The *Phytophthora infestans* avirulence gene *PiAvr4* and its potato counterpart *R4*

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General introduction

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INTRODUCTION

The central themes in this thesis are the avirulence (*Avr*) gene *PiAvr4* in the oomycete pathogen *Phytophthora infestans* and recognition in potato of the Avr4 protein mediated by the resistance gene *R4*. *P. infestans* causes late blight on potato and tomato and is the pathogen that was responsible for the Irish potato famine in the mid-nineteenth century. It is one of many oomycete species that causes severe problems, not only in agriculture but also in natural environments and on indigenous trees and shrubs. *Phytophthora* and other oomycetes have been the subject of numerous investigations but in spite of intensive study, the tools and tactics exploited by these successful plant pathogens are still enigmatic. In recent years genomics boosted oomycete research, and genomic data uncovered a treasure trove for plant pathologists, genome biologists and evolutionary biologists alike. A major breakthrough was the discovery of the RXLR-dEER class of effector proteins. This introduction presents some of the latest discoveries and insights in oomycete biology and pathology. The first part gives a brief overview of oomycete plant pathogens, highlights the species that currently feature as model organisms and mentions some of the tactics used by plants to defend themselves against oomycetes. Subsequently we summarize the genomic resources available for oomycete research and the molecular tools that can be used to study gene function. We also describe how genomics has accelerated gene discovery. The last section of this chapter presents the scope of this thesis.

BIOLOGY AND PATHOLOGY OF OOMYCETES

Branches in the tree of life

Oomycetes, also known as water molds, resemble fungi in many ways. Like fungi, oomycetes have a global distribution and prosper in quite diverse environments. They can live as (hemi-) biotrophic or necrotrophic pathogens in association with plants (see Box 1), animals, or other microbes, but also as saprophytes feeding on decaying matter. Currently, at least 800 oomycete species are known but depending on the definition of a species this number might actually reach 1500 (Dick, 2001). Nevertheless, the species richness seems low when compared to the number of fungal species known to date; 30,000 basidiomycete species have been described and ascomycetes reach a similar number (James et al., 2006; Kirk et al., 2001). It is likely that there are many oomycetes out there yet to be discovered. In this respect the genus *Phytophthora* is illustrative. In the last ten years at least 18

new species have been described expanding the genus to nearly 95 members (Blair et al., 2007; http://PhytophthoraDB.org, accessed December 2008).

Box 1. Lifestyles of plant pathogenic oomycetes

Plant pathogens can be divided into groups based on the different strategies they employ to colonize plants (Agrios, 2004). Obligate biotrophs grow and reproduce in living plant tissue and obtain energy by uptake of plant nutrients. They are entirely dependent on living cells and can therefore only survive by establishing a nondestructive relationship with their host. Among the obligate biotrophic oomycetes are the downy mildews (e.g. *Hyaloperonospora parasitica* and *Bremia lactucae*) and white rusts (e.g. *Albugo candida*). Necrotrophic pathogens feed on dead cell material. Before colonizing plant tissue, necrotrophs kill cells by secreting toxins or cell wall degrading enzymes. A third strategy is an intermediate lifestyle called hemibiotrophy. The first stage of hemibiotrophy is based on an intimate relationship of the pathogen with its host. Later in the infection process, hemibiotrophic pathogens switch from their initial biotrophic to a destructive necrotrophic lifestyle. The oomycete genera *Pythium* and *Phytophthora* comprise both necrotrophs and hemibiotrophs.

Due to their shared morphology (filamentous, branched somatic structures that bear spores) oomycetes and fungi were traditionally classified in the same kingdom, the Fungi (Erwin and Ribeiro, 1996). In the 'Five Kingdom' classification of Margulis and Schwartz (2000) the two groups were separated over two kingdoms: Protoctista and Fungi, the first being a highly diverse group including both unikonts and bikonts. In recent years new insights based on molecular phylogeny and comparative genomics have again reshaped the tree of life. In 2005 Keeling et al. (2005) launched the five supergroups of eukaryotes. In this classification the ascomycete and basidiomycete fungi are grouped together with animals and Amoebozoa (e.g. slime molds) in the supergroup Unikonts (Fig. 1). Oomycetes with their biflagellated zoospores are typical bikonts. They fall within the supergroup Chromalveolates and the lineage Stramenopiles (Cavalier-Smith, 1999). Since the stramenopile lineage also contains photosynthetic organisms like diatoms and brown algae it is thought that the common ancestor is a photosynthetic life-form that obtained its chloroplast by secondary endosymbiosis, presumably from red algae in the bikont supergroup Plantae (Cavalier-Smith, 2002) (Fig. 1). The loss of plastids in the non-photosynthetic stramenopile taxa is supported by whole genome comparisons that revealed numerous photosynthesis-related genes shared by *Phytophthora* and diatoms (Tyler et al., 2006). Most of these genes encode mitochondria-targeted proteins with close homology to proteins targeted to chloroplasts in photosynthetic organisms.

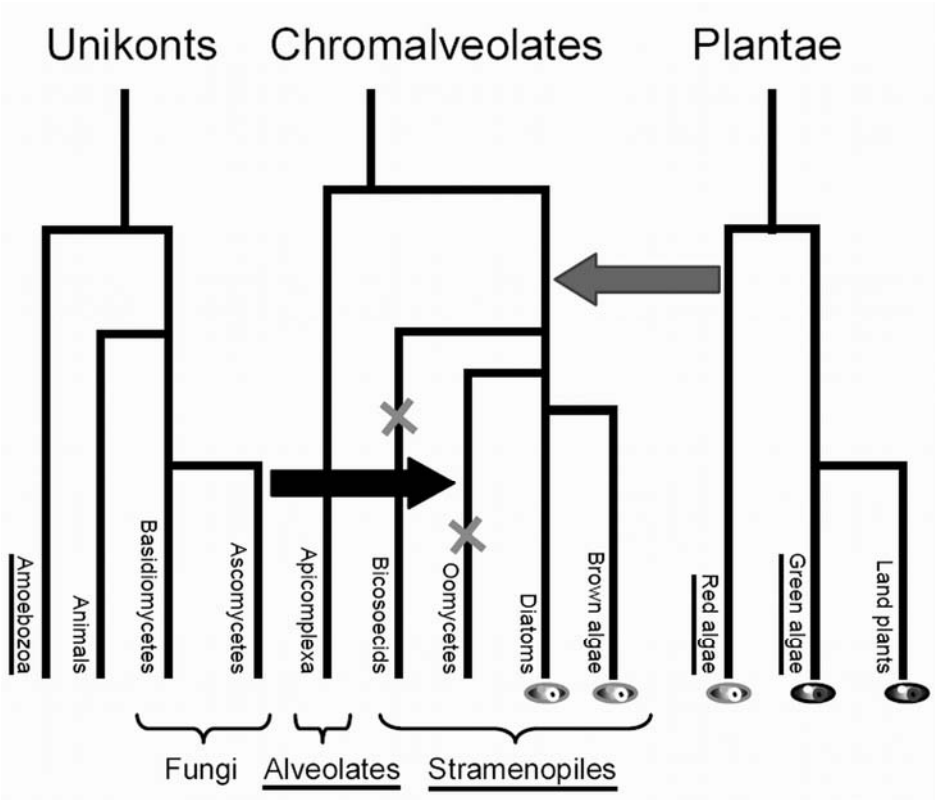

Figure 1. Plastid gain-and-loss and intra-eukaryotic horizontal gene transfer (HGT). This simplified phylogenetic tree shows three of the five eukaryotic supergroups as defined by Keeling et al. (2005). Oomycetes and Apicomplexa (a.o. *Plasmodium)* belong to the supergroup Chromalveolates. The supergroup Unikonts comprises, a.o., animals and fungi, and the supergroup Plantae includes red and green algae, and land plants. Two of the four bikont supergroups, Rhizaria and Excavates, are not shown. For simplicity, not all lineages are depicted. Major branches are underlined. During evolution the Chromalveolates acquired a photosynthetic plastid (👁) most likely originating from a red alga (grey arrow). This plastid was lost in the oomycete lineage (**x**) but retained in other chromealveolates that are phototrophic, e.g., the brown alga *Laminaria digitata* (kelp). *Phytophthora* genomes still carry many genes reminiscent of a phototrophic origin (Tyler et al., 2006). The black arrow represents a horizontal gene transfer event from ascomycetes to oomycetes. Genes found in oomycetes that are thought to have a fungal origin have putative functions in osmotrophy (Richards et al., 2006).

Endosymbiosis can explain how genes with high similarity to bacterial genes end up in eukaryotes. In contrast, the processes underlying horizontal gene transfer (HGT) among eukaryotes are not so easy to trace. Nevertheless, as more and more genomes are being sequenced evidence for intraeukaryotic HGT is accumulating. In a thorough study Richards et al. (2006) found strong evidence for HGT from ascomycete fungi to oomycetes. The genes involved presumably have functions related to an osmotrophic lifestyle and could explain convergent evolution of osmotrophy coupled to filamentous growth in two distinct eukaryotic lineages. Convergent evolution probably also shaped the pathogenic behavior of oomycetes and fungi but whether, and to what extent, HGT is involved is still questionable (Latijnhouwers et al., 2003).

Table 1 Oomycete diseases and model systems

Species	Host plants	Disease type	Lifestyle	Model species
Phytophthora infestans	Potato and tomato	Late blight	hemi-biotrophic	yes
Phytophthora sojae	Soybean	Damping-off and root rot	hemi-biotrophic	yes
Phytophthora ramorum	Several trees and bushes (e.g. oak, rhododendron)	Sudden oak death, canopy dieback	hemi-biotrophic	
Phytophthora nicotianae **syn.** ***Phytophthora parasitica***	Multiple hosts, including citrus, castor bean and tobacco	Leaf and stem blight, root rot	hemi-biotrophic	
Phytophthora brassicae	Several brassicaceous plants, including *Arabidopsis thaliana*	Leaf blight	hemi-biotrophic	potential
Phytophthora capsici	Multiple hosts, including cucurbits and *Capsicum* peppers	Leaf blight, fruit, stem and root rot	hemi-biotrophic	potential
Phytophthora palmivora	Large host range, including cacao and rubber tree	Leaf blight, fruit and root rot	hemi-biotrophic	
Phytophthora cinnamomi	Extremely large host range, exceeding 3000 species, including several crops	Root rot, dieback	necrotrophic/ saprophytic	
Albugo candida [a]	Several brassicaceous plants, like mustard and *A. thaliana*	White rust	obligate biotrophic	
Bremia lactucae	Lettuce and several close related species	Downy mildew	obligate biotrophic	
Hyaloperonospora parasitica	Several brassicaceous plants, including *A. thaliana*	Downy mildew	obligate biotrophic	yes
Plasmopara viticola	Grapevine	Downy mildew	obligate biotrophic	
Plasmopara halstedii	Asteraceae, including sunflower	Downy mildew	obligate biotrophic	

Table 1 Continued

Species	Host plants	Disease type	Lifestyle	Model species
Peronospora destructor	Monocots of the *Allium* family, like onion and garlic	Downy mildew	obligate biotrophic	
Pseudoperonospora cubensis	Several Curcubitaceae, like cucumbers	Downy mildew	obligate biotrophic	
Sclerospora graminicola	Several monocots, maize, sorghum and pearl millet	Downy mildew	obligate biotrophic	
Pythium ultimum	Multiple dicots (e.g. potato) and monocots (e.g. turf grass)	Damping-off	necrotrophic	
Aphanomyces euteiches [b]	Several legumes, including peas, alfalfa, *Medicago truncatula* and clover	Root rot	necrotrophic	potential
Aphanomyces cochlioides [b]	Sugar beet	Root rot	necrotrophic	

[a] Belongs to the order Albuginales.
[b] Belongs to the order Saprolegniales.

Typical features of oomycetes

Molecular phylogeny has now firmly established the distinct taxonomic positions of fungi and oomycetes but also before the genomics era mycologists recognized several features that are characteristic for oomycetes (Erwin and Ribeiro, 1996). Among these are cell walls that lack chitin, but are composed of a mix of cellulosic compounds and glycans, hyphae that lack septa (so called coenocytic mycelium) and have diploid nuclei (instead of haploid as found in fungi), stacked Golgi cisternae (versus unstacked in fungi), tubular mitochondrial cisternae (versus disc-like in fungi) and sterol auxotrophy. Most characteristic for oomycetes are the zoospores, the free-swimming asexual spores that are propelled by two unequal flagellae and explain why a moist environment is most favorable for these water molds. One of the flagella has lateral hair-like structures called mastigonemes that contain the ß-1,3-glucan mycolaminarin, an energy storage molecule that is also found in brown algae and diatoms (Feofilova, 2001). Literally, oomycetes means “egg fungi”, a name based on the egg-shaped resting spores, named oospores. Oomycetes can be either homothallic or heterothallic. Sexual reproduction is initiated upon release of hormones that trigger the formation of gametangia (♀

oogonium and ♂ antheridium), in which meiosis takes place. The diploid oospores are produced as a result of oogamous fertilization when a haploid oosphere fuses to a haploid gamete. Thick-walled oospores are most durable propagules that can survive harsh environmental conditions and are important for the generation and maintenance of genetic variation in a population. For a recent review on sexual reproduction in oomycetes see Judelson (2007).

Oomycete diseases and model systems

The plant pathogenic oomycetes are remarkably diverse and exhibit lifestyles ranging from obligate biotrophic to necrotrophic (Box 1; Table 1). Oomycete diseases occur on nearly every agricultural crop across the globe and many of the economically important species were spread unwittingly by humans. The late blight pathogen *Phytophthora infestans,* renowned for its impact on history, caused the dreadful Irish potato famine in the 1840s. The journey from its origin to Europe and the subsequent global distribution was undoubtedly supported by potato trading. For a long time Mexico was considered as the center of origin of *P. infestans* but recent findings raise doubts and point to South America (Gómez-Alpizar et al., 2007; Grünwald and Flier, 2005). In the late 1870s another severe oomycete disease appeared in Europe when lice-resistant (*Phylloxera*) grapevine rootstocks were imported from the US to France. Unfortunately, the rootstocks carried with them the downy mildew pathogen *Plasmopara viticola*. The disease was first observed in France in 1878 and in successive years it was found in almost every vineyard in Europe (Gobbin et al., 2006). Another oomycete first described in the nineteenth century is *Bremia lactucae,* the causal agent of lettuce downy mildew (Regel, 1843). *B. lactucae* probably originates from Eastern Europe and the Near East, where the majority of the wild lettuce species is found. Nowadays, lettuce downy mildew occurs worldwide and is one of the major problems in lettuce cultivation. During the history of lettuce breeding several resistance (*R*) genes have been introgressed into commercial cultivars. However, the resistance did not last, probably due to rapid genetic adaptation of the pathogen (Lebeda and Zinkernagel, 2003). Defeat of resistance by oomycete pathogens is not uncommon. Also *P. infestans* is notorious in that respect (Wastie, 1991).

Unintended movement of oomycete plant pathogens is also the cause of severe diseases that disturb complete ecosystems. One illustrative example is Sudden Oak Death (SOD) caused by *Phytophthora ramorum.* This species originates from the Chinese highlands, and most likely made its entry into Europe, Northern America and Australia via trade of ornamental plants (Goheen et al., 2006; Werres et al., 2001). In California, SOD emerged about a decade ago and killed many of the tanoaks (*Lithocarpus* spp.) and coast live oaks (*Quercus*

agrifolia) that dominate in coastal forests. This disease not only harms its host plants but also indirectly changes avian species diversity (Monahan and Koenig, 2006). Another example is dieback in native Australian woods caused by *Phytophthora cinnamomi.* This species has an extremely wide-host range (over 3000 plant species) and is believed to have originated near Papua New Guinea but now has a worldwide distribution. It was accidentally introduced into Australia around the 1920s. Dieback is threatening multiple endangered 'red list' plant species (http://www.iucnredlist.org) and also has a severe impact on some agricultural crops (Hardham, 2005; Shearer et al., 2007).

