR8 (Table 3). The presence of *R8* in Ma*R9* confirms the conclusion of our previous study (Kim et al. 2012). We further tested all AVR8 responding genotypes using *R8* flanking markers, and found that *S. microdontum* spp. *gigantophyllum* genotypes GIG712-6 and GIG715-4 had distinctive marker patterns, which lack the target bands that linked to the *R8* resistance (data not shown). This suggests that AVR8 recognition specificity of these plants may be conferred by *R* gene(s) different from *R8*.

Table 3. AVR8 response in a selection of *Solanum* germplasm material and the Mastenbroek late blight differential set

Species	Genotype[a]	Series [b]	Country of origin	Avr8	pK7WG2	R3a/ Avr3a	Resistance (IPO-C field) [c]
S. species	spp114-5[f]	*Demissa*	BOL	+	-	+	R
S. demissum	DMS345-1	*Demissa*	MEX	+	-	+	R
S. demissum	DMS343-1	*Demissa*	MEX	+	-	+	R
S. demissum	DMS344-18	*Demissa*	GTM	+	-	+	R
S. demissum	DMS585-7	*Demissa*	MEX	+	-	+	R
S. demissum	DMS585-1	*Demissa*	MEX	+	-	+	R
S. microdontum gigantophyllum	GIG712-6	*Tuberosa*	BOL	+	-	+	R
S. microdontum gigantophyllum	GIG715-4	*Tuberosa*	ARG	+	-	+	M
S. schenckii	SNK213-1	*Demissa*	MEX	+	-	+	R
S. species	spp891-1[g]	*Tuberosa*	BOL	+	-	+	R
S. tarijense	TAR852-5	*Yungasensa*	BOL	+	-	+	M
S. stoloniferum	STO389-4	*Longipedicellata*	MEX	+	-	+	R
(S. demissum)[d]	Ma*R1*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R2*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R3*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R4*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R5*		(MEX)[e]	-	-	-	M
(S. demissum)[d]	Ma*R6*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R7*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R8*		(MEX)[e]	+	-	+	R
(S. demissum)[d]	Ma*R9*		(MEX)[e]	+	-	+	R
(S. demissum)[d]	Ma*R10*		(MEX)[e]	-	-	+	S
(S. demissum)[d]	Ma*R11*		(MEX)[e]	-	-	+	S

[a] The three letter code represents the *Solanum* species (Simmonds 1962). The first number represents the CBSG number for the accession followed by a genotype number. MEX: Mexico, BOL: Bolivia, ARG: Argentina, GTM: Guatemala. [b] Classified according to Hawkes (1990). [c] R (highly resistant): >8.5, M (moderately resistant): 4.5-8.0, phenotype data for two years' field trials (Vleeshouwers et al. 2011b). [d,e] The resistance of potato differential set is originating from *S. demissum* from Mexico. [f] Unclassified *Solanum*, previously named *S. astleyi*. [g] Unclassified *Solanum*, previously named *S. ugentii*.

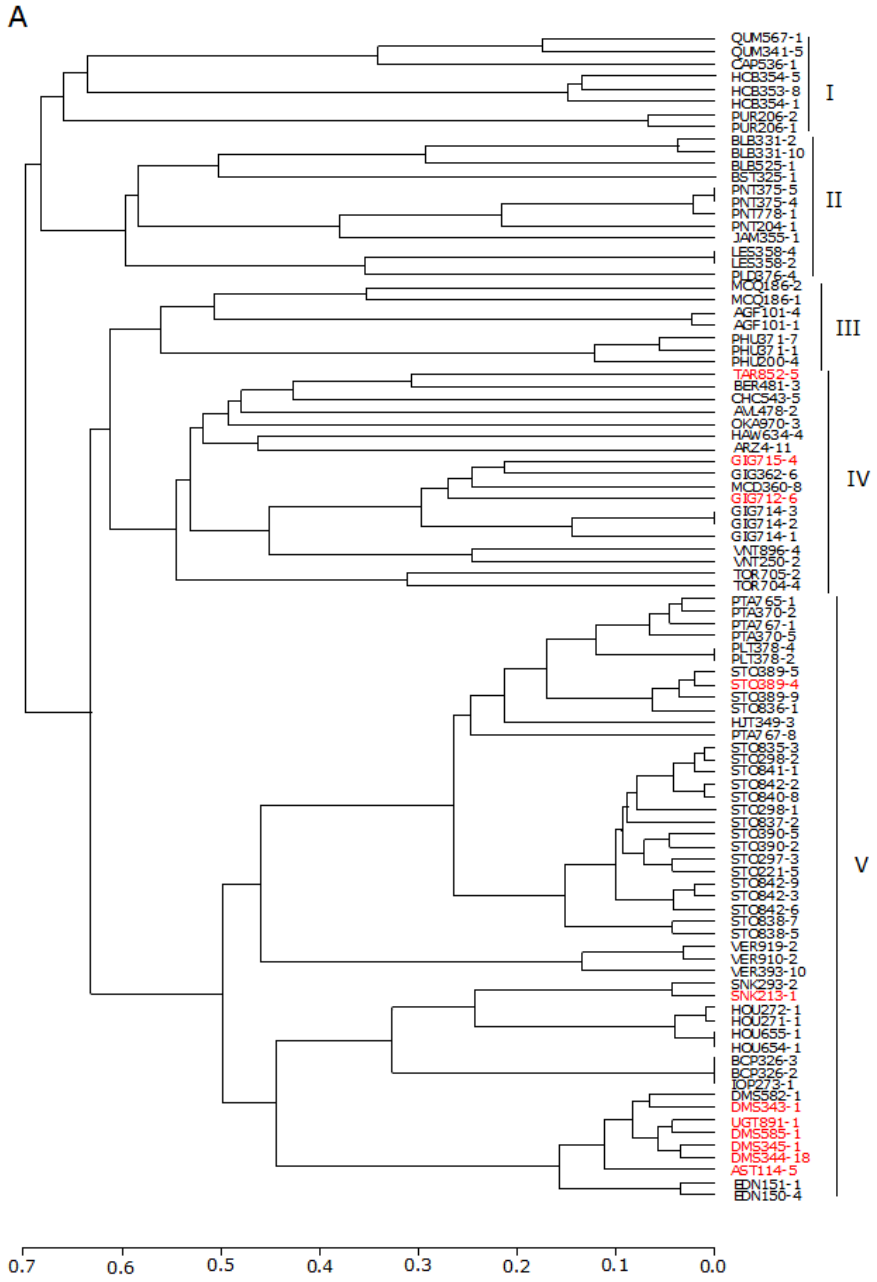
Response to Avr8 occurs in Central and South American Solanum species

An AFLP-based phylogenetic tree of the *Solanum* species that were tested for response to AVR8 was created using the Sol*R*gene database (Figure 4A; Jacobs et al. 2011; Vleeshouwers et al. 2011b). Basically, the tested wild genotypes were arranged into five different groups (Figure 4A). Group I and IV included genotypes from South America while groups II and V are from Central America. Genotypes from both Central and South America belong to group III. The AVR8 responding genotypes belonged to groups IV and V, which consist of genotypes derived from Mexico (Central America) and from Bolivia and Argentina (South America) (Figure 4B, Table 3). All five *S. demissum* genotypes were responsive to AVR8*.* The two unclassified genotypes that also respond to AVR8 were classified with *S. demissum* (based on AFLP patterns, Jacobs 2008). From the Mexican polyploid species *S. stoloniferum* and *S. schenckii*, additional responsive genotypes were identified. From South American origin, genotypes of *S. tarijense* and *S. microdontum gigantophyllum* were found to respond to AVR8 (Figure 4B). In summary, we detected response to AVR8 in various *Solanum* species that originate from both the Central and the South American centre of diversity of *Solanum* section *Petota*.

Discussion

Effectoromics identifies Avr8

In this study, we employed the *P. infestans* genome sequence and the complete catalogue of RXLR effector genes to apply effectoromics in combination with genetic analysis. This gives another successful illustration of effectoromics and complements the catalogue of *R-Avr* pairs of the potato-*P.infestans* pathosystem with the duo *R8-Avr8*. The *Avr8* gene fits most of the criteria that are typical for known *Avr* genes of *P. infestans*, i.e., it encodes a typical RXLR effector protein consisting of a N-terminal domain with a signal peptide and a RXLR motif for protein targeting inside plant cells and a C-terminal domain for effector recognition (Win et al. 2007).


A
QUM567-1
QUM341-5
CAP536-1
HCB354-5
HCB353-8
HCB354-1
PUR206-2
PUR206-1
I
BLB331-2
BLB331-10
BLB525-1
BST325-1
PNT375-5
PNT375-4
PNT778-1
PNT204-1
JAM355-1
LES358-4
LES358-2
PLD376-4
II
MCQ186-2
MCQ186-1
AGF101-4
AGF101-1
PHU071-7
PHU071-1
PHU000-4
III
TAR852-5
BER481-3
CHC543-5
AML478-2
OKA970-3
HAW634-4
ARZ4-11
GIG715-4
GIG362-6
MCD360-8
GIG712-6
GIG714-3
GIG714-2
GIG714-1
VNT896-4
VNT250-2
TOR705-2
TOR704-4
IV
PTA765-1
PTA370-2
PTA767-1
PTA370-5
PLT378-4
PLT378-2
STO389-5
STO389-4
STO389-9
STO836-1
HJT349-3
PTA767-8
STO835-3
STO298-2
STO841-1
STO842-2
STO840-8
STO298-1
STO837-2
STO390-5
STO390-2
STO297-3
STO221-5
STO842-9
STO842-3
STO842-6
STO836-7
STO838-5
VER919-2
VER910-2
VER393-10
SNK293-2
SNK213-1
HOU272-1
HOU271-1
HOU655-1
HOU654-1
BCP326-3
BCP326-2
IOP273-1
DMS582-1
DMS343-1
UGT891-1
DMS585-1
DMS345-1
DMS344-18
AST114-5
EDN151-1
EDN150-4
V
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0.6
0.5
0.4
0.3
0.2
0.1
0.0

B

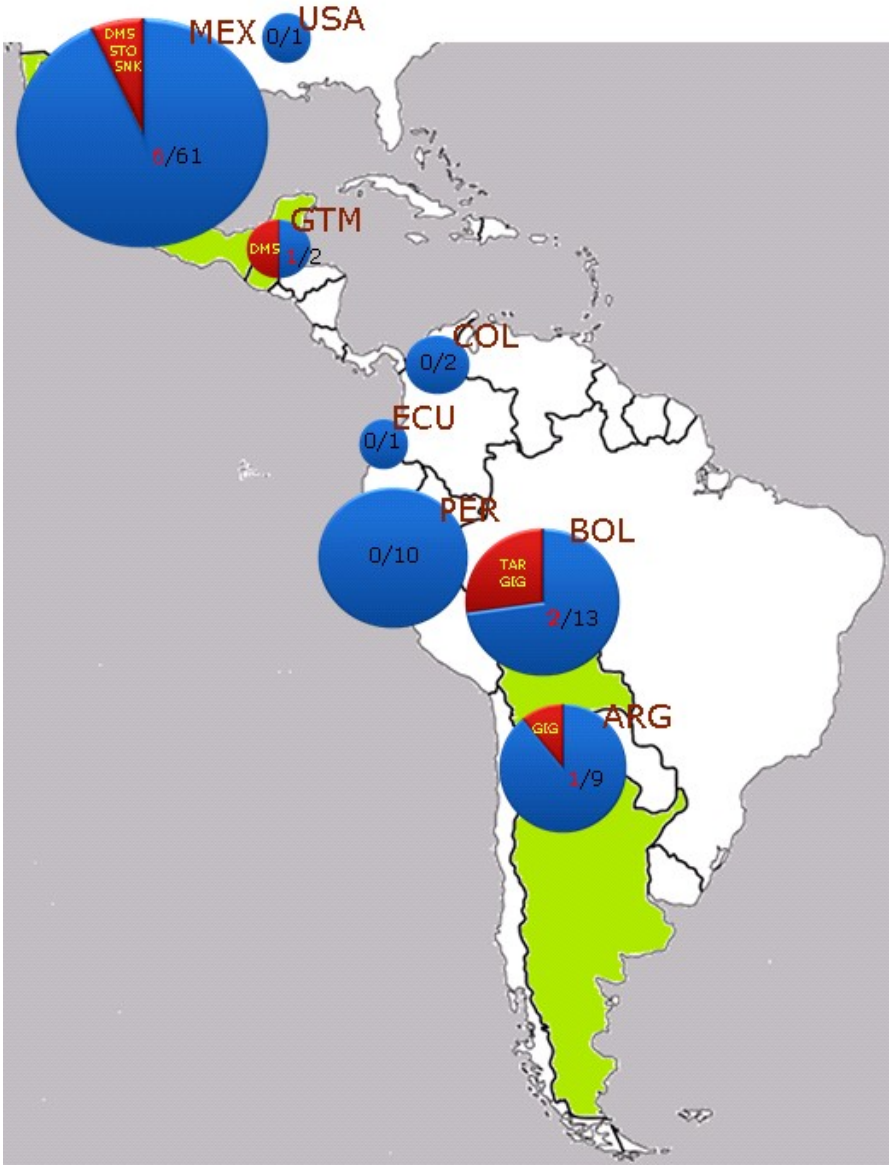

Figure 4. **AVR8-responding *Solanum* germplasm accessions are distributed both in Central and South America**. **A**. The phylogenetic tree was assembled using the AFLP fingerprinting data generated by Jacobs et al. (2008). All fingerprints can be obtained from

Sol*R*gene database website. Some genotypes, e.g., TUQ299-4 (previously named DMS299-4), DMS585-7, and CHN544-2 (previously named STO554-2), were not included in the phylogenetic tree because these are not available in Sol*R*gene database. Genotypes in red color respond to *Avr8*. **B**. Geographical overview of *Avr8*-responding *Solanum* species. The bubble size represents number of genotypes tested, and red sector the number of AVR8 responding genotypes. DMS; *S. demissum*, TAR; *S. tarijense,* GIG; *S. microdontum gigantophyllum,* USA; United States, MEX; Mexico, GTM; Guatemala, COL; Colombia, ECU; Ecuador, PER; Peru, BOL; Bolivia, ARG; Argentina. The unclassified *Solanum*, previously named *S. astleyi* and *S. ugentii* were not included.

Also, a putative WY domain is detected, a structural folding of the protein that is conserved in clade of the Peronosporales (Win et al. 2012). The *Avr8* gene resides in a gene-sparse repeat-rich region (GSR) of the *P. infestans* genome, where elevated levels of evolution occur (Raffaele et al. 2010). Also, expression of *Avr8* is induced at the early biotrophic phase of the interaction with potato. However, expression of *Avr8* peaks earlier than the other known avirulence effectors of *P. infestans,* i.e. 16 versus 48 hours after inoculation, respectively (Haas et al. 2009; Vleeshouwers et al. 2011b; Figure 2D). Currently, we are performing qRT-PCRs on inoculated potato tissue with *P. infestans* isolate IPO-C to test whether these microarray data on the reference isolate T30-4 (Haas et al. 2009) can be confirmed (data not shown). It remains to be investigated whether a dissimilar expression pattern is related to the typical phenotype of *R8* specific resistance that is mainly detected in field conditions.

R8 confers qualitative and quantitative resistance

Previously, we described a major contribution of *R8* to resistance in Ma*R8* and Ma*R9* which were analysed by "on site" virulence monitoring (Kim et al. 2012). It was found that plants containing *R8* had a similar delay in the onset of late blight symptoms as was observed in cultivar Sarpo Mira. In this study, we have shown that AVR8 recognition in Sarpo Mira maps to the *R8* locus. Besides, the *R8* specific resistance phenotype is clearer when performing whole plant assays, either in climate cells or field trials, compared to detached leaf assays (Jo et al. 2011; Orłowska et al. 2012). Whereas Ma*R8* and Ma*R9* populations showed a clear segregation of *R8* specific qualitative resistance in the field, the Sarpo Mira population showed a quantitative field resistance phenotype (Rietman et al. 2012). In contrast, the response to AVR8 was always a clear, qualitative trait in all three sources. Altogether these data strongly suggest that *Rpi-Smira2* is the same gene as *R8*. Our findings support the proposition that quantitative resistances could be controlled by NB-LRR type of resistance genes similar to other qualitative *Rpi* genes (Leonards-Shippers et al. 1994; Gebhardt and Valkonen 2001; Bradshaw et al. 2006; Tan et al. 2008). Isolation of the *R8* gene will

be essential to confirm this hypothesis. For breeding purposes, the molecular marker CDP3 can easily be used to introgress the *R8* mediated resistance into potato cultivars through marker-assisted breeding. Also, the functional marker *Avr8* for *R8* specific resistance can be exploited in breeding programs and for deployment of the *R8* in practical potato production. The qualitative or quantitative variation patterns for the capability of *R8* to provide resistance against late blight in segregating populations might be explained by differences in binding affinities of the AVR8 toward the target protein and/or the R8 protein (van der Hoorn and Kamoun 2008). Also, different genetic backgrounds can influence the outcome of resistance phenotypes (Cao et al. 2007). Dissection of the underlying molecular mechanism will provide research tools to convert difficult-to-follow and weak field resistance into a qualitative type of relatively strong resistance.

Functional germplasm screens reveal R genes interacting with Avr8 from Central and South America.

Functional screens with effectors open up new possibilities to facilitate the discovery of novel *R* genes or *R* gene homologs with potentially altered recognition specificities. The distribution of *R* genes in natural populations is not easy to study phenotypically in disease assays, because resistance to one particular pathogen isolate might result from the presence of multiple different *R* genes. Such examples are available (Verzaux 2010; Kim et al. 2012). Taking advantage of *Avr* genes to determine whether a corresponding *R* gene is present is the most preferred option, but a prerequisite is obviously that the corresponding *Avr* genes are available. Our study shows that AVR8 recognition specificity is present in all tested resistant genotypes of the Central American hexaploid species *Solanum demissum*, suggesting that (homologs of) the *S. demissum*-derived *R8* are highly abundant in this species that has been a major source of late blight resistance breeding thus far. Two unclassified species that were previously named *S. astleyi* and *S. ugentii,* also grouped with the *S. demissum* clade (Jacobs 2008), and most likely are related to *S. demissum.* AVR8 was also recognized in other Mexican *Solanum*, i.e., *S. schenckii* and *S. stoloniferum*. These species are classified in *Demissa* or *Yungasensa* (Hawkes 1990), and grouped together in polyploid Mexican *Solanum* (Jacobs 2008). We expect that the response to AVR8 in those plants is most likely caused by a conserved *R8* or a functional homolog of *R8*. Also from Mexican origin are two late blight resistance genes *Rpi-blb1* and *Rpi-blb2* from *S. bulbocastanum* (Vleeshouwers et al. 2008; Wang et al. 2008; Lokossou et al. 2010). In addition, from the *R2* late blight resistance gene family that occurs in various

Mexican *Solanum* species, up to 10 functional *R2* homologs are known (Lokossou 2010; Champouret 2010). However, in contrast to *R2, Rpi-blb1* and *Rpi-blb2* that are all geographically restricted to Mexico, AVR8 was also recognized in accessions from South American *Solanum* species *S. tarijense* and *S. microdontum gigantophyllum* (Table 3; Figure 4B) and whether this is mediated by a functional homolog of *R8* remains to be investigated further. Since it was hypothesized that *S. demissum* was derived by amphiploidy through hybridization and bilateral sexual polyploidization between an unidentified South American diploid species as the female and *S. albicans* as the male parent (Spooner et al. 1995; Nakagawa and Hosaka 2002; Pendinen et al. 2012), a common ancestor containing the ancient *R8* could might have spread from South America to Central America. Alternatively, the AVR8 response in South American *Solanum* that group closer to cultivated potato (*Tuberosa*) (Jacobs 2008, Hawkes 1990, Sol*R*gene) may be caused by a different *R* gene. It is possible that distinct R proteins can be recognized by the same effector protein. This has for example been noted for AVR2, being recognized by R2 of the major late blight cluster on chromosome 4 (Lokossou et al. 2009; Champouret 2010) as well as by another *R* gene that is localized on a different chromosome and belongs to a different family (unpublished data). Furthermore, in **chapter 5** of this thesis, evidence is provided that R9a and Rpi-blb2, two highly divergent R proteins recognise members of the same effector family. Similar to the hypothesis that a single AVR protein can be recognized by more R proteins, there are also reports that R proteins confer dual resistance specificity to diverse pathogens (Vos et al. 1998; Lozano-Torres et al. 2012).

R8 perspectives in potato resistance breeding

R8 may provide a broad spectrum of effective potato late blight resistance, as suggested in previous studies (Kim et al. 2012; Wang et al. 2012). Most of the currently characterized *P. infestans* isolates worldwide are not *Race 8*, which suggests that *R8* may confer a broad spectrum resistance (Rivera-Peña et al. 1990; Zhang and Kim 2007; Guo et al. 2009; Vargas et al. 2009; Harbaoui et al. 2012). However, we could imagine that since multiple *R* genes are present in the Ma*R8* differential, it is likely to be a certain over-estimation of virulence to the sole *R8*. To assess the percentage of virulence towards *R8* in the *P. infestans* population more accurately, studies on the genetic variation of *Avr8* are in progress. So far, a functional copy of *Avr8* is represented in all *P. infestans* strains tested, which would be in line with the expectation that *R8* confers resistance to a broad spectrum of *P. infestans* isolates. Sarpo Mira that has retained its resistance in the field over many years contains a

similar pyramid of *R* genes as Ma*R8*, i.e., *R3a, R3b, R4, Rpi-Smira2*, and only one extra (yet defeated) *Rpi-Smira1* gene was detected. Furthermore, in the context of agricultural deployment, besides Sarpo Mira, two potato cultivars, C88 (Li et al. 2010) and Missaukee (Douches et al. 2010), that have been described to display durable late blight resistance through multiple years of commercial cultivation, were also found to contain the *R8* markers (data not shown), demonstrating potential durability of late blight resistance for *R8*. To confirm this hypothesis, agroinfiltrations with AVR8 in these plants should be performed. Future deployment of *R8* as a broad spectrum and potentially durable *R* gene in combination with other *R* genes by cisgenic potato transformation (Jacobsen and Schouten 2007) is expected to provide durable late blight resistant potato cultivars.

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Chapter 4

The *R9* resistance to *Phytophthora infestans* in potato is conferred by two closely linked *R* genes and maps adjacent to the *R8* locus on chromosome IX

The *R9* resistance to *Phytophthora infestans* in potato is conferred by two closely linked *R* genes and maps adjacent to the *R8* locus on chromosome IX

Kwang Ryong Jo[1,2,3], Tok-Yong Kim[3], Richard GF Visser[1], Evert Jacobsen[1], Jack H. Vossen[1]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, P.O. Box 16, 6700 AA, Wageningen, The Netherlands, [2] Graduate School Experimental Plant Sciences, The Netherlands, [3] Research Institute of Agrobiology, Academy of Agricultural Sciences, Pyongyang, DPRK

Abstract

Genetic disease resistance is a promising tool in the fight against late blight of potato (*Solanum tuberosum*), a devastating disease caused by the oomycete pathogen *Phytophthora infestans*. The late blight differential set plant Ma*R9* provides durable resistance to a broad spectrum of late blight strains. The genetic basis of this resistance is brought about by at least seven late blight resistance genes derived from *S. demissum* including the resistance genes *R8* and *R9*. BC1 populations derived from Ma*R9* were challenged with the incompatible *P. infestans* isolate IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) and the progeny could be categorised as resistant and susceptible in both field trials and whole plant assays in a climate cell. Based on marker analysis and effector-responsiveness, two populations (3151 and 3154) in which the *R8* resistance was absent were selected. Remarkably, the PCR marker 184-81, which was closely linked to *R8*, fully co-segregated with the resistance in populations 3154 and 3151. The *R* gene responsible for the observed resistance in population 3154 is referred to as *R9a*. In population 3151, besides *R9a*, another tightly linked *R* gene was inferred in terms of the availability of six plants that were resistant but lacked the marker 184-81 and this resistance gene is referred to as *R9b*. The map positions of both genes on the distal end of the lower arm of chromosome IX were confirmed using CAPS marker GP101 and SSR marker Stm1021. Using population 3154, *R* gene cluster directed profiling (CDP) was carried out on ten resistant and ten susceptible progeny plants, revealing six closely linked markers. $CDP^{Tm^2}2$ flanked the *R9a* on the proximal side (2.9 cM) and had the same sequence as $CDP^{Tm^2}2$ found in the previous *R8* mapping. $CDP^{Tm^2}6$ and $CDP^{Tm^2}7$ fully co-segregated with the resistance and had high homology to *Tm-2²*, showing that *R9a* locates in a *Tm-2²* cluster of NBS-LRR genes and, most likely will be a member of the *Tm-2² R* gene family. $CDP^{Sw5}8$, $CDP^{Sw5}9$ and $CDP^{Sw5}10$ flanked the *R9a* at the distal end (5.8 cM) and belonged to the *Sw-5* cluster. Furthermore,

marker analysis using population 3151 showed that *R9a* located proximal to *R9b*. Future implications for employing both *R9* resistance genes in breeding are discussed.

Introduction

Late blight of potato (*Solanum tuberosum*) is a devastating disease caused by the oomycete pathogen *Phytophthora infestans* (*Pi*) (Fry 2008; Haverkort et al. 2009). This pathogen is a diploid, heterothallic fungus-like organism with two mating types (A1 and A2). Over the past several decades, with the increased incidence of late blight epidemics worldwide, the coexistence of both mating types has been observed throughout the world rather than the confinement inside Mexico (Fry et al. 1993; Goodwin et al. 1994). In accordance with this, there is a possibility of sexual recombination and increased genetic diversity among progeny of the pathogen which increases the difficulty in late blight control. Furthermore, developing resistance to fungicides in *Pi* populations, which has been demonstrated by widespread resistance to metalaxyl, a key component of fungicides for potato production, requires more frequent applications during the season to control late blight, causing contamination harmful to both humans and environment (Deahl et al. 1993; Goodwin et al. 1994; Grünwald et al. 2001). Genetic disease resistance has long been considered a promising method for the management of late blight as an alternative to fungicides and healthy seed tubers for late blight control. The Mexican hexaploid wild *Solanum* species, *Solanum demissum*, has been the most widely used source of resistance to *P. infestans* since the early part of the last century when the first crosses between *S. tuberosum* and *S. demissum* were carried out (Umaerus et al. 1983). Breeding activities have mainly focused on the high resistance conferred by dominant major resistance (*R*) genes because they were easy to transfer and follow. Presently, many cultivars with *S. demissum* in their pedigree are available (Umaerus et al. 1983; Swiezynski et al. 1991). Eleven race-specific *R* genes, named *R1–R11*, have been proposed in *S. demissum* and introduced into potatoes (Black et al. 1953; Malcolmson and Black 1966; Ewing et al. 2001). A current international set of potato *R* gene differentials that initially included clonal selections made individually by W. Black (Scotland) and C. Mastenbroek (the Netherlands), and by W.R. Mills and L.C. Peterson (USA) and afterwards was amended by Black et al. (1953), comprises 11 clones originating from *S. demissum* (Trognitz and Trognitz 2007) for the detection of late blight virulence factors. The differential set collected by Mastenbroek (1952) is also known as the Mastenbroek differential set: Ma*R1* to Ma*R11*. Ma*R1* to Ma*R4* were developed by Mastenbroek and the other *R* gene differentials are identical to the

Scottish differential set developed by Black (Huang 2005). Seven genes controlling late blight resistance within this differential set have been mapped: *R1* on chromosome V (Leonards-Schippers et al. 1992), *R2* on chromosome IV (Li et al. 1998), *R3a, R3b, R4, R6* and *R7* on chromosome XI (El-Kharbotly et al. 1996; Huang et al. 2005; Verzaux 2010) and *R8* on chromosome IX (Jo et al. 2011). Although the differential set was initially thought to represent single late blight resistance factors, many exceptions have been observed: *R1* was also found in the Ma*R5*, Ma*R6* and Ma*R9* differentials (Trognitz and Trognitz 2007) and the Ma*R3* differential plant contained two *R* genes, *R3a* and *R3b* (Huang et al. 2005). Even in the differentials Ma*R8* and Ma*R9*, respectively, at least four (*R3a, R3b, R4* and *R8*) and seven (*R1, Rpi-abpt1, R3a, R3b, R4, R8* and *R9*) *R* genes were present (Kim et al. 2012). In the past, *R* genes from Ma*R1*, Ma*R2*, Ma*R3*, Ma*R4* and Ma*R10* were overcome rapidly (Wastie 1991), but *S. demissum* is still considered a valuable source for resistance (Niederhauser and Mills 1953; Colon et al. 1995). Especially, the Ma*R8* and *MaR9* have been reported to show broad spectrum resistance both under laboratory and under field conditions (Fry and Goodwin 1997; Swiezynski et al. 2000; Haynes et al. 2002; Bisognin et al. 2002; Zhang and Kim 2007). Recently, it was shown that this broad spectrum resistance is a result of natural *R* gene stacking and/or a result of individual *R* genes like *R8*, using a "de-stacking" approach and an "on site" *Pi* virulence monitoring system (Kim et al. 2012). Stacking of multiple late blight *R* genes in different *Solanum* accessions has been revealed (Verzaux 2010) and is most likely a natural defence strategy against the highly flexible late blight pathogen. Also for late blight resistance breeding, stacking of multiple *R* genes seems mandatory in order to provide sufficient durability. So far, over 20 functional late blight *R* genes have been cloned and all belong to the CC-NB-LRR class. These include four *Solanum demissum* genes (*R1* (Ballvora et al. 2002)*, R2* (Lokossou et al. 2009)*, R3a* (Huang et al. 2005)*,* and *R3b* (Li et al. 2011)) and *Rpi* genes derived from wild *Solanum* species like *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005; Lokossou et al. 2009), *S. stoloniferum* and *S. papita* (Vleeshouwers et al. 2008), *S. venturii* and *S. mochiquense* (Pel et al. 2009; Foster et al. 2009). In our previous studies (Jo et al. 2011; Kim et al. 2012), it was shown that Ma*R9* differential contained additional *R* gene(s), referred to as *R9* which were not mapped or cloned. The present study describes the genetic dissection of the *R9* resistance in potato differential set plant Ma*R9.* It is conferred by two *R* genes just near the locus coding for *R8*. Thereby, the previous suggestion that *R9* would be an allelic variant of *R3* on chromosome XI (Huang et al. 2005) could be rejected.

Materials and methods

- Plant materials

The Ma*R9* differential, corresponding to 2573(2) and LB 1(Black et al. 1953; Malcolmson and Black 1966), was crossed as female parent with the cultivar Concurrent. BC1 populations were generated by crossing the resistant F1 progenies as female parents with susceptible cultivar Katahdin as a male parent and two populations were selected (3151 and 3154) which lacked the *R8* marker (Jo et al. 2011). Both F1 and BC1 male parental cultivars lack *R* genes that cause incompatibility of the *P. infestans* IPO-C isolate. Seeds were sown under sterile conditions, and plants were maintained in *in vitro* culture and propagated for multiple field trials and for the whole plant assays in a climate cell.

- *Phytophthora infestans* isolate and late blight resistance tests

Phytophthora infestans isolate IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) was used in both field trials and whole plant climate cell assays. Field trials were done as described by Jo et al. (2011). For whole plant climate cell assays, seedlings from population 3151 and 3154 were planted in pots and grown in the greenhouse at 22°C with a 10h day/14h night photoperiod and a relative humidity of 70 – 80%. One month after growth of plants, they were transferred into a growth chamber and inoculated. Inoculum preparation and inoculation were performed essentially as described by Vleeshouwers et al. (1999). Three leaves per plant for two plants of each of the BC1 population clones were inoculated. Seven days after inoculation with 10µl droplets of inoculum (5×10^4 zoospores/ml) in a cooled climate cell maintained at 15°C and 100% humidity with a photoperiod of 16h/8h day/night, plants could be classified in two groups, resistant (no symptoms, limited hypersensitive (HR) lesions or spreading HR lesions) or susceptible (sporulating lesions). There was a complete agreement with field trial results as a clear segregation of resistance and susceptibility two weeks after spraying of the zoospore suspension.

- Agroinfiltration assay

Two leaves per plant for three plants of each of the BC1 population clones were infiltrated with the following constructs: *Avr8* (**Chapter 3**), *R3a* and *Avr3a* (Bos et al. 2006) as the positive control, and empty pK7FWG2.0 (Karimi et al. 2002) as the negative control. Agroinfiltration assay was carried out as described by Vleeshouwers

et al. (2008). *Agrobacterium tumefaciens* strain AGL1, pSoup, and pVirG cells from glycerol stocks was grown in 3 ml of LB medium supplemented with appropriate antibiotics at 28°C overnight. The next day, the cultures were transferred to 15 ml of YEB medium (5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g yeast extract, 2 ml 1 M $MgSO_4$ in 1 liter of milli-Q water) supplemented with antibiotics, 10 µl of 200mM acetosyringone and 1000 µl of 1M MES. On the third day, the cells were harvested and resuspended in MMA solution (20 g sucrose, 5 g MS salts and 1.95 g MES in 1 liter of distilled water, adjusted to pH5.6) supplemented with 1ml of 200 mM acetosyringone to a final OD_{600} of 0.3. Leaves of 4- to 5- weeks old plants were infiltrated with this suspension. Responses were scored 3 to 4 days after infiltration.

- DNA isolation and marker analysis

Total genomic DNA was isolated from young leaves as described by Fulton et al. (1995). The Retsch machine (RETSCH Inc., Hannover, Germany) was used to grind young plant materials frozen in liquid nitrogen. For the identification of *R* gene specific clusters, a modification of the NBS profiling protocol of van der Linden et al. (2004) was carried out as described by Jo et al. (2011). The restriction ligation reaction was done using *Mse*I restriction enzyme and eight *Tm-2*[2] primers (Tm1R, Tm2F, Tm3F, Tm3R, Tm6F, Tm15F, Tm15R, and Tm19F) described by Verzaux (2010) were used for the successive PCR reactions. For *Sw-5*-CDP, seven specific primers were designed on cluster specific conserved domains encoding CC and LRR after the alignment of *Sw-5* sequences available from NCBI (http://www.ncbi.nlm.nih.gov/). The *Sw-5* specific primers described by Dianese et al. (2010) were also used for *Sw-5*-CDP. The CDP primers were used in combination with a labelled adapter primer (fluorescent dye IRD700) and labelled *R* gene targeted PCR products were separated on a denaturing polyacrylamide gel using a NEN® IR^2 DNA analyser (LI-COR® Biosciences, Lincoln, NE, USA). A set of the ten resistant and the ten susceptible BC1 plants including parents was used to obtain CDP markers linked to resistance in mapping population. If linked CDP markers were found, a second round of CDP was applied to all the remaining individuals of a segregating population. Fragments were excised as described in the Odyssey® manual for band extraction (Westburg, The Netherlands) and re-amplified with the specific profiling primer and the adaptor primer. PCR products were checked on polyacrylamide gels and fragments were cloned into the pGEM-T Easy vector (Promega, USA) prior to sequencing with M13 primers. Sequencing was carried out with the BigDye Terminator kit and an ABI 3700 automated sequencer from Applied Biosystems (USA). The marker nomenclature followed as described by Jo et al. (2011).

PCR reactions for GP101, 184-81, and Stm1021 were performed using DreamTaq™ polymerase (Fermentas) in a standard PCR program (94°C for 60 s followed by 30 cycles of 94°C for 30 s, 58°C for 60 s, 72°C for 90 s and a final extension time of 5 min at 72°C). In order to screen for cleaved amplified polymorphic sequences (CAPS), PCR was done using primers listed in Table 1 and subsequently PCR products were digested using the restriction enzymes listed in Table 1. For SSR, a labelled forward primer (fluorescent dye IRD800) was used to enable visualization on a denaturing polyacrylamide gel using a NEN® IR^2 DNA analyser (LI-COR® Biosciences, Lincoln, NE, USA). *Tm-2*-like or *Sw-5*-like sequences available from NCBI (http://www.ncbi.nlm.nih.gov/) and *S. tuberosum Group Phureja* DM1-3 516R44 (CIP801092) Genome Annotation v3.4 (based on v3 superscaffolds) PGSC_DM_v3.4_gene.fasta available from the Potato Genome Sequencing Consortium (PGSC) were collected and aligned using DNASTAR SeqMan Pro™ (DNASTAR. Inc.).

- Map construction and comparison

Co-segregating, simplex-inherited CDP markers from the tetraploid female parent (Ma*R9*) were scored as dominant markers (Wu et al. 1992). The marker order was determined by TetraploidMap (Hackett and Luo 2003). The map distance was calculated based on the frequency of the recombination between markers. Publicly available potato and tomato genetic maps from SGN (http://sgn.cornell.edu/) and GABI (http://www.gabipd.org/database/) databases, and physical map (Jupe et al. 2012) were included for comparison of marker positions and synteny.

Results

Development of mapping populations containing R9 but lacking R8

In order to generate BC1 populations with a reduced *R* gene content, eight IPO-C resistant F1 plants derived from a cross between Ma*R9* and cv. Concurrent were selected and crossed with susceptible cultivar Katahdin (Kim et al. 2012). The BC1 populations were tested in field trials inoculated with IPO-C in the potato growing seasons of 2010 and 2011. Five BC1 populations showed a black and white segregation of susceptibility and resistance. To select BC1 populations without *R8* but segregating for the resistance, they were tested for the presence of *R8* using *R8* marker, CDP3 that fully co-segregated with resistance (Jo et al. 2011). The progeny of two populations (3151 and 3154) lacked the CDP3 marker. To further confirm the

absence of *R8* from both populations the response of the BC1 plants to AVR8, matching *R8*, was tested (**Chapter 3**).

Table 1. Markers used for mapping of *R9* in the BC1 populations (Ma*R9* x Concurrent) x Katahdin

Marker	Primer sequence (5'→3')	Marker type	Tm[a] (°C)	Product size (bp)	References
$CDP^{Tm2}2$	Tm19F: GCCAAATAGTATTGTCAAGCTC	CDP/ *Mse*I	55	120bp	Jo et al (2011)
$CDP^{Tm2}6$	Tm1R: CATTTCTCTCTGGAGCCAATC	CDP/ *Mse*I	55	375bp	Verzaux (2010)
$CDP^{Tm2}7$	Tm2F: CAAGTTTGTCGCAGAGATTGA	CDP/ *Mse*I	55	430bp	Verzaux (2010)
$CDP^{Sw5}8$	Sw3856F: AAGGATGCGACCGTATTGACCTCAT	CDP/ *Mse*I	55	118bp	This study
$CDP^{Sw5}9$	Sw3856F: AAGGATGCGACCGTATTGACCTCAT	CDP/ *Mse*I	55	237bp	This study
$CDP^{Sw5}10$	Sw3856F: AAGGATGCGACCGTATTGACCTCAT	CDP/ *Mse*I	55	277bp	This study
184-81	184-81F: CCACCGTATGCTCCGCCGTC 184-81R: GTTCCACTTAGCCTTGTCTTGCTCA	CAPS/ *Rsa*I	58	480bp	Jo et al. (2011)
GP101	GP101F: GGCATTTCTATGGTATCAGAG GP101R: GCTTAACATGCAAAGGTTAAA	CAPS/ *Bsp*LI	58	750bp	GABI
Stm1021	Stm1021F: GGAGTCAAAGTTTGCTCACATC Stm1021R: CACCCTCAACCCCCATATC	SSR	58	210bp	Collins et al. (1999)

Sequences of adapter and adapter primer for CDP profiling

*Mse*I adapter	*Mse*-ad-top: CCCGAAAGTATAGATCCCAT *Mse*-ad-bottom: TAATGGGATCTATACTT	van der Linden et al. (2004)
Adapter primer	ACTCGATTCTCAACCCGAAAG	

[a] annealing temperature

As expected, none of the BC1 progeny produced a HR in response to AVR8. According to the marker analysis and effector-responsiveness, we concluded that these BC1 populations did not contain *R8* and that the resistance in these plants must be conferred by an additional *R* gene which will be referred to as the *R9* gene(s).

Whole plant late blight resistance tests in climate cells

In previous studies related to *R8* material (Jo et al. 2011; Kim et al. 2012), we described the discrepancy between DLA and field trial results for *Phytophthora infestans* isolate IPO-C. Populations 3151 and 3154 were tested in field trials in 2010 and 2011 and a clear black and white segregation of susceptibility and resistance was found. For more efficiently testing the segregation of resistance to IPO-C, additional

genotypes of these populations were used in a newly developed whole plant assays that could be performed in a climate cell. The outcome of this assay fully matched with results of the field assays in 2010 and 2011. The total population sizes now reached 104 and 69 plants, respectively. In both populations the resistance segregated in a 1:1 fashion (χ^2= 0.5, p >0.05 in population 3154, χ^2= 0.7, p >0.05 in population 3151), indicating the expected simplex based inheritance (Table 2).

Table 2. Segregation of disease resistance and marker 184-81 in two BC1 populations (Ma*R9* x Concurrent) x Katahdin

population	184-81 / phenotype	ab	aa
3154 (total 69)	R	32	0
	S	0	37
3151 (total 104)	R	47	6
	S	0	51

In population 3154, the resistance to IPO-C is fully co-segregating with marker 184-81, whereas in population 3151 six plants were observed which were resistant but had no marker band, suggesting the presence of another closely linked resistance gene. R: resistant, S: susceptible, ab: presence of the marker band, aa: absence of the marker band

Molecular marker analysis of segregating BC1 population

As described above, the *R8* marker was absent from both BC1 populations. Interestingly, when the 184-81 marker that flanked the *R8* gene at 1 cM distance was tested in the BC1 populations, we found that this marker fully co-segregated with the resistance in population 3154. This suggests that like *R8*, *R9* locates on chromosome IX. In order to verify this finding, we set out to develop additional closely linked markers near the *R9* gene. Known markers (GP101, S2g3, TG591A, GP41, CT220, T0521, S1d11, S1d5-a, T1065, TG328, TG424, St_At3g23400) from the SGN and GABI databases on the long arm of chromosome IX were selected and tested for linked polymorphisms. CAPS marker GP101, was found polymorphic and located 2.9 cM proximal (two recombinants) relative to *R9* in population 3154 (Figure 2). SSR marker Stm1021 which is present in RH9 BIN65 of the SH x RH map (Van Os et al. 2006) mapped at 20.3 cM (14 recombinants) proximal to *R9*. In this interval of chromosome IX, two *R* gene clusters (C42 and C43) are known (Jupe et al. 2012). These clusters

were targeted for *R* gene-CDP. Using eight *Tm-2²* primers, population 3154 was screened for linked markers. Three markers, CDP^{Tm2}2 (120bp), CDP^{Tm2}6 (375bp) and CDP^{Tm2}7 (430bp) were identified (Figure 1) that mapped in close proximity to *R9.*

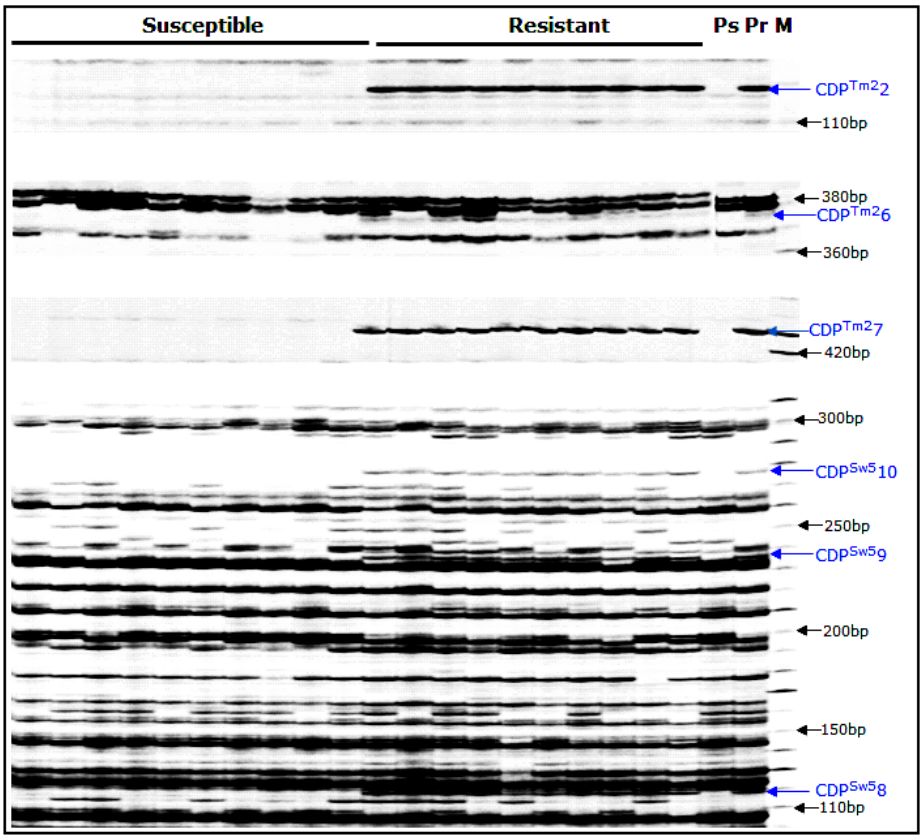

Figure 1. **CDP profiles of BC1 population 3154 showing ten susceptible clones and ten resistant clones, including parental clones.** Pr: resistant parent (Ma*R9*), Ps: susceptible parent (Katahdin), M: molecular weight marker. The arrows in blue and in black on the right side point out linked markers and the bands size of molecular weight marker, respectively.

CDP^{Tm2}2 marker is at 1.5 cM distance (one recombinant), proximal from *R9* and the other two markers fully co-segregated with the resistance in population 3154 (Figure 1 and Figure 2). Using *Sw-5*-CDP, three linked markers were found; CDP^{Sw5}8, CDP^{Sw5}9 and CDP^{Sw5}10 (Figure 1). All CDP^{Sw5} markers were located at 5.8 cM (4 recombinants) to the opposite side (distal) of the CDP^{Tm2} markers from the *R9* gene (Figure 2). The CDP markers were excised from the gel and subjected to sequence analysis. The

sequence of CDP^{Tm2}2 was identical to CDP^{Tm2}2 found in mapping *R8* gene (Jo et al. 2011; Genbank accession number JF317285.1). All three CDP^{Tm2} markers identified showed similarity to *Tm-2*^{2} and showed 92% identity to PGSC0003DMG402020585. This is a non-TIR-NB-LRR gene which locates in the *Tm-2*-like cluster C42 (Jupe et al. 2012). CDP^{Sw5}8, CDP^{Sw5}9 and CDP^{Sw5}10 were confirmed to be similar to *Sw-5*, a *S. lycopersicon tospovirus* resistance gene (Brommonschenkel and Tanksley 1997; Spassova et al. 2001).

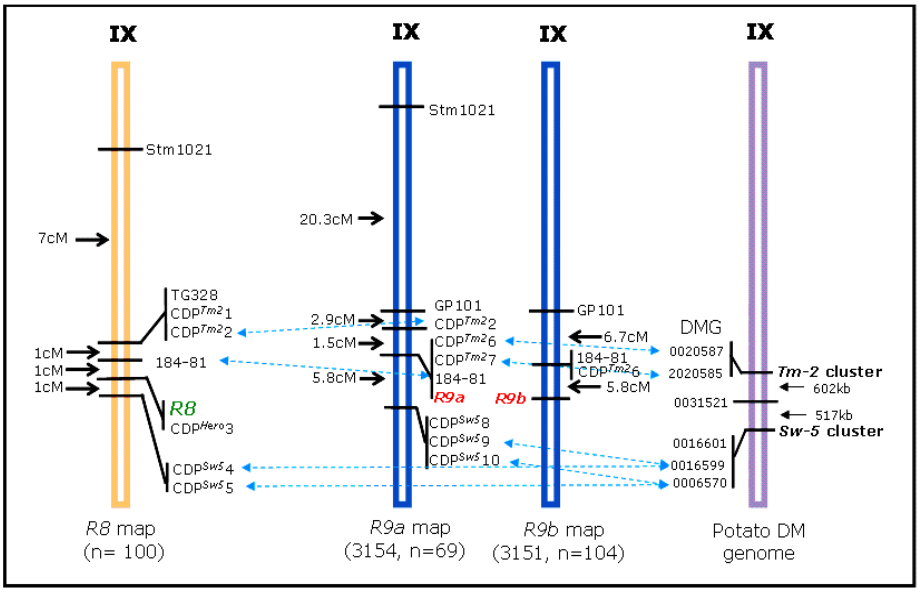

Figure 2. **Comparision of different maps of the distal end of potato chromosome IX.** Blue dotted arrows indicate similar or identical markers/sequences. Genetic distances in centimorgan are indicated by black arrows.

When the CDP^{Sw5} markers from the *R9* and *R8* maps were compared with the physical map of unique DMGs encoding for NB-LRR type genes (Jupe et al. 2012), they were found in cluster C43. Marker CDP^{Sw5}10 in *R9* map and CDP^{Sw5}4 in *R8* map had 70% and 85% of identity to DMG400016601, respectively. There is a good agreement between the relative positions of the *Tm-2*^{2} and *Sw-5* homologous markers identified in the Ma*R9*-derived BC1 population and the *R8* map (Jo et al. 2011) and DMG maps (Figure

2). In conclusion, *R9* resides on the telomeric end of the southern arm of chromosome IX and locates in or near a *Tm-2²* cluster.

R9 consists of two closely linked genes

In population 3151, six out of 53 resistant plants did not contain the markers 184-81, CDP^{Tm2}6 and CDP^{Tm2}7, which were fully co-segregating with the *R9* resistance in population 3154, while none of the susceptible plants contained these markers (Table 2). This could be caused by recombination between the *R9* gene and the marker. Alternatively, it can suggest the presence of an additional closely linked *R* gene in population 3151, that might be absent from population 3154. If the first hypothesis would be correct, two types of recombinants, namely, marker present & susceptible and marker absent & resistant, must be observed with the same ratios in population 3151. Otherwise, if the *R9* resistance would be conferred by two closely linked genes, only one type of phenotype for recombinants, marker absent & resistant, could occur. In population 3151, the fact that none of the susceptible plants contained the marker 184-81 strongly supports that the *R9* resistance is conferred by two closely linked genes. We referred to the resistance which was described in population 3154 as *R9a* and the additional resistance gene observed in population 3151 as *R9b*. When population 3151 was tested with markers used in *R9a* mapping, seven recombinants were found between GP101 and 181-84. These seven recombinants were different from the recombinants between *R9a* and *R9b* showing that *R9b* resistance locates distal to *R9a*.