Since *Phytophthora* diseases are so important and wide spread it is not surprising that they are intensively studied from different angles, the front runners being *P*. *infestans* (potato blight) and *Phytophthora sojae* (soybean root and stem rot). Also downy mildews are favorite subjects of study, in particular in the area of molecular plant-microbe interactions. However, their obligate nature makes these organisms more difficult to handle. The many destructive *Pythium* species (> 125) are mostly soil-borne pathogens and primarily necrotrophs, and hence less attractive as a model for studying the intimate relation between host and pathogen. Table 1 gives an overview of a variety of oomycete pathogens. To choose a pathosystem that fulfills all requirements for a model system for molecular plant-microbe interaction research is nearly impossible. In a recent review Lamour et al. (2007) compared the five species for which the genomes have been sequenced - *P*. *sojae*, *P*. *ramorum*, *P*. *infestans*, *Phytophthora capsici* and *Hyaloperonospora parasitica* -, and listed the pros and cons of these five as model organisms for oomycete research. Attractive features include the high potential of *P*. *capsici* for genetic studies*,* the capability of *H*. *parasitica* to infect the model plant Arabidopsis and gene inactivation in *P*. *infestans* by gene silencing. In their comparison Lamour et al. (2007) did not consider *Phytophthora brassicae.* It is true that its genome has yet to be sequenced but its potential as model species lies in the fact that, similar to *H. parasitica,* it is a pathogen of Arabidopsis and that different strains show differential interactions with various ecotypes (Roetschi et al., 2001). The advantage over *H*. *parasitica* is its non-obligate nature and amenability to DNA transformation (Si-Ammour et al., 2003). In that respect, the non-obligate *Aphanomyces euteiches* also has the potential to become a model species, because one of its hosts is *Medicago truncatula* which is a model species for legumes and research on beneficial plant-microbe interactions (Gaulin et al., 2007).

PLANT DEFENSES AGAINST OOMYCETES AND OTHER PATHOGENS

Plants have developed several defense responses towards invading oomycetes and other pathogens. At the molecular level they include degrading enzymes that are targeted against pathogen-derived compounds, pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), and R proteins that recognize race-specific Avr factors. The recognition of a PAMP as a non-self molecule results in PAMP-triggered immunity (PTI). Typical PAMPs, or microbe-associated molecular patterns (MAMPs), are integral parts of the pathogen cell architecture, mostly cell wall components and are often conserved across species. A microbial pathogen can not easily discard PAMPs and will produce effectors to suppress PTI. The deployment of these effectors subsequently results in effector-triggered susceptibility (ETS). As defense against these effectors plants have evolved molecular receptors, R proteins that in turn mediate effector-triggered immunity (ETI). The co-evolution of pathogen effectors and host R proteins is described in the zig-zag model (Jones and Dangl, 2006) (Fig. 2). R proteins and effectors - the latter are named Avr factors when recognized by R proteins- interact in a gene-for-gene manner; one particular *R* gene is responsible for the recognition of a single *Avr* gene product. Contrary to PAMPs, most Avr factors can easily be modified without a direct penalty on the pathogen's viability or even its virulence on a susceptible host. Therefore, pathogens can continuously adapt their effectors to evade recognition, which in the plant will result in the co-evolution of *R* genes. *R* genes against oomycete plant pathogens have been identified in a wide range of plant species, and several have been cloned.

Based on their sequence and overall structure plant R proteins can be divided in six major groups (Martin et al., 2003). Two groups encode a wide range of unrelated proteins including the first cloned *R* gene, *Hm1* from maize, which inactivates a toxin of the fungus *Cochliobolus carbonum* (Johal and Briggs, 1992). A third group contains only a single gene, the rice *Xa21* gene that encodes a receptor-like kinase resembling PRRs, and mediating resistance to *Xanthomonas oryzae* (Song et al., 1995). A fourth group contains the tomato *Cf* genes that code for receptor-like proteins (RLPs). Most *R* genes belong to the remaining two groups which contain genes that encode nuclear binding site-leucine rich repeat (NBS-LRR) proteins, one with an N-terminal Toll and Interleukin 1 receptor (TIR) domain and the other with an N-terminal coiled-coil (CC) domain. All *R* genes that have been described to mediate resistance to oomycete plant pathogens encode NBS-LRR proteins (Ballvora et al., 2002; Bhattacharyya et al., 2005; Bittner-Eddy et al., 2000; Botella et al., 1998; Gao et al., 2005; Huang et al., 2005a; McDowell et al., 1998; Parker et al., 1997; Sandhu et al., 2004; Shen et al., 2002; Slusarenko and

Schlaich, 2003; Song et al., 2003; van der Vossen et al., 2003; van der Vossen et al., 2005; Wroblewski et al., 2007). In recent years a few examples of co-evolution of *R* genes and their cognate oomycete *Avr* genes have been described, and these gene pairs apparently behave as predicted by the zig-zag model (Allen et al., 2004; Rehmany et al., 2005; Jones and Dangl, 2006).

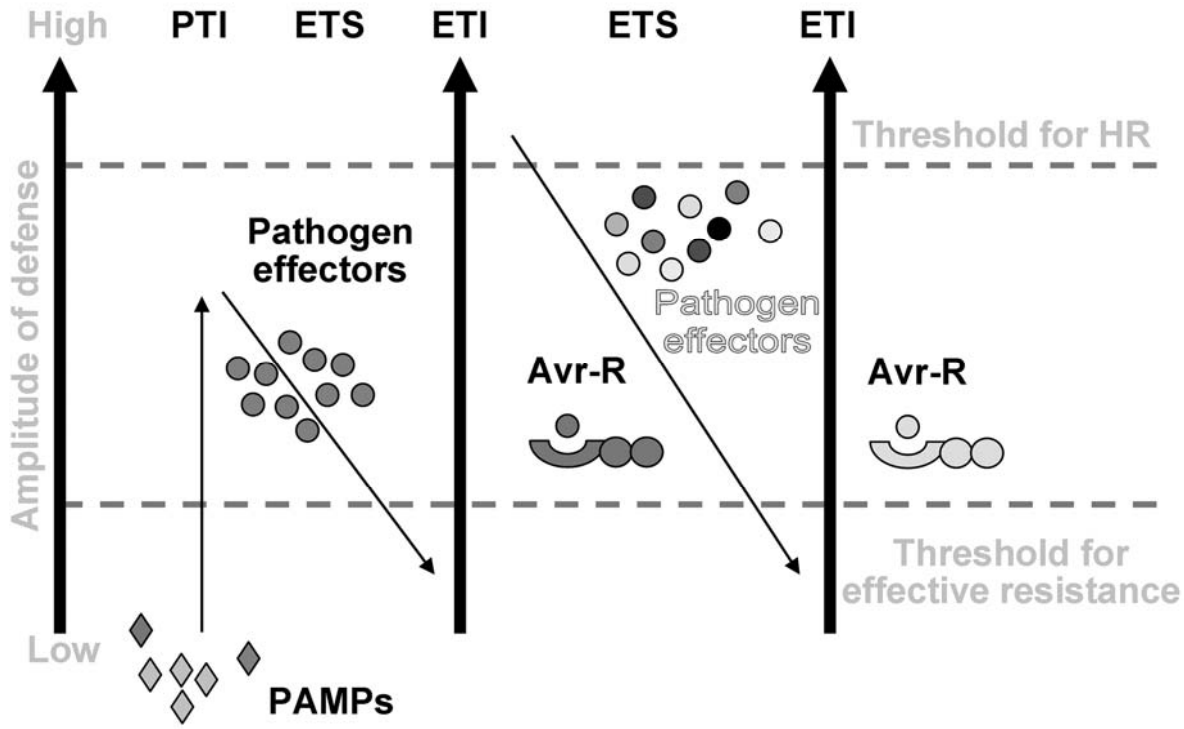

Figure 2. Zig-zag model illustrating the amplitude of defense of a host plant to pathogen attack and the subsequent arms race (adapted from Jones and Dangl, 2006). After pathogen attack the plant recognizes pathogen-associated molecular patterns (PAMPs). This recognition results in PAMP-triggered immunity (PTI) which inhibits pathogen growth. To suppress PTI the pathogen delivers effectors resulting in loss of resistance in the plant or effector-triggered susceptibility (ETS). In response plant resistance proteins (typically NBS-LRR proteins) will specifically recognize these pathogen effectors. The latter results in an effector-triggered immunity (ETI) which can be observed as a hypersensitive response (HR). Subsequently, the pathogen will modify its effectors to evade ETI which forces the plant to evolve new resistance specificities.

GENOMIC RESOURCES

The rise of genomics has had a major impact on oomycete research. In the early 1990s when the discipline of molecular phytopathology saw the light, research on oomycete pathogens lagged behind. The disadvantage of a diploid genome was one reason; homologous recombination, applicable in many fungi to make gene knock-outs and mutant libraries for reverse genetics, is out of reach for an oomycete researcher. The large genome sizes of oomycetes and hence, the

inability to clearly separate chromosomes on agarose gels (Howlett, 1989; Tooley and Carras, 1992), as well as the lack of easy scorable phenotypic markers for genetic analyses were other barriers that made these organisms less attractive for basic research. Not without reason Shaw referred to *Phytophthora* and related species as 'a nightmare for the fungal geneticist' (Shaw, 1983). These disadvantages though, were a stimulus to search for alternatives and the leap to genomics as an instrument to tackle *Phytophthora* was made in an early stage. As a result, the genomes of five oomycete species have now been sequenced and it is very likely that more will be sequenced in the next few years.

EST and BAC libraries

Genomics activities started off with small scale expressed sequence tag (EST) projects (Kamoun et al., 1999; Qutob et al., 2000) that were followed by the release of more extensive EST datasets (Randall et al., 2005; Torto-Alalibo et al., 2007). The *P. infestans* EST resource with over 94.000 ESTs generated from different isolates, developmental stages and culturing conditions, is still one the largest available for any plant pathogen (http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html, accessed December 2008). Table 2 summarizes the genomic resources for oomycete plant pathogens and lists the number of ESTs currently deposited in GenBank.

BAC libraries have been constructed for *P. infestans* (Randall and Judelson, 1999; Whisson et al., 2001), *P. sojae* (Zhang et al., 2006), *Phytophthora nicotianae* (Shan and Hardham, 2004) and *H. parasitica* (Rehmany et al., 2003). Zhang et al. (2006) obtained the first genome wide physical map of a *Phytophthora* species by integrating *P. sojae* BAC contigs with the genome sequence. BAC libraries have been instrumental for positional cloning, in particular of genes located at *Avr* loci (Allen et al., 2004; Armstrong et al., 2005; Jiang et al., 2006c; Rehmany et al., 2005; Shan et al., 2004). Also, the mating type locus of *P. infestans* was mapped to a 60-70 kb region by exploiting BAC clones (Randall et al., 2003). Several other cloning efforts were aided by the availability of BAC libraries (Laxalt et al., 2002; Meijer et al., 2005), and restriction and sequence analyses of individual BACs provided a first glimpse of the complexity and distribution of gene families (Armstrong et al., 2005; Jiang et al., 2005; Liu et al., 2004) and repeat elements such as tRNA-related short interspersed elements (SINEs), retrotransposons and DNA transposons (Ah Fong and Judelson, 2004; Jiang et al., 2005; Whisson et al., 2005a).

Genome sequencing projects

To date genome sequencing of five oomycetes has been completed (Table 2). Four of these are *Phytophthora* species, number five is *H. parasitica*, a downy mildew species. Their genome sizes vary from 65 up to 250 Mb and are overall larger than those of an average fungal genome. The first oomycete genome sequence project actually handled two species in parallel, *P. sojae* and *P. ramorum* (Govers and Gijzen, 2006; Tyler et al., 2006), enabled the application of powerful analyses based on comparative genomics. This immediately revealed a high level of conserved synteny between the two species; but interestingly, the synteny appeared to be interrupted at loci harboring genes encoding effectors with putative functions in interactions with host plants (Jiang et al., 2006a; Jiang et al., 2008). Preliminary analyses including the three other sequenced genomes suggest that large regions of conserved synteny occur throughout the genus *Phytophthora* and often even extend to *H. parasitica* (R.H.Y. Jiang and H.J.G. Meijer, personal communication). One example of a region of conserved synteny between *P. infestans* and *H. parasitica* that is interrupted by an effector gene (i.e. Avr3a) was published by Armstrong et al. (2005).

The phenomenon of conserved synteny also helps the gene calling process. Over 20 % of the predicted gene models in oomycetes are not known in other species but the existence of orthologs often arranged in similar orders along the scaffolds is an indication that the gene models represent real genes. As shown in Table 2 the predicted number of gene models seems more or less colinear with genome size. Automated gene prediction is mostly based on training sets. The training set of oomycete genes is still relatively small and therefore the current genome browsers often show incorrect models. Handsaker et al. (2007) developed a gene calling program called Orthosearch (http://www.broad.mit.edu/ annotation/ genome/phytophthora_infestans/GeneFinding.html) that makes use of the DNA conservation between *Phytophthora* species and could help to improve the gene prediction process. To avoid inaccurate models ending up in the literature and being propagated it is important to check the coherence of existing data by comparing genome sequences of related species with ESTs and/or proteome data.

Table 2 Genomic resources for plant pathogenic oomycetes

Species	Genome size (Mb)	Gene models	ESTs[a]	Genome sequencing and assembly status	URL[b]
Phytophthora infestans	237	18262	94091[c]	Completed	http://www.broad.mit.edu/annotation/genome/phytophthora_infestans
Phytophthora sojae	95	19027	28357[d]	Completed	http://genome.jgi-sf.org/Physo1_1
Phytophthora ramorum	65	15743	—	Completed	http://genome.jgi-psf.org/Phyra1_1
Phytophthora nicotianae/ Phytophthora parasitica	n.d.	n.d.	553/6328[e]	—	—
Phytophthora brassicae	≈ 75[f]	n.d.	12922	—	—
Phytophthora capsici	65	n.d.	9	In progress	http://www.jgi.doe.gov
Hyaloperonospora parasitica	75	14726	46[g]	In progress	http://genome.wustl.edu/
Plasmopara halstedii	n.d.	n.d.	145	—	—
Pythium ultimum	n.d.	n.d.	9727	—	—
Aphanomyces euteiches	n.d.	n.d.	18684[h]	—	http://www.polebio.scsv.ups-tlse.fr/aphano/
Aphanomyces cochlioides	n.d.	n.d.	3599	—	—

n.d., not determined
[a] http://www.ncbi.nlm.nih.gov/dbEST/.
[b] see also omgn website: http://pmgn.vbi.vt.edu/.
[c] Kamoun et al., 1999; Randall et al., 2005.
[d] Qutob et al., 2000; Torto-Alalibo et al., 2007.
[e] Panabières et al., 2005; Le Berre et al., 2008.
[f] Roetschi et al., 2001.
[g] Casimiro et al., 2006
[h] Gaulin et al., 2008; Madoui et al., 2007.

MOLECULAR TOOLBOX

A prerequisite to investigate gene function is the ability to modify or mutate a gene in the organism from which it originates. As alternative one can express the gene of interest in another organism (heterologous expression) and monitor the activity; for example, complementation of a mutant phenotype in yeast or triggering defense responses in plants. In this section we first describe the state of the art with respect to DNA transformation of oomycetes and the use of reporter genes. Subsequently, we present some of the approaches that have been used to study the functions of oomycete genes.