Discussion

Mapping of R9

In this study, we describe the mapping of the *R9* resistance to *P. infestans* isolate IPO-C from the late blight differential Ma*R9* originating from *S. demissum.* *R9* also resides on the distal end of chromosome IX like *R8*. Both genes have been suggested to locate on chromosome XI as allelic variants of *R3* in a previous study (Huang et al. 2005) because of the presence of the *R3a* haplotype in the *R8* and *R9* plants. In retrospect, the presence of the haplotype was associated with the presence of the *R3a* gene itself that was a contaminant of the *R8* and *R9* differentials (Kim et al. 2012) rather than with the location of the *R8* and *R9* genes on this haplotype. We employed a *R* gene "destacking" (making offspring plants containing different *R* gene combinations) approach using marker analysis and effector response. The resulting BC1 populations

were analysed using *R* gene cluster directed profiling (CDP) strategies for mapping *R9*. It was essential to give a clear discrimination between *R9* and *R8* because Ma*R9* contained *R8* as well as *R9* and both *R8* and *R9* are avirulent to the same *Pi* isolate IPO-C. Analysis of the presence or absence of *R8* in mapping populations using the *R8* marker alone was not sufficient. Another molecular tool to further confirm the absence of *R8* was required, especially since *R9* was physically closely linked to *R8. R8* gene activity can efficiently be assessed by expressing the *Avr8* gene *in planta*. It was confirmed that populations lacking the *R8* marker indeed were not responsive to AVR8, so it could be concluded that the *R8* gene could not be responsible for the resistance to IPO-C in the populations. Using the "destacked" populations the *R9* gene could be mapped. By comparing the results from populations 3154 and 3151 it could be determined that *R9* is encoded by two closely linked genes. Apparently the parent of population 3154 underwent a recombination between the *R9a* and *R9b* genes. Although the *R9* resistance to *Phytophthora infestans* in potato is conferred by two tightly linked *R* genes and maps adjacent to the *R8* locus on chromosome IX, the fine mapping for two genes remains to be done. Currently, for fine mapping of *R9b* but also for confirming the two gene hypothesis, new BC2 populations are generated using BC1 plants like 3151-03, 3151-41 or 3151-55 that lack the *R8* gene and the *R9a* markers CDP^{Tm2}6 and CDP^{Tm2}7. The presence of two closely linked genes was also found in the Ma*R3* plant; *R3a* and *R3b* located at 0.4 cM from each other at the telomere of chromosome XI (Huang et al. 2005). The molecular markers linked to each other in *R9* and *R8* maps (Stm1021, CDP^{Tm2}2 and 184-81) are collinear and they had the same polymorphic patterns, suggesting that Ma*R8* and Ma*R9* could be derived from the same accession of *S. demissum* (Mastenbroek 1952; Malcomson et al. 1966).

Whole plant climate cell assay

It is of interest to note that the whole plant assay gives the same results as those of field trials for resistance characterization. Although the whole plant assays in climate cells have been used in mapping of genes for resistance to late blight present in the potato differentials of Black (Bradshaw et al. 2006), no comparisons with field trials were made. Furthermore, most studies involved in comparisons between detached leaf assay, whole plant glasshouse assay and field trials have been used for specific potato clones rather than mapping populations (Steward et al. 1983; Steward and Bradshaw 2001). Previously we described that there was a remarkable discrepancy between resistance to IPO-C in detached leaf assays (DLA) and in field trials for F1 and BC1 populations and only field trials gave consistent phenotyping results for resistance

(Kim et al. 2012). For more efficiently testing populations for segregation of resistance to IPO-C, a whole plant assay was developed that could be performed in a climate cell. The outcome of this assay fully matched with that of the field assays. Therefore, such a reliable whole plant assay in a climate cell would be essential for further studying plant-pathogen interactions at the physiological or molecular level, e.g., to unravel whole plant-dependent resistance expression in populations derived from Ma*R8*, Ma*R9* and cv. Sarpo Mira (Kim et al. 2012; Rietman et al. 2012).

The long arm of chromosome IX is a hot spot for resistance

The long arm of chromosomes IX is a hot spot for resistance in *Solanaceous* genomes (Sliwka et al. 2006; Pel 2009). The resistance genes clustering to the long arm of chromosome IX contain the late blight resistance gene *Rpi-moq1* of *S. mochiquense* (Smilde et al. 2005), *Ph-3*, a major QTL for late blight resistance in tomato (Chungwongse et al. 2002), *Sw-5*, a tomato gene for tospoviruses resistance (Brommonschenkel and Tanksley 1997), *Nx* encoding hypersensitive resistance to Potato virus X of *S. phureja* (Tommiska et al. 1998) and *Gpa6*, a QTL for potato resistance to *Globodera pallida* (Rouppe van der Voort et al. 2000). *Rpi-phu1* (Sliwka et al. 2006), derived most likely from *S. phureja* and conferring broad-spectrum resistance to late blight has been identified in a similar region of the gene *Gm* conferring resistance to Potato virus M on potato chromosome IX (Marczewski et al. 2006), as well as a QTL for resistance to *Erwinia carotovora* ssp. *atroseptica* (Zimnoch-Guzowska et al. 2000). Recently, three *R* genes have been identified from *S.x edinense*, a natural pentaploid hybrid between *S. demissum* and the South American cultivated potato *S. tuberosum* spp. *andigena*, among which *Rpi-edn2* controlling the resistance to IPO-C mapped in a similar region of *R9* (Verzaux 2010). *Rpi-dlc1*, from *S. dulcamara*, a *Solanum* species native to Europe, mapped in the proximity of *Rpi-mcq1* (Golas et al. 2010). The long arm of chromosome IX features two large heterogeneous clusters (Jupe et al. 2012). Cluster 42 harbours eight TIR-NB-LRR genes that are separated by eight homologues of *Rpi-vnt1* and *Tm-2*^{2}, whereas more distal cluster C43 contains 15 homologues of the Tospovirus resistance gene *Sw-5* (Jupe et al. 2012). Therefore, all genes on the long arm of chromosomes IX could reside either in the *Tm-2*^{2} cluster or the *Sw-5* cluster so that *R* gene cloning by allele mining would be encouraged, although it will require more sophisticated bioinformatics for quick and accurate cloning.

Implications for late blight resistance breeding

The utility of the *R9* resistance in combination with *R8* gene would be of importance in the context of late blight resistance breeding of potato. Although it was unknown to what extent *R9* genes would be durable for the resistance to late blight, the combination of these genes could most likely contribute to durability due to combinations of all other *R* genes which could be expected from previous work described by Kim et al. (2012). Two approaches can be used to produce potato clones with both *R9* and *R8*; marker assisted selection and cisgenic strategy. Marker-assisted selection allows us to effectively transfer the multiple *R* genes into potato using traditional breeding methods, an alternative to deploying the *R* gene through genetic transformation which has been a non-stop controversial issue. The facts that *R9* and *R8* genes reside in *Tm-2²*- and/or *Sw-5* clusters which are physically separated by a maximum distance of 1120kb (Jupe et al. 2012; Figure 2), and that they are naturally located on different chromatids, provide a potential challenge to recombining them into coupling phase, as demonstrated by Robbins et al. (2010). This would result in a chromosome fragment containing several *R* genes which can include *R8, R9a* and *R9b*. As a consequence, possible genes with a negative effect (linkage drag) located between *R9a* and *R9b* (or between *R9* and *R8*) will not be lost by backcrossing. Cisgenic breeding based on the introduction of natural *R* genes isolated only from crossable species using marker free transformation technology, would not encounter the problem of linkage drag that could not be removed.

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Chapter 5

Cloning and characterization of the potato late blight resistance gene *R9a* showing an overlapping recognition spectrum with the resistance gene *Rpi-blb2*

Cloning and characterization of the potato late blight resistance gene *R9a* showing an overlapping recognition spectrum with the resistance gene *Rpi-blb2*

Kwang Ryong Jo[1,2,3], Minh Hoa Tran[1], Estelle Verzaux[1], Yong-Gi Paek[1,3], Gert van Arkel[1], Geert Kessel, Trudy vd Bosch[4], Marieke Forch[4], Hendrik Rietman[1], Tok-Yong Kim[3], Richard GF Visser[1], Evert Jacobsen[1], Nick de Vetten[5], Jack Vossen[1]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, P.O. Box 16, 6700 AA, Wageningen, The Netherlands, [2] Graduate School Experimental Plant Sciences, The Netherlands, [3] Research Institute of Agrobiology, Academy of Agricultural Sciences, Pyongyang, DPRK, [4] Plant Research International, Biointeractions and Plant Health, Wageningen University and Research Center, P.O. Box 16, 6700 AA, Wageningen, The Netherlands, [5] Averis Seeds B.V. Valtherblokken Zuid 40, 7876 TC Valthermond

Summary

There is an increasing necessity to develop potatoes that possess durable resistance to late blight, caused by the oomycete pathogen *Phytophthora infestans*, as more virulent and fungicide resistant isolates of the pathogen are rapidly emerging. Here, we describe the positional cloning of the *R9a* gene from the differential plant Ma*R9* which confers broad spectrum late blight resistance. Using backcross mapping populations, the *R9a* late blight resistance (*R*) gene was previously mapped near a cluster of *Tm-2*^{2} homologous sequences at the distal end of the long arm of chromosome IX. A bacterial artificial chromosome (BAC) library derived from the differential plant Ma*R9* was screened with co-segregating *R* gene cluster directed profiling (CDP) markers whereby two overlapping BAC clones carrying CDP markers were obtained. Sequence annotation of the complete insert of these BAC clones identified two complete *R* gene analogs (*RGA9.1* and *RGA9.2*) of the NB-LRR class of plant *R* genes in one BAC clone. Two *RGAs* including their natural regulatory transcriptional elements were subcloned by long-range PCR into a binary vector for plant transformation. Complementation analyses showed that *RGA9.1* was able to complement the susceptible phenotype in cultivar Desiree. The gene, designated *R9a*, encodes a CC-NB-LRR protein sharing 82% identity at the amino acid level with Rpi-mcq1 from *S. mochiquense*, 78% with Rpi-vnt1.1 from *S. venturii* and 74% identity with Tm-2^2 from *S. lycopersicum*, respectively. Further analyses of 107 *Solanum* genotypes for the presence of a *R9a*-

specific marker, co-segregation of *R9a* marker with IPO-C resistance in a *S.x edinense* derived population, and a similar pattern of the isolate resistance spectrum in *R9a* and *Rpi-edn2* materials strongly support the notion that *Rpi-edn2* and *R9a* are functional homologs. Agroinfiltration-based effector screens for identifying the *Avr* genes matching the *R9a* gene were performed, leading to the discovery of Avrblb2 homologs which trigger *R9a* mediated hypersensitivity in *Nicotiana benthamiana*. Resistance profiling with 54 *P. infestans* isolates showed that Ma*R9* and *S.x edinense* accessions had similar resistance spectra as the *Rpi-blb2* containing cultivar Bionica. This is in line with the observation that similar *Avr* effectors are recognised by the *R* genes from these genotypes.

Introduction

The Irish potato famine oomycete pathogen *Phytophthora infestans*, the causal agent of late blight, remains the most important constraint in potato producing regions of the world. It might cause the complete destruction of the foliage and tubers of potato if meteorological conditions are conducive to the onset and spread of an epidemic (Fry and Goodwin, 1997). Although fungicide applications provide reasonable levels of late blight control, they impose high input costs to the farmer, are detrimental to humans and the environment and increase the capacity of the pathogen to develop resistance to the active ingredients of fungicides applied (Goodwin et al. 1996; Grünwald et al. 2001). The increased genetic diversity among progeny due to sexual recombination between two mating types (A1 and A2) in many parts of the world (Fry et al. 1993; Goodwin et al. 1994) enhances the necessity to develop potatoes that possess durable late blight resistance. Therefore, breeders have been extremely interested in creating durable resistance in potato cultivars as an alternative to the use of fungicides. During the 20th century, breeding for late blight resistance concentrated on using major dominant resistance (*R*) genes from the Mexican wild species *Solanum demissum* as a source of extreme resistance. This led to the identification of eleven *S. demissum R* genes (Black et al. 1953; Malcolmson and Black 1966; Malcolmson 1969; Bradshaw et al. 2006). However, resistance obtained by introgression breeding of *S. demissum R* genes, *R1, R2, R3,* and *R10* was overcome rapidly in European potato varieties (Malcolmson 1969; Wastie 1991). This motivated to focus on the introgression of additional late blight resistance (*Rpi*) genes for stacking in order to increase durability. Efforts to identify broad-spectrum resistance derived from diverse wild *Solanum* species besides *S. demissum* have revealed a wealth of major resistance to *P. infestans* (*Rpi)* genes which have a potential value in agricultural deployment (Hawkes

1990; Jansky 2000; Hoekstra 2009; Vleeshouwers et al. 2011a). During the last decades, much progress has been made in the identification, mapping and cloning of *Rpi* genes against late blight. Eleven race-specific *R* genes, named *R1–R11*, have been proposed in *S. demissum* and introduced into potatoes (Black et al. 1953; Malcolmson and Black 1966; Ewing et al. 2001). Internationally, a set of potato *R* gene differentials comprising 11 clones is a standard for the late blight resistance factors originating from *S. demissum* (Trognitz and Trognitz 2007) and allows the discrimination of avirulence phenotypes in *P. infestans* populations. The Dutch differential set collected by Mastenbroek (1952) is referred to as Ma*R1* to Ma*R11*. Ma*R1* to Ma*R4* are different, and Ma*R5* to Ma*R11* are identical to the Scottish differentials developed by Black (Huang et al. 2005). Seven genes controlling late blight resistance within this differential set have been mapped: *R1* on chromosome V (Leonards-Schippers et al. 1992), *R2* on chromosome IV (Li et al. 1998), *R3a, R3b, R4, R6* and *R7* on chromosome XI (El-Kharbotly et al. 1996; Huang et al. 2005; Verzaux 2010) and *R8* on chromosome IX (Jo et al. 2011). Very recently, two closely linked genes, named *R9a* and *R9b,* were identified from the Ma*R9* differential plant and mapped near the *R8* locus on chromosome IX (**Chapter 4**). Besides, *Rpi* genes from other wild potato species have been identified and mapped. So far, over 20 functional late blight *R* genes have been cloned and all belong to the CC-NB-LRR class. These include four *Solanum demissum* genes, *R1* (Ballvora et al. 2002)*, R2* (Lokossou et al. 2009)*, R3a* (Huang et al. 2005)*,* and *R3b* (Li et al. 2011) and *Rpi* genes derived from wild *Solanum* species, e.g., *RB/Rpi-blb1*, *Rpi-blb2* and *Rpi-blb3* from *S. bulbocastanum* (Song et al. 2003; van der Vossen et al. 2003, 2005; Lokossou et al. 2009), *Rpi-sto1* and *Rpi-pta1* from *S. stoloniferum* and *S. papita* (Vleeshouwers et al. 2008), *Rpi-vnt1* from *S. venturii* (Pel et al. 2009; Foster et al. 2009) and *Rpi-mcq1*, also known as *Rpi-moc1* (Smilde et al. 2005) from *S. mochiqense* (Foster et al. 2009), and *Rpi-chc1* from *S. chacoense* (Vossen et al. 2012). To clone these *Rpi* genes, conventional map-based cloning approaches or candidate gene cloning approaches have been used (Vleeshouwers et al. 2008; Pel et al. 2009; Lokossou et al. 2010). Important to exploit durability of *Rpi* genes is an understanding of their interactions with effectors from *P. infestans*. In recent years, the rapid development of effectoromics enabled the discovery of more than eight *Rpi* and *Avr* gene pairs for the potato-*P. infestans* pathosystem*,* opening up new possibilities to facilitate unravelling of the underlying resistance mechanism, *R* gene cloning and resistance breeding (Vleeshouwers et al. 2011a; Rietman 2011). Here, we report molecular cloning and characterization of the *R9a* gene from the differential plant Ma*R9*. The gene was cloned by exploiting co-segregating *R* gene cluster directed profiling (CDP) markers for

positional cloning without the construction of a high resolution map and encodes a CC-NB-LRR protein. We also found that *Avrblb2* homologs are recognized by *R9a* and isolate recognition spectra are used to study overlap in recognition specificity.

Materials and methods

- Plant material

The potato differential plant Ma*R9,* corresponding to 2573(2), was used for bacterial artificial chromosome (BAC) library construction. *S.x edinense* P. Berthault accession edn150-4, *S.x edinense*-derived clone JV1, and the cultivar Bionica were used for agroinfiltration and resistance screening. *S.x edinense* population (population code 7727) and Ma*R9* population (code 3151) were used for marker analysis. The wild *Solanum* plant materials which were used for germplasm screens and potato cultivars including cv Desiree, which was used for transformation, were maintained *in vitro* at WageningenUR Plant Breeding. Information on species, origin, collection site, GPS coodinates and other genebank code are accessible on the Sol*R*gene database (http://www.plantbreeding.wur.nl/SolRgenes/).

- Bacterial artificial chromosome library construction and screening

Genomic DNA from Ma*R9* was used for the construction of a BAC library. High-molecular weight DNA preparation and cloning into pCC1 BAC library was carried out as described previously (Rouppe van der Voort et al. 1999). Approximately 174000 clones with an average insert size of 85 kb, corresponding to 4 haploid genome equivalents, were obtained. The BAC clones were stored as 576 bacterial pools containing approximately 300 white colonies each. These were generated by scraping the colonies from the agar plates into LB medium containing 18 % glycerol and 12.5 µg ml^{-1} chloramphenicol. These so-called simple pools were stored at -80ºC. Profiling markers described in mapping the *R9* resistance (**Chapter 4**) were used to screen the BAC library, bacteria corresponding to positive pools were diluted and plated on LB agar plate containing chloramphenicol (12.5 µg ml^{-1}). Individual white colonies were picked into 384-well microtitre plates and single positive BAC clones were subsequently identified by a second round of marker screening. A modification of the NBS profiling protocol of van der Linden et al. (2004) was carried out as described in Jo et al. (2011). The concentrations of DNA for all samples were adjusted to 300ng/µl prior to profiling experiments. Polymorphisms were detected using polyacrylamide gel electrophoresis. Fragments were prepared using a labelled primer (fluorescent dye

IRD700) to enable visualization on a denaturing polyacrylamide gel using a NEN® IR2 DNA analyser (LI-COR® Biosciences, Lincoln, NE, USA). Markers used in *R9a* gene cloning were shown in Table 1.

- DNA sequencing and computer analysis

BAC clone sequencing was carried out using a shotgun strategy. Fragmentation, library production, 454 sequencing (GS FLX titanium system) and contig assembly was performed at Macrogen (Korea). Gene structures were predicted using FGENESH2.6 (Softberry) and protein sequences were deduced by translation of ORF using the standard genetic code. Multiple sequence alignments and phylogenetic analyses were conducted using CLUSTALX 1.81 (Thompson et al. 1997) available in the MegAlign Lasergene 9.0 software package (DNASTAR Inc., USA). The search for genes homologous to *R9a* was carried out using the Basic Local Alignment Search Tool (BLAST) in publically available sequence databases. Conserved domains were identified using Swiss-Prot (InterProScan, EMBL-EBI, ExPASy, SAPS).

- Subcloning of candidate genes

Long-range PCR reactions for subcloning *RGA9.1* and *RGA9.2* were performed using the primers in Table 1 and Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc,) in a simple PCR program (98°C for 30 s followed by 34 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 5.5 min and a final extension time of 15 min at 72°C). The resulting PCR products was subjected to ethidium bromide-containing 1% agarose gel electrophoresis in 0.5 x TBE buffer at a field strength of 105 V cm^{-1} for 60 min and the target size of band was cut on UV illuminator. From the gel slice, DNA was extracted using Zymoclean™ Gel DNA Recovery Kit. After DNA concentration was measured, the PCR DNA was digested with *Asc*I and *Sbf*I and the digested products were purified using QIAEX®II Gel Extraction Kit. The digested PCR products for *RGA9.1* and *RGA9.2* were ligated to the *Asc*I and *Sbf*I digested and dephosphorylated binary vector pBINPLUS (van Engelen et al. 1995). Ligation product were transformed to ElectroMAX *E.coli* DH10B competent cells (Life technologies, Paisley, UK). The binary constructs were sequenced using a primer walking strategy (700 bp by 700 bp) to confirm that no mutations were introduced. Primers were designed using Primer Select from the Lasergene 9.0 software package (DNASTAR Inc., USA).

- Phytophthora infestans isolates and late blight resistance tests

Detached leaf assays (DLAs) were performed according to Vleeshouwers et al. (2000) using *P. infestans* isolates IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) and 89148-09 (race 0). In field trials only isolate IPO-C was used. Field trials for the transgenic plants were performed as described earlier (Jo et al. 2011) in the growing season of 2012 in Wageningen, The Netherlands. Disease assessments were made in four replicates per genotype by observing leaves infested to late blight lesions at multiple time points after inoculation. For late blight isolate resistance spectrum analysis multiple *P. infestans* isolates were used that were collected at many different locations in The Netherlands in the year 2010. Pure cultures were produced from *P. infestans* infected foliar samples and stored in Liquid Nitrogen. Prior to the detached leaf assays the isolates were grown on greenhouse grown potato leaves for seven days. Sporangia were washed off to produce a sporangial suspension containing 2 x 10^4 sporangia/ml, which were used to inoculate the detached leaves using spray application. The inoculated leaves were incubated for 7 days at 15°C in petri dishes with 1% agar in water to maintain high humidity 16/8 hrs light /dark. The extent to which the leaves were covered with late blight lesions was estimated and expressed as a percentage of the total leaf area. Also the extent to which the lesions showed sporulation was assessed micro-and macroscopically and expressed on scale from 0 (no sporulation) to 1 (strongly reduced sporulation) to 2 (full sporulation as observed macroscopically).

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

Agroinfiltration was performed essentially as previously described (Vleeshouwers and Rietman 2009). All binary plasmids were transformed to *A. tumefaciens* strain AGL1 with an additional plasmid borne copy of VirG. Two leaves per plant and three replicates of 4 weeks old *N. benthamiana* seedlings were agroinfiltrated. A mixture of *R3b* and *Avr3b* (Li et al. 2011) were used as the positive control and empty pK7WG2 (Karimi et al. 2002) was used as a negative control. *Agrobacterium* clones harbouring *R9a* gene candidates, *Rpi-chc1* (Vossen et al. 2012), *Rpi-vnt1.1* (Pel et al. 2009) and *Rpi-blb2* (van der Vossen et al. 2005) were infiltrated alone and in a 1:1 mixture with the effectors *Avr3b, Avrchc1, Avrvnt1,* or any of the candidate *Avr9a* genes listed in Table 2*. A. tumefaciens* strains from frozen glycerol stocks were grown overnight at 28°C in 3 ml of LB medium supplemented with appropriate antibiotics. The next day these cultures were used to inoculate 15 ml of YEB medium (5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g yeast extract, 2 ml 1 M $MgSO_4$ in 1 liter of milliQ water) supplemented with antibiotics, 10 µl/l of 200mM acetosyringone and 1000 µl/l of 1 M MES. On the third day, the cells were harvested and resuspended to a

final OD_{600} of 0.2 in MMA (20 g sucrose, 5 g MS salts and 1.95 g MES in 1 liter of distilled water, adjusted to pH5.6 with KOH) supplemented with 1 ml/l of 200 mM acetosyringone in DMSO. Responses were scored 3 to 4 days after infiltration.

- *Transformation of potato*

Binary plasmids harbouring candidate genes were transferred to *A. tumefaciens* strain AGL1 (Lazo et al. 1991) containing the helper plasmid pVirG (van der Fits et al. 2000). The stability of these clones in *Agrobacterium* was tested and overnight cultures of the transformed *A. tumefaciens* strain were used to transform susceptible cultivar Desiree (Heilersig et al. 2006). The kanamycin resistant regenerants (transgenic events) were analysed by PCR to determine the presence of the desired *R9a*. Two or four plants per transgenic event were transferred to the greenhouse. These plants were either used for detached leaf assays or for successive planting in the field.

Results

BAC landing

In order to clone the *R9a* gene, the co-segregating markers $CDP^{Tm2}6$ and $CDP^{Tm2}7$ (**Chapter 4**) were used to select BACs from a library containing the *R9a* genomic segment. Two BAC pools, 2H10 and 2G12 were positive for $CDP^{Tm2}6$ and $CDP^{Tm2}7$, respectively. The marker containing BACs were isolated from both pools. After both BAC clones were sequenced it turned out that the BACs had an overlap of 15kb identical sequence. Putative genes were predicted using the Softberry FGENESH web application. Annotation of the complete inserts of these BAC clones identified two complete *RGAs* of the NB-LRR class of plant *R* genes and several partial *RGAs*. The two complete RGAs, named RGA9.1 and RGA9.2, shared 89.5% amino acid identity, were located on BAC 2H10 and contained uninterrupted open reading frames encoding proteins of 863 and 862 amino acids, respectively (Figure 1). Interestingly, *RGA9.1* contained marker $CDP^{Tm2}6$ while $CDP^{Tm2}7$ was located in a partial *RGA* in BAC 2G12.

Complementation analysis

RGA9.1 and *RGA9.2* including their predicted regulatory transcriptional elements were amplified by PCR from the corresponding BAC clone resulting in 7012bp and 4374bp of fragments, respectively. The restriction sites attached to the primers allowed efficient cloning in the modified multiple cloning site of the binary vector pRIAB1.2 and pBINPLUS, respectively.

Table 1. Primers used in this study

Primer	Primer sequence (5'→3')	Remarks
CDP^{Tm2}6	Tm1R: CATTTCTCTCTGGAGCCAATC	BAC screen
CDP^{Tm2}7	Tm2F: CAAGTTTGTCGCAGAGATTGA	BAC screen
RGA9.1-2H10-*Sbf*I-F	tgacctgcaggGAGCATGAAAGTGAAGACGAGCAG	subcloning
RGA-9.1-2H10-*Asc*I-R	tggcggcgcgccAGGCTCGCACAACGGGCTATT	subcloning
RGA-9.2-2H10-*Sfr*I-F	gcccgggcAACTTGCAACGCACGTTCCTAGA	subcloning
RGA-9.2-2H10-*Sbf*I-R	cctgcaggCCGGTCCCGTCCAGGGTGTA	subcloning
RGA9.1-1F	ATTATGTATGTTGCTTTATCTGG	resequencing
RGA9.1-11F	TGAGGCCAAAAGGTAAAC	resequencing
RGA9.1-12F	CATTTACTTGACATTATTAGGAGACTT	resequencing
RGA9.1-13F	TTTCACAACAGCCAAGAGCAGGAG	resequencing
RGA9.1-6F	GACTCTTGCATATTTTCTCATTTG	resequencing
RGA9.1-14R	GATGTCTCAATTGTGTAGTCTTCC	resequencing
RGA9.1-15F	ACGACACTGGAGGTTCTAAAG	resequencing
RGA9.1-8F	AACATACACTGGCACTTCACTTTG	resequencing
pRIAB1.2_PASSA_R	TCGCGTAACTTAGGACTTGTGC	resequencing
RGA-9.2-1F	AAACCTTTAACTTGTGTCCAGAGAT	resequencing
RGA-9.2-2F	GATAAAAAGAAGGGTTGCGGACATTGAC	resequencing
RGA-9.2-3F	CAGGATGGATGTGCTAAGGTATTGA	resequencing
RGA-9.2-4F	AAACGTGTACTCATTGCCTCCTAA	resequencing
RGA-9.2-5F	TCTTCTGATGCCTGTTTGTAATAA	resequencing
Sequences of adapter and adapter primer for CDP profiling		
*Mse*I adapter	*Mse*-ad-top: CCCGAAAGTATAGATCCCAT *Mse*-ad-bottom: TAATGGGATCTATACTT	van der Linden et al. (2004)
Adapter primer	ACTCGATTCTCAACCCGAAAG	

Small letters in primer sequences indicate restriction sites for cloning.

Figure 1. **Schematic diagram of the predicted gene structure for CDP^{Tm2}6-positive BAC clone 2H10**

These constructs were transformed to the susceptible cultivar Desiree through *Agrobacterium*-mediated transformation. Primary transformants (events) harbouring

the two gene analogs, *RGA9.1* and *RGA9.2*, were tested for resistance to *P. infestans* isolates IPO-C and 89148-09 in duplicate experiments of detached leaf assays. All *RGA9.2*-containing events and the Desiree control plants were susceptible to both isolates IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11) and 89148-09 (race 0). On the other hand, *RGA9.1* was capable to complement the susceptible phenotype in cv. Desiree for the isolate 89148-09 (Table 2; Figure 2A).

Table 2. Complementation of late blight susceptibility in potato

RGA	Total	DLA89148-09		DLA IPO-C		Field IPO-C	
RGA9.1	38	R	37	R	4	R	36
		S	1	M	30	S	0
				S	2		
RGA9.2	5	R	0	R	0		
		S	5	S	5		
Desiree	3	R	0	R	0		
		S	3	S	3		

R: no symptom or small HR on the inoculated spots, M: big HR on the inoculated spots, S: sporulating lesions

37 out of 38 *RGA9.1* containing events were fully resistant to 89148-09. However, IPO-C did induce large HR lesions, far beyond the inoculum droplet, pointing at trailing necrosis. Remarkably, in 34 out of 36 no sporulation was observed, while in Desiree control plants full sporulation was observed. (Table 2; Figure 2A). This is consistent with the previous study (**Chapter 4**). It had been described before that F1 and BC1 plants containing the *R9a* gene could not convey resistance to IPO-C in detached leaf assays. The resistance could, however, be readily detected in whole plant assays. Therefore, these transgenic events were tested in a whole plant field trial. The 36 plants showing full resistance to 89148-09 were also fully resistant to IPO-C in a field assay (Table 2; Figure 2B). Because of the presented genetic complementation data and the type of resistance provided by *RGA9.1,* it is concluded that *RGA9.1* is the *R9a* gene.

R9a gene structure and putative amino acid sequence

The *R9a* gene consists of one exon containing a single open reading frame which encodes a predicted protein of 863 amino acids.

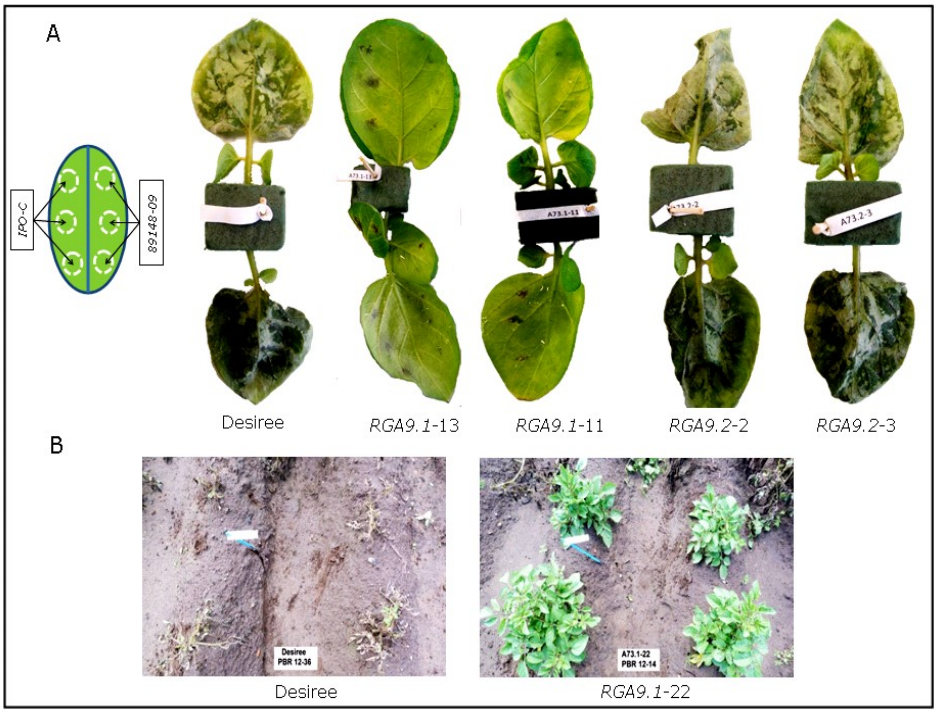

Figure 2. **Genetic complementation for *R9a* mediated late blight resistance in potato. A**. Detached leaf assays. Two different *P. infestans* isolates were inoculated in each leaf as indicated in the diagram. **B**. Desiree and a transgenic *RGA9.1* event *RGA9.1*-22 in a field trial, 3 weeks after inoculation.

The protein sequence of R9a harbors several conserved motifs of the CC-NB-ARC-LRR class of R proteins (Figure 3). A coiled coil (CC) domain is located in the N-terminal part of the protein between amino acids 1 and 153. In the first 153 residues 3 putative heptad motifs composed of hydrophobic residues could be recognized in R9a. A NB-ARC (nucleotide binding site, apoptosis, *R* gene products, CED-4) domain could be recognized in the amino acid stretch between residues 163-445 (van der Biezen and Jones 1998). The subdomains (Bendahmane et al. 2002) Kinase 1a (P-loop), Kinase-2, Kinase 3a, GxPL (where x is any amino acid) and RNBS-D could be found, while subdomains RNBS-C and MHD appeared to be absent. The C-terminal half of R9a comprises 16 LRRs of irregular size that only loosely fit the consensus sequence LxxLxxLxxLxLxxC/N/Sx(x)LxxLPxx (where x is any amino acid and L can be I, L, P, H, M or V; McHale et al. 2006). At the protein level, R9a shares 82% identity with Rpi-

mcq1 (Foster et al. 2010; Jones et al 2009), and 78% with Rpi-vnt1.1. Lower percentage of homology was found with *Tm-2²* sharing 74% identity, showing that R9a defines a new subclass of the Tm-2² protein family. A phylogenetic analysis of R protein analogs from GenBank and other sources was carried out and confirms that R9a locates in a distinct clade of Tm-2² like protein sequences.

Figure 3. **Sequence of the deduced protein product encoded by *R9a*.** The complete amino acid sequence of R9a is shown. Putative coiled-coil (CC) domains (hxxhcxc; h-hydrophobic residues, c-charged residues, x; any residues) are highlighted in bold and first and fourth hydrophobic residues of each of heptad repeat are underlined. Conserved motifs in the NB-ARC domain are overlined and presented in blue. Amino acid residues matching the consensus LxxLxxLxxLxLxxC/N/S(x)xLxxLP of putative leucine-rich repeats (LRRs) are shown in red.

Apart from the sequences of known R proteins, also three sequences of proteins with unknown function, deriving from a paralog mining strategy and from whole genome sequencing, respectively, were found to locate in this Tm-2^2 like clade. This clade is clearly distinct from other functionally validated *Rpi* genes as supported by bootstrap values (Figure 4).

Abundance of R9a in wild germplasm

To investigate the abundance of *R9a* in the wild germplasm, 107 *Solanum* genotypes consisting of geographically and phylogenetically diverse wild *Solanum* (Sol*R*gene database) were tested for the presence of *R9a* specific markers.Remarkably, only one out of 17 *S. demissum* genotypes was found positive for the marker in the *R9a* gene ($CDP^{Tm2}6$), while all three *S.x edinense* genotypes and eight *S. stoloniferum* genotypes were positive for the presence of the *R9a* marker (Table 3). This suggests that *R9a* distributes in the Mexican area. In order to test whether *R9a* could effectively tag late blight resistance in the germplasm, F1 population 7727 that had edn150-4 as a resistant parent was tested with the *R9a* marker. Indeed, the *R9a* marker co-segregated with IPO-C resistance in this population. Previously, the resistance to IPO-C from different *S.x edinense* genotypes (edn151-1 and edn150-4) was mapped to a *Tm-2* cluster on chromosome IX (Verzaux 2010).

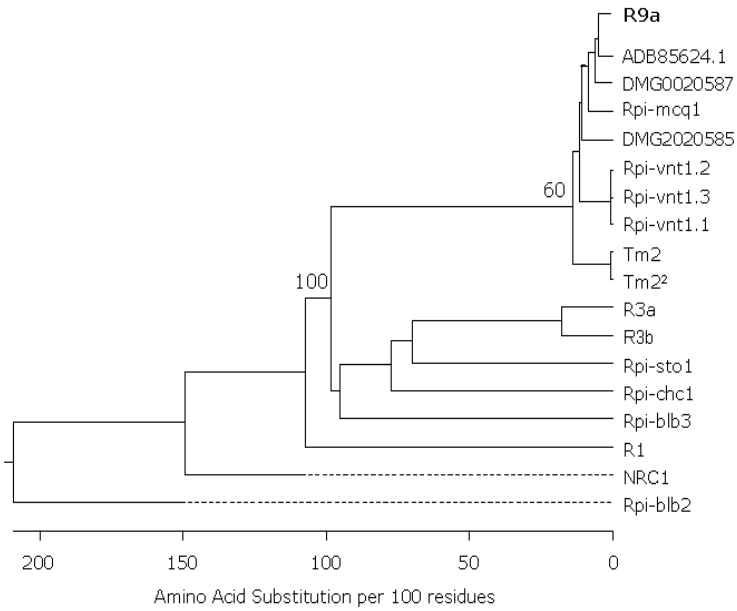

Figure 4. **Phylogenetic analysis of Rpi proteins and related sequences.** Tm2^2-like protein analogues were retrieved from GenBank (ADB85624.1, sequence from *Solanum okadae* accession 970-3 obtained in *Rpi-vnt1* paralog mining study) and other sources (DMG sequences from the potato genome sequencing project). Alignments were carried out according to the Clustal W algorithm. Bootstrapping was performed using 1,000 jumbles and seed=111. Only relevant bootstrap values are shown.

Table 3. Germplasm screens for the *R9a*-specific marker CDP^{Tm2}6

Species	Number of accessions	Number of genotypes with origin of country	*R9a* marker positive genotypes[a]
S. acaule	4	3 (BOL), 1 (PER)	
S. albicans	2	2 (PER)	
S. brachycarpum	2	2 (MEX)	
S. colombianum	1	1 (COL)	
S. demissum	9	6 (?), 1 (GTM), 10 (MEX)	dms582-1
S.x edinense	2	3 (MEX)	edn150-4, edn151-1, edn151-3
S. fendleri	1	1 (USA)	
S. guerreroense	1	2 (MEX)	
S. hjertingii	5	6 (MEX)	
S. hougasii	4	9 (MEX)	
S. longiconicum	2	2 (COS)	
S. polytrichon	4	6 (MEX)	
S. acaule punae	1	1 (PER)	
S. papita	5	10 (MEX)	
S. schenckii	1	1 (MEX)	
S. weberbaueri	1	1 (?)	
S. stoloniferum	14	40 (MEX)	sto221-5, sto390-8, sto554-2, sto554-4, sto554-5, sto837-2, sto837-7, sto840-8

[a] The three letter code represents the *Solanum* species (Simmonds 1962). The first number represents the CBSG number for the accession followed by a genotype number. MEX: Mexico, BOL: Bolivia, GTM: Guatemala, COS: Costa Rica, USA: United States, PER: Peru, COL: Colombia, ? Not known

The resistance in DLA of genotype edn150-4 was not very strong and therefore not suitable for this type of analysis. *S.x edinense* breeding material JV1, which was also positive for the *R9a* marker, had much higher levels of resistance in DLA and was chosen for this comparative study. The chromosomal position of *Rpi-edn2* is similar to that of *R9a*, suggesting that the two genes are functional homologs. Further confirmation of this idea was provided by a comparison of the isolate resistance spectrum of *R9a* and *Rpi-edn2* material (Table 4). 54 isolates that were collected in The Netherlands in the year 2010 were applied to Ma*R9* and JV1. Fifteen isolates were able to overcome Ma*R9* resistance in DLA. Fourteen of these isolates were also able to overcome resistance in JV1 *Rpi-edn2* material.

Table 4. Overview of resistance screening with a set of 54 *P. infestans* isolates

Genotype	NL10001	NL10003	NL10004	NL10047	NL10076	NL10078	NL10080	NL10083	NL10087	NL10098	NL10112	NL10144	NL10147	NL10148	NL10150	NL10154	NL10157	NL10170
JV1	-	-	-	+	-	+	+	-	+	+	+	+	+	-	-	-	-	-
Ma*R9*	-	-	-	+	-	+	+	-	+	+	+	+	+	-	-	-	-	-
Bionica	-	-	-	+	-	+	+	-	+	+	+	+	+	-	-	-	-	-
Desiree	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Genotype	NL10175	NL10216	NL10218	NL10247	NL10250	NL10252	NL10253	NL10260	NL10275	NL10277	NL10289	NL10314	NL10321	NL10333	NL10361	NL10378	NL10379	NL10380
JV1	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Ma*R9*	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Bionica	+	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-
Desiree	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Genotype	NL10422	NL10434	NL10454	NI10459	NL10461	NL10472	NL10474	NL10480	NL10483	NL10485	NL10486	PP10-0790	PP10-0797	PP10-0835	NL07434	NL08645	NL08797	PP10-0787
JV1	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Ma*R9*	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-
Bionica	-	-	+	-	-	+	-	+	-	-	-	+	+	+	-	-	-	-
Desiree	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ : sporulating lesions, −: no or non-sporulating lesions. The differential isolates were highlighted.

This huge overlap in recognition specificity strongly supports the idea that *Rpi-edn2* and *R9a* are functional homologs.

R9a recognizes Avrblb2 homologs

To identify the *P. infestans* component that is recognized by *R9a* and *Rpi-edn2*, an effectoromics approach was pursued. Since the Ma*R9* plant was not highly amenable to agroinfiltrations, initially two *S.x edinense* genotypes JV1 and edn150-4 were tested for the recognition of potential *Avr* genes among a genome wide collection of RXLR effectors. JV1 showed a specific response to 18 effectors and edn150-4 specifically responded to 21 effectors (Table 5). 11 effectors were overlapping between the genotypes. An additional nine effectors had only been tested on one of both *S.x edinense* genotypes. Among this set of 20 effectors were *Avr2*, members of the *Avrblb2* and members of the *Avrchc1* family. It had already been shown that *S.x edinense* genotype 150-4 showed AVR2 response (Verzaux 2010). Using molecular markers in a F1 population it was shown that the AVR2 response mapped to chromosome 4 and that the cognate *R* gene, most likely a *R2* homolog, was distinct from the *Rpi-edn2* gene. In order to test if any of these 20 effectors were recognised by *R9a*, we performed a co-agroinfiltration experiment. There was no response to any of the known *Avrs,* members of the *Avrchc1* family members or *Avr2*, showing that indeed these responses were caused by recognition through different genes. Two effectors gave very strong HR upon co-expression with *R9a* and three other effectors also induced a specific HR response, albeit not the entire infiltrated area underwent cell death (referred to as an intermediate response in Table 5). In addition, there was one effector showing only weak HR upon co-infiltration. Interestingly, the two effectors providing strong HR and two of the effectors providing an intermediate response

belonged to the *Avrblb2* family. In order to further confirm the *R9a* gene specificity towards the Avrblb2 family, we set out to test all *Avrblb2* family members from the T30-4 genome by co-infiltration with *R9a*. First we tested how the different constructs reacted when they were infiltrated alone.

Table 5. Effectors that trigger cell death response in the edn150-4 and JV1 or upon co-infiltration with *R9a* in *N. benthamiana*.

Gene ID	Function	Response in edn150-4	Response in JV1	Co-infiltration with *R9a*
PITG_18683	*Avrblb2*	-	Strong	Strong
PITG_04090	*Avrblb2-like*	-	Strong	No
PITG_00582		No	Strong	-
PITG_05750		No	Strong	-
PITG_14788		No	Strong	-
PITG_23117		Strong	-	No
PITG_12731		Strong	-	No
PITG_22972		Strong	-	No
PITG_20336	*Avrchc1*	Strong	-	No
PITG_04169		Strong	-	No
PITG_09616		Strong	No	-
PITG_09716		Strong	No	-
PITG_23131		Strong	No	-
PITG_16235	*Avrchc2*	Strong	Strong	No
PITG_00774		Strong	Strong	No
PITG_10540		Strong	Strong	No
PITG_20300	*Avrblb2 69A*	Strong	Strong	Intermediate
PITG_20301	*Avrblb2 69F*	Strong	Strong	intermediate
PITG_20303	*Avrblb2 69F*	Strong	Strong	Strong
PITG_22880		Strong	Strong	Intermediate
PITG_04097		Strong	Strong	No
PITG_15039		Strong	Strong	No
PITG_15152		Strong	Strong	Weak
PITG_22870	*Avr2*	Strong	Strong	No
PITG_23074		Strong	Weak	No
PITG_16726		Strong	Weak	No

- : Not tested

None of the six Avrblb2 family members alone did cause a HR response but PITG_20303 did cause chlorosis in the infiltrated area (Table 6; Figure 5). Also infiltration of *R9a* alone caused only slight chlorosis. To rule out that synthetic enhancement of chlorotic responses, potentially culminated into a HR, *R9a* was co-infiltrated with *Avrchc1*, which also caused chlorosis. This combination just caused chlorosis and no HR was observed. Interestingly, the homologous *Rpi-vnt1* was

infiltrated in *N. benthamiana* and also here a chlorosis response was observed. When *Rpi-vnt1* was combined with PITG_20303, no synthetic enhancement was observed. When *R9a* was combined with the six different *Avrblb2* homologs, clearly a HR response was observed except for PITG_04090. *RGA9.2*, *Rpi-chc1* and *Rpi-vnt1* did not recognize any of the *Avrblb2* homologs (Table 6). When the Avrblb2 recognition spectrum of *R9a* was compared to *Rpi-blb2,* a partially overlapping pattern was found.

Table 6. Overlapping effector recognition spectrum between two late blight resistance genes *R9a* and *Rpi-blb2*

Gene ID	Function	RXLR	Effector alone	*R9a*	*RGA9.2*	*Rpi-blb2*	*Rpi-chc1*	*Rpi-vnt1*
PITG_20301	*Avr9a*	RSLR	-	HR	-	-	-	-*
PITG_20303	*Avr9a*	RSLR	-*	HR	-*	-*	-*	-*
PITG_20300	*Avrblb2*	RSLR	-	HR	-	HR	-	-*
PITG_04090	*Avrblb2*	RSLR	-	-*	-	HR	-	-*
PITG_04085	*Avrblb2*	RSLR	-	HR	-	HR	-	-*
PITG_18683	*Avrblb2*	RSLR	-	HR	-	HR	-	-*
PITG_20934	*Avrchc1*	RSLR-EER	-*	-*	-*	-*	HR	-*
PITG_16294	*Avrvnt1*	RLLR-EEK	-	-*	-	-	-	HR
R gene alone				-*	-	-	-	-*

* Infiltrated area showed chlorosis

PITG_18683, PITG_04090 and PITG_20300 but not PITG_20301 and PITG_20303 were recognised by *Rpi-blb2* (Table 6; Figure 5), which is in agreement with the results in a previous study (Oh et al. 2009). One effector, PITG_04090, was exclusively perceived by *Rpi-blb2* (Table 5, 6; Figure 5). Two effectors, PITG_20301 and PITG_20303, were exclusively recognised by R9a and had phenylalanine (F) residue in the positively selected amino acid 69 (Figure 6; Oh et al. 2009). Here, it was found that PITG_04085 which was not mentioned by Oh et al. (2009) was also perceived by *Rpi-blb2* (Table 6; Figure 5).

R9a/Rpi-edn2 has a similar resistance spectrum to Rpi-blb2

Now that it was clear that an overlapping set of effectors was recognised by *R9a* and *Rpi-blb2*, we compared the isolate recognition spectrum of *R9a*/*Rpi-edn2* and *Rpi-blb2*. Three genotypes, JV1, Ma*R9*, and cultivar Bionica, which contain the *Rpi-edn2, R9a*

and *Rpi-blb2* resistance genes, respectively, were selected and inoculated with 54 *P. infestans* isolates (Table 4). As was noted before, Ma*R9* had a highly similar *Pi* isolate resistance spectrum to JV1. Also the resistance spectrum of Bionica was highly similar. Only three out of 54 isolates (highlighted in Table 4) showed a differential virulence spectrum between Ma*R9* and JV1.

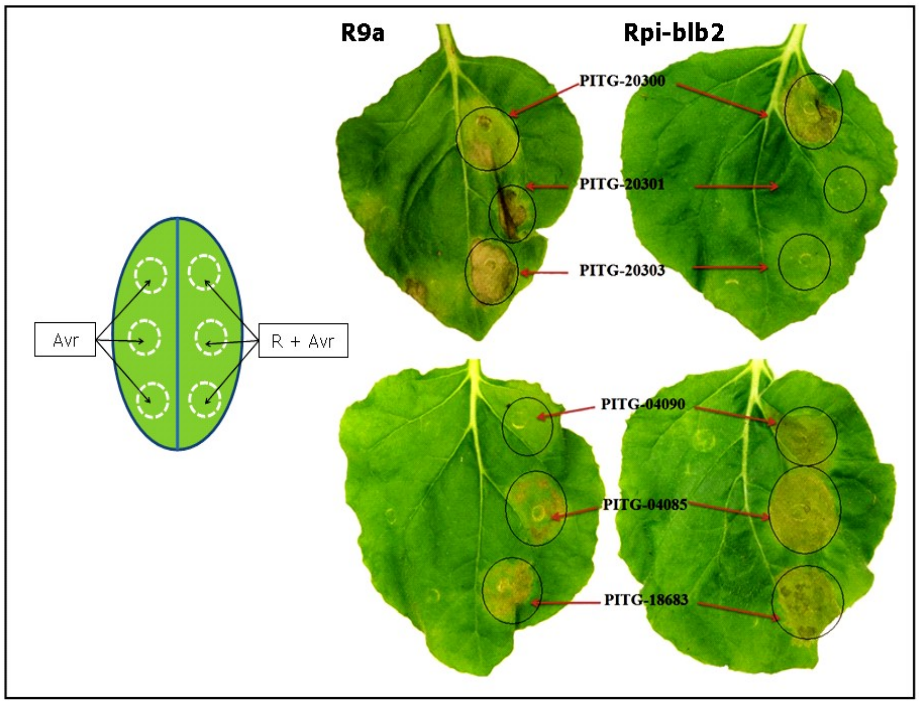

Figure 5. **Co-infiltration of *R9a* or *Rpi-blb2* with *Avrblb2* homologs in *N. benthamina*.** The right and left pairs of leaves showed differential HR responses when the same *Avrblb2* homologs were co-infiltrated with *R9a* or *Rpi-blb2*, respectively.

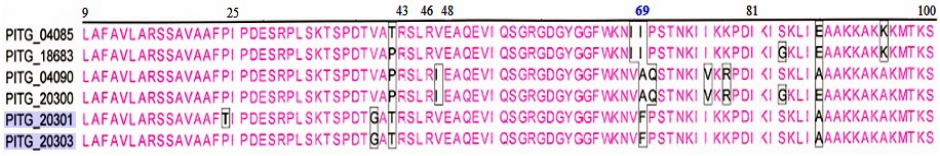

Figure 6. **The Avrblb2 homologs that are recognized by R9a have phenylalanine (F) residue in the positively selected amino acid 69**. The black residues indicate sequence differences between effector proteins.