DNA transformation

The methods that have been described for DNA transformation of oomycetes are primarily based on experiments involving *Phytophthora* species and in particular *P. infestans*. To date five methods are available. The most commonly used transformation method is based on transformation of protoplasts using polyethylene glycol (PEG) and $CaCl_2$, a procedure that is also used for transformation of yeast, fungal, plant and mammalian cells. PEG-mediated transformation has already been described for several oomycete species including *P. infestans* (Judelson et al., 1993; van West et al., 1999a) *Phytophthora palmivora* (van West et al., 1999b), *Phytophthora parasitica* (Bottin et al., 1999) *P. sojae*, *Phytophthora citricola* and *Pythium aphanidermatum* (McLeod et al., 2008). The transformation efficiency ranges from 0.1 and 2 transformants per microgram of DNA. Transformation by means of electroporation of zoospores was successful for *P. infestans* (Latijnhouwers and Govers, 2003) and *Py. aphanidermatum* (Weiland, 2003). The transformation efficiency was comparable to that of PEG-mediated transformation. The third method is *Agrobacterium*-mediated transformation. Using this method Vijn and Govers (2003) obtained stable transformants of *P. infestans*, *P. palmivora* and *Pythium ultimum*. Contrary to PEG transformation, *Agrobacterium*-mediated transformation does not require protoplasts. Instead, a mixture of zoospores and *Agrobacterium* is plated and after co-cultivation for a few days the colonies are transferred to selective medium. In the transformants obtained by this method the transgene copy number is relatively low compared to the other protocols, i.e. one to two copies, while PEG-mediated transformation and zoospore electroporation mostly result in transformants that have multiple copies of the transgene. A fourth method that has been successfully used to transform *P. infestans* is microprojectile bombardment of germinated asexual sporangia, zoospores, and mycelium (Cvitanich and Judelson, 2003). The transformation efficiency of this method is higher than that of the PEG-mediated transformation

(14 transformants per microgram of DNA), but a disadvantage is that the transformants are generally heterokaryons that carry both wild-type and transformed nuclei. More recently a mechanoperforation method was developed for transient transformation of the sunflower downy mildew pathogen *Plasmopara halstedii* (Hammer et al., 2007). This method is particularly interesting, especially when it can be exploited to obtain stable transformants of obligate pathogens, like the downy mildew *H. parasitica*.

Several selectable markers such as geneticin (G-418), hygromycin, and streptomycin are available which allows one to perform multiple transformations on a single isolate. Co-transformation of a plasmid carrying a selectable marker gene together with a plasmid carrying a gene of interest can generate up to 50% of the transformants carrying both plasmids (van West et al., 1999a). Transformation vectors have to contain oomycete promoters and terminators to regulate the expression of the gene of interest. The promoters of the genes *ham34* and *hsp70*, which originate from *B. lactucae*, are suitable for constitutive expression of transgenes both in *P. infestans* and other oomycetes (Judelson et al., 1991).

Reporter genes

So far genes coding for β-glucuronidase (GUS), green fluorescent protein (GFP) and monomeric red fluorescent protein (mRFP) have been used as reporter genes in *Phytophthora*. For example transformants that produce GUS or GFP have been used in infection assays to visualize the infection process (van West et al., 1999b; Whisson et al., 2007) or to quantify pathogen biomass during infection and hence to compare infection efficiencies (Kamoun et al., 1998a). GUS has also been used as reporter gene to monitor the activity of the promoter of the *ipiO* gene (van West et al., 1998) and the *P. infestans* cell cycle regulator *cdc14* (Ah Fong et al., 2007). For the latter several construct were tested that carried truncated versions of the *cdc14* promoter, thereby pinpointing motifs in the promoter that are essential for transcription. The differences in sexual preference of several *P. infestans* strains were investigated by mating GUS producing strains with wild-type strains (Judelson, 1997). Lately, stable transformants carrying chimeric constructs of the effector gene *Avr3a* and the reporter gene *mRFP1*, were used to determine the cellular localization of the mRFP-tagged Avr3a (Whisson et al., 2007).

Gene silencing

One of the approaches to determine gene function is analyzing changes in phenotype in mutants in which the gene of interest has been inactivated (loss of function), either through disruption or through gene silencing. Gene disruption in oomycetes is currently not feasible due to low rates of homologous recombination

during transformation and the diploid state of these organisms. Gene silencing in *Phytophthora* species has been achieved by the introduction of sense, anti-sense and hairpin constructs. In a comparative study of different silencing methods in *P. infestans* Ah Fong et al. (2008) observed that hairpin constructs are the most efficient way of inducing silencing. Silencing has been described for a number of *P. infestans* genes including the elicitin gene *inf1* (Kamoun et al., 1998b; van West et al., 1999a), the heterotrimeric Gα and Gβ subunit genes *Pigpa1* (Latijnhouwers et al., 2004) and *Pigpb1* (Latijnhouwers and Govers, 2003) and the cell cycle regulator gene *cdc14* (Ah Fong and Judelson, 2003). In all cases several transformants in which the target genes were silenced showed the same change in phenotype thereby providing the proof that gene silencing was the cause of the observed mutation. This was also the case with silencing a cellulose-binding elicitor lectin (*CBEL*) gene in *P. parasitica* var. *nicotianae* (Gaulin et al., 2002). Several silenced transformants showed a reduction in the attachment to cellulose as well as increased amounts of polysaccharides that were deposited on the cell wall. Whisson et al. (2005b) developed a method for transient gene silencing in *P. infestans*. *In vitro* synthesized dsRNA was introduced into *Phytophthora* protoplasts and after 12-17 days a reduction in gene expression was observed. Silencing of the *inf1* and the marker gene *gfp* resulted in a decreased production of the elicitin INF1 and decreased green fluorescence respectively. Silencing of the *cdc14* was phenotypically confirmed by a reduction in sporangia formation in silenced strains.

Overall, the efficiency of silencing is highly dependent on the target that one wants to silence. For some genes, such as the *inf1* gene in *P. infestans*, it is relatively easy to obtain silenced transformants (van West et al., 1999a; Ah Fong et al., 2008) whereas for other genes it is much more tedious or even impossible. The many unsuccessful attempts are usually not published but through personal communications (Peter J.I. van de Vondervoort) we know that, for example, the many efforts to silence the elicitin gene *inf2* and the phospholipase D gene *PiPXTM-PLD* in *P. infestans* failed.

TILLING

Another approach to obtain loss of function mutants is targeting induced local lesions in genomes (TILLING). TILLING was initially developed in Arabidopsis and involves random mutagenesis with a mutagen causing point mutations and subsequent screening of the mutant library for mutation in the gene of interest. To obtain homozygous mutants several progeny of the primary mutant are obtained and homozygous mutant progeny is recovered. For Arabidopsis and tomato (McCallum et al., 2000; Menda et al., 2004) several TILLING libraries are now available and these have been used successfully to recover mutants.

The Lamour Lab at the University of Tennessee has taken the initiative to develop TILLING resources for two *Phytophthora* species, *P. sojae* and *P. capsici* (Lamour et al., 2007). In an initial screen of a *P. sojae* TILLING library, mutations were found for the genes *PsojNIP* and *PsPXTM-PLD* and knock-outs were generated from oospore progeny (Lamour et al., 2006). The *PsojNIP* mutants did not show a change in phenotype compared to wild-type strains, but the *PsPXTM-PLD* mutants showed a reduction in mycelial growth. Not all oomycete species are suitable for TILLING. Requirements for TILLING include the possibility to grow a large number of mutant strains and the ability to perform crosses. Hence TILLING is not suitable for *P. infestans* and *P. ramorum* nor for obligate biotrophs such as *H. parasitica,* of which mutant libraries are impractical to maintain.

Heterologous expression systems

Heterologous expression of a gene of interest is a tool to produce large quantities of relatively pure protein. The produced protein can be used to study its biochemical characteristics such as structure, enzymatic activity, effector activity, etc. One major disadvantage of heterologous expression, especially in prokaryotic expression systems, is that posttranslational modifications of a protein can be different when it is produced in a different species. Nevertheless, when the effectors CBEL from *P. parasitica* var. *nicotianae* and INF1 from *P. infestans* were produced in *Escherichia coli* they retained their activity as effector. Both induced necrosis when infiltrated in tobacco leaves (Gaulin et al., 2006; Kamoun et al., 1997).

The methylotrophic yeast *Pichia pastoris* is often used as a eukaryotic heterologous expression system for protein production. For instance, elicitins from *P. infestans* produced in *P. pastoris* were used to validate cell death responses on different *Solanum* accessions (Vleeshouwers et al., 2006). The avirulence activity of the *P. sojae* Avr1b protein produced in *P. pastoris* was monitored by infiltrating it in the leaves of soybean plants with and without the *R* gene *Rps*1b (Shan et al., 2004).

Complementation assays

Another method of determining the function of a given gene is through complementation assays. Such assays require a mutant or a strain that lacks the predicted function of the gene of interest. A few studies describe complementation of gene function in a *Phytophthora* background. In other studies yeast mutants are used as recipient strains. *Avr* genes are the kind of genes that one has to test in a *Phytophthora* background. Two recent papers describe experiments where specific races of *P. infestans* and *P. sojae* are complemented with the genes *Avr3a* and

Avr1b-1 respectively, resulting in a gain of avirulence on *R3a* potato or *Rps*1b soybean plants (Whisson et al., 2007; Dou et al., 2008b). The authors also introduced mutations in certain regions of the *Avr* genes and could thereby determine which regions and which amino acid residues were essential for avirulence function.

Budding yeast (*Saccharomyces cerevisiae*) is a model organism for which many well defined mutants are available. The functionality of a cell cycle regulator encoded by the *Picdc14* gene of *P. infestans* and an H^+-ATPase encoded by *PnPMA1* of *P. nicotianae* was demonstrated by their ability to complement yeast strains mutated in *Cdc14* and *PMA1*, respectively (Ah Fong and Judelson, 2003 (Shan et al., 2006). Also an ABC transporter gene from *P. sojae, pdr1,* was able to partially restore strains that were mutated for the drug resistance transporters PDR5 and SNQ2 gene in yeast (Connolly et al., 2005).

***In planta* expression**

Since most effector proteins of plant pathogenic oomycetes fulfill their function *in planta*, several assays have been developed in which effector activity is monitored by expressing these effector genes in different plant species. For expression of oomycete effector genes *in planta* transient expression assays such as agroinfiltration, also referred to as *Agrobacterium tumefaciens* transient expression assay (ATTA), and agroinfection are standard techniques. Both assays are based on gene transfer mediated by *A. tumefaciens* and often *Nicotiana benthamiana* is used as a host for initial screenings. Typically agroinfection constructs are based on a binary potato virus X (PVX) vector (Jones et al., 1999; Takken et al., 2000). Agroinfection was used to screen *P. sojae* cDNAs for elicitor activity on *N. benthamiana*, a non-host for *P. sojae*, resulting in the identification *PsojNIP*, a gene encoding a necrosis-inducing protein (Qutob et al., 2002). Similarly, a *P. infestans* cDNA library was screened on *N. benthamiana* leading to the identification of *crn1* and *crn2*. Also these two genes encode proteins that elicit necrosis on a broad range of plant species (Torto et al., 2003). Agroinfiltration was used to validate the avirulence function of Avr3a from *P. infestans* (Armstrong et al., 2005). Agroinfiltration has several advantages over agroinfection; it is possible to infiltrate a larger leaf surface; there is no necessity for the intermediate PVX step and it is possible to co-infiltrate a mixture of constructs. Unfortunately, agroinfiltration-based assays are not feasible on plants that respond with a necrosis to infiltration of a wild-type *Agrobacterium* strain as is often observed in potato. Agroinfiltration has been reported for several solanaceous species and lettuce but proved to be less reliable for Arabidopsis (Wroblewski et al., 2005). An advantage of agroinfection is that it can be performed on plants that do not respond to the produced effector. In

these plants a systemic infection will develop and the transgenic virus particles that are produced, which can be harvested and directly inoculated on a plant of interest (Kooman-Gersmann et al., 1997; Kamoun et al., 1999).

An alternative approach for *in planta* expression is the use of biolistics. Co-bombardment was used to show that several *ATR1*^{NdWsB} alleles are differentially recognized by *RPP1* genes from two Arabidopsis accessions (Rehmany et al., 2005). Also, the use of plants containing effector genes as stably integrated transgenes is an option to monitor effector activity and is especially useful when the effector gene is regulated by an inducible promoter. Hitherto, stable *in planta* expression has been used for fungal effector genes (Van Esse et al., 2007) and more recently for oomycete effector genes (Bouwmeester et al., in preparation).

THE IMPACT OF GENOMICS ON GENE DISCOVERY

An incentive to sequence a genome is to accelerate gene discovery. Before the genomics era the quest for the mechanisms that underlie pathogenicity in oomycetes already lead to the identification of a number of oomycete genes and gene families with putative roles in the interaction with host plants. For gene isolation in oomycetes several strategies were used ranging from unbiased approaches, such as differential screening and positional cloning, to more biased approaches aimed at homologs of fungal pathogenicity genes or via reverse genetics starting with the purification of a protein that shows toxic or necrotic activity on a host plant. This section gives examples that demonstrate how genomics has influenced gene discovery in oomycetes.

From expression pattern to gene

The first *Phytophthora* genes to be identified were selected based on expression patterns (Pieterse et al., 1991; Pieterse et al., 1993a). These so called *in planta* induced (*ipi*) genes showed induced or specific expression during growth of the pathogen in association with its host (Pieterse et al., 1993b). Many years later, when comparison with the whole gene repertoire of *Phytophthora* became feasible, one of the *ipi* genes, *ipiO,* turned out to be member of the superfamily of RXLR-dEER effectors. In an elegant bio-informatic analysis, the relation of *ipiO* to another oomycete *Avr* gene, *Avr1b*-1, was shown (Jiang et al., 2008). Similar to several other RXLR-dEER effectors, IPI-O functions as an *Avr* gene in a gene-for-gene manner with the *R* genes *Rpi-blb1* and *Rpi-sto1* (Vleeshouwers et al., 2008).

From protein to gene

Reverse genetics, successfully used to clone several *Avr* genes from fungal plant pathogens (Westerink, 2004), was applied to clone the first elicitin gene named *para1* (Kamoun et al., 1993). Elicitin was initially identified as a highly abundant toxic compound in culture filtrates of *P. parasitica* that causes necrosis on tobacco (Ricci et al., 1989). The elicitin genes *para1* and *inf1,* from *P. parasitica* and *P. infestans* respectively, are single copy genes that encode 10 kDa secreted proteins with a core elicitin domain (Kamoun et al., 1993; Kamoun et al., 1997). From a small scale EST inventory it became evident that the 10 kDa elicitins belong to a larger protein family not only comprising soluble extracellular elicitins but also elicitins anchored to the membrane or cell wall (Kamoun et al., 1999). Subsequently, whole genome sequencing revealed that elicitins (ELI) and elicitin-like (ELL) genes make up a large, complex and highly conserved family with 17 clades that existed prior to the divergence of *Phytophthora* species from a common ancestor (Jiang et al., 2006b). Members of the four ELI clades all have elicitor activity on tobacco comparable to the ELI1 elicitins INF1 and PARA1, whereas ELL proteins are not active as elicitors (A. van 't Slot, P. van de Vondervoort and FG, unpublished). The intrinsic functions of ELI and ELL proteins are still unknown. However, ELIs can bind sterols and since *Phytophthora* is a sterol auxotroph, ELIs possibly function as carrier proteins to acquire sterols from the environment. The whole genome perspective tells us that the simplistic view that a single abundant component secreted in culture filtrate in an artificial environment (i.e. *in vitro* growth) is responsible for elicitin activity in the natural situation should be revisited.

A reverse genetics approach was also used to clone the gene encoding a 42 kDa extracellular glycoprotein from *Phytophthora megasperma* (Sacks et al., 1995) that is now identified as a transglutaminase with the PAMP pep-13 as the active site (Brunner et al., 2002). Other examples are the elicitors CBEL from *P. parasitica* (Mateos et al., 1997) in which two cellulose binding domains (CBDs) act as PAMPs (Gaulin et al., 2006), and NPP1 from *P. parasitica,* another putative PAMP (Fellbrich et al., 2002), belonging to the class of Nep1-like proteins or NLPs (Necrosis and ethylene inducing protein-like proteins). NLPs have a wide distribution across taxa and a broad spectrum of activity on plants (Gijzen and Nürnberger, 2006). In bacteria and fungi NLPs are encoded by one or two genes but, in contrast, in *Phytophthora* species large families of NPP genes are found (Tyler et al., 2006). As a last example we mention the toxin PcF from *Phytophthora cactorum* (Orsomando et al., 2001). Again a single gene was cloned by reverse genetics but genomics data revealed a much more complex situation. *P. infestans* homologs of PcF were named Scr74 (secreted cysteine-rich protein). The *scr74* family has at least ten members, some of which are clustered in the genome. They

are highly polymorphic and under diversifying selection (Liu et al., 2004). Also in *P. sojae*, *PcF* is a multicopy family but in *P. ramorum* very few *PcF* genes are detected (Tyler et al., 2006).