Ma*R9* and JV1 displayed the same resistance spectrum except for two isolates. The isolate NL10480 was virulent to JV1 but not to Ma*R9*. Another isolate, PP10-0797, was virulent to Ma*R9* but not to JV1. These results support our previous conclusion that despite their huge sequence differences, *R9a* is a functional homolog of *Rpi-blb2*.

Discussion

The R gene cluster directed profiling (CDP) approach produces specific markers landing near the gene of interest.

Map-based cloning has been widely used to isolate genes from different organisms (Chi et al. 2008). The principle is to systematically narrow down the genetic interval containing an allele of interest by sequentially excluding all the other regions in the genome (Lukowitz et al. 2000; Pâcurar et al. 2012). This can be achieved by a level of marker saturation which is equivalent to a physical spacing of markers that is less than the average insert size of the genome libraries such as a BAC library (Brugmans et al. 2006). The process of a targeted saturation with markers for BAC landing is commonly performed for map-based cloning and is laborious and time consuming. From our previous work, we experienced that CDP increases the chance to discover co-segregating, even fully co-segregating, profiling markers in specific chromosomal regions representing *R* gene clusters (Jo et al. 2011; Chapter 4). The more cosegregating markers can be found, the higher the chance that BAC landing or PCR-based allele mining could succeed and here we show that CDP enabled to select BAC clone(s) carrying the target *R* gene without the need to perform high resolution mapping.

R9a or Rpi-edn2 is Tm-2 homolog at the distal end of the long arm of chromosome IX.

There are several supporting data that *R9a* and *Rpi-edn2* are functional homologs on the same locus. Previously, the resistance to *P. infestans* isolate IPO-C conferred by *Rpi-edn2* from *S.x edinense,* which is a natural hybrid between the Mexican *Solanum demissum* and the South American *S. tuberosum* spp. *andigena*, was mapped (Verzaux 2010). The *R9a* marker cosegregated with IPO-C resistance in *S.x edinense* population 7727 and Ma*R9* BC1 population (population code 3151; Chapter 4). In addition, *R9a* has a similar resistance spectrum to *Rpi-edn2* (Table 4). The cloned and/or mapped *R* genes which include *Tm-2*^{2}, *Rpi-vnt1* and *Rpi-mcq1* (formerly *Rpi-moc1*) reside at three *Tm-2*^{2} clusters that are spread across the long arm of chromosome IX (Pel et al. 2009). Genome sequencing revealed that eight TIR-NB-

LRRs and nine non-TIR-NB-LRRs locate at the distal end of the long arm (Jupe et al. 2012), of which genes of non-TIR-NB-LRRs group shared 74.9%-86.7% of amino acid identity with R9a. Taken together, this suggests that four functional *R* genes, *Tm-2*^{2}, *Rpi-vnt1, Rpi-mcq1,* and *Rpi-edn2/R9a* have a common origin and have evolved through gene duplication and translocation events. However, there are clear grouping differences between these *R* genes in terms of low bootstrap values, suggesting that they have been subject to diversifying selection. This is additionally supported by the fact that, for two *Rpi* genes *Rpi-vnt1* and *Rpi-mcq1*, spectrum analyses with *P. infestans* isolates and functional assays using effectors showed that they have distinct recognition specificities (H. Rietman, unpublished results). However, whether *R9a* and *Rpi-edn2* are really functional homologs remains to be confirmed.

The AVRblb2 family triggers R9a-mediated hypersensitivity.

It is interesting that the Avrblb2 family is recognized by R9a. Oh et al. (2009) described the *Avrblb2* family that induced hypersensitive cell death specifically in the presence of the *S. bulbocastanum* late blight resistance gene *Rpi-blb2* using the PVX-based assay and co-agroinfiltration in *N. benthamiana*. *Avrblb2* belongs to a multi-gene family with at least seven duplicated copies in the genome of *P. infestans* strain T30–4 (Haas et al. 2009). Regarding the positively selected amino acid at position 69 of Avrblb2 which is critical for activation of *Rpi-blb2* hypersensitivity, the Avrblb2 homologs that are recognized by *Rpi-blb2* have Val-69, Ala-69, or Ile-69, whereas homologs, e.g., PITG_20301 and PITG_20303, that are not recognized by *Rpi-blb2* have Phe-69. Surprisingly, we found that PITG_20301 and PITG_20303 with Phe-69 are recognized by *R9a* (Figure 5, 6). Weak hypersensitive responses of *R9a* with the functional *Avrblb2* could result from flexible structure-function relationships in different domains for the *R9a* gene (Bai et al. 2011). The similar resistance spectrum between Ma*R9* or JV1 and Bionica could be explained by the coexistence of *Avrblb2*^{Phe69} variants with avirulence copies of the gene in the genome of several isolates of *P. infestans* (Oh et al. 2009; Vleeshouwers et al. 2011a). The systematic structure-function analysis of the CC-NB-LRR gene *R9a* activity in disease resistance remains to be performed. Also, the effector composition of *R9a* breaking isolates remains to be determined. Meanwhile, although effectors PITG_20301 and PITG_20303 are expected for *Avr9a*/*Avredn2, R-Avr* interactions by co-segregation of responses to the effectors with resistance to *P. infestans* isolates in segregating populations should be validated.

R9a origin and implications in potato breeding

As the case for *Rpi* genes such as *Rpi-blb2, Rpi-vnt1*, and *Rpi-mcq1,* the low distribution of the *R9a* resistant allele through the section *Petota* and the confinement of the gene to a geographic area in Mexico (Table 3) suggest that this *R* gene emerged recently. For *R8*, the majority of the *S. demissum* genotypes were positive and only one positive genotype was from *S. stoloniferum* (**Chapter 3**). Here it is the other way around. It is therefore remarkable that *R9a* came into the differential Ma*R9* which is derived from *S. demissum*. Although Ma*R9* contains both *R8* and *R9a/R9b*, it is most unlikely that *R9a* is originating from *S. demissum. S. demissum* is more similar to *S. acaule* than to the other polyploidy Mexican species *S. stoloniferum* which is most similar to *S. verrucosum* and together they cluster with species from series *Tuberosa* group III (from Bolivia, Argentina and Chile) (Bonierbale et al. 1990; Jacobs 2008). Further analysis remains to be done for the *R9a* origin. Also, it is most unlikely that *R9a* has widely been used in agriculture, even though the pentaploid *S.x edinense* confers resistance to a broad spectrum of isolates from potato growing areas in Europe and was included in breeding programs in 1914 (Verzaux 2010). *R9a* might have a wider resistance spectrum compared to *Rpi-blb2* which is considered a desired gene for breeding because of effective resistance against many *P. infestans* isolates (van der Vossen et al. 2005; van Berloo et al. 2005; Haverkort et al. 2009) (Table 4). Still, virulence to *P. infestans* has been found in *R9a*- or *Rpi-blb2*- containing *Solanum* species and thus combinations of several *R* genes are preferred to achieve durable resistance. A genetically engineered potato cultivar into which broad spectrum *Rpi* genes such as *R9a* are introduced by cisgenic transformation (Jacobsen and Schouten 2007) is expected to provide reasonable production under the occurrence of late blight endemics for a long term.

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Chapter 6

Development of late blight resistant potatoes by cisgenic stacking

Development of late blight resistant potatoes by cisgenic stacking

Kwang Ryong Jo[1,2,3], Tok-Yong Kim[3], Chol-Jun Kim[3], Marjan Bergervoet[1], Sung-Jin Kim[3], Maarten A. Jongsma[4], Richard GF Visser[1], Evert Jacobsen[1], Jack Vossen[1]

[1] Wageningen UR Plant Breeding, Wageningen University & Research Centre, P.O. Box 16, 6700 AA, Wageningen, The Netherlands, [2] Graduate School Experimental Plant Sciences, Wageningen University, The Netherlands, [3] Research Institute of Agrobiology, Academy of Agricultural Sciences, Pyongyang, DPRK, [4] Plant Research International, Wageningen University and Research Centre, Wageningen, The Netherlands

Summary

The introduction of multiple resistance (*R*) genes with different spectra into potato by cisgenesis is considered an attracting approach for the production of highly resistant potatoes that bypass most biosafety issues associated with transgenic crops. Cisgenesis comprises the transfer of native genes to the same or crossable plant species. Here we report the production of cisgenic potato plants by introducing two broad spectrum potato late blight resistance (*Rpi*) genes, *Rpi-sto1* from *Solanum stoloniferum* and *Rpi-vnt1.1* from *Solanum venturii* into four potato cultivars, Atlantic, Bintje, Doip1, and Potae9. Resistance profiles for the varieties with five *P. infestans* isolates were determined. Single *Rpi* gene-containing transgenic plants for all varieties were obtained and used as references. *Agrobacterium*-mediated transformation with a construct containing two *Rpi* genes (*Rpi-vnt1* and *Rpi-sto1*) under non-selectable marker conditions (cisgenesis) was compared to kanamycin assisted selection (transgenesis) in terms of transformation frequency, vector backbone integration, and copy number. Also, the different time tracks to obtain the PCR-positive shoots were studied for cisgensis. This study allowed the selection of cisgenic potatoes with two stacked *Rpi*-genes. Through further analyses involving phenotypic evaluations in the greenhouse, agroinfiltration of avirulence (*Avr*) genes and detached leaf assays, the selection of cisgenic plants was narrowed down to eight independent events. Two cisgenic transformants of cv. Altantic and four cisgenic transformants of cv. Bintje, were developed that showed broad spectrum late blight resistance due to the activity of both cisgenic *Rpi* genes. In two cisgenic transformants the existing late blight

resistance spectrum of cv. Potae9 has been broadened by adding two additional *Rpi* genes. Considerations and future work for cisgenesis are discussed.

Introduction

Genetic disease resistance is considered an attractive method for sustainable management to late blight. Breeding at the beginning of the twentieth century concentrated on major dominant late blight resistance (*Rpi)* genes from the Mexican wild species *Solanum demissum* and led to the identification of eleven *Rpi* genes (Muller and Black 1951; Malcolmson and Black 1966; Malcolmson 1969; Bradshaw et al. 2006). However, rapid breakdown of resistance in potato varieties containing *S. demissum Rpi* genes *R1, R2, R3,* and *R10* (Malcolmson 1969; Wastie 1991) has sparked an increased focus on the introgression of multiple broad spectrum *Rpi* genes in order to impart durability to commercial varieties. It has turned out in various crops that stacking of multiple resistance (*R*) genes is necessary to provide satisfactory resistance in the field (Que et al. 2010). Also, the availability of cloned *Rpi* genes from diverse wild *Solanum* species allows breeders to introduce *Rpi* gene stacks into potato varieties using genetic engineering (Song et al. 2003; van der Vossen et al. 2003; van der Vossen et al. 2005; Vleeshouwers et al. 2008; Lokossou et al. 2009; Pel et al. 2009; Vossen et al. 2012). Genetic engineering does not only circumvent the problem of linkage drag, in addition it can speed up the introgression of the *Rpi* gene (Jacobsen and Schouten 2007). The ethical framework provided by cisgenesis, in which only natural genes from the same or crossable species are used, allows sustainable applications of genetic engineering techniques and allows efficient deployment of the *Rpi* genes. Although the used *Rpi* genes provide resistance to broad spectra of late blight strains, the predominant agricultural deployment of only one *Rpi* gene can cause the appearance of new virulent strains. In the absence of chemical controls this might even result in the destruction of an entire harvest (Strange and Scott 2005). Therefore, the use of combinations of *Rpi* genes with different spectra must be pursued to increase durability of resistance and thereby providing food security under no or little fungicide application. *Rpi* gene stacking might be achieved by genetic crossings but the desired cultivar characteristics will never be fully recovered due to the high level of heterozygosity in potato. Addition of stacks of *Rpi* genes to existing (resistant) cultivars is therefore an attractive idea and could prevent future breaking of resistance. Recently, transgenic stacking of three broad spectrum potato late blight resistance genes (*Rpi-sto1*, *Rpi-vnt1.1* and *Rpi-blb3*) was described (Zhu et al. 2012). The potato plants were transformed with native *Rpi* genes from crossable species and

could therefore be considered as cisgenes. However, these plants are considered as "transgenic" as the selectable marker gene, NPTII, was of bacterial origin. In this study, we performed experiments for the production of several cisgenic potato varieties by *Agrobacterium*-mediated transformation in the absence of a selectable marker gene. After the absence of vector backbone integration was confirmed, these potatoes are claimed as "true cisgenic" because of the absence of any foreign (non potato) genes. This is the first scientific report on the production and functional evaluation of cisgenic *R* gene stacking in potatoes.

Materials and methods

- Plant material

The potato cultivars Atlantic, Desiree, Bintje, Doip1 and Potae9 were clonally maintained *in vitro* using Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose at 20°C at Wageningen UR Plant Breeding, Wageningen, The Netherlands. The two cultivars from DPR Korea, Doip1 and Potae9, which are resistant to late blight, were used for testing their reaction to certain late blight isolates and for transformation experiments to broaden their resistance spectrum.

- *Phytophthora infestans* isolates and late blight resistance tests

Five *Phytophthora infestans* isolates were used in Detached Leaf Assays (DLAs); EC1 (race 3, 4, 7, 11), IPO-C (race 1, 2, 3, 4, 5, 6, 7, 10, 11), 90128 (race 1, 3, 4, 7, 8, 10, 11), pic99189 (race 1, 2, 5, 7, 10, 11) and DHD11 (race 1, 2, 3, 4, 6, 7, 10, 11). The DLAs were performed as described by Vleeshouwers et al. (1999).

- Vector construction

The *Rpi* genes used in our study are genomic fragments from *S. venturii,* e.g., *Rpi-vnt1.1* (Foster et al. 2009; Pel et al. 2009) and *S. stoloniferum,* e.g., *Rpi-sto1* (Vleeshouwers et al. 2008). These fragments comprise the entire genes including their native promoters and terminators. In order to subclone *Rpi-sto1,* long-range PCR reactions were carried out with primers that had the *Asc*I and *Sbf*I restriction sites attached to their 5' ends. Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc.) was used in a PCR reaction (program: 98°C for 30 s followed by 34 cycles of 98°C for 10 s, 62°C for 30 s, 72°C for 5.5 min and a final extension time of 15 min at 72°C). The resulting PCR products of the right target size were isolated from

an agarose gel and DNA was extracted using Zymoclean™ Gel DNA Recovery Kit. Afterwards DNA concentration was measured, the DNA was double-digested with restriction enzymes and the digested products were purified using QIAEX®II Gel Extraction Kit. Subsequently, the digested PCR products were ligated to the double digested and dephosphorylated binary vector pBINAW2, a modified version of pBINPLUS that lacks the *NPTII* gene between the T-DNA borders (Dr. A. Wolters, Plant Breeding, personal communication). The ligation mixture was transformed to ElectroMAX *E.coli* DH10b competent cells (Life technologies, Paisley, UK). To this construct the *Rpi-vnt1.1* gene was added using a *Sbf*I fragment from the pBINPLUS: *Rpi-blb3*:*Rpi-vnt1.1*:*Rpi-sto1* described by Zhu et al. (2012). The clone with the desired *Rpi-vnt1.1* insert orientation (in tandem with *Rpi-sto1*) was selected (Figure 2). Subsequently, tests for stability of the *Rpi* genes in *Agrobacterium* and functionality of the *Rpi* genes in *N. benthamiana* were carried out, which confirmed stability and activity of those constructs in further experiments.

- Potato transformation

Transformation was essentially performed as described by Visser (1991). Internodes of 2-5 mm in length were cut from thick stems of 4-week-old *in vitro*-grown plants and were used as explants in transformation experiments. After pre-culture on R3B medium (MS + 3% Sucrose + 0.8% Agar + 4 mg/ml NAA + 1 mg/ml BAP, pH5.8) with 1.5 ml of PACM (MS + 3% Sucrose + 0.2% Caseine hydrolysate + 1 mg/ml 2,4-D + 1 mg/ml Kinetine, pH6.5) for two days, explants were inoculated with *Agrobacterium* strain AGL1+VirG which were resuspended in LB medium to an OD_{600} of 0.2. After a 2 day cocultivation period, the explants were transferred to ZCVK medium (MS + 2% Sucrose + 0.8% Agar + 1 mg/ml Zeatine + 200 mg/ml Cefotaxime + 200 mg/ml Vancomycine, pH5.8) for regeneration of shoots. Explants were transferred to fresh medium every two weeks. Shoots were transferred to CK medium (MS + 2% Sucrose + 0.8% Agar + 200 mg/ml Cefotaxime + 200 mg/ml Vancomycine, pH5.8) to induce root formation. For transgenesis, 100 mg/ml Kanamycin was added to ZCVK medium and CK medium for selection of transgenic shoots. For marker free transformation, to guarantee the independent transformation events for individual shoots, only shoots regenerating from physically separated positions on each explant were collected. Three weeks later, the rooted plantlets were analysed by PCR to determine the presence of the desired *Rpi* genes. The transformation frequency was calculated as a percentage of the number of *Rpi* gene-PCR positive shoots over the total number of tested shoots.

- Functional tests of resistance (*Rpi*) genes

Agroinfiltration was performed as previously described (Vleeshouwers and Rietman 2009). Two leaves per plant from three copies of each of the transformants were infiltrated with the following constructs: two effectors (*Avrvnt1* and *IpiO=Avrsto1*) (Pel 2010; Vleeshouwers et al. 2008), *R3a* (Huang et al. 2005) and *Avr3a* (Armstrong et al. 2005) as the positive control and empty pK7WG2 (Karimi et al. 2002) as the negative control. *Agrobacterium tumefaciens* strain from glycerol stocks was grown in 3 ml of LB medium supplemented with appropriate antibiotics at 28°C overnight. The next day, the cultures were transferred to 15 ml of YEB medium (5 g beef extract, 5 g bacteriological peptone, 5 g sucrose, 1 g yeast extract, 2 ml 1 M $MgSO_4$ in 1 litre of milli-Q water) supplemented with antibiotics, 10 µl of 200 mM acetosyringone and 1000 µl of 1 M MES. On the third day, the cells were harvested and resuspended in MMA solution (20 g sucrose, 5 g MS salts and 1.95 g MES in 1 litre of distilled water, adjusted to pH5.6) supplemented with 1 ml of 200 mM acetosyringone to a final OD_{600} of 0.3. Leaves of 4- to 5-weeks old, greenhouse-grown, plants were infiltrated with this suspension. Responses were scored 3 to 4 days after infiltration.

- DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA was isolated from young leaves as described by Fulton et al. (1995). The Retsch machine (RETSCH Inc., Hannover, Germany) was used to grind young plant materials frozen in liquid nitrogen. Primers used for analysis of *Rpi* genes, vector backbone integration and T-DNA copy number determination are listed in Table 1. A pooled sampling method was exploited for PCR analysis of shoots in marker free transformation. DNA extraction was carried out first by pooling one small leaf from each of ten shoots. If in this first round pools were found which were PCR-positive for both *Rpi* genes and PCR-negative for backbone integration, a second round of PCR was carried out on genomic DNA of individual shoots within the pools. PCR reactions for *Rpi-sto1, Rpi-vnt1.1,* NPTIII, trfA, ColE1, oriV and traJ were performed using DreamTaq™ polymerase (Fermentas) in a standard PCR program (94°C for 60 s followed by 30 cycles of 94°C for 30 s, 58°C for 60 s, 72°C for 90 s and a final extension time of 5 min at 72°C). To determine *Rpi* gene copy number, DNA was quantified using a NanoDrop® Spectrophotometer ND-1000 (ISOGEN Life Sciences). Reactions were conducted in a 10 µl total volume that contained 8-10 ng of genomic DNA, 5 µl of iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), 3 mM forward primer and 3 mM reverse primer, using CFX96™ Real-Time System (Bio-Rad, Hercules, CA, USA). The quantitative PCR assays underwent an initial denaturing step

of 3 min at 94 °C followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. DNA input in the different samples was normalized relative to EF1-α primers (Nicot et al. 2005). Copy numbers were calculated using the formula $2^{(Ctx-Ct1)}$ where Ct1 is the threshold value of a control plant harbouring a single copy of a single *Rpi* gene and Ctx is the threshold value of the plant to be tested.

Results

Generation of single Rpi gene-containing transgenic potatoes and their resistance profiles

Table 1. Primers used for PCR analysis of transformants

Gene ID		Sequences (5'-3')	Fragment size (bp)	Remarks
Rpi-vnt1.1	forward	ATGAATTATTGTGTTTACAAGACTTG	1100	gene detection
	reverse	AGCATTGGCCCAATTATCATTAAC		
Rpi-sto1	forward	ACCAAGGCCACAAGATTCTC	890	
	reverse	CCTGCGGTTCGGTTAATACA		
tetA	forward	CTGCTAGGTAGCCCGATACG	396	backbone
	reverse	CCGAGAACATTGGTTCCTGT		
trfA	forward	CGTCAACAAGGACGTGAAGA	146	
	reverse	CCTGGCAAAGCTCGTAGAAC		
NPTIII	forward	GAAAGCTGCCTGTTCCAAAG	162	
	reverse	GAAAGAGCCTGATGCACTCC		
ColE1	forward	ATAAGTGCCCTGCGGTATTG	246	
	reverse	GCAGCCCTGGTTAAAAACAA		
oriV	forward	TGCGGCGAGCGGTATCAG	1045	
	reverse	CTTCTTGATGGAGCGCATGGG		
traJ	forward	ACGAAGAGCGATTGAGGAAA	260	
	reverse	CAAGCTCGTCCTGCTTCTCT		
EF1-α	forward	ATTGGAAACGGATATGCTCCA		copy number
	reverse	TCCTTACCTGAACGCCTGTCA		
Rpi-sto1	forward	TTCAATTGTGTTGCGCACTAG		
	reverse	GCTTGATCAGTTGTGGACATC		
Rpi-vnt1.1	forward	ATGAATTATTGTGTTTACAAGACTTG		
	reverse	CAGCCATCTCCTTTAATTTTTC		

First, we tested resistance spectra of the selected varieties to be used for transformation with five *Pi* isolates with variable virulence spectra and aggressiveness. Cvs. Atlantic and Bintje were susceptible to all tested isolates while cvs. Doip1 and Potae9 were resistant to two isolates (Table 2). Resistance to EC1 and 90128 for Doip1 and Potae9 was in agreement with the presence of *R2* or a functional homolog

in these varieties that was found from AVR2 response experiments which were performed earlier. We used two constructs containing a single *Rpi* gene, pBINPLUS: NPTII-*Rpi-vnt1.1* and pBINPLUS: NPTII-*Rpi-sto1* for transformation of four varieties. The single *Rpi* gene-containing transgenic plants were obtained and tested for the functional expression of the introduced *Rpi* genes using agroinfiltration with the cognate *Avr* genes (Table 2).

Table 2. List of transgenic reference plants obtained by single *Rpi* gene transformation

Host plant	Constructs	Plant ID	PCR		Agroinfiltration		DLA				
			vnt1.1	*sto1*	*vnt1.1*	*sto1*	EC1	IPO-C	DHD11	90128	pic99189
Atlantic	n	WT	—	—	—	—	S	S	S	S	S
	pBINPLUS:NPTII-*Rpi-vnt1.1*	H13-2	+	—	+	—	S	R	R	R	R
	pBINPLUS:NPTII-*Rpi-sto1*	H9-10	—	+	—	+	R	R	R	R	S
Bintje	n	WT	—	—	—	—	S	S	S	S	S
	pBINPLUS:NPTII-*Rpi-vnt1.1*	F13-10	+	—	+	—	S	R	R	R	R
	pBINPLUS:NPTII-*Rpi-sto1*	F9-4	—	+	—	+	R	R	R	R	S
Doip1	n	WT	—	—	—	—	R	S	S	R	S
	pBINPLUS:NPTII-*Rpi-sto1*	S9-1	—	+	—	+	R	R	R	R	S
Potae9	n	WT	—	—	—	—	R	S	S	R	S
	pBINPLUS:NPTII-*Rpi-vnt1.1*	W13-8	+	—	+	—	R	R	R	R	R
	pBINPLUS:NPTII-*Rpi-sto1*	W9-1	—	+	—	+	R	R	R	R	S

n= no transformation; WT= wild type; —= not detected; += PCR positive; R=resistant; S= susceptible

Subsequently, the plants showing a hypersensitive response were subjected to late blight inoculation using DLA (Table 2; Figure 1). As expected, the transgenic plants showed resistance to at least four of the five tested *Pi* isolates. EC1 and pic99189 were described previously to break the *Rpi-vnt1.1* and *Rpi-sto1* mediated resistances, respectively (Pel 2010; Vleeshouwers et al. 2008). Transgenic Atlantic and Bintje plants harbouring the *Rpi-vnt1.1* gene were susceptible to isolate EC1 while the Potae9 and Doip1 transgenics containing *Rpi-vnt1.1* were resistant to EC1. The *Rpi-sto1*-containing plants were susceptible to isolate pic99189. Now it was clear that both *Rpi-vnt1.1* and *Rpi-sto1* were able to confer resistance in the selected varieties, these two genes could be combined in cisgenic *Rpi* gene stacks in the selected varieties.

Comparision of cisgenesis with transgenesis

Cisgenesis excludes antibiotic resistance marker assisted transformation since the genes encoding the antibiotic resistance derive from non crossable species.

Figure 1. Detached leaf assays of transgenic potatoes transformed with single *Rpi* gene constructs in cutivars Atlantic and Bintje. The wild type of Atlantic or Bintje was susceptible to the four *Pi* isolates tested. *Rpi-vnt1.1* containing transgenic plants were susceptible to EC1 and *Rpi-sto1* containing transgenic plants were susceptible to pic99189.

Table 3. Selection of transgenic plants using kanamycin assisted transformations with single *Rpi* gene constructs

Construct	Variety	Plant ID	# exp	% sht	# sht	% rt	% PCR+	% freq	% bbf	# bbf plants DLA	# bbf R plants DLA
pBINPLUS:*vnt1.1*	Atlantic	H13	200	76	30[a]	100	100	76	40	12	12
pBINPLUS:*sto1*		H09	200	66	30[a]	100	100	66	50	15	15
pBINPLUS:*vnt1.1*	Bintje	F13	200	13	26[b]	76.9	100	10	40	8	8
pBINPLUS:*sto1*		F09	200	10	20[b]	100	100	10	45	9	9
pBINPLUS:*sto1*	Doip1	S09	200	100	30[a]	100	100	100	43	13	13
pBINPLUS:vnt*1.1*	Potae9	W13	200	16	31[b]	83.7	100	13	47	12	12
pBINPLUS:*sto1*		W09	200	19	37[b]	61.2	100	11	39	9	9

exp: number of explants. % sht: percentage of number of shoots over number of explants. # sht: number of rooted shoots tested by PCR. % rt: percentage of number of rooted shoots over the number of shoots. % PCR+: percentage of PCR positive shoots over the number of shoots. % freq: transformation frequency, calculated by %sht x (%rt/100) x (%PCR+/100). %bbf: percentage of backbone free plants out of plants tested. # bbf plants DLA: number of backbone free plants tested in detached leaf assays. #bbf R plants DLA: number of resistant plants in detached leaf assays. [a] Among regenerated shoots, only 30 plants were tested by PCR. [b] All regenerated shoots were tested by PCR.

We therefore pursued a marker free transformation and successive selection of cisgenic events using PCR. For gathering technical information on cisgenesis, we compared transformation frequency in cisgenesis with that of transgenesis. For transgenesis, transformation frequency was considerably different depending upon genotypes (Table 3).

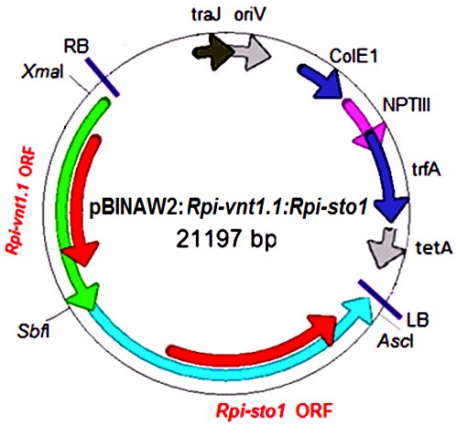

Figure 2. Schematic diagram of the marker free double gene construct pBINAW2: *Rpi-vnt1.1:Rpi-sto1.* In light green and light blue arrows the *Rpi-vnt1.1* and *Rpi-sto1* genes are shown, respectively. The red arrows indicate the coding regions of *Rpi-vnt1.1* or *Rpi-sto1*. Unique restriction enzyme recognition sites *Xma*I, *Sbf*I and *Asc*I are shown. RB: right border of T-DNA, LB: left border of T-DNA, TetA, trfA, NPTIII, ColE1, oriV and traJ are vector backbone sequences for plasmid stability and replication in bacterial hosts *Agrobacterium tumefaciens* and *Escherichia coli*.

In two varieties, Atlantic and Doip1, high regeneration and transformation frequency was observed whereas the other two varieties, Bintje and Potae9 had low regeneration and therefore low transformation frequencies. As expected, transformation frequency for marker free transformation was much lower (Table 4A). It ranged from 0.3% to 2.4% per regenerated shoot. Differences in the transformation frequencies depending on genotypes could not be observed in this experiment. In case of cv. Doip1, transformation frequency under antibiotic free conditions was very low, contrary to 100% shooting under antibiotic conditions. Meanwhile, the frequencies of the PCR-positive plantlets that were calculated from the number of shoots collected under non-

selectable conditions in different time ranges showed that PCR-positive shoots could be obtained between 1.5 and 3 months after co-cultivation (Table 4B).

Table 4. Overview of cisgenic transformation with a marker free construct containing two *Rpi* genes (*Rpi-vnt1.1:Rpi-sto1*)

A. Transformation frequency for cisgenesis

Constructs	Host plant	# exp	# sht	# PCR+	% freq	# bbf
pBINAW:*Rpi-vnt1.1:Rpi-sto1*	Atlantic	200	497	0/0/12*	2.4	9
pBINAW:*Rpi-vnt1.1:Rpi-sto1*	Doip1	200	774	1/0/2*	0.3	1
pBINAW:*Rpi-vnt1.1:Rpi-sto1*	Bintje	200	590	2/0/6*	1.0	5
pBINAW:*Rpi-vnt1.1:Rpi-sto1*	Potae9	200	428	0/0/7*	1.6	5

exp: number of explants. # sht: number of rooted shoots tested by PCR. # PCR+: number of PCR positive shoots. %freq: transformation frequency, calculated by percentage of number of PCR-positive shoots over the number of rooted shoots. #bbf: number of backbone free plants. *: number of shoots containing *Rpi-vnt1.1, Rpi-sto1* and both genes, respectively.

B. Identification of PCR-positive shoots in different time ranges after cisgenic transformation

Construct	Host plants	31-50 days	51-70 days	71-90 days	91-110 days	111-130 days	Total*
pBINAW: *Rpi-vnt1.1:Rpi-sto1*	Atlantic	4/197	4/174	4/111	0/15	0/0	12/497 (2.4%)
pBINAW: *Rpi-vnt1.1:Rpi-sto1*	Doip1	0/200	0/187	1/164	1/128	1/69	2/774 (0.3%)
pBINAW: *Rpi-vnt1.1:Rpi-sto1*	Bintje	2/199	3/194	1/2/165	0/32	0/0	6/590 (1.0%)
pBINAW: *Rpi-vnt1.1:Rpi-sto1*	Potae9	4/183	2/143	1/78	0/24	0/0	7/428 (1.6%)

* Number of PCR-positive shoots carrying both genes over the number of shoots, expressed as percentages between brackets. The numbers in red and blue indicate number of PCR-positive shoots for both of genes or a single gene in different time course, respectively.

Generally, this is about half a month quicker than selectable marker-assisted transformation. For Doip1, PCR positive shoots could be collected about two months later contrary to those of the other three varieties. When shoot harvest in cisgenesis

was performed over five different time points, all PCR positive events were originating from different explants, indicating that all insertion events were independent. The backbone integration frequency associated with marker free transformation (26%, Table 4A) was lower than that for transgenesis (57%; Table 4) and in most transformants the entire vector backbone was integrated into the potato genome (Figure 3).

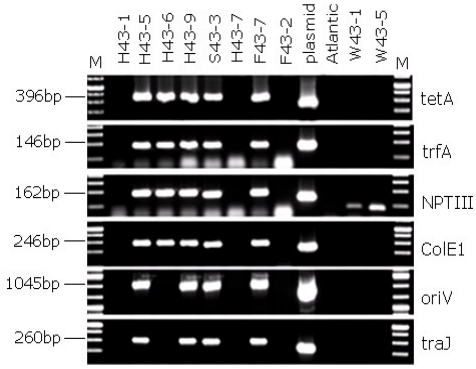

Figure 3. **Vector backbone integration after cisgenic transformation.** Atlantic (H), Doip1 (S), Bintje (F) and Potae9 (W), were transformed with construct 43 (pBINAW2: *Rpi-vnt1.1:Rpi-sto1*). PCR analysis was performed using primers specific for tetA, trfA, NPTIII, ColE1, oriV and traJ to detect vector backbone integration. The plasmid pBINAW2: *Rpi-vnt1.1:Rpi-sto1* was used as a positive control and the untransformed cv. Atlantic as a negative control. M: molecular weight marker.

The T-DNA copy number was compared between single *Rpi* gene-containing transgenic plants and the cisgenic plants containing both *Rpi* genes. The *Rpi-vnt1.1* copy number ranged from one to eight and two or three copies were predominant whereas the *Rpi-sto1* copy number ranged from one to four. The mean copy number was about 1 to 2 (Table 5).

Production and validation of cisgenic plants with two stacked Rpi genes

Totally, 27 transformants were obtained after marker free transformation of the binary plasmid containing both *Rpi-vnt1.1* and *Rpi-sto1* between the T-DNA borders. These 27 plants were selected using gene-specific PCR analysis. Out of these, seven plants contained vector backbone (Table 4A). The remaining 20 plants were transferred to the greenhouse for further tests. Three weeks after transfer to the greenhouse, five plants displayed abnormal phenotypes that consisted of curly leaves and growth-retardation (Figure 4). The plants with these aberrant phenotypes were eliminated and the remaining 15 plants were tested for AVR responsiveness using agroinfiltration. Five plants expressed only the *Rpi-vnt1.1* gene while eight plants expressed both genes (Table 5). The latter eight plants for Atlantic, Bintje and Potae9

displayed resistance to all *Pi* isolates tested in DLA. There were two plants (H43-4 and H43-12) that contained *Rpi-vnt1.1* but showed no resistance.

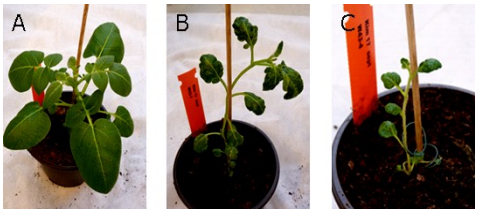

Figure 4. Phenotypes after marker free transformation of cv. Potae9. A: non-transformed Potae9, **B**: curly leaf plant, **C**: dwarf plant.

Table 5. Characterization of marker free transformants carrying two *Rpi* genes in different potato cultivars

			PCR				Greenhouse	Agroinfiltration		DLA				
No	Plant ID	Host plant	*Rpi-vnt1.1*	*Rpi-sto1*	copy number[a]	back bone	abnormal type	*Rpi-vnt1.1*	*Rpi-sto1*	EC1	IPO-C	DHD11	90128	pic99189
1	H43-1	Atlantic	+	+	1/0	no		+	-	S	R	R	R	R
2	H43-2	Atlantic	+	+	1/1	no	curly leaf	n	n	n	n	n	n	n
3	H43-3	Atlantic	+	+	1/0	no	curly leaf	n	n	n	n	n	n	n
4	H43-4	Atlantic	+	+	1/0	no		+	-	S	S	S	S	S
5	H43-7	Atlantic	+	+	4/2	no		+	+	R	R	R	R	R
6	H43-8	Atlantic	+	+	4/1	no		+	+	R	R	R	R	R
7	H43-10	Atlantic	+	+	1/0	no		+	-	S	R	R	R	R
8	H43-11	Atlantic	+	+	3/1	no	curly leaf	n	n	n	n	n	n	n
9	H43-12	Atlantic	+	+	1/0	no		+	-	S	S	S	S	S
10	S43-2	Doip1	+	+	3/1	no		-	-	R	S	S	R	S
11	F43-1	Bintje	+	+	2/0	no		+	-	S	R	R	R	R
12	F43-2	Bintje	+	+	2/1	no		+	+	R	R	R	R	R
13	F43-3	Bintje	+	+	2/1	no		+	+	R	R	R	R	R
14	F43-4	Bintje	+	+	2/1	no		+	+	R	R	R	R	R
15	F43-5	Bintje	+	+	2/2	no		+	+	R	R	R	R	R
16	W43-1	Potae9	+	+	3/1	no		+	+	R	R	R	R	R
17	W43-2	Potae9	+	+	3/1	no	curly leaf	n	n	n	n	n	n	n
18	W43-3	Potae9	+	+	3/0	no		+	-	S	R	R	R	R
19	W43-4	Potae9	+	+	3/0	no	dwarf	n	n	n	n	n	n	n
20	W43-5	Potae9	+	+	3/1	no		+	+	R	R	R	R	R

[a]: copy number for *Rpi-vnt1.1* or *Rpi-sto1*, respectively. "0" means that no estimates were made. This probably reflects incomplete integration of genes of interest because primers for determination of copy number were designed in terminator regions. Functional cisgenic plants were highlighted. n: no data, The aberrant plants were not tested further. R: resistant to the indicated isolate in detached leaf assays (DLA). S: susceptible to the indicated isolate in DLA.

Figure 5 shows an example of the validation of functional expression for both transferred genes in event H43-7 (Atlantic background) by agroinfiltration and resistance assays in the DLA. This plant displayed HR responses for both genes and

showed broad spectrum resistance for all tested *Pi* isolates. This demonstrates that stacking of genes with different resistance spectra leads to complementary effects on susceptibility for the single gene-containing transgenic plants as reference (Table 2). The same can be seen for two cisgenic transformants of cv. Potae9. Here, the two *Rpi* genes are complementing the spectrum of the resistance that was already present in these plants.

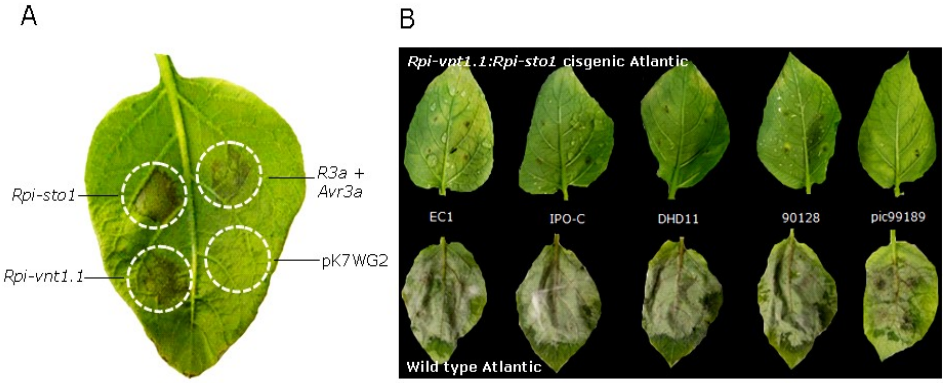

Figure 5. **Functional validation of cisgenic transformants by agroinfiltration and resistance assays. A**. *Avrvnt1*- and *Avrsto1*-induced hypersensitive responses in cisgenic transformant H43-7 (*Rpi-vnt1.1*:*Rpi-sto1* in Atlantic background). *Avrvnt1 and Avrsto1* were infiltrated in cisgenic plants. A 1:1 mixture of *R3a* and *Avr3a* and pK7WG2 were infiltrated as positive and negative controls, respectively. **B**. Detached leaf assays for cisgenic transformant H43-7. Different isolates are shown in the middle. Cisgenic transformants are shown on the top and the wild type Atlantic on the bottom of the panel.

Discussion

Cisgenesis, as a new tool for traditional plant breeding, is a method that uses genetic modification technology to introduce natural genes from the plant species itself or from crossable plant species into crops (Jacobsen and Schouten 2008). Therefore, any alien gene can be avoided in the end product which is causal to many environmental and consumers' concerns about GM food crops. In order to produce crops with stacks of cisgenes, two issues must be refined: 1. stacking methods and 2. methods to exclude vector backbone genes and sequences of foreign origin from transformed

plants. With respect to methods to generate marker free transformed potatoes, transformation by marker free vectors and subsequent regeneration in medium without antibiotics followed by PCR-based selection of transformed plants seems to be preferred. Alternatives involving co-transformation of cis and transgenes or site specific recombination techniques pose disadvantages such as the requirement of sexual crossing and the remnant sequences of foreign origin, respectively (de Vetten et al. 2003; Joshi 2010). In this study, the average marker free transformation frequency was 1.3% and was only slightly genotype dependent, while regeneration ability during transgenesis using NPTII resistance, was much more genotype-dependent. Considering the observation that all the regenerated shoots after antibiotic assisted selection are PCR positive, there is a need to make clear that transformation frequencies between transgenesis and cisgenesis have different meanings. The former transformation frequency reflects gene expression per se in terms of antibiotic resistant plants whereas in the latter, it indicates the presence of genes. Therefore, in transgenesis, regeneration efficiency under antibiotic regime is the same or similar as transformation frequency (Table 3). In cisgenesis, seven plants out of 15 cisgenic plants that were transferred into the greenhouse did not sufficiently express both or one cisgene as observed using agroinfiltration of the corresponding *Avr* genes (Table 5). It suggests that all cells under culture conditions without antibiotic selection have the same capability to regenerate and the transformation frequency reflects percentage of the target gene-integrated cells among *Agrobacterium*-infected cells. This study was not set up to observe differences in transformation efficiency between transgenesis and cisgenesis depending on the T-DNA size. From experiences in our laboratory, however, we know that it takes much longer regeneration time to get long-sized T-DNA-introduced-potato. Therefore, for stacking of more than two genes in cisgenic transformation, the effect of an increased insert size (e.g., >20kb) on transformation frequency remains to be tested. Transformation frequency of cv. Doip1 using our transformation and regeneration protocol under antibiotic conditions (Table 3) reaches a relatively high frequency. It means that cv. Doip1 can widely be used in potato transformation. Nonetheless, in cisgenesis, transformation frequency of cv. Doip1 under antibiotic free conditions was very low and also the harvest time of PCR positive shoots was very late. The high speed of regeneration of this variety might be connected with low transformation efficiency under non selective conditions. Shoots might have regenerated before the T-DNA was transferred. In terms of vector backbone integration, cisgenesis apparently produces a lower percentage of vector backbone integrations compared to transgenesis. This suggests that exclusive antibiotic selection of transformants by the presence of NPTII gene flanked to the left

border of T-DNA would stimulate higher levels of backbone integration. The T-DNA copy number of the transferred genes in cisgenic transformation ranged from one to four. Considering estimates of the transferred multiple T-DNA copy number, using Q-PCR should only be interpreted as approximate (Bubner and Baldwin 2004). For more accurate estimations of multiple integrations, the copy number analysis via Southern hybridization might be needed. Observations of plants (H43-4 and H43-12) that contained *Rpi-vnt1.1* but showed no resistance can probably be explained by insufficient transcript due to insertion at a less active region of the genome (Bradeen et al. 2009). In this study, we observed some cisgenic plants differing morphologically from wild type cultivars in the greenhouse. This is a generally observed phenomenon after plant tissue culture and is one of the considerations in transformation-based molecular breeding schemes (Heeres et al. 2002). As 2-3 shoots per explant can be collected and 30 independent transformed plants are required considering backbone integration and expression, it is recommended that between 1000-1500 explants are to be treated in a single batch cisgenic experiment of potato. The efficiency of PCR analysis can be improved by a factor 10 (by pooling ten shoots), so that the labour intensity for cisgenesis is considered reasonable compared to the transgenic work. It takes less than one year to obtain cisgenic potatoes from the *Rpi* gene construct preparation to performing DLAs. Considering 2-3 years' field trials, it takes totally 3-4 years to produce cisgenic potatoes highly resistant to late blight which can be released for seed tuber multiplication. This time span is remarkable compared to the conventional breeding scheme. The cisgenic potatoes selected in this study will be further tested for several years to evaluate whether the transferred *Rpi* genes are stably expressed over many vegetative cycles. Chimeras and epigenetic silencing are issues that could affect stability of resistance. Also agronomic performance needs to be assessed and confirmed in multiple growing seasons.

Acknowledgments

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Chapter 7

General discussion

Parts are taken from the published paper: **Kim HJ, Lee HR, Jo KR et al. (2012)** Broad spectrum late blight resistance in potato differential set plants Ma*R8* and Ma*R9* is conferred by multiple stacked *R* genes. Theor Appl Genet 124:923–935

General discussion

The main objective of this thesis is to uncover the underlying mechanisms of the multi-year-persisting resistance in the field, also referred to as durability, in some potato clones and cultivars and describe how to deploy that resistance in potato cultivars. In this research we found strong indications that the durability of the resistance to late blight mostly associates with the stacking of multiple resistance (*R*) genes. In previous studies, some wild *Solanum* species highly resistant to late blight were found to contain several *R* genes (Verzaux 2010). This so called "natural stacking" might explain the durability of their resistance. Similarly, here we also refer to gene stacking that has occurred spontaneously via conventional breeding efforts as "natural stacking". In contrast, the directed gene stacking by using biotechnological tools, such as genetic engineering or marker-assisted selection, we refer to as "molecular stacking". In a narrow sense, gene stacking by cisgenic transformation described in **Chapter 6** is named "cisgenic stacking". The isolate recognition spectrum of each of these individual *R* genes is not necessarily broad spectrum and some of them are even considered as "defeated" *R* genes. However the combination of these genes sums up to broad spectrum and contributes in that way to durability. The model plants for broad spectrum late blight resistance included potato differential set plants Ma*R8* and Ma*R9* and potato cultivar Sarpo Mira. A common factor in all these plants was the *R8* resistance gene. Our research led to the discovery of the new effector *Avr8*. Also, the *R9* resistance appeared to be encoded by two closely linked genes on chromosome IX. One of these genes, *R9a*, has been cloned and its recognition spectrum was studied. We discuss which could be the best or favourable option(s) as to how to select, combine, and deploy *R* genes for achieving durable late blight resistance in potato cultivars. One approach towards the introduction of cisgenic *R* gene stacks has been investigated and provides material that can be used for future durability studies.

A rationale for durability of late blight resistance

Durability indicates how long resistance lasts since release of a specific resistant variety or formation of a specific resistant genotype. The oomycete pathogen *Phytophthora infestans* (Mont) de Bary is able to quickly evolve and thereby to overcome *R* gene mediated resistance. The question therefore arises whether it is possible to obtain durable *R* gene mediated resistance. And if so, how can it be realized and maintained? Answers might be found in the nature of the *R* genes or

their cognate *Avr* gene. Theoretically, there are four classes of R-AVR interactions: one R protein to one AVR protein (Class I), one R protein to multiple AVR proteins (Class II), multiple R proteins to one AVR protein (Class III), and multiple R proteins to multiple AVR proteins (Class IV) (Table 1). Besides, considering accessory proteins following guard/decoy models, there could be more types of R-AVR interactions. Also, the class to which each *R-Avr* pair belongs could be provisional because other new *R* gene(s) can be identified by extended germplasm screens.

Table 1. Classification of R-AVR interactions

Class	R/Avr pair	Resistance			Avirulence		References
		Functional R allele	Functional R variant	Resistance spectrum	Avirulent variant	Avr abundance	
I	R1/ Avr1	unknown	unknown	narrow	1	low	Ballvora et al. 2002, Tyler 2009
	R3a/ Avr3a	R3a Rpi-sto2 cph541-2[a]	unknown	narrow	2 alleles Avr3a^{KI}, Avr3a^{KIL}	low	Huang 2005 Armstrong et al. 2009 Champouret 2009
	R3b/ Avr3b	unknown	unknown	narrow	1	low	Li et al. 2011 Rietman 2011
	R4/ Avr4	Rpi-edn3[b]	unknown	narrow	1	low	van Poppel et al. 2008
	Rpi-vnt1/ Avrvnt1	Rpi-vnt1.1, Rpi-vnt1.2, Rpi-vnt1.3	unknown	broad	3 alleles 2 copies	high	Foster et al. 2009 Pel 2010
II	Rpi-blb1/ Avrblb1	Rpi-blb1/RB Rpi-sto1 Rpi-pta1	unknown	broad	7 4 copies[g] ipiO1, ipiO2 ipiO3, ipiO5, ipiO7, ipiO8, ipiOm2	high	Song et al. 2003, van der Vossen et al. 2003, Vleeshouwers et al. 2008, Champouret 2009
III	R8/ Avr8	Up to 10[c]	gig712-6[e] gig715-4[e]	broad	1	high	Chapter 3
IV	R2/Avr2	R2 R2-like, Rpi-blb3, Rpi-abpt	Rpi-edn1, Rpi-snk1, Rpi-hjt1	Interm-ediate	5 2 copies[g] PiAvr2 PEXRD11 PITG_21949, PITG_21645, PITG_13940[h]	medium	Lokossou et al. 2009 Champouret 2009
	Rpi-blb2/ Avrblb2	unknown	R9a	broad	24 7 copies[g]	high	van der Vossen et al. 2005, Oh et al. 2009, Chapter 5
	R9a/ Avrblb2	Rpi-edn2[d]	Rpi-blb2	broad	24 7 copies[g]	high	Chapter 5

[a] Most likely R3a homolog is present in the genotype cph541-2 from agroinfiltration data (Champouret 2010). cph, *S. cardiophyllum* [b] Potentially the *Rpi-edn3* gene from *Sx. edinense* is a functional allele (Veruzaux 2010). [c] Functional *R8* homologs by agroinfiltration of *Avr8* (Chapter 3). [d] Potentially the *Rpi-edn2* gene from *S x. edinense* is a functional allele (Chapter 5). [e] Avr8 responding genotypes gig712-6 and gig715-4 might contain quite different *R* gene from *R8* on chromosome IX based on haplotype analysis near the *R8* locus but not confirmed yet (Chapter 3). gig, *S. microdontum gigantophylum.* [g] Copy number of paralogs in the strain T30-4 genome. [h] *R2* variants show a differential recognition to PTIG_13940 (Champouret et al. 2010).