From homolog to gene

The infection strategies of oomycetes and fungi have much in common (Latijnhouwers et al., 2003) and therefore it is logical to investigate whether genes involved in host-pathogen interactions are also similar. The approach to isolate homologs of fungal pathogenicity genes by PCR amplification using degenerate primers was, for example, used by Götesson et al. (2002) and Wu et al. (2008) to clone polygalacturonase genes from *P. cinnamomi* and *P. parasitica.* Cell wall degrading enzymes are important for the pathogenicity of many plant pathogenic fungi (ten Have et al., 2002) and it is likely that necrotrophic oomycetes also make use of such enzymes. Laxalt et al. (2002) used degenerated PCR primers to clone the *P. infestans* gene coding for the Gα subunit of the heterotrimeric G-protein (i.e. *gpa1*). G-protein-mediated signal transduction is the most ubiquitous and best studied signaling pathway among eukaryotes and in several plant pathogenic fungi the α and β G-protein subunits appear to be crucial for virulence. In *P. infestans* silencing of *gpa1* results in non-pathogenic mutants (Latijnhouwers et al., 2004) and silencing of *gpb1*, the gene encoding the G-protein β subunit, severely effects sporulation (Latijnhouwers and Govers, 2003). With the emergence of EST databases and genome sequences it is no longer necessary to design the optimal degenerated PCR primers and to face the challenge of finding the perfect PCR conditions to clone the homologs. In the *Phytophthora* EST databases several candidate genes that resemble known fungal pathogenicity genes have been identified and can be readily used for functional analysis (Qutob et al., 2002; Randall et al., 2005; Torto-Alalibo et al., 2007).

From domain to gene

Mining the databases for certain motifs that represent catalytic domains of enzymes is another strategy that can lead to novel candidates. This was nicely demonstrated by Meijer and Govers (2006) who made an inventory of genes involved in phospholipid signaling in *Phytophthora.* They found several novelties including a family of genes encoding secreted proteins with a phospholipase D (PLD) catalytic domain. Similar proteins are present in downy mildews (H.J.G. Meijer, personal communication) but have, as yet, not been found in other plant pathogens or other eukaryotes. It is therefore interesting to investigate how wide spread these enzymes are in oomycetes and whether they have a function in pathogenicity. Another novel class of proteins that deserves attention comprises

the G-protein coupled receptor- phosphatidylinositol (phosphate) kinases (GPCR-PIPKs), which are composed of an N-terminal seven transmembrane domain that is typical for GPCRs and a C-terminal PIPK catalytic domain. *Phytophthora* spp. and *H. parasitica* each have twelve GPCR-PIPKs which are slightly different. However, outside oomycetes only one homolog has been identified so far (Bakthavatsalam et al., 2006). This is RpkA in *Dictyostelium discoideum* that is essential for cell density sensing (Bakthavatsalam et al., 2007). Such a feature might also be important for oomycete pathogens when, for example, zoospores aggregate and prepare to invade the host. Involvement of G-protein signaling in this process was already demonstrated by Latijnhouwers et al. (2004) who found that zoospores of *P. infestans* transformants lacking the G-protein α subunit (generated through silencing of *gpa1*), have lost the ability to autoaggregate.

From map position to gene

For organisms that are a 'geneticist's nightmare' (Shaw, 1983) gene discovery based on positional cloning is not the most logical approach to follow. Nevertheless, before the genomics era several groups started to generate molecular genetic linkage maps with the aim to clone genes with a scorable phenotype but no clue about the gene product or stage of expression. Often their genes of interest were *Avr* genes that interact in a gene-for-gene manner with plant *R* genes. In addition, the mating type locus is a target for positional cloning (Randall et al., 2003). Species for which molecular genetic linkage maps have been generated are *P. infestans* (Carter et al., 1999; van der Lee et al., 2001; van der Lee et al., 2004; van der Lee et al., 1997; Randall et al., 2003), *P. sojae* (MacGregor et al., 2002; May et al., 2002; Whisson et al., 2004; Whisson et al., 1995), *H. parasitica* (Rehmany et al., 2003) and *B. lactucae* (Sicard et al., 2003). These long- term investments paid off and at least a handful of *Avr* genes have been isolated based on their map positions. One example where cloning primarily relied on RAPD and RFLP markers, a large number of recombinants and chromosome walking, was *Avr1b*-1 from *P. sojae* (Shan et al., 2004). *P. sojae* is homothallic and once F1 hybrids between two different races have been identified, it is straightforward to obtain F2 mapping populations by selfing the F1 hybrids (May et al., 2002; Tyler et al., 1995). In most cases, however, the genetically linked markers were not sufficient to locate the gene; the distance was too large or too few recombinants were available. Additional markers obtained by transcriptional profiling such as cDNA-AFLP (Guo et al., 2006) or by suppression subtractive hybridization (Bittner-Eddy et al., 2003), were needed to nail down the gene of interest and in several cases genomics resources helped to speed up the gene isolation. To find a candidate gene at the *P. infestans Avr3b-Avr10-Avr11* locus,

Jiang et al. (2006c) made use of a *Phytophthora* GeneChip with over 18,000 unigenes (Judelson et al., 2008). The chip was used for transcriptional profiling and comparative genomic hybridization (array-CGH) and this provided additional markers that were crucial for identifying the *Pi3.4* gene.

From effector activity to gene

Nearly all the *Avr* genes obtained by positional cloning encode proteins that belong to the superfamily of RXLR-dEER effectors (Rehmany et al., 2005; Whisson et al., 2007; Jiang et al., 2008). This finding stimulated the use of a gene discovery approach based on *in planta* expression of effector genes. The principle of this approach was first described by Torto et al. (2003), who mined *P. infestans* ESTs for sequences encoding secreted proteins and cloned these in binary PVX vectors for *in planta* expression in *N. benthamiana*. This high throughput approach resulted in the identification of *crn1* and *crn2* (crinkling and necrosis). When expressed *in planta crn1* and *crn2* cause necrosis and *crn2* also induces expression of defense response genes in tomato. The ESTs that were tested by Torto et al. (2003) encode a variety of secreted proteins including cell wall degrading enzymes, proteases, a chitinase and elicitins (ELI and ELL proteins). With the prediction that a putative *Avr* gene is likely a member of the RXLR-dEER family Vleeshouwers et al. (2008) developed a more dedicated approach concentrating exclusively on RXLR-dEER effectors. The corresponding ESTs were extracted from the *P. infestans* EST database and cloned into a binary PVX vector. In a high throughput screening a broad range of wild *Solanum* accessions showing resistance to late blight was toothpick-inoculated with the *Agrobacterium* strains carrying recombinant PVX constructs in a binary plasmid. Effectors inducing necrosis were retested on F1 progeny obtained by crossing a responsive, resistant accession with a non-responsive, susceptible potato line. If the progeny showed co-segregation of resistance and response to the effector, the resistant accession was further investigated for the presence of an *R* gene that specifically recognizes the RXLR-dEER effector. This approach, dubbed as 'effector genomics', resulted in the identification of an *R* gene in *Solanum stoloniferum* that is responsible for recognition of IPI-O. The *R* gene, *Rpi-sto1*, turned out to be the ortholog of *Rpi-blb1,* and hence *ipiO* is now known to function as *Avr-blb1* and *Avr-sto1* (Vleeshouwers et al., 2008).

Effector genomics is clearly a powerful approach that will soon result in the identification of many more effectors matching the wide range of *R* genes present in the *Solanum* gene pool or in any gene pool that is exploited for crop breeding. It is, however, a very biased approach; only genes that have certain characteristics such as signal peptide sequences and particular domains known to be involved in

effector function, are taken into account. Despite the fact that positional cloning is tedious it should not be discarded in the gene discovery process. The *pi3.4* gene located at the *Avr3b-Avr10-Avr11* locus in *P. infestans* is an illustrative example of a gene that would have been missed by effector genomics (Jiang et al., 2006c; Qutob et al., 2006a). *Pi3.4* encodes a protein of 1956 amino acids with regulatory domains characteristic for transcription factors. In isolates avirulent on *R3b*, *R10* and *R11* potato plants, truncated versions of *Pi3.4* are amplified with up to 25 copies located in tandem and adjacent to the full length copy of *Pi3.4.* Sequence comparison revealed that the amplification may generate modular diversity and assist in the assembly of novel full length genes via unequal crossing over. Such amplification is not found in virulent isolates. The current hypothesis is that *Pi3.4* is a transcriptional regulator that influences expression of effector genes e.g. RXLR-dEER genes. Copy number variation and modular diversity as observed at the *Avr3b-Avr10-Avr11* locus could be a mechanism for pathogens to quickly adapt to the environment.

SCOPE OF THIS THESIS

Phytophthora infestans, the causal agent of potato and tomato late blight, was the first species described in the genus *Phytophthora* and hence can be considered as the type species of the genus. The pathogen was responsible for the Irish Potato Famine in the 1840's that resulted in millions of deaths in Ireland, and also led millions of people to emigrate (Wax, 2007). *P. infestans* was first identified as the cause of late blight by Anton de Bary (1876). Intensive efforts to solve the late blight problem has increased the basic knowledge on oomycete plant pathogens and potato *R* genes but did not yet result in effective strategies for controlling the disease (Turner, 2005). In the last decade, genomics has expanded the knowledge on *P. infestans* and other oomycetes and may offer new opportunities to counter late blight (Birch and Whisson, 2001). As described in this introduction (**Chapter 1**)**,** many of the tools that are used for oomycete research were initially developed for *P. infestans* or can be applied to study *P. infestans*. This thesis describes the cloning and characterization of a *Phytophthora infestans Avr* gene. The gene is named *PiAvr4.* It encodes a race-specific elicitor and has a gene-for-gene interaction with a major *R* gene ($R4^{Ma}$) from *Solanum demissum*.

Several of the genomic resources and molecular tools that are available for *P. infestans* were used for the cloning and characterization of the *Avr* gene *PiAvr4 (***Chapter 2**). Similar to other oomycete avirulence genes, *PiAvr4* was obtained via a positional cloning approach by exploiting genetic markers and transcriptome markers for BAC marker landing. To investigate whether *PiAvr4* encodes an elicitor that is recognized by potato plants carrying the $R4^{Ma}$ resistance gene, DNA transformation of virulent isolates and *in planta* expression studies were performed. Avr4 belongs to the RXLR-dEER effector family, a family of rapidly evolving proteins that share the conserved RXLR and dEER motifs in the N-terminal part. The C-terminal part that has effector function also contains typical motifs designated as W and Y motifs. Agroinfection assays using constructs carrying W and Y in several combinations were performed on potato plants to determine which region of the C-terminal part of PiAvr4 triggers an $R4^{Ma}$-specific resistance response (**Chapter 3**). Mining of the *Phytophthora* genome sequences was performed to find homologs of *PiAvr4* in other species and the genomic region surrounding *PiAvr4* was analyzed. The RXLR-dEER domain is important for effector trafficking and plays a role in targeting effectors to the host cell. *P. infestans* was transformed with chimeric constructs carrying the RXLR-dEER genes *PiAvr4* and *ipiO* fused to red fluorescent protein gene (**Chapter 4**). Fluorescence was monitored in several stages of the *P. infestans* life cycle and during infection.

The effector PiAvr4 interacts with its cognate potato resistance protein R4. The gene-for-gene recognition specificity of *R4* potato differentials was studied in infection assays and by *in planta* expression of *PiAvr4* using agroinfection (**Chapter 5**). This revealed different recognition specificities in potato differentials labeled as *R4*. **Chapter 6** describes the use of NBS profiling and bulked segregant analysis to identify markers linked to the resistance gene $R4^{Ma}$. NBS profiling specifically targets genes encoding NBS-LRR proteins, a class of genes that comprises all *R* genes conferring resistance to oomycete plant pathogens, cloned so far.

The general discussion (**Chapter 7**) presents an overview of the secretome of oomycete plant pathogens and the role of secreted proteins in pathogenesis. The major focus is on the RXLR-dEER effector family. Finally, the experimental data described in chapter 2 to 6 are placed in a broader context and the implications of this research with respect to gene-for-gene interactions, effector recognition and late blight resistance breeding are discussed.

CHAPTER 2

The *Phytophthora infestans* avirulence gene *Avr4* encodes an RXLR-dEER effector

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SUMMARY

Resistance in potato against the oomycete *Phytophthora infestans* is conditioned by resistance (*R*) genes that are introgressed from wild *Solanum* species into cultivated potato. According to the gene-for-gene model, proteins encoded by *R* genes recognize race-specific effectors resulting in a hypersensitive response (HR). We isolated *P. infestans* avirulence gene *PiAvr4* using a combined approach of genetic mapping, transcriptional profiling and BAC marker landing. *PiAvr4* encodes a 287 amino acid protein that belongs to a superfamily of effectors sharing the putative host cell targeting motif RXLR-dEER. Transformation of *P. infestans* race 4 strains with *PiAvr4* resulted in transformants that were avirulent on *R4* potato plants, demonstrating that *PiAvr4* is responsible for eliciting *R4*-mediated resistance. Moreover, expression of *PiAvr4* in *R4* plants using PVX-agroinfection and agroinfiltration showed that PiAvr4 itself is the effector that elicits HR on *R4* but not *r0* plants. The presence of the RXLR-dEER motif suggested intracellular recognition of PiAvr4. This was confirmed in agroinfiltration assays but not with PVX-agroinfection. Since there was always recognition of PiAvr4 retaining the signal peptide, extracellular recognition can not be excluded. Deletion of the RXLR-dEER domain neither stimulated nor prevented elicitor activity of PiAvr4. Race 4 strains have frame shift mutations in *PiAvr4* that result in truncated peptides; hence, PiAvr4 is apparently not crucial for virulence.

INTRODUCTION

Plants are attacked by a variety of pathogens and have, as a countermeasure, developed defense mechanisms that result in innate immunity (Jones and Takemoto, 2004; Nürnberger et al., 2004; Chisholm et al., 2006). In most cases, pathogen attack is unsuccessful; the plant will not host the pathogen (a non-host interaction) and the pathogen is unable to overcome the defense barriers. However, when the pathogen encounters a suitable host, it is able to overcome or suppress the defense barriers and can successfully invade and colonize the plant tissues. The most devastating pathogen on potato (*Solanum tuberosum*) is *Phytophthora infestans,* the oomycete that causes late blight and was responsible for the Irish potato famine in the 1840s (Govers and Latijnhouwers, 2004). To combat this disease, breeders have introduced resistance (*R*) genes from wild *Solanum* species into cultivated potatoes. Proteins encoded by these *R* genes can recognize specific races of the pathogen. This triggers defense responses often leading to a hypersensitive response (HR) that arrests growth of the pathogen. Potato and *P. infestans* interact according to the gene-for-gene model (Flor, 1971) which predicts that recognition is governed by direct or indirect interaction of an R protein with its corresponding effector, the product of an avirulence (*Avr*) gene. If either the *R* gene or the *Avr* gene is absent or non-functional, the interaction is compatible and the host susceptible. This model also predicts that the durability of an *R* gene is highly dependent on the stability or role of its cognate effector. That is, if an *Avr* gene can easily mutate without fitness penalty, the pathogen can circumvent recognition and resistance is overcome.