In Table 1, class I includes one copy of *Avr* genes recognized by rapidly evolving type I *R* genes *R1-Avr1*, *R3a-Avr3a, R3b-Avr3b* and *R4-Avr4* pairs. Frequency in *Pi* populations for these AVRs is low. The *R* genes have already been defeated and thus have a limited agricultural value. A Rpi-vnt1-Avrvnt1 pair belonging to class I displays broad spectrum resistance which is caused by abundance of different *Avrvnt1* alleles in most *P. infestans* isolates. *R-Avr* pairs belonging to class II in which one R protein recognizes multiple AVR proteins are interesting in explaining broad spectrum resistance to late blight. There is only one example, *Rpi-blb1* and its allelic variants *Rpi-sto1* and *Rpi-pta1*. *Rpi-blb1*, also known as *RB*, was originally described as a broad spectrum *R* gene conferring race-nonspecific resistance to all the tested *Pi* isolates (van der Vossen et al. 2003). *Avrblb1* belongs to the IpiO family which can be divided into three classes (Champouret et al. 2009). The known seven avirulent effectors of the IpiO family are present in most *Pi* isolates collected worldwide. *Avrblb1* differs from the *Avr* genes recognized by *R* genes belonging to class I, which are mostly defeated, since it occurs as an expanded gene family. Several family members, occuring as paralogs, are targeted by allelic variants of *Rpi-blb1*, suggesting the emergence of virulent races has low chances because multiple independent mutations or segmental deletions should occur for *P. infestans* to become virulent on the cognate *R* gene-containing potatoes (Oh et al. 2009). The fact that only one *R* gene recognising members of this effector family has been identified so far suggests there has not been an extensive arms/race co-evolution based on this R-Avr interaction. The *R8-Avr8* pair probably belongs to class III because haplotype analysis near the *R8* locus for some AVR8 responding genotypes showed that AVR8 might be recognized by quite different *R* genes as well as *R8* (**Chapter 3**). Although there is only one active copy of *Avr8* in the T30-4 genome, which appears to be non polymorphic among different *Pi* isolates (data not shown), the observation that the *Avr8* gene is present in all thus far sequenced *P. infestans* isolates could explain why *R8* is broad spectrum for many *Pi* isolates (**Chapter 3**). Although *Avr8* was not selected as a core effector in the study of Cooke et al. (2012) because of altered expression pattern during infection as compared to the other

known *Avr* genes, we should note that *R8* is also considered a target for durable resistance breeding, similar to *R* genes recognising the other core effectors. The first example of class IV interaction came from Champouret (2010). The highly homologous R proteins R2, Rpi-abpt1, and Rpi-blb3 on the one hand and Rpi-hjt1 and Rpi-snk1 on the other hand recognised overlapping spectra of effectors. Rpi-blb2-Avrblb2 and R9a-Avrblb2 are the first examples of a class IV interaction where the *R* proteins have less than 30% homology but still an overlapping set of the same effector family (Avrblb2) is recognised (Table 1). *Avrblb2* as a multi-gene family has at least seven duplicated copies in *Pi* strain T30–4 reference genome (Haas et al. 2009). 24 avirulent variants for Avrblb2 were observed and are present in all *Pi* strains tested (Oh et al. 2009). It is remarkable that R9a also recognizes Avrblb2^{Phe69} variants that are not perceived by Rpi-blb2 (**Chapter 5**). *R-Avr* pairs belonging to the class IV are interesting for broad spectrum resistance because their existence suggests a long history of co-evolution. The recurrent recognition of the same effector family could mean that the plant's innate immune system has discovered the Achilles heel of *Phytophthora*.

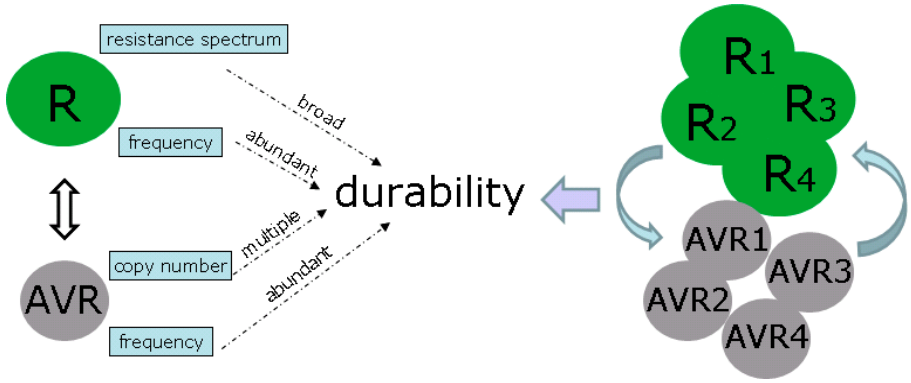

Figure 1. **Durability is relative to the combination of components defined by each counterpart for a R-AVR pair.** On the left side, a R-AVR pair is shown. The contribution of a plant R protein to durability relies on its nature which comprises broad spectrum and abundance of functional homologs. As a counterpart of the durable R protein, pathogen AVR protein that possesses multiple copy number and abundant frequency among most *P. infestans* isolates could be optional for durability. On the right side, stacking of different durable R proteins with different resistance spectra recognizes a different suite of *P. infestans* effectors from potentially all *P. infestans* isolates, enabling to produce appropriate durable resistance.

Although *R2* has been exploited in agriculture but has been overcome by late blight in the field, it has been shown to still confer resistance in some geographical regions (Pilet et al. 2005; Vleeshouwers et al. 2011a). Recently, Wang et al. (2012) and Li (2012) independently reported that Ma*R2* together with Ma*R8* and Ma*R9* was rarely infected for 72 isolates collected from the potato fields of the Heilongjiang Province of China between 2004 and 2008. Also in the Netherlands the Ma*R2* clone still provides higher levels of resistance than *R0* plants (Table 2). These findings imply that *R2* is a *R* gene that when combined with other *R* genes, might support durability. Given the mentioned R-AVR interactions, we can deduce some important points for durability (Figure 1). The copy number variation and abundance of the *Avr* gene in *Pi* are major components for producing durability. As a counterpart of such an *Avr* gene, potato *R* genes that display broad spectrum resistance and have probably abundant functional homologs among various wild *Solanum* species could be optional for *R* gene combinations providing durability. Up to now, the ideal durable *R* gene that is resistant to all *Pi* isolates was not found. Even if such a *R* gene would be available, exposure to strong selection pressure under large scale agricultural deployment could lead to *R* gene breakdown due to the co-evolutionary predominance of virulent isolates caused by mutation, silencing or suppression by another effector. For instance, *Rpi-blb2* and *R9a* breaking isolates were found in the *Pi* population of the Netherlands at low frequency (**Chapter 6**; Förch et al. 2010). Also, virulent races on *Rpi-blb1*–containing potatoes have been identified in Mexico (Champouret et al. 2009) and the Netherlands (Dr. G. Kessel, Plant research international WUR, personal communication). Therefore, the logical idea to combine multiple *R* genes with different spectra would be preferred to achieve durability. In order to get supporting information on resistance over time and thus durability for *R* gene stacks, resistant potato clones and varieties with one or multiple *R* genes can be tested in late blight epidemic fields of the Netherlands for several years (see next section).

Do (natural) *R* gene stacks impart durability?

Once (potentially) durable *R* genes are identified, pyramiding or stacking of these *R* genes with different resistance spectra recognizing different *Pi* effectors commonly present in the *Pi* population will enable to produce appropriate resistance for a long term. Currently, *R* genes with different broad spectra are available and cloning of broad spectrum or durable *R* genes is continually underway. This will open up the possibility to produce and maintain durable late blight resistant potatoes. The

combination of *R8* and *R9* genes identified in our study is suggested to enable durable resistance to late blight. The efficacy of the *R8* or *R9* resistance has been shown in detail in six-year-field trials (Table 2). Ma*R8* and Ma*R9* were exposed in sequential years to high *Pi* infection pressure in trap fields in major potato growing areas in the Netherlands. The onset of late blight symptoms was observed and every year Ma*R8* and Ma*R9* performed significantly better than control cultivar Bintje and in most cases also significantly better than Ma*R1*, Ma*R2*, Ma*R3*, and Ma*R4* plants (Table 2). In the last three years, also comparisons were made to cv. Sarpo Mira. In 2009, Ma*R9* showed a similar delay as cv. Sarpo Mira, and in 2010 and 2011 Ma*R8* showed a similar delay as cv. Sarpo Mira. In 2010 and 2011, Ma*R9* outperformed cv. Sarpo Mira. This was in agreement with other comparisons made between Ma*R8* and Ma*R9* and for cv. Sarpo Mira in Western Europe and Northern Africa (Chmielarz et al. 2010; Corbiere et al. 2010; White and Shaw 2010). Using a combination of studies including *Avr* responsiveness, *R* gene-specific molecular markers, candidate gene cloning and segregation analysis of F1 populations, it was possible to show that *R3a, R3b, R4,* and *R8* resistance genes were present in Ma*R8* and that *R1, Rpi-abpt1* (an allelic variant of *R2*)*, R3a, R3b, R4, R8, R9a* and *R9b* were present in Ma*R9*. Such an extensive *R* gene stacking in Ma*R8* and Ma*R9* caused broad spectrum resistance. These studies show that the more *R* genes are present in a plant the broader the isolate resistance spectrum is (Kim et al. 2012). From 2010, also F1 clones from Ma*R8*, Ma*R9* and *S.x edinense* accession 150-4 were included in these studies. These "destacked" plants had various combinations of *R* genes. Plant 3020-18 that only contained *R8* was analyzed for two years (Table 2) and in the first year there was no difference between the *R8* alone and the Ma*R8* stack. However, in the following year field trials showed that the *R8* alone was not as effective as the Ma*R8* stack, suggesting that the "defeated" *R* genes provided some contribution to the resistance spectrum towards the *Pi* population in The Netherlands. However, this needs further analysis for several more years and also the high dependence on genetic background of *R8* (Kim et al. 2012) might influence this result. In 2010 and 2011, the *Rpi-blb2*-containing cultivar Toluca was included in the field trials. In both years Toluca showed similar delays as the plants containing *R9a*, being in line with our data for resistance spectra of Ma*R9* (**Chapter 5**). Nonetheless, we expect the combined effect of *R9a* and *Rpi-blb2* would be bigger than their individual effects because the stack could recognize a complementary suite of effectors and potentially even the entire Avrblb2 family (**Chapter 5**). In 2011, Ma*R2* showed stronger delays compared to those in the first three years of the experiments. *Rpi-edn1*, a resistance gene with

an overlapping effector recognition pattern with *R2*, was present in *S.x edinense* 150-4 derived plants 7727-102 and 7727-158 that also contained *Rpi-edn2*.

Table 2. On site *Pi* virulence monitoring

Year	Genotype	*R* gene content	Calculated delay in *Pi* symptoms (days)	Relative delay in *Pi* symptoms*
2006	Bintje	–	4	a · ·
	Ma*R1*	1	4	a · ·
	Ma*R2*	2	8	a b ·
	Ma*R3*	3a, 3b	4	a · ·
	Ma*R4*	4	7	a b ·
	Ma*R8*	3a, 3b, 4, 8	37	· b ·
	Ma*R9*	1, abpt, 3a, 3b, 4, 8, 9a, 9b	66	· · c
2007	Bintje	–	1	a · · ·
	Ma*R1*	1	2	a b · ·
	Ma*R2*	2	2	a b · ·
	Ma*R3*	3a, 3b	2	a b · ·
	Ma*R4*	4	1	a · · ·
	Ma*R8*	3a, 3b, 4, 8	13	· · c d
	Ma*R9*	1, abpt, 3a, 3b, 4, 8, 9a, 9b	No lesions**	· · · d
2008	Bintje	–	3	a b · ·
	Ma*R1*	1	1	a · · ·
	Ma*R2*	2	3	a b · ·
	Ma*R3*	3a, 3b	2	a b · ·
	Ma*R4*	4	2	a b · ·
	Ma*R8*	3a, 3b, 4, 8	24	· · c d
	Ma*R9*	1, abpt, 3a, 3b, 4, 8, 9a, 9b	15	· · c ·
2009	Bintje	–	1	a · ·
	Ma*R8*	3a, 3b, 4, 8	113	· b ·
	Ma*R9*	1, abpt, 3a, 3b, 4, 8, 9a, 9b	No lesions**	· · c
	Sarpo Mira	3a, 3b, 4, smira1, 8	No lesions**	· · c
2010	Bintje	–	3	a · · ·
	3020-18	8	50	· b · ·
	Ma*R8*	3a, 3b, 4, 8	50	· b · ·
	Sarpo Mira	3a, 3b, 4, smira1, 8	51	· b · ·
	Toluca	blb2	50	. b . .
	3025-53	3a, 3b, 4, 8, 9	193	· · c ·
	Ma*R9*	1, abpt, 3a, 3b, 4, 8, 9a, 9b	No lesions**	· · · d
2011	Bintje	–	2	a · · · · · · ·
	3151-3	9b	9	· b c · · · · ·
	Ma*R2*	2	12	· b c d · · · ·
	3020-18	8	15	· · c d e · ·
	edn7727-104	edn2	19	· · · d e · ·
	edn7727-148	10, edn2	22	· · · d e · ·
	3025-53	3a, 3b, 4, 8, 9a, 9b?***	19	· · · d e · ·
	Toluca	blb2	18	· · · d e · ·
	3025-1	abpt, 3a, 3b, 4, 9a, 9b?***	23	· · · d e · ·
	3025-43	3a, 3b, 8	19	· · · d e · ·
	3025-48	3a, 3b, 4, 8, 9b?***	36	· · · · e f ·
	Sarpo Mira	3a, 3b, 4, smira1, 8	44	· · · · e f ·
	Ma*R8*	3a, 3b, 4, 8	43	· · · · e f ·
	edn7727-102	edn1, 4, edn2	40	· · · · e f ·
	edn7727-158	edn1, 10, edn2	40	· · · · e f ·
	Ma*R9*	1, abpt, 3a, 3b, 4, 8, 9a, 9b	114	· · · · · f g
	edn150-4	edn1, 4, edn2	No lesions**	· · · · · · g

* The onset of *Pi* symptoms was compared between different genotypes using T test. Significant differences in delay between different genotypes are represented by different letters. If ranges of letters are overlapping there was no significant difference observed between the genotypes in this particular year. ** In case no lesions were found on the tested plants during the course of the experiment, calculations for delay according to censor showed an unrealistic figure, which was replaced by "No lesions".

These plants outperformed *Rpi-edn1*-lacking ones, suggesting the importance of *Rpi-edn1* for durability in stacks. Ma*R9* is a good example to demonstrate that high levels of resistance can be obtained by natural stacks that consist of broad spectrum *R* genes, like *R8* and *R9,* and moderately broad spectrum *R* gene *Rpi-abpt*, which is an allelic variant of *R2*. Sarpo Mira that has retained its resistance in the field over many years contains a similar pyramid of *R* genes as Ma*R8*, i.e., *R3a, R3b, R4, Rpi-Smira2*, and one gene, *Rpi-Smira1,* whose presence has not been detected in the Ma*R8* plant. Besides Sarpo Mira, two potato cultivars, C88 (Li et al. 2010) and Missaukee (Douches et al. 2010), that have been described to display durable late blight resistance through multiple years of commercial cultivation, were also found to contain the *R8* markers, demonstrating potential durability of late blight resistance for *R8* (**Chapter 3**). To confirm this hypothesis agroinfiltrations with AVR8 in these plants should be tested. However, as *Pi* populations should evolve and evade *R8* recognition during long term cultivation, it would be wise to stack additional *R* genes on top of the *R8* gene. Future deployment of *R8* in combination with other *R* genes by cisgenic potato transformation (Jacobsen and Schouten 2007) is expected to provide durable late blight resistant potato cultivars. We propose a breeding program by which similar *R* gene combinations, e.g., cisgenic stacks of *R2, R8, R9a* and *R9b*, as those in Ma*R9* are selected and introduced into potato cultivars by cisgenic transformation. This, potentially, is an effective way to produce durable late blight resistant potatoes. When these *R* gene stacks are deployed in potato cultivation a virulence monitoring system is required. Selection of individuals with different known *R* gene combinations, using *R* gene "destacking" will provide tester plants for "on site" virulence monitoring. Monitoring of virulence towards the *R* genes involved within the local *Pi* populations can be linked to a fungicide spray advice. A system of tester plants with one *R* gene is essential to determine the virulence spectrum of individual *Pi* isolates. The differential set Ma*R1*-Ma*R11* has long been considered to contain one *R* gene per tester plant. However, it was shown in other studies, and also in our own study, that several plants from the differential set contained multiple resistance genes. Ma*R3* contained two resistance genes (*R3a, R3b*) (Huang et al. 2005), Ma*R5* and Ma*R6* on top of the *R5* and *R6* genes the *R1*

gene (Trognitz and Trognitz 2007), Ma*R8* four *R* genes (*R3a, R3b, R4, R8*), Ma*R9* eight *R* genes (*R1, Rpi-abpt1, R3a, R3b, R4, R8, R9a, R9b*) (Kim et al. 2012) and Ma*R10* contained two *R* genes (*R3b, R10*) (Rietman 2011). Our unpublished data show that Ma*R5*, Ma*R6*, Ma*R7* and Ma*R10* also contain multiple *R* genes. These findings call for an update of the differential set. An improved or "purified" differential set will allow a more accurate analysis of *Pi* isolate virulence spectra. This could be done by crossing the original differential set plants or their offspring to cultivars without *R* genes. So far, it was possible to select plants that contained only *R8* and only *R9b* (Table 2). Such "purified" differential plants will be very useful for future "on site" virulence monitoring. A drawback of the purified differential set will be the differences in plant physiology and in particular the maturity type. An alternative to a purified differential set will be a transgenic differential set that contains the different single or combinations of *R* genes in the same genetic background.

Deployment of *R* genes: cisgenic stacking

As a consequence of accumulation of huge amounts of sequence information and development of fast evolving bioinformatics tools by the recent rapid advancements in the field of genomics, possibilities to speed up identification, characterization and isolation of previously unknown or under-utilized sources of genetic variation in *Solanum* have become available. As a result now some promising genes that confer broad spectrum resistance to late blight are more readily available for deployment in agriculture. *R* genes can be introduced through two different breeding programs; introgression breeding and genetic modification. With respect to methods to generate cisgenic potato plants, marker free gene delivery by *Agrobacterium tumefaciens* and subsequent regeneration in medium without antibiotics followed by PCR-based selection of transformed plants is feasible. Simultaneous stacking using multiple *R* genes in one vector seems to be preferred. Alternatives involving co-transformation of cis- and trans- genes or site specific recombination techniques pose disadvantages such as the requirement of sexual crossing and the remnant sequences of foreign origin, respectively. It takes less than one year to obtain potato plants with cisgenic *R* gene stacks (Figure 2; **Chapter 6**). Considering 2-3 years' laboratory, greenhouse and field trials, it takes 3-4 years to validate cisgenic potatoes highly resistant to late blight in field trials which can enter a seed potato production scheme. Such a time frame for the development of late blight resistant potatoes by cisgenic stacking is remarkably fast compared to that of conventional

resistant varieties, Bionica and Toluca, which as a result of a very complicated plant breeding program lasted over 40 years (www.Agrico.com; Haverkort et al. 2009).

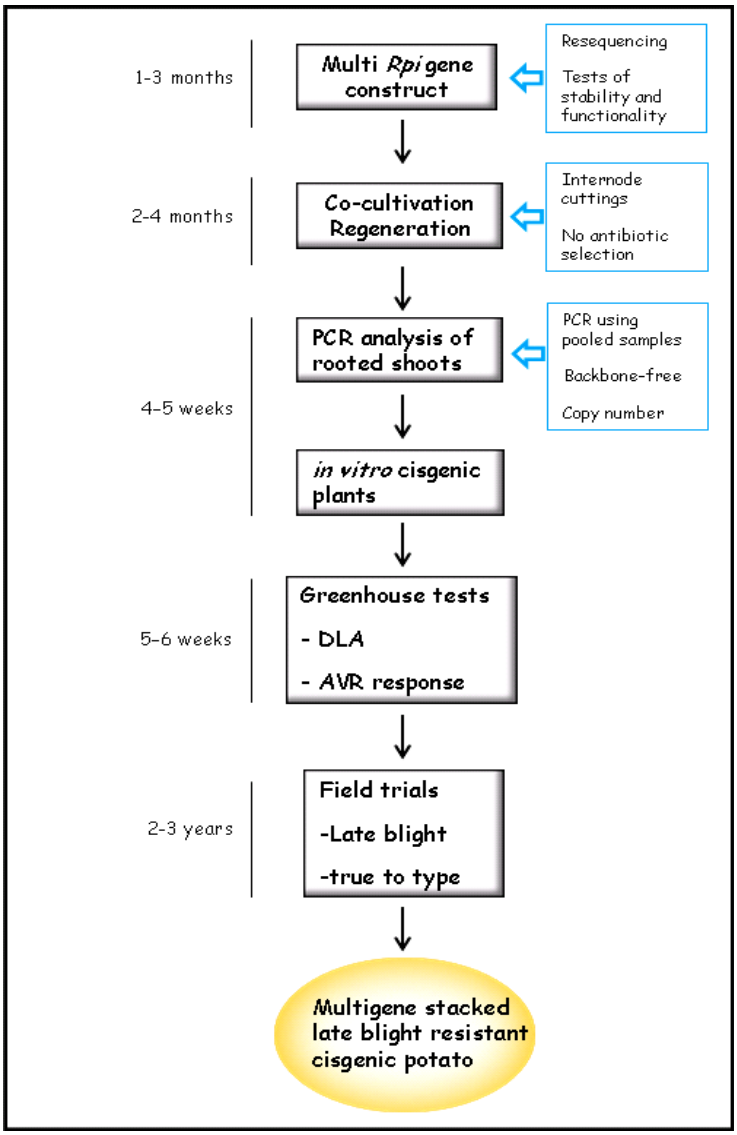

Figure 2. Pipeline for the production of late blight resistant cisgenic potatoes (see text)

The first cross breeding for introgressing the resistance genes from wild potatoes took place in 1959 and it took until 2005 before the varieties were available for seed multiplication. These varieties contain the same, single broad spectrum resistance gene *Rpi-blb2* from *S. bulbocastanum* and are still used on a small scale; almost only in organic farming. The example of Bionica and Toluca shows that stacking of *R* genes through the interspecific cross breeding schemes would be more complicated and slower. Although breeding durable late blight resistant potatoes by cisgenic stacking is efficient, both technical and regulatory issues remain to be refined in terms of the integrated position in the genome and the subsequent expression level of the resistance genes. Expression of a *R* gene is closely correlated with the resistance phenotype and plants with multiple integration events are typically more resistant than those with fewer copies of the *R* gene (Kramer et al. 2009; Bradeen et al. 2009). A low expression of native promoter *R* gene in tuber tissue requires tuber-specific promoter with high expression levels in tubers to trigger an effective resistance response (Pel 2009). This is a very difficult task and the achievement by higher copy numbers of the *R* gene driven by its native promoter or by replacing the native promoter with a stronger plant derived promoter would be possible, however, resulting in an intragenic or even transgenic approach. Also, targeted integration of a *R* gene into the potato genome using novel technologies like TAL effector recognition sites (Scholze and Boch 2011) remains to be done in the context of regulatory issues. The *R* gene stack is a valuable trait because it confers broad spectrum and potentially durable resistance to *P. infestans* with no impact on yield in tested transgenic/cisgenic cultivars (data not shown). The deployment of potatoes that require little or no fungicide input whose costs are estimated at more than €5.2 billion/year worldwide (Haverkort et al. 2009) and remain resistant even under heavy disease pressure conditions should significantly impact costs associated with growing potato anywhere in the world (Halterman et al. 2010).