P. infestans is notorious for its adaptive ability in response to *R* genes (Wastie, 1991). The first attempts to breed late blight resistant potatoes were disappointing. The 11 *R* genes that were introgressed in potato from the *Solanum demissum* (Black et al., 1953; Malcolmson and Black, 1966; Mastenbroek, 1953) were all defeated within five years and breeders lost confidence in this approach. Lately, interest in *R* gene breeding has revived and two *R* genes from durable resistant *Solanum bulbocastanum* accessions were cloned (Song et al., 2003; van der Vossen et al., 2003; 2005). Key to a better understanding of the molecular basis of late blight resistance is dissecting R protein-effector interactions and, thus, there is a need to have more cloned *R* and *Avr* genes available to study these interactions. Besides the two *S. bulbocastanum R* genes, named *Rpi-blb1* and *Rpi-blb2* (Song et al., 2003; van der Vossen et al., 2003; 2005), a few other late blight *R* genes have been cloned, including two from *S. demissum*, *R1* (Ballvora et al., 2002) and *R3a* (Huang et al., 2005a). They all encode NBS-LRR type R proteins that are predicted to reside in the cytoplasm. So far, only for *R3a* has the

corresponding *Avr* gene been isolated. *Avr3a* was identified by association genetics and with the help of the cloned *R3a* gene its avirulence function was demonstrated (Armstrong et al., 2005). Avr3a belongs to a large, oomycete-specific family of highly divergent effectors that share a conserved domain named RXLR-dEER (Rehmany et al., 2005; Tyler et al., 2006). Recent analyses have suggested the RXLR-dEER genes in *Phytophthora ramorum* and *P. sojae* to be derived from a common ancestor (Jiang et al., 2008). The RXLR motif is also present in effectors encoded by *Avr1b*-1 from *P. sojae* (Shan et al., 2004), and *ATR1*^{NdWsB} (Rehmany et al., 2005) and *ATR13* (Allen et al., 2004) from the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica.* These latter three were obtained by map-based cloning and, similar to the late blight *R* genes, the *R* genes recognizing *ATR1*^{NdWsB} and *ATR13* (i.e., *RPP1* and *RPP13* respectively), encode NBS-LRR proteins (Catanzariti et al., 2007). As yet, conserved domains have not been found in any of the fungal or bacterial Avr effectors that interact with NBS-LRR type R proteins (Kjemtrup et al., 2000; Birch et al., 2006; Catanzariti et al., 2007). It is therefore remarkable that nearly all oomycete *Avr* loci that were cloned by unbiased approaches encode effector genes belonging to the RXLR-dEER family. The one exception is the *Avr3b-Avr10-Avr11* locus in *P. infestans*. This locus harbors the *Pi3.4* gene that encodes a putative regulatory protein (Jiang et al., 2006c).

In order to isolate *P. infestans Avr* genes, we initially followed a map-based cloning approach and positioned several *Avr* genes on high density genetic linkage maps (van der Lee et al., 2001). A *P. infestans* bacterial artificial chromosome (BAC) library was made from a progeny predicted to contain all six *Avr* alleles segregating in this population (Whisson et al*.,* 2001). Since the map resolution was insufficient for chromosome walking, additional markers were obtained using expression profiling. To identify *Avr*-associated transcripts, transcriptional profiling using cDNA-AFLP was performed on pools of F1 progeny segregating for avirulence. *Avr* genes are expected to be expressed prior to penetration of the host and therefore RNA from germinated cysts was used as cDNA-AFLP template (Guo et al., 2006). Here we describe cloning and functional characterization of the *P. infestans Avr4* gene, the counterpart of the late blight resistance locus from *S. demissum* that is denoted as *R4* (Black et al., 1953; Mastenbroek, 1953). Previously, the *Avr4* locus was positioned on linkage group A2-a with nine AFLP markers in close vicinity (van der Lee et al., 2001). Added to that were two cDNA-AFLP markers that were specific for strains avirulent on *R4* plants. In this study, the AFLP and cDNA-AFLP markers were used for BAC landing and a gene located at the *Avr4* locus was identified. *PiAvr4* encodes a typical oomycete RXLR effector molecule, elicits a hypersensitive response when expressed in *R4* potato plants*,*

and can complement the avr4 phenotype in race 4 strains. Taken together, these results show that recognition of PiAvr4 underlies *R4*-mediated resistance in potato.

RESULTS

Two cDNA-AFLP fragments co-segregate with the *Avr4* locus and are derived from the same gene

By cDNA-AFLP we previously identified 23 *Avr4*-associated Transcript Derived Fragments (TDFs). Two of these, TDF4.1s and TDF4.2s, showed 100% association with the AVR4 phenotype in 18 F1 progeny of a mapping population (cross 71) (Guo et al., 2006). BLASTN screening of an EST database (Randall et al., 2005) with the TDF4.1s and TDF4.2s sequences resulted in a match of both TDFs to PH051G10, an EST of 748 nucleotides and no homology to known genes.

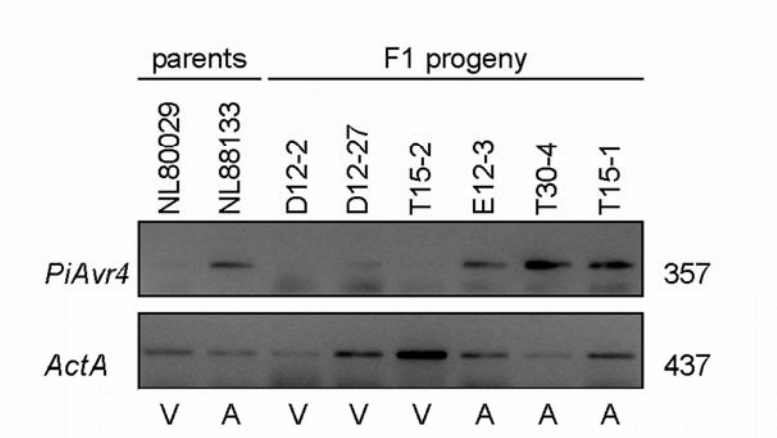

Figure 1. Expression of a putative *PiAvr4* gene in the parental isolates NL80029 and NL88133, and seven F1 progeny. RT-PCR products obtained with primers specific for PH051G10 (upper panel) and the actin A gene *Act*A (lower panel), and total RNA isolated from germinated cysts as template. Amplicon sizes (right) are in base pairs. A and V refer to the phenotype on *R4* plants: avirulent or virulent, respectively.

To confirm that expression of the gene corresponding to PH051G10 is associated with the AVR4 phenotype we performed RT-PCR expression analysis with primers specific for PH051G10. Transcripts were found in germinated cysts of all tested avirulent progeny of cross 71 and parental isolate NL88133, but not in virulent progeny nor parental isolate NL80029. Occasionally, a faint band was observed in a virulent F1 strain, e.g. D12-27, but the transcript level was always much lower than in avirulent strains (Fig. 1 and data not shown). This shows that in the mapping population expression in germinated cysts of a gene represented by TDF4.1s, TDF4.2s and EST PH051G10 co-segregates with the *Avr4* locus.

Avr4-linked AFLP markers and a candidate *Avr4* gene are located on a single BAC contig

Previously, we used a mapping population of 76 F1 progeny (cross 71) to position the *Avr4* locus on linkage group A2-a and identified nine AFLP markers closely linked to *Avr4* (van der Lee et al., 2001). Five of these were used to screen a 10x coverage BAC library of strain T30-4, an F1 progeny of cross 71 that is avirulent on *R4* plants. This gave nine positive BACs that could be assembled in one contig (Fig. 2A; Table S1). Subsequently, these BACs were screened by PCR with primers derived from EST PH051G10. Seven of the nine were positive and this was confirmed by Southern hybridization with TDF4.1s and TDF4.2s probes. This demonstrated that the candidate *Avr4* gene and the AFLP markers genetically linked to *Avr4*, are located on this BAC contig.

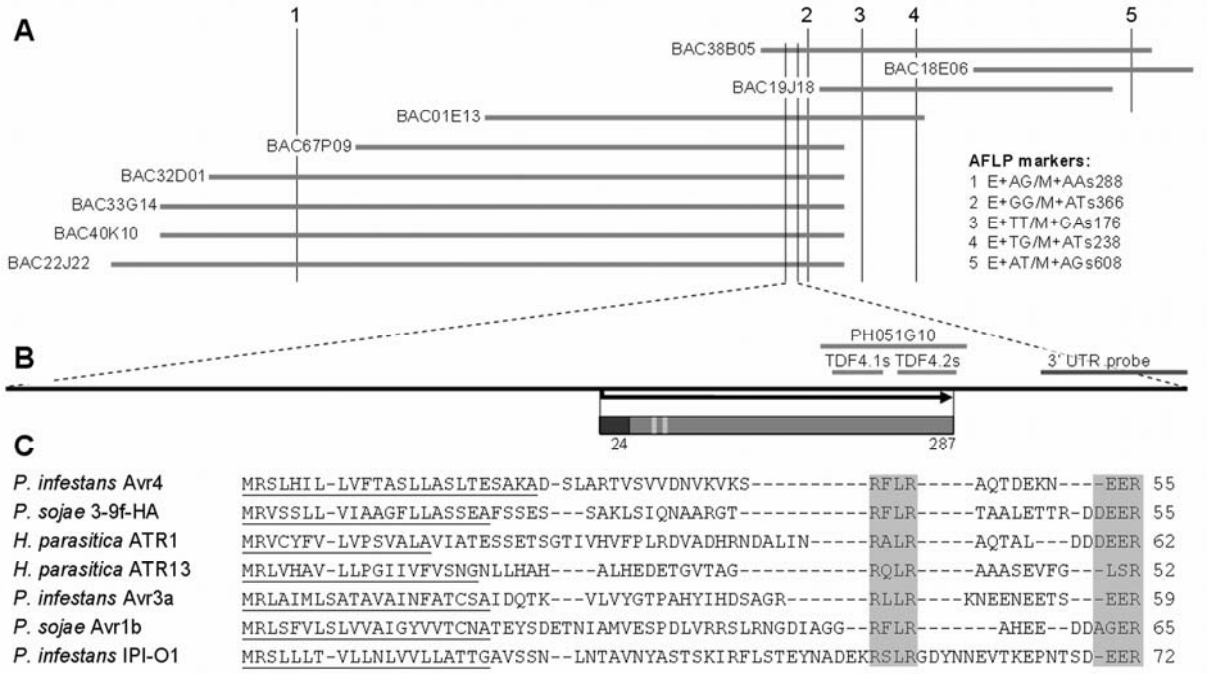

Figure 2. A. BAC contig spanning the *Avr4* locus. The nine BACs were selected by screening the BAC library with *Avr4*-linked AFLP markers, indicated by 1-5. The sizes of the BACs are estimated and not drawn on scale. **B.** The 2890 bp *Bam*HI fragment carrying *PiAvr4*. The open reading frame of 861 bp (black arrow) starts at bp position 1455. The black line represents the 3'UTR probe (a 465 bp *Sal*I-*Bam*HI fragment). The bar represents PiAvr4 with the predicted SP of 24 amino acids (black box) and the RXLR and dEER motifs (light grey). **C.** Alignment of the N-terminal portion of oomycete effector proteins. Shown are *P. infestans* PiA*vr4*, *P. sojae* 3-9f-HA (accession AY183415), *H. parasitica* ATR1 (AY842877), *H. parasitica* ATR13 (AY785306), *P. infestans* AVR3a (AJ893357), *P. sojae* AVR1b (AY426744) and *P. infestans* IPI-O1 (L23939). Sequences were manually aligned. The RXLR-dEER motif is highlighted and the predicted SPs are underlined.

PiAvr4 encodes an RXLR-dEER protein

From three overlapping BACs (BAC01E13, BAC38B05 and BAC40K10) we subcloned a 2.9 kb *Bam*HI fragment that hybridized to both TDF4.1s and TDF4.2s. Sequencing revealed a single 861 bp open reading frame (ORF) encoding a 287 amino acid protein with a predicted 24 amino acid signal peptide (SP) (Fig. 2B). Overall the protein showed no homology to any known protein but contained a conserved motif that had been identified in several oomycete Avr factors encoded by genes interacting in a 'gene-for-gene' manner with *R* genes (Rehmany et al., 2005). Manual alignment showed that the relative position of the conserved RXLR-dEER motif with respect to the SP is comparable. Our candidate Avr4 protein also followed that pattern (Fig. 2C) and we tentatively named the gene *PiAvr4.* In the PiAvr4 protein, the conserved motif starts with RFLR at amino acid position 18 of the mature protein and ends at position 31 with a dEER region lacking the aspartic acid residue (D).

The 'virulent' allele at the *Avr4* locus encodes a truncated protein

Strain T30-4 is heterozygous at the *Avr4* locus. To identify the virulent allele of *PiAvr4* we hybridized the BAC library with a 3'UTR probe. Sixteen *Avr4* positive BACs were identified, nine of which were not selected in the screening with *Avr4*-linked AFLP markers. PCR, Southern hybridization, restriction analysis and sequencing revealed that six of the nine carry an allelic variant that is located on a 3.8 kb *Sal*I fragment, while three BACs fit in the contig and carry *PiAvr4* (Fig. 2, Table S1). Compared to *PiAvr4*, the allelic variant, i.e. *Piavr4,* has fifteen single nucleotide polymorphisms (SNPs) and two single base pair deletions (ΔT^{12} and ΔT^{196}) (Fig. S1). The two deletions cause frame shift mutations and premature stop codons resulting in a truncated protein of only 17 amino acids that probably is not functional.

The promoter regions in the two alleles are similar up to around 500 bp upstream of the start codon, but further upstream the sequences diverge dramatically (Fig. S1). Between -500 and +1 there are five SNPs and nine indels ranging from 1 to 10 bp. In the promoter, the 16 nucleotide oomycete core consensus sequence that surrounds the putative transcription start site (TSS) is located between -38 and -23 relative to the ATG start codon (Pieterse et al., 1994; McLeod et al., 2004). Apart from a one bp indel at the 3' end, the core consensus TSS sequence is identical in the two alleles. Downstream of the stop codon of *PiAvr4* 107 bp of the 3' UTR were compared to the equivalent portion of the *Piavr4* allele and only one SNP was found.

Table 1 Infection efficiency (IE) and lesion growth rate (LGR) of *Phytophthora infestans* donor and recipient strains (F1 progeny), *PiAvr4* transformants and field isolates on cultivars Bintje (*r0*) and Isola (*R4*).

strain	origin	*PiAvr4* mRNA[a]	Bintje (*r0*)		Isola (*R4*)	
			IE[b]	LGR[c]	IE	LGR
T30-4	progeny of cross 71	+	100	0.79	25	0.14
D12-2	progeny of cross 71	-	100	0.73	75	0.51
T35-3	progeny of cross 71	-	100	0.83	100	0.53
DA4.2	D12-2 transformant	+	100	0.55	17	0.15
TA2.3	T35-3 transformant	+	100	0.28	17	0.15
TA5.4	T35-3 transformant	+	100	0.42	0	0
TA5.5	T35-3 transformant	+	100	1.15	0	0
NL80029	field isolate; A1 parent of cross 71	-	75	0.6	100	0.5
NL88133	field isolate; A2 parent of cross 71	+	100	0.8	0	0
NL88069[d]	field isolate	+	100	0.9	100	0.46
IPO-0[e]	field isolate	+	100	0.94	0	0

[a] Transcripts were detected by RT-PCR on mRNA isolated from germinated cysts using primers matching *PiAvr4*.
[b] Infection efficiency is expressed as the percentage of successful infections per plant genotype.
[c] Lesion growth rate of successful infections expressed in cm day^{-1}. LGR was determined by comparing the infected area on day 3 and day 5 in a single experiment using six independent inoculations per tested isolate.
[d] In NL88069 only allele XIII has been identified; this allele has a frame shift mutation resulting in a truncated protein (Fig. 5).
[e] IPO-0 contains virulent allele V and avirulent allele II (Fig. 5).

Complementation of race 4 strains with *PiAvr4* results in a change in phenotype

To determine if *PiAvr4* is the avirulence gene interacting with the *R4* gene, we transformed race 4 strains with a plasmid carrying *PiAvr4* under control of its native promoter and monitored the ability of the transformants to infect *R4* plants. Plasmid pTA23.9 contains a 2.9 kb *Bam*HI fragment from BAC01E13, and harbors the *PiAvr4* ORF with 1454 bp upstream of the ATG and 572 bp downstream of the stop codon. As recipient strains we used T35-3 and D12-2, two F1 progeny of cross 71 and siblings of T30-4. Four transformants showed a change in phenotype: TA2.3, DA4.2, TA5.4 and TA5.5 were still pathogenic on Bintje, a susceptible cultivar lacking *R* genes (*r0*), but were no longer able to infect *R4* plants (Fig. 3, Table 1). Inoculation on two different *R4* plants, the clone Cebeco44-31-5 and cultivar Isola, resulted in an HR whereas inoculation with the recipient strains resulted in sporulating lesions. With each of the transformants the infection assays were repeated at least three times and the avirulent phenotypes on *R4* plants were

always confirmed. In all cases the transformants could easily infect and colonize Bintje but the lesion growth rate was slightly reduced when compared to the wild-type recipient strains (Table 1). To confirm expression of the transgene we performed RT-PCR expression analysis and showed the presence of *PiAvr4* transcripts in germinated cysts of the transformants. These transcripts were not detected in the non-transformed recipient strains T35-3 and D12-2 (Table 1). Altogether, these results demonstrate that *PiAvr4* conditions avirulence of *P. infestans* on potato plants carrying *R4*.

Expression of *PiAvr4* in *R4* plants results in a hypersensitive response

To investigate if PiAvr4 itself has elicitor activity we expressed *PiAvr4 in planta* by means of agroinfection and compared the responses in different solanaceous plants. The ORF of *PiAvr4* was cloned in the binary PVX vector pGR106 and transformed into *Agrobacterium tumefaciens* strain GV3101. Toothpick inoculation of *A. tumefaciens* carrying pGR106-Avr4$^{1-287}$ resulted in an HR on *R4* plants within 13 days post-inoculation (dpi) whereas Bintje (*r0*) and the *R3a* potato clone SW8540-025 showed no response (Fig. 4). Also, on *Nicotiana tabacum* and *Nicotiana clevelandii,* non-host species that show no macroscopic responses upon drop inoculation with *P. infestans* zoospores*,* there was no visible response. As positive control we used pGR106-CRN2. CRN2, which is a general necrosis inducing elicitor (Torto et al*.,* 2003), elicited responses on *N. tabacum* and *N. clevelandii* as well as on all tested potato lines, including *R4* plants and Bintje (Fig. 4). Inoculation with strains carrying the empty vector pGR106 gave no response. These results show that PiAvr4 is an elicitor of HR on plants carrying *R4*.

Since PiAvr4 has an RXLR-dEER motif that may function as a host targeting signal (HTS) (Whisson et al., 2007), we anticipated that in the agroinfection assays the SP would not be required for HR-inducing activity. To test this we constructed plasmid pGR106-Avr4$^{25-287}$ that lacks the sequence for the SP. Surprisingly, none of the *R4* plants responded with a visibly detectable HR to agroinfection with strains carrying pGR106-Avr4$^{25-287}$ (Fig. 4), and neither did any of the control lines tested. Two independent pGR106-Avr4$^{25-287}$ constructs of which the identity was confirmed by DNA sequencing, gave comparable results. To exclude the possibility that the native SP of PiAvr4 in pGR106-Avr4$^{1-287}$ does not function properly in plants we replaced it with the tobacco PR1a SP. Agroinfection with strains carrying pGR106-Avr4$^{SP(PR1a)-25-287}$ gave the same result as the strains carrying pGR106-Avr4$^{1-287}$, i.e., an HR on *R4* plants and no response on Bintje or *N. clevelandii* (Fig. 4).

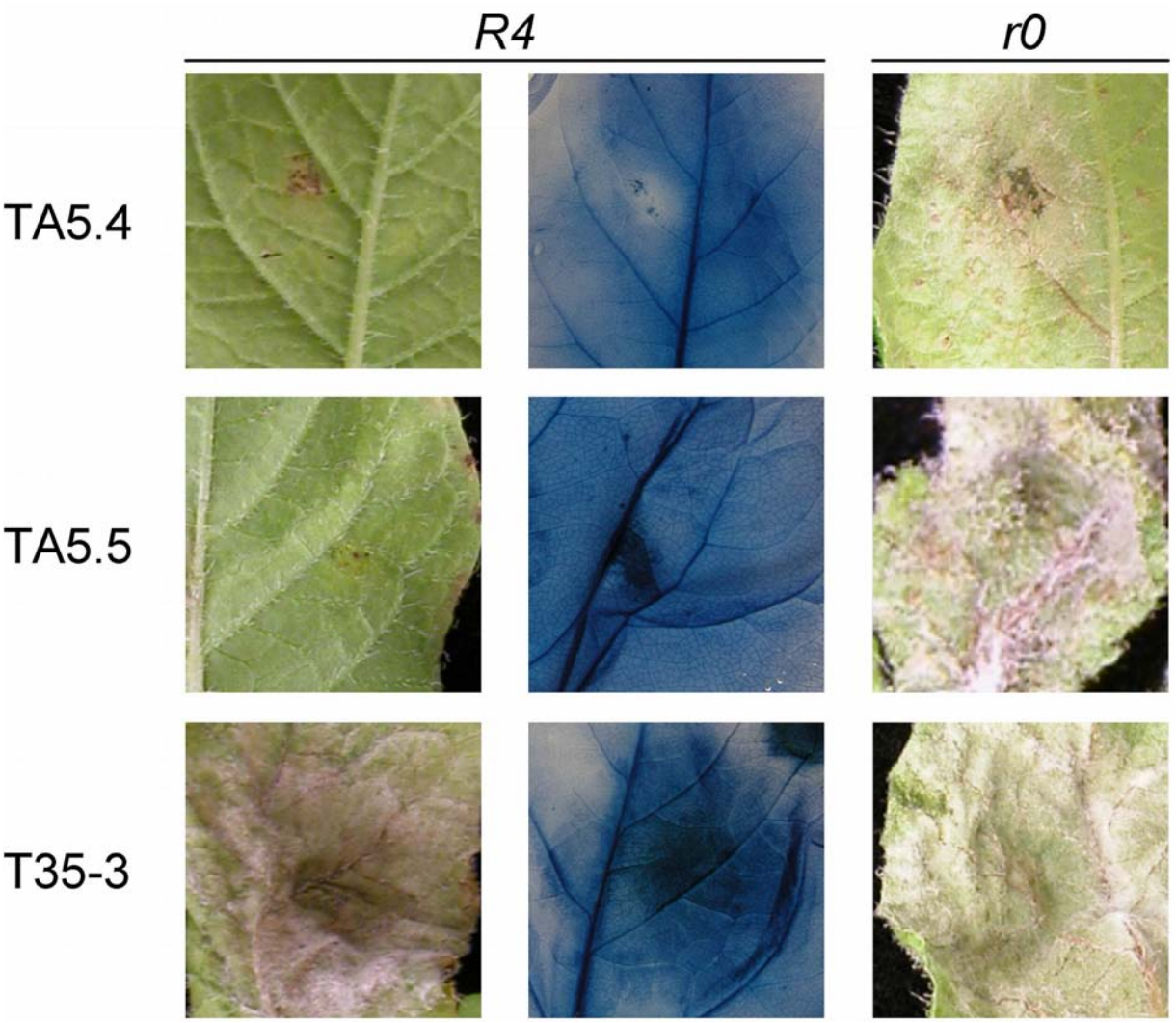

Figure 3. Virulence phenotypes of recipient strain T35-3 and transformants complemented with *PiAvr4* (TA5.4 and TA5.5) on leaves of potato cultivar Bintje (*r0*) and Cebeco44-31-5 (*R4*). The middle column shows the *R4* leaves displayed in the left column after trypan blue staining. Leaves were spot-inoculated with equal numbers of zoospores. Photographs were taken 5 dpi.

Since the absence of any visible response with constructs without SP could be due to an extremely fast and strong HR that immediately blocks spread of virus particles, we tested the HR-inducing activity with two other *in planta* expression assays, virus inoculation and agroinfiltration. Recombinant virus particles were obtained from systemically infected *N. clevelandii* leaves and inoculated on potato. After 5 days the primary inoculated leaves of *R4* plants showed necrosis when inoculated with recombinant virus particles derived from pGR106-Avr4$^{1\text{-}287}$, but there was no response upon inoculation with particles from pGR106-Avr4$^{25\text{-}287}$ or pGR106 (Fig. S2). Bintje showed no response in the primary inoculated leaves with any of the constructs but always showed systemic virus spread. Systemic virus spread was also observed in *R4* plants inoculated with pGR106-Avr4$^{25\text{-}287}$ or

pGR106 but not in *R4* plants inoculated with pGR106-Avr4$^{1-287}$. As in the agroinfection assay, the SP seems to be required for elicitor activity. For agroinfiltration, Avr4$^{1-287}$ and Avr4$^{25-287}$ were cloned in the binary vector pGRAB and transformed into *A. tumefaciens* strain AGL1. Infiltration in leaves of *R4* plants resulted in an HR within 2 dpi irrespective of the presence or absence of the SP (Fig. 4). Infiltration in Bintje (*r0*) and *N. benthamiana* showed no responses and was comparable to the empty vector control. In contrast, the positive control, pGRAB-CRN2, elicited responses on all tested plants (Fig. 4). Infiltration with a strain carrying the empty vector pGRAB gave no response. These results show that the different *in planta* expression systems gave conflicting data with respect to the requirement for the SP but the conclusion that PiAvr4 is an elicitor of HR specifically on plants carrying *R4* was substantiated.

The RXLR-dEER motif is not required for HR-inducing activity of PiAvr4

The observation that secretion of PiAvr4 to the extracellular space does not abolish elicitor activity is in conflict with the assumption that *Phytophthora* delivers the RXLR-dEER effectors inside the host cell at the site where they are presumed to perform their function. We may assume that *in planta* expression of the full length *PiAvr4* construct (pGR106-Avr4$^{1-287}$) or the construct with the PR1a SP (pGR106-Avr4$^{SP(PR1a)-25-287}$) results in maturation of the protein in the endoplasmic reticulum and the Golgi, followed by secretion from the cell whereby the SP is removed. If there is no further processing after cleavage of the SP, the mature PiAvr4 retains the RXLR-dEER motif. Since this motif could be responsible for translocating PiAvr4 across the membrane to an intracellular location, we generated and tested constructs in which a region of 41 amino acids (25-65) comprising the RXLR-dEER motif is deleted. Agroinfection with pGR106-Avr4$^{SP(PR1a)-66-287}$ resulted in a strong necrotic response specifically on *R4* plants. This response was comparable to the response with the full length construct pGR106-Avr4$^{1-287}$ or pGR106-Avr4$^{SP(PR1a)-25-287}$ (Fig. 4). This shows that in these agroinfection assays the RXLR-dEER motif neither stimulates nor prevents elicitor activity of PiAvr4. The finding that the absence of the RXLR-dEER motif does not abolish elicitor activity could point to extracellular recognition of PiAvr4, implying another re-uptake mechanism that is unknown, or indicate that some PiAvr4 is mis-targeted during secretion, and is retained in the cell.

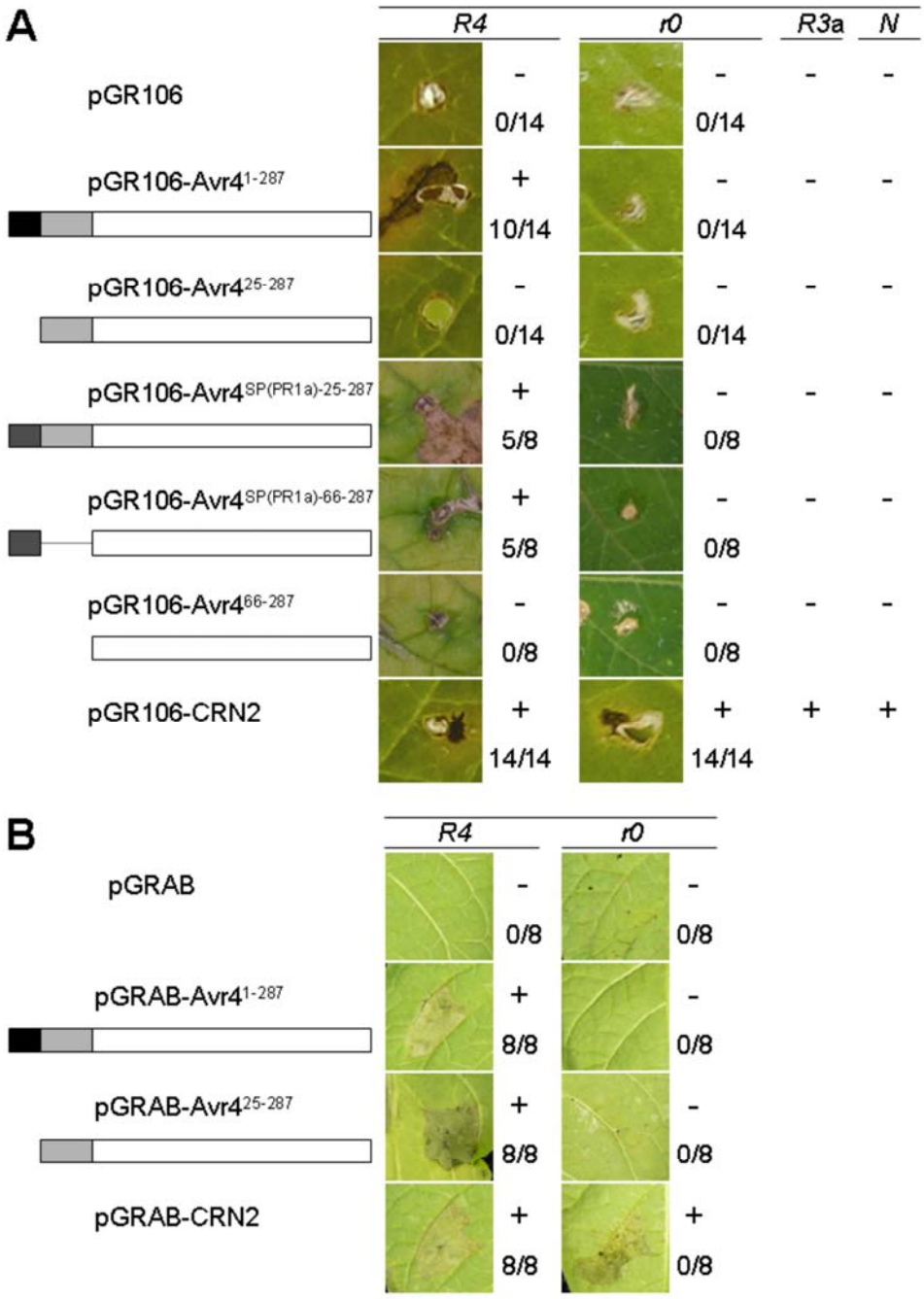

Figure 4. Expression of *PiAvr4* in plants. **A,** Agroinfection and **B,** agroinfiltration of potato line Cebeco44-31-5 (*R4*) and cultivar Bintje (*r0*) by toothpick inoculation with strains carrying pGR106 constructs (in **A**) and with *A. tumefaciens* strains carrying pGRAB constructs (in **B**). + indicates a necrotic response and - no visual response. The numbers show the ratio of '+' responses and the total number of toothpick inoculations (in **A**) or the ratio of '+' responses and the total number of agroinfiltrations (in **B**) in a typical experiment. Pictures were taken 18 dpi (in **A**) or 3 dpi (in **B**). Controls for agroinfection included inoculations on potato line SW8540-025 (*R3a*), and two *Nicotiana* species, *N. tabacum* and *N. clevelandii* (*N*), which responded similarly. In the schematic representation of the constructs, the SP of PiAvr4 is shown in black, the SP of PR1a in dark gray, the RXLR-dEER region (aa 25-65) in light gray and the C-terminal region (aa 66 to 287) as an open bar. CRN2, cloned in pGR106-CRN2 and pGRAB-CRN2, is a universal elicitor that is used as positive control.

PiAvr4 homologs

BLASTN searches of the *P. infestans* T30-4 genome sequence (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html), confirmed the presence of the two alleles that we identified in this study. In addition, a number of distant homologs were retrieved. These are located on other supercontigs, and similar to *PiAvr4*, these homologs have the HTS and belong to the RXLR-dEER superfamily.

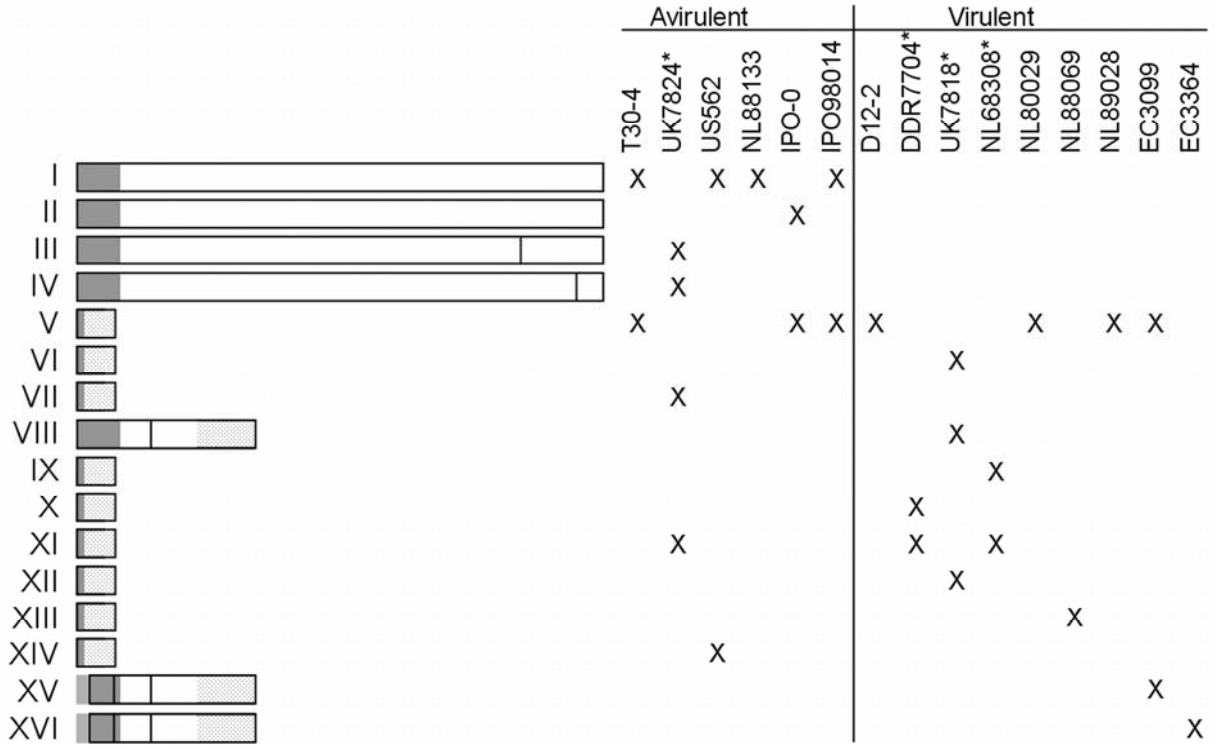

Figure 5. *PiAvr4* alleles identified in *P. infestans* field isolates. Shown are the proteins encoded by sixteen different alleles numbered I-XVI of which the polymorphisms at the DNA level are provided as Supplementary Information (Figure S2). PiAvr4 encoded by allele I is shown as a bar with the signal peptide shaded (dark gray). A vertical black line (|) represents an amino acid change with respect to PiAvr4. A dotted bar (⁞) is an out of frame ORF. In alleles XV and XVI the light gray shaded area preceding the bar represents 22 nucleotides of which the sequence was not determined. The isolates are grouped according to their phenotype on *R4* plants. Isolates marked by * originate from the clonal population that existed prior to the introduction of the A2 mating type in Europe.

Southern blot hybridizations and PCR amplifications on genomic DNA isolated from a variety of *Phytophthora* species revealed *PiAvr4* homologs in the closely related species *Phytophthora phaseoli, Phytophthora andina*, *Phytophthora mirabilis* and *Phytophthora ipomoeae* but in species belonging to other clades, no homologs could be identified. The lack of Southern hybridization of *PiAvr4* to *P. sojae* and *P. ramorum* DNA was supported by the apparent absence (by BLASTN) of a homologous DNA sequence in the genome sequences of these oomycetes

and *H. parasitica*. Nevertheless, a TBLASTN search in *Phytophthora* EST sequence databases revealed the presence of a *P. sojae* EST exhibiting modest similarity to *PiAvr4.* This EST named 3-9f-HA (accession AY183415) encodes an RXLR-dEER protein and corresponds to a sequence on Scaffold 9 of the *P. sojae* genome (sequence assembly 3.0) (Qutob et al., 2002). In total five close homologs of PiAvr4 were identified by BLASTP searches in the genomes of *P. infestans*, *P. sojae* and *P. ramorum* (E value < 1e-5, sequence identity > 30%). In the *H. parasitica* genome sequences no homologs we detected.

***PiAvr4* polymorphism in *Phytophthora infestans* field isolates**

The frame shift mutation in the virulent allele of strain T30-4 likely leads to a non-functional Avr4 protein. To investigate the allele variation in the *P. infestans* population and to correlate this with the Avr4 phenotype, we analyzed *PiAvr4* alleles in field isolates. PCR amplification from genomic DNA of T30-4 with *PiAvr4*-specific primers resulted solely in fragments derived from the virulent or avirulent allele at the *Avr4* locus. *PiAvr4*-specific primers were used to amplify *PiAvr4* alleles from genomic DNA and the amplicons were cloned and sequenced. The primers match to the first and the last twenty nucleotides of the ORF and, as a consequence, SNPs that may occur in regions 1-20 and 845-864 can not be identified. From 15 isolates, 16 different alleles were cloned, I to XVI*,* and 18 polymorphic sites were found, 17 of which were already identified in the two alleles of T30-4 (Fig. S3). Of the 16 alleles only four (I to IV) have a full length ORF (Fig. 5). All isolates that are avirulent on *R4* plants have at least one allele with a full-length ORF. In contrast, none of the isolates that are virulent on *R4* have an intact copy of *PiAvr4*. The absence of alleles with a full length ORF was confirmed by direct sequencing and cloning of the PCR products generated from genomic DNA. Direct sequencing of PCR products generated from genomic DNA of avirulent strains resulted in unreadable chromatograms because of mixtures of avirulent and frame shifted virulent alleles. This was the case, for example, with NL88133 which, in addition to allele I, is known to have a virulent allele based on genetic analysis (van der Lee et al., 2001) and Southern hybridization. The virulent isolates with only one identified allele could be homozygous at the *Avr4* locus, as expected for NL80029 and NL88069. This is supported by the finding that PH051G10, the EST that originates from NL88069, is identical to allele XIII cloned from NL88069. Several of the isolates tested were obtained prior to the introduction of the A2 mating type in Europe and belong to the ‘old’ clonal population. Despite their uniform US-1 genotype, these isolates have different *PiAvr4* alleles, and the allele distribution is consistent with their phenotype. The multiple alleles in isolates UK7824 and UK7818 could be the result of gene duplication or reflect polyploidy, a

known phenomenon in *P. infestans* (Tooley and Therrien, 1987). One of the two Ecuadorian (EC3364) isolates was originally isolated from tree tomato (*Solanum betaceum*) (Adler et al., 2004) but is pathogenic on potato.

DISCUSSION

Through a combined approach of genetic linkage mapping (van der Lee et al., 2001), transcriptional profiling (Guo et al., 2006) and BAC marker landing (Whisson et al., 2001) we have cloned an *Avr* gene from *P. infestans* that functions according to the gene-for-gene model with an *R* gene from *Solanum demissum.* Isolates carrying *PiAvr*4 elicit a HR on *R4* potato plants*,* whereas race 4 isolates with a frame shift mutation in the *PiAvr4* ORF are not recognized and can colonize *R4* plants. The definitive proof comes from complementation of race 4 isolates with *PiAvr4*; transgenic race 4 isolates were pathogenic on *r0* plants but avirulent on *R4* plants, demonstrating that recognition was conferred by a single gene.

In laboratory assays race 4 isolates with a disrupted *PiAvr4* ORF are as virulent as isolates carrying an intact copy of *PiAvr4.* Therefore, PiAvr4 by itself is not an essential virulence component, although more subtle effects in nature can not be excluded. Loss of Avr4 is not compensated by close homologs, since these do not exist. Loss of Avr4 is not compensated by close homologs, since these do not exist, but could be compensated, however, by sequence-unrelated effectors that are functionally redundant. *PiAvr4* belongs to a family of over 500 *P. infestans* genes encoding potentially secreted proteins containing the RXLR motif (R.H.Y. Jiang, personal communication; Whisson et al., 2007). Similarly, large families are present in other *Phytophthora* spp. (Tyler et al*.,* 2006; Jiang et al., 2008) and the downy mildew *H. parasitica* (R.H.Y. Jiang and B.M. Tyler, personal communication). Key features are found in the N-terminus of the proteins: a SP followed by a conserved domain that has two characteristic motifs RXLR and dEER. The C-terminal domains, however, are highly divergent and as a consequence the RXLR-dEER superfamily is one of the most divergent families in the *Phytophthora* secretome (Jiang et al., 2008). Nevertheless, some of the RXLR-dEER proteins do have recognizable orthologs in distantly related *Phytophthora* species (Jiang et al., 2008).

In recent years evidence has accumulated that, similar to the PEXEL motif in secreted proteins of the malaria parasite *Plasmodium falciparum* (Przyborski and Lanzer, 2004), the RXLR-dEER domain is a HTS that is responsible for targeting pathogen proteins to the host cytoplasm (Whisson et al., 2007; Dou et al., 2008b). Since all oomycete *Avr* genes that have been characterized to date encode RXLR-

dEER proteins (reviewed in Bouwmeester et al., 2009) it is anticipated that the HTS plays a crucial role in their function as *Avr* factors. Moreover, the fact that all known cognate *R* genes of oomycete *Avr* genes encode intracellular NBS-LRR proteins (Dangl and Jones, 2001) makes it conceivable that oomycete Avr factors are targeted to the host cell. In recent years, evidence that not only bacterial plant pathogens but also fungi (Ellis et al., 2007a) deliver effectors to the host cytoplasm, or even the nucleus, is accumulating. Examples are (putative) Avr factors from rust fungi (Dodds et al., 2004; Catanzariti et al., 2005; Kemen et al., 2005) and powdery mildews. Most strikingly is *Blumeria graminis* AVR_{A10}, a protein which lacks a SP but is nevertheless secreted by the fungus and targeted to the host nucleus. It forms a complex with the barley R protein MLA10 and WRKY transcription factors resulting in de-repression of PAMP-triggered basal defense (Ridout et al., 2006; Shen et al., 2007). Recently, Whisson et al. (2007) described an elegant approach to demonstrate that intracellular delivery of the oomycete Avr protein Avr3a is necessary for recognition by its cognate R protein. In that study the Type III secretion system of the bacterial pathogen *Pectobacterium atrosepticum* was exploited to deliver the C-terminal domain of the Avr protein into the host cell. By comparing wild type strains and Type III secretion mutants of *P. atrosepticum* it could be shown that Avr3a has to be targeted to the inside of the cell in order to be recognized by the R protein R3a. Two other recent studies used the *Pseudomonas syringae* Type III secretion system to target ATR13 into Arabidopsis cells (Sohn et al., 2007; Rentel et al., 2008) and both showed that intracellular targeting is also required for recognition of ATR13.

Prior to the discovery of the RXLR-dEER motif, Shan et al. (2004) published the positional cloning of the first oomycete *Avr* gene. To prove that the *P. sojae Avr1b*-1 gene was indeed an *Avr* gene, they infiltrated Avr1b protein, obtained by heterologous expression, into the apoplast (Shan et al., 2004). The *R* gene-mediated response that was observed suggested extracellular recognition of the Avr1b protein. More recent data have shown that transformation of a virulent *P. sojae* race with *Avr1b*-1 can complement the avirulent phenotype whereas *Avr1b*-1 mutated in the RXLR or dEER motif can not (Dou et al., 2008b), thus suggesting host cell targeting by virtue of the HTS and intracellular recognition of the Avr1b protein. In retrospect, the initial observations by Shan et al. (2004) point to an intrinsic transport mechanism embodied within Avr1b and independent of other pathogen proteins, as has been suggested for AvrM and AvrP4 from the flax rust pathogen *Melampsora lini* (Catanzariti et al., 2005). Hypothetically, an RXLR-dEER effector could be capable of inducing membrane channels or modulating the host endocytosis machinery for its own benefit. Interestingly, the Tyler lab has recently

shown that the HTS of Avr1b by itself can govern uptake of Avr1b into plant cells (Dou et al., 2008b; Govers and Bouwmeester 2008).

As we have observed in this study, the transient *in planta* expression system that is used to monitor effector activity can be misleading. Since *R4* is not cloned, we were constrained to perform our assays on potato differentials. Hence, we chose a method that was previously optimized for potato, i.e., PVX agroinfection (Vleeshouwers et al., 2006). In these agroinfection assays we never saw a response with *PiAvr4* when expressed without an SP and also the virus inoculations pointed towards a requirement for an SP. With agroinfiltration, however, there was no difference between a construct with or without SP. *In planta* expression assays in potato using agroinfection or agroinfiltration are quite challenging. With agroinfection PVX replication rates and stability of the constructs may vary. With agroinfiltration there are often non-specific responses and the success rate is highly dependent on the *A. tumefaciens* strain, the potato line and the conditions of the plants. The *A. tumefaciens* strain that we used though, did not give non-specific responses on potato. Others who have used different *in planta* expression assays in parallel also found conflicting results with respect to the requirement of an SP. In some cases when the *R* gene was available, agroinfection and co-infiltration of *Avr* and *R* gene was used (Armstrong et al., 2005); in other cases particle bombardment on plants with an *R* locus (Allen et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005; Dou et al., 2008a). Qutob et al. (2006b) showed that particle bombardment can distinguish between intra- and extracellular recognition. They tested a NEP-like protein (NLP) on Arabidopsis and, as expected for NLP, only constructs with SP were recognized. In the case of particle bombardment of *R3a* plants with *Avr3a,* only constructs lacking the SP elicited a HR (Armstrong et al*.,* 2005) leading to the conclusion that Avr3a is recognized intracellularly. In later experiments, however, co-infiltration of *Avr3a* and *R3a* in *Nicotiana benthamiana* showed no differences in necrotic response between constructs with and without SP (Bos et al., 2006). In the case of particle bombardment of Arabidopsis with *H. parasitica ATR1*^{NdWsB} the cell death response was stronger when the SP was removed (Rehmany et al., 2005) whereas *H. parasitica ATR13* with and without SP gave similar responses (Allen et al., 2004). However, when ATR13 with SP was targeted by *P. syringae* via Type III secretion into Arabidopsis cells, it did not elicit an HR (Rentel et al., 2008).

In the PVX agroinfection assays, *PiAvr4* without SP does not elicit a HR in *R4* plant. This suggests that an unprocessed, intracellular PiAvr4 effector does not activate defence and that the SP is required to facilitate secretion of PiAvr4 to reach the site where receptors are located. In contrast, the agroinfiltration assays show unequivocal recognition of PiAvr4 with and without the SP, suggesting that

the protein is also recognized inside the plant cell. Because of these conflicting data we can not draw firm conclusions about the primary site of recognition of PiAvr4, extracellular or intracellular. We can conclude though, that the presence or absence of the RXLR-dEER domain did not change the response. If, similar to the HTS of Avr1b (Dou et al., 2008b), the HTS of Avr4 by itself is capable of directing the uptake then our experimental data are in favour of extracellular recognition, but this needs further experimentation.

What then happens in a natural infection? And what is the function of the RXLR-dEER domain if PiAvr4 is not necessarily targeted to the cytoplasm? One could speculate that the host target of PiAvr4 may be a plant protein that is located extracellularly. We do not know the nature of R4, but it may differ from other late blight R proteins and resemble, for example, receptor-like kinases like the Cf or Xa21 R proteins (Dangl and Jones, 2001) that have a transmembrane region and an extracellular LRR domain. Alternatively, the host target may be a plant protein that mediates interaction between PiAvr4 and R4, i.e., a so-called virulence target, The first identified putative virulence target for an RXLR-dEER effector (IPI-O) is a membrane spanning protein with an extracellular domain with binding affinity for IPI-O and an intracellular kinase (Gouget et al., 2006). Another possibility to be considered when using transient *in planta* assays is re-uptake of PiAvr4 after secretion. However, since deletion of the RXLR-dEER domain from a construct with SP did not change the response, re-uptake mediated by the RXLR-dEER domain is not likely to occur. A third alternative may be that PiAvr4 has multiple host proteins as targets, some of which may be located intracellularly and others extracellularly, and that interaction with an extracellular target is perceived by R4. This may explain why the RXLR-dEER domain is intact and has not degraded through drift.

It is noteworthy to mention that the virulent alleles of *PiAvr4* have disrupted ORFs, in contrast to other oomycete *Avr* genes where point mutations or transcript levels determine the phenotype (summarized in Bouwmeester et al., 2009). The fact that PiAvr4 does not seem to be crucial for virulence raises the question if, in a compatible interaction between *r0* plants and isolates expressing *PiAvr4,* PiAvr4 has a function as effector and is internalized. Even though the RXLR motif and the dEER motif in PiAvr4 are conserved, one can not exclude the possibility that mutations surrounding the motifs have disabled the HTS and uptake is no longer possible. Recent experimental evidence showed that residues adjacent to the RXLR motif are crucial for the effector trafficking (Dou et al., 2008b). HMM scores of RXLR-dEER domains vary considerably and could have a predictive value for the function of the HTS (Jiang et al., 2008; Dou et al., 2008b). In *P. sojae* and *P. ramorum* almost 10% of the predicted RXLR-dEER genes are truncated (Jiang et

al., 2008) and in *P. infestans* many partial ORFs are also found (R.H.Y. Jiang, personal communication). Apparently, this reservoir of truncated RXLR-dEER genes does not harm the organism. *PiAvr4* is an example of a gene that is expressed in several field isolates, but is mutated without an obvious fitness penalty in other isolates.

To address the site of recognition of PiAvr4 and the role of the RXLR-dEER domain in PiAvr4 more detailed experiments are required. As has been shown for *P. infestans* Avr3a (Whisson et al., 2007) and *P. sojae* Avr1b (Dou et al., 2008b) cell biological analyses, using fluorescent tags, such as monomeric red fluorescent protein (mRFP) or green fluorescent protein (GFP), will help to localize PiAvr4, and mutational analysis of the RXLR-dEER domain will address the role of the HTS in PiAvr4.

MATERIALS AND METHODS

Phytophthora infestans isolates, and nomenclature of phenotypes and genes

The cross 71 mapping population, the two parental isolates NL80029 and NL88133 and the F1 progeny were described previously (Drenth et al., 1995; van der Lee et al., 1997; Guo et al., 2006; Jiang et al., 2006c). The genomic T30-4 BAC library was described by Whisson et al. (2001). The nomenclature of phenotypes is according to van der Lee et al. (2001). Isolates avirulent (a.o. NL88133 and T30-4) or virulent (a.o. NL80029) on *R4* plants have the AVR4 and avr4 phenotype, respectively. Genes and proteins are named according to the system proposed by the Oomycete Molecular Genetics Network (Govers and Gijzen, 2006). The gene cloned from the avirulence locus *Avr4* is designated as *PiAvr4* and the encoded protein as PiAvr4; *Piavr4* is the gene located at the recessive allele. *P. infestans* field isolates were retrieved from our own culture collection or kindly provided by colleagues.

Phytophthora infestans growth conditions

P. infestans was maintained on rye sucrose agar (RSA) (Caten and Jinks, 1968) at 18°C. Germinated cysts for RNA isolation were obtained as described by van West et al. (1998). Zoospores were released by flooding 10 day-old mycelium with ice cold water followed by 3 hour incubation at 4°C. Released zoospores were encysted by shaking and germinated in water for 2-4 hours at room temperature. Germinated cysts were concentrated by centrifugation for 5 minutes at 4000 x G, frozen in liquid N_2 and stored at -80°C. Mycelium for DNA and RNA isolation was obtained by growing *P. infestans* in liquid clarified rye sucrose (RS) medium for 7 days at 18°C. Dried mycelium was quickly frozen and stored at -80°C.

Nucleic acid manipulations

DNA and RNA isolation were performed according to established procedures. DNA extraction buffer (0.2 M Tris, pH=8.5, 0.25 M NaCl, 25 mM EDTA, 2% SDS) and 3 mm glass beads were added to frozen mycelium and the mixture was grinded in a FastPrep® instrument (Qbiogene, Carlsbad, CA, USA). Three phenol/chloroform extractions were performed, followed by an RNAse treatment and DNA precipitation. For RNA isolation, glass beads and TRIzol (Invitrogen, Carlsbad, CA, USA) were added to the frozen samples and

these were homogenized in the Fastprep® instrument. Further extraction was performed according to the manufacturers' procedure.

For RT-PCR we used the SuperScript™ III One-Step RT-PCR System (Invitrogen). Primer combinations RTAvr4F and RTAvr4R, and RTActAF and RTActAR (Table S2) were used to amplify *PiAvr4* and actin gene *ActA,* respectively. cDNA synthesis was performed at 50°C for 30 min followed by amplification cycles of 15 s at 94°C, 30 s at 50°C and 30 s at 74°C. 27 cycles were required for *ActA* amplification and 32 cycles for *PiAvr4* amplification.

DNA digestion, agarose gel electrophoresis, Southern blotting to Hybond-N+ membranes (Amersham Biosciences, Piscataway, NJ, USA) and hybridizations were performed according to standard procedures (Sambrook and Russell, 2001). Hybridization probes were ^{32}P-labeled by random primer labeling (Prime-a-gene labeling system, Promega, Madison, WI, USA).

BAC library screening and cloning

The BAC library was screened with *Avr4*-linked AFLP markers (van der Lee et al., 2001) using a three-dimensional pooling strategy (Whisson et al., 2001). Hybridization of the BAC library and BAC fingerprint blots, BAC DNA isolation and digestion and BAC contig building were performed as described (Jiang et al., 2005; Jiang et al., 2006c). Hybridization probes were a 295 bp fragment from EST PH051G10 (NCBI accession number CV920942) and a 465 bp *Bam*HI-*Sal*I restriction fragment located at the 3' UTR of *PiAvr4*. For subcloning and sequencing we used standard procedures (Sambrook and Russell, 2001). Sequence data have been submitted to GenBank under accession numbers EF672354 and EF672355.

Cloning of *PiAvr4* alleles

PiAvr4 alleles were amplified from genomic DNA isolated from different *P. infestans* isolates by *Pfu* proofreading polymerase (Promega) with primers PiAvr4F and PiAvr4R. DNA was amplified in a thermal cycler in 35 cycles of 60 s at 94°C, 60 s at 55°C and 150 s at 72°C. The obtained fragments were gel-purified and cloned into the pGEM-T Easy vector (Promega) according to the manufacturers' descriptions. Direct sequencing of PCR bands was performed using primers PiAvr4seqF and PiAvr4seqR. Cloned fragments were sequenced using standard M13 primers. Sequence files were assembled and analyzed using the Vector NTI 10 software package.

DNA transformation of *Phytophthora infestans*

P. infestans isolates D12-2 and T35-3 were stably transformed using the PEG protoplast transformation protocol described by van West et al. (1998). Protoplasts were obtained by a mixture of lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) (5 mg mL^{-1}) and cellulase from *Trichoderma reesei* (Sigma-Aldrich) (2 mg mL^{-1}). For transformation we constructed plasmid pTA23.9 that carries *PiAvr4* and a geneticin (G418) resistance gene. To generate pTA23.9, a 2890 bp *Bam*HI fragment derived from BAC01E13 and carrying *PiAvr4* (Fig. 2) was cloned into pTH209SK, a vector with a pBS II SK+ backbone that caries a 5' HSP70::NPTII::3' HAM34 cassette. Transformants were selected on RSA supplemented with 3 µg mL^{-1} geneticin. Transformants appeared after 9-20 days.

Plant genotypes and growth conditions

R4 potato clone Cebeco44-31-5 (Black et al., 1953; Mastenbroek, 1953) from the Mastenbroek differentials, and cultivar Isola (*R4*) were used for infection assays. As control plants cultivar Bintje (*r0*) and clone SW8540-025 (*R3a*) (Huang et al., 2005a) were used. Plants were grown *in vitro* for 4 weeks in climate chambers on MS30 medium (16 hours light, 8 hours dark, 20°C) and transferred to potting soil in a greenhouse, where they were grown for an additional 4-6 weeks (16 hours light 21°C, 8 hours dark, 19°C).

Virulence assays
Virulence phenotypes of *P. infestans* isolates and transformants were determined in assays performed as described by Vleeshouwers et al. (1999). Ten µL of a zoospore suspension (100 spores μL^{-1}) were spotted on the abaxial side of detached potato leaves. Lesion development was monitored up to 6 days post inoculation (dpi). Infection efficiency (IE) and lesion growth rate (LGR) were determined as described (Vleeshouwers et al., 1999).

Binary constructs, agroinfection and agroinfiltration
For *in planta* expression of *PiAvr4* binary PVX constructs were made in vector pGR106 (Jones et al., 1999). Fragments were amplified by PCR and overlap PCR with the appropriate primers (Tables S2 and S4). The amplicons were digested and cloned into pGR106. The constructs were than transformed to *Agrobacterium tumefaciens* strain GV3101, which carries the pSoup helper plasmid (Hellens et al., 2000).

Agroinfection assays were performed as described for potato (Vleeshouwers et al., 2006) and *Nicotiana* (Huitema et al., 2005). Briefly, *A. tumefaciens* strains containing the binary PVX constructs were grown for 2 days on LBman agar medium (10 g bacto trypton, 5 g yeast extract, 2.5 g NaCl, 10 g mannitol and 15 g daishin agar per liter) supplemented with antibiotics (10 µg mL^{-1} rifampicin and 100 µg mL^{-1} kanamycin). Toothpicks were used to transfer bacteria to the leaves and to pierce the leaf creating wounded tissue. Responses were monitored up to 4 weeks post inoculation.

For agroinfiltration assays, *PiAvr4* constructs were cloned in vector pGRAB (Whisson et al., 2007). The obtained constructs were than transformed into *Agrobacterium tumefaciens* strain AGL1, which carries the pSoup helper plasmid (Hellens et al., 2000).

Agroinfiltration assays were performed as described previously (van der Hoorn et al., 2000). Briefly, *A. tumefaciens* strains containing the binary constructs were grown in 100 mL YEB medium (5 g beef extract, 5 g bacto trypton, 5 g sucrose and 1 g yeast extract per liter) supplemented with 20 µM acetosyringone, 10 mM MES, 10 µg mL^{-1} rifampicin and 100 µg mL^{-1} kanamycin. When the OD_{600} reached 0.8, cells were centrifuged and resuspended in MMA (5 g MS salts, 20 g sucrose and 1,95 g MES per liter, pH 5.6) to an OD_{600} of 2.0. Virulence was induced by incubating the resuspended bacteria at room temperature for 1 hour. Leaves of 2-3 week old potato plants were infiltrated with the bacterial suspension. Responses were monitored up to 1 week post inoculation.

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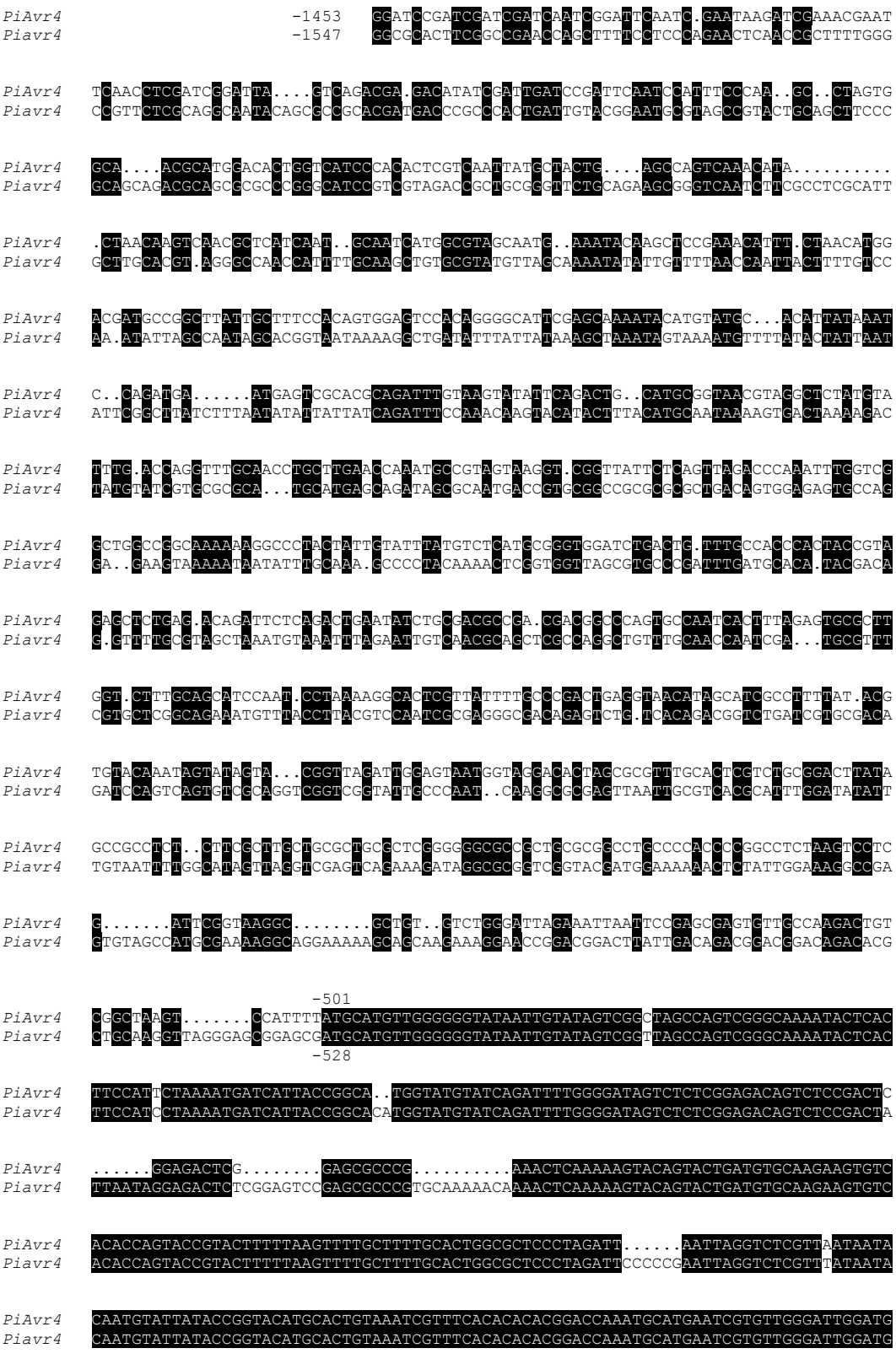