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Summary

The potato, which receives an increased attention as a food crop, has long been in threats from the oomycete *Phytophthora infestans*, the causal agent of late blight. This disease still remains the most important constraint in potato producing regions of the world. It might cause the complete destruction of the foliage and tubers of potato if meteorological conditions are conducive to the onset and spread of late blight epidemics. Although fungicides applications provide sufficient levels of late blight control, they impose high input costs to the farmer, are detrimental to human and environment and increase the capacity of the pathogen to develop resistance to the active ingredients of fungicides applied. The increased genetic diversity in *P. infestans* populations due to sexual recombination between two mating types in many parts of the world and the emergence of fungicide resistant strains poses the necessity to develop potatoes that possess high levels of durable resistance as an alternative to the use of fungicides. Clones Ma*R8* and Ma*R9* from the Mastenbroek differential set, used to assess virulence towards *R* genes, have been known for their strong resistance to *P. infestans*. This also holds for cultivar Sarpo Mira which has retained resistance in the field over several years without fungicide applications. Uncovering genetic basis of such, partly naturally-formed, late blight resistance is the prerequisite for the implementation of durable resistance in a breeding scheme. In this study, Ma*R8*, Ma*R9* and cv. Sarpo Mira were used as plant materials for unveiling durability of late blight resistance in potato. First, F1 mapping populations from crosses between these resistant materials with susceptible parents were assessed for late blight resistance in field trials and in detached leaf assays (DLA) after inoculation with an incompatible *P. infestans* isolate IPO-C. A 1:1 segregation of resistance and susceptibility was observed in the Ma*R8* derived-F1 population in field trials, but not in detached leaf assays. NBS profiling and *R* gene cluster directed profiling (CDP), followed by marker landing in the newly sequenced potato genome, referred to as "anchored scaffold approach", led to the mapping of *R8* at a new locus on chromosome IX rather than on chromosome XI, the previously suggested chromosomal position (**Chapter 2**). The *R* gene mediated resistance reaction in potato is a consequence of an (in)direct interaction between the pathogen *Avr* and host *R* gene product that leads to a hypersensitive cell death (HR). We screened a wide collection of RXLR effectors of *P. infestans* for eliciting cell death in the differential potato Ma*R8* by agroinfiltration (**Chapter 3**). *R8*-specific cell death to one effector PITG_07558, termed AVR8, co-segregated with the *R8*-mediated resistance to *P. infestans* isolate IPO-C in a F1 population. From the notion that *Avr8* is identical to effector *AvrSmira2* that was

previously found to associate with field resistance in cultivar Sarpo Mira, we performed genetic mapping studies in a Sarpo Mira-based F1 population and indeed *Rpi-Smira2* localized in the *R8* locus. To investigate the geographical and phylogenetic origin of *R8* in the *Solanum* gene pool, we conducted functional screens for AVR8 responsiveness in 98 wild genotypes (72 accessions of 40 species) of *Solanum* section *Petota*. We identified twelve AVR8 responding *Solanum* accessions originating both from Central and South America. Interestingly, our study involving late blight resistance from the differential plant Ma*R9* described that it is near the *R8* locus on chromosome IX (**Chapter 4**). An integrated approach combining 1. a *R* gene "de-stacking" approach using *R* gene specific marker analysis and effector responses, 2. the whole plant climate cell assay, and 3. CDP profiling enabled a clear picture for the presence of two closely linked genes, termed *R9a* and *R9b.* It was shown that *R9a* locates in a *Tm-2*^{2} cluster of NB-LRR genes and, most likely will be a member of the *Tm-2*^{2} *R* gene family (**Chapter 4**). The identified fully co-segregating *Tm-2 like* CDP markers were used to select the *R9a* gene-containing BAC clone, demonstrating the possibility of BAC landing by marker saturation in the targeted chromosomal regions (**Chapter 5**). For cloning *R9a* gene, a bacterial artificial chromosome (BAC) library derived from the differential plant Ma*R9,* was screened with co-segregating *R* gene CDP markers whereby two overlapping BAC clones carrying CDP markers were obtained. Sequence annotation of the complete insert of these BAC clones identified the presence of two complete *R* gene analogs (*RGA9.1* and *RGA9.2*) of the NB-LRR class in one BAC clone. Two *RGAs,* including their natural regulatory transcriptional elements, were subcloned by long-range PCR into a binary vector for plant transformation. After transformation, it was found that *RGA9.1* was able to complement the susceptible phenotype in cultivar Desiree. *RGA9.1*, now designated *R9a,* encodes a CC-NB-LRR protein of the Tm2 family, where the LRR consensus is only loosely fitted. Agroinfiltration-based effector screens for identifying the *Avr*genes matching the *R9a* gene was performed, leading to the discovery of *Avrblb2* homologs which trigger *R9a* mediated hypersensitivity in *Nicotiana benthamiana* (**Chapter 5**). Resistance profiling with 54 *P. infestans* isolates showed that Ma*R9* and *S.xedinense* accessions had similar resistance spectra as the *Rpi-blb2* containing cultivar Bionica. Transformation of potato with resistance genes and antibiotic resistance markers encounters consumers' criticism. These criticisms are considerably less if only resistance genes from crossable species, and no antibiotic resistance selection marker is used. Genes deriving from crossable species are referred to as cisgenes. For the production of cisgenic potatoes with a broader resistance spectrum and potential durability, *Agrobacterium*-mediated marker free transformation and PCR selection of transformants was performed. This way four

potato cultivars (Atlantic, Bintje, Potae9 and Doip1) were successfully transformed with a construct containing two cisgenic *Rpi* genes (*Rpi-vnt1* from *Solanum venturii* and *Rpi-sto1* from *Solanum stoloniferum*) (**Chapter 6**). Resistance assays in untransformed varieties with five *P. infestans* isolates showed that cvs. Potae9 and Doip1 were already resistant to certain isolates. Single *Rpi* gene containing transgenic plants for all 4 varieties were obtained and used as references. Marker free transformation with a construct containing two *Rpi* genes (cisgenesis) was compared to kanamycin assisted selection (transgenesis) in terms of regeneration and transformation frequency, vector backbone integration, and T-DNA copy number. In addition, the different time tracks to harvest regenerated shoots for the selection of PCR positive regenerants for one or both *Rpi*-genes were studied. Through further analyses involving phenotypic evaluations in the greenhouse, agroinfiltration of avirulence (*Avr*) genes and detached leaf assays, totally eight cisgenic plants were selected. Two cisgenic plants of cv. Altantic and four of cv. Bintje, were selected that showed broad spectrum late blight resistance due to the activity of both *Rpi* genes. Based on characterization of two cisgenic transformants of cv. Potae9, it was demonstrated that the existing late blight resistance spectrum has been broadened by adding the two *Rpi* genes. Finally, results from this study are discussed in terms of genetic and molecular mechanism of durability and cisgenic deployment to address the challenges of the durable resistant potato variety development (**Chapter 7**). We pursue possible options for durability in the nature of the *R* genes or their cognate *Avr* genes. The comparative analysis of several features of available R-AVR pairs shows that major components for producing durability are the copy number variation in the *P. infestans* genome and abundance of the *Avr* gene in different isolates. As a counterpart of such an *Avr* gene, potato *R* genes that display broad spectrum resistance and often have abundant functional homologs among various wild *Solanum* species could be optional for *R* gene combinations providing durability. Multiple years' on-site-monitoring of resistance spectrum in natural *R* gene stacks demonstrates that stacking of several broad spectrum *Rpi* genes or even "defeated" *R* genes could sum up to high levels of resistance potentially capable to provide durability to commercial potato cultivars. Our data about acquirement of complementary resistance spectrum by cisgenic introduction of two broad spectrum resistance genes into cultivars support a first step into that direction.

개 요

식량작물로서 관심이 계속 커지고 있는 감자는 오래전부터 역병의 발병요인인 oomycete *Phytophthora infestans* 의 위협을 받아왔다. 이 병은 지금도 세계적으로 감자를 생산하는 지역들에서 피해를 주는 가장 중요한 병으로 되고 있다. 기상조건이 역병의 발생과 만연에 유리하면 감자의 잎줄기 및 덩이줄기의 완전한 고사를 초래할수 있다. 농약처리로 합당한 수준에서 역병을 구제할수 있지만 이 방법은 농민들에게 있어서 투자비용이 많이 들고 사람과 환경에 해로우며 시비한 농약의 활성성분에 대해 병원균이 저항성을 획득할수 능력을 높여준다. 세계 많은 지역들에서 두 교배형들사이의 유성재조합에 기인하는 역병균집단에서 유전적다양성의 증대는 농약의 리용을 대신하는 방법으로서 높은 수준의 지속저항성을 가지는 감자를 육성할 필요성을 제기한다. Mastenbroek 의 한조의 판별계통 가운데서 영양계 Ma*R8* 및 Ma*R9* 는 역병에 강한 저항성을 가지는것으로 알려져 있다. 첫 진정한 역병저항성감자품종으로서 Sarpo Mira 는 농약을 치지 않고 여러 해동안 포전에서 저항성을 유지하였다. 이러한 부분적으로 자연적으로 형성된 역병저항성의 유전적기초를 밝히는것은 지속저항성육종의 전제조건으로 된다. Ma*R8*, Ma*R9* 그리고 품종 Sarpo Mira 를 감자에서 역병저항성의 지속성을 해명하기 위한 식물체재료로 리용하였다. 우선 이 저항성재료들과 감수성친들을 섞붙임하여 얻은 F1 집단들을 불친화성의 역병균그루 IPO-C 로 접종한 후 포전시험 및 잎검정법 (DLA) 으로 역병저항성을 평가하였다. 저항성 및 감수성의 1:1 분리가 포전시험에서 Ma*R8* 기원의 F1 집단에서 관찰되였지만 잎검정법에서는 관찰되지 않았다. NBS profiling 법, anchored scaffold 법, 그리고 *R* gene cluster directed profiling (CDP) 법을 리용하여 *R8* 을 선행연구에서 제기된 염색체 XI 이 아니라 염색체 IX 의 새로운 유전자자리에 자리잡고 있다는것을 밝히였다 (**제 2 장**). 역병저항성의 감자육성에서 병원균인식의존성의 병저항성의 중요성으로부터 아그로침투법에 의해 판별감자 Ma*R8* 에서 세포치사를 시동하는 역병균의 RXLR 효과체들을 선발하였다 (**제 3 장**). AVR8 로 이름지은 한개의 효과체 PITG_07558 에 대한 *R8*-특이적인 세포치사반응은 F1 집단에서 역병균그루 IPO-C 에 대한 *R8* 유전자에 의한 저항성과 공분리하였다. *Avr8* 이 선행연구에서 품종 Sarpo Mira 의 포전저항성과 련관이 있다고 밝혀진 효과체 *AvrSmira2* 와 동일하다는 사실로부터 Sarpo Mira 의 F1 집단에서 유전지도작성연구를 진행한 결과 실제로 *Rpi-Smira2* 은 *R8* 유전자자리에 위치하였다. *Solanum* 유전자원에서 *R8* 의 지리적 및 계통분류적 기원을 연구하기 위하여 *Solanum* section *Petota* 의 98 개의 야생종유전자형들 (40 종의 72 수집계통) 에서 *AVR8* 반응성에 대한 기능성선발을 진행하였다. 중앙아메리카와 남아메리카에서 기원하는 12 개의 AVR8 에 반응하는 *Solanum* 계통들을 동정하였다. 판별식물체 Ma*R9* 에서 역병저항성에 관한 연구결과 그것이 염색체 IX 우의 *R8* 유전자자리근방에 자리 잡고있다는것을 해명하였다 (**제 4 장**). 분자표식자분석과 효과체반응을 결합한 저항성유전자 "집적해제" 방법, 완전식물체기후실검정법, 그리고 CDP profiling 법을 종합적으로 리용하여 *R9a* 및 *R9b* 로 이름지은 두개의 밀접히 련관된 유전자들의 존재에 대한 명확한 표상을 얻을수 있었다. *R9a* 는 NBS-LRR 유전자들의 *Tm-2²* 클라스터에 위치한다는것이 밝혀졌는데 그 유전자는 모름지기 *Tm-*

2²R 유전자족의 한 성원일수 있다 (**제 4 장**). 우리의 연구에서 동정된 완전히 공분리하는 *Tm-2* 류사 CDP 표식자들은 *R9a* 유전자를 포함하는 BAC 클론을 선발하는데 리용되였는데 이것은 목적하는 염색체령역들에서 표식자포화에 의한 BAC landing 의 가능성을 보여주었다 (**제 5 장**). *R9a* 유전자를 클로닝하기 위하여 판별식물체 Ma*R9* 로부터 얻은 세균인공염색체 (BAC) 도서관을 공분리하는 CDP 표식자들을 가지고 선발하여 CDP 표식자들을 포함하는 두개의 중복되는 BAC 클론들을 얻었다. 이 BAC 클론들의 완전한 삽입단편의 염기배렬해석 및 배렬특성결정에 의해 하나의 BAC 클론에서 NB-LRR 형의 두개의 완전한 *R* 유전자류사체들 (*RGA9.1* 및 *RGA9.2*)의 존재를 동정하였다. 자체의 전사조절요소들을 포함하는 두개의 저항성유전자류사체들을 long-range PCR 법에 의해 클로닝하고 식물형질전환이원운반체에 삽입하였다. 형질전환에 의한 유전자의 기능성분석(상보성분석)은 *RGA9.1* 이 품종 Desiree 에서 감수성표현형을 저항성으로 바꿀수 있다는것을 보여주었다. *R9a* 로 이름지은 이 유전자는 CC-NB-LRR 단백질을 암호화 한다. *R9a* 유전자에 대한 *Avr* 유전자들을 동정하기 위해 아그로침투에 기초한 효과체선발을 진행하여 *Nicotiana benthamiana* 에서 *R9a*-중개의 과민감반응을 시동하는 *Avrblb2* 상동체들을 발견하였다 (**제 5 장**). 54 개의 역병균그루들을 가지고 진행된 저항성스펙트르분석은 Ma*R9* 및 *S.x edinense* 계통들이 *Rpi-blb2* 유전자를 가지고 있는 품종 Bionica 와 비슷한 저항성스펙트르를 가진다는것을 보여주었다. 저항성스펙트르가 넓어지고 지속저항성을 가지며 유전자전이작물과 관련된 생물안전성문제들을 극복한 저항성이 매우 높은 유전자변이감자를 얻기 위하여 무항생제조건하에서 선발표식자 없는 두개 유전자의 구조물 (*Solanum venturii* 로부터 *Rpi-vnt1, Solanum stoloniferum* 로부터 *Rpi-sto1*)을 가지고 *Agrobacterium* 형질전환을 4 개의 감자품종들 (Atlantic, Bintje, Potae9, Doip1)에서 진행하였다 (**제 6 장**). 5 개의 역병균을 가지고 비형질전환품종들에서 진행된 저항성스펙트르분석은 품종 Potae9 와 Doip1 이 이미 일련의 균들에 저항성을 나타낸다는것을 보여주었다. 4 개의 모든 품종들에서 한개의 *Rpi* 유전자를 가지는 유전자전이식물체들을 얻었으며 대조식물체로 리용되였다. 항생제선발표식자가 없는 조건에서 두개의 *Rpi* 유전자들을 포함하는 구조물을 가지고 진행된 아그로박테리움형질전환 (cisgenesis) 을 식물체분화 및 형질전환 빈도, 운반체골격의 통합, 꼬삐수에 관하여 카나마이신도움선발 (transgenesis) 과 비교하였다. *Avr* 유전자들의 아그로침투, 잎검정법을 통하여 모두 8 개의 기능성의 동질유전자전이감자클론들을 선발하였으며 이 클론들은 저항성유전자들의 집적으로 인한 명백한 보완성의 저항성을 나타냈다. 마감장 (**제 7 장**) 에서는 감자품종에 지속저항성을 부여하기 위해 저항성유전자들을 선택하고 조합하며 농업실천에 리용하는데서 가장 좋은 또는 유망한 방도가 무엇인가 하는 문제들을 우리의 연구자료에 근거하여 론리적서술을 주었다.

Samenvatting

De aardappel, die steeds vaker gebruikt wordt als consumptiegewas, wordt al geruime tijd bedreigd door *Phytophthora infestans*, de veroorzaker van de beruchte aardappelziekte. Wereldwijd blijft deze ziekte de belangrijkste beperkende factor in veel aardappel producerende regio's. De aardappelziekte kan het loof en de knollen vernietigen als de weersomstandigheden gunstig zijn voor het vestigen en verspreiden van een epidemie. Hoewel deze ziekte te controleren is met het spuiten van fungiciden, zijn de toedieningen kostbaar voor de teler, schadelijk voor mens en omgeving en bovendien kan de ziekteverwekker resistentie ontwikkelen tegen de toegediende middelen. De wereldwijd toegenomen genetische diversiteit van *P. infestans* populaties, als gevolg van sexuele recombinatie tussen twee paringstypen en het ontstaan van fungicide resistente stammen brengt de noodzaak met zich mee om aardappelrassen te ontwikkelen die een hoge mate van duurzame resistentie hebben waardoor het gebruik van fungiciden drastisch beperkt kan worden. De aardappel klonen Ma*R8* en Ma*R9* uit de Mastenbroek tester set, waarmee virulentie tegen bepaalde *R* genen vastgesteld kan worden, staan bekend om hun sterke en duurzame resistentie tegen *P. infestans*. Dit geldt ook voor het ras Sarpo Mira dat zijn resistentie in het veld al verschillende jaren behoudt zonder dat fungiciden gespoten worden. Het ophelderen van de genetische factoren achter zo'n min of meer natuurlijk gevormde ziekteresistentie is een voorwaarde om tot duurzame resistentie in veredelingsprogramma's te komen. In dit onderzoek zijn de tester klonen Ma*R8*, Ma*R9* en Sarpo Mira gebruikt om de achtergrond van zo'n duurzame resistentie op te helderen. Allereerst zijn er F1 populaties, gemaakt door deze resistente klonen met vatbare ouders te kruisen, getest op hun resistentie tegen de aardappelziekte onder zowel veld- als laboratorium (detached leaf assay; DLA) omstandigheden waarbij geïnoculeerd werd met het incompatibele *P. infestans* isolaat IPO-C. In de Ma*R8* afgeleide F1 populatie werd een 1:1 uitsplitsing van resistentie en vatbaarheid gevonden in veldtoetsen maar niet in DLA toetsen. NBS profiling en *R* gen cluster gerichte "profiling" (cluster directed profiling; CDP) gevolgd door het laten "landen" van deze merkers in het recent opgehelderde aardappel genoom, heeft geleid tot het karteren van *R8* op een nieuwe genetische plek op chromosoom 9 en niet op chromosoom XI zoals in eerder onderzoek gesuggereerd werd (**Hoofdstuk 2**). De *R* gen gemedieerde resistentiereactie in aardappel gaat gepaard met celdood (overgevoeligheidsreactie; HR) en is het gevolg van een (in)directe interactie tussen het product van het *R* gen en het product van het bijbehorende avirulentie (*Avr*) gen van het pathogeen. Middels agroinfiltratie hebben we een grote collectie "RXLR

effectoren" van *P. infestans* gescreened op de mogelijkheid om celdood uit te lokken in de Ma*R8* plant (**Hoofdstuk 3**). De effector PITG_07558, omgedoopt tot AVR8, gaf deze specifieke HR reactie die steeds samen uit splitste met de *R8* resistentie tegen het *P. infestans* isolaat IPO-C in de betreffende F1 populatie. Het bleek dat *Avr8* identiek was aan de effector *AvrSmira2* waarvan eerder een associatie was aangetoond met het *Rpi-Smira2* gen dat in dit ras veldresistentie geeft. Na kruising van het ras Sarpo Mira met een vatbare plant liet genetische kartering in deze F1 populatie zien dat *Rpi-Smira2* inderdaad in het *R8* gebied ligt. Om de geografische oorsprong en afstamming van *R8* in de *Solanum* soorten terug te zoeken, is de reactie op AVR8 getest in 98 genotypen (72 accessies van 40 wilde soorten) van *Solanum* sectie Petota. Er werden twaalf accessies gevonden, afkomstig uit zuid- en midden Amerika, die op AVR8 reageerden. Uit het onderzoek aan tester set plant Ma*R9* bleek dat de *R9* resistentie ook op chromosoom 9, dicht bij de *R8* resistentie lag (**Hoofdstuk 4**). Door een geïntegreerde benadering waarin gebruik werd gemaakt van 1. *R* gen "ontstapeling" middels specifieke *R* gen merker analyse en AVR responsen, 2. een resistentietoets van de gehele plant in een klimaatcel, en 3. fijnkartering middels CDP, werd een duidelijk beeld verkregen van twee nauw gekoppelde genen, *R9a* en *R9b*. Verder werd aangetoond dat *R9a* in een cluster ligt van NB-LRR genen met *Tm-2* homologie en waarschijnlijk zelf onderdeel is van deze familie (**Hoofdstuk 4**). De geïdentificeerde *Tm-2*^{2} achtige CDP merkers, die volledig samen uitsplitsten met de *R9a* resistentie werden gebruikt om *R9a* bevattende DNA fragmenten (BAC klonen) te selecteren. Deze aanpak illustreerde de mogelijkheden die CDP biedt om sterke merkerverzadiging in geselecteerde chromosomale gebieden te bereiken (**Hoofdstuk 5**). Om het *R9a* gen te kloneren werd een BAC kloon bank, afgeleid van Ma*R9*, gescreened op de aanwezigheid van de twee CDP merkers die volledig samen uit splitste met de *R9a* resistentie. Twee overlappende BAC klonen werden geselecteerd met de twee CDP merkers. Annotatie van de sequentie van de inserts van deze BAC klonen toonde de aanwezigheid van twee complete *R* gen analogen (RGA) van de NB-LRR klasse, *RGA9.1* en *RGA9.2*, aan in het insert van één van beide BACs. Middels "long range" PCR werden deze twee RGAs, inclusief hun natuurlijke transcriptioneel regulatoire elementen, gesubcloneerd in een binaire vector voor plant transformatie. Na transformatie werd duidelijk dat *RGA9.1* het vatbare fenotype van het ras Desiree kon complementeren. *RGA9.1*, omgedoopt tot *R9a*, codeert voor een CC-NB-LRR eiwit uit de Tm-2 familie waarin de LRR consensus sequentie maar losjes past. Een agroinfiltratie gebaseerde effector screen, gericht op de identificatie van de *Avr* die bij *R9a* hoort, werd uitgevoerd en leidde tot de ontdekking dat *Avrblb2* homologen een *R9a* gemedieerde overgevoeligheidsreactie in tabak uit konden lokken (**Hoofdstuk 5**).

Een resistentie spectrum profilering uitgevoerd met 54 *P. infestans* isolaten toonde aan dat Ma*R9* en *S. edinense* planten een vergelijkbaar spectrum hebben als het *Rpi-blb2* bevattende ras Bionica. Transformatie van aardappel met resistentiegenen en een antibioticumresistentie merker stuit op bezwaren van de consument. Deze bezwaren zijn aanzienlijk kleiner als alleen de plant gerelateerde resistentiegenen uit kruisbare soorten gebruikt worden. Zulke genen uit kruisbare soorten worden cisgenen genoemd. Voor het genereren van cisgene aardappels met een breder resistentie spectrum, mogelijk leidend tot duurzame resistentie, werd een *Agrobacterium* gemedieerde merkervrije transformatie, gevolgd door PCR selectie op de ingebrachte resistentiegenen, uitgevoerd. Op deze manier werden vier aardappelrassen (Atlantic, Bintje, Potae9 en Doip1) succesvol voorzien van een construct waarop twee cisgene resistentiegenen liggen (*Rpi-vnt1* van *Solanum venturii* en *Rpi-sto1* van *Solanum stoloniferum*) (**Chapter 6**). Resistentie onderzoek met vijf *P. infestans* isolaten van de niet getransformeerde rassen liet zien dat de rassen Potae9 en Doip1 al resistent waren tegen bepaalde isolaten. Van alle vier de rassen werden transgene planten met enkelvoudige resistentie genen gemaakt die als referentieplant dienden. Merkervrije transformatie met een construct met twee *R* genen (cisgenese) werd vergeleken met kanamycine gestuurde transformatie (transgenese) op grond van regeneratie en transformatie frequentie, ongewenste klonerings vector integratie (vector backbone) en T-DNA kopie getal. Ook werd er naar het tijdspad gekeken waarlangs PCR positieve scheuten met één of beide *R*genen geoogst werden. Door middel van verdere analyses zoals fenotypische evaluatie in de kas, AVR reacties en resistentie in DLA toetsen, werden acht cisgene planten geselecteerd. Twee cisgene planten, afgeleid van het ras Atlantic, en vier cisgene planten afgeleid van het ras Bintje hadden een breed resistentie spectrum verkregen door de activiteit van de beide ingebrachte *R*genen. De twee transformanten van het ras Potae9 lieten een verbreding van het bestaande resistentie spectrum zien door de toevoeging van de twee *R* genen. Uiteindelijk, werden de uitkomsten van dit onderzoek aangaande de genetische en moleculaire ontrafeling van duurzame resistentie tegen *P. infestans* en de toepassing van cisgenese bediscussieerd om de uitdaging tot het ontwikkelen van een duurzaam resistent aardappelras aan te kunnen gaan (**Hoofdstuk 7**). Aanwijzingen voor duurzaamheid worden gezocht in de aard van de *R* genen en de *Avrs* die herkend worden. Een vergelijkende analyse van diverse eigenschappen van beschikbare R-Avr paren laat zien dat de variatie in het kopiegetal in het *P. infestans* genoom en de veelvuldigheid van het *Avr* gen in de *P. infestans* populatie belangrijke indicatoren kunnen zijn voor duurzaamheid. Aan de andere kant kunnen *R* genen met een breed resistentie spectrum, die vaak meerdere functionele homologen hebben in

verschillende *Solanum* accessies, in stapelingen gecombineerd worden om duurzaamheid te bewerkstelligen. Meerjarige virulentie monitoring op locatie toont aan dat stapeling van breed spectrum *R* genen en soms zelfs stapeling van doorbroken *R* genen op kunnen tellen tot zeer hoge resistentie niveaus die meer duurzaamheid zouden kunnen brengen in commerciële aardappelrassen. Het aanbrengen van een complementair resistentie spectrum in bestaande rassen door de cisgene introductie van twee *R* genen, zoals hier beschreven, ondersteunt een eerste stap in die richting.

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About the author

Kwang Ryong Jo was born on October 30th, 1970 in North Korea. He studied molecular biology at the Agricultural University, Pyongyang, North Korea, from 1987 to 1993. He acquired his BSc in Plant breeding in 1993. He obtained his MSc degree in crop genetics and genomics at the Academy of Agricultural Sciences, in 2001. During his master his six-month-internship was carried out at the plant biotechnology laboratory, Institute of Genetics, Beijing, China. He also had the opportunity to receive another internship and educational excursions in Germany for four months of the year 2002. He worked for the Research Institute of Agrobiology, Academy of Agricultural Sciences, Pyongyang, North Korea, in the field of crop biotechnology. In 2010, he started a sandwich PhD at WageningenUR Plant Breeding under the supervision of Prof. Evert Jacobsen and Dr. Jack Vossen in the Netherlands and Dr. Tok-Yong Kim in North Korea. This program was financially supported by the European Commission (EuropeAid project DCI-FOOD/2009/218-671), and the Dutch Ministry of Agriculture, Nature and Fisheries (International Cooperation projects BO-10-010-112 and BO-10-001-200).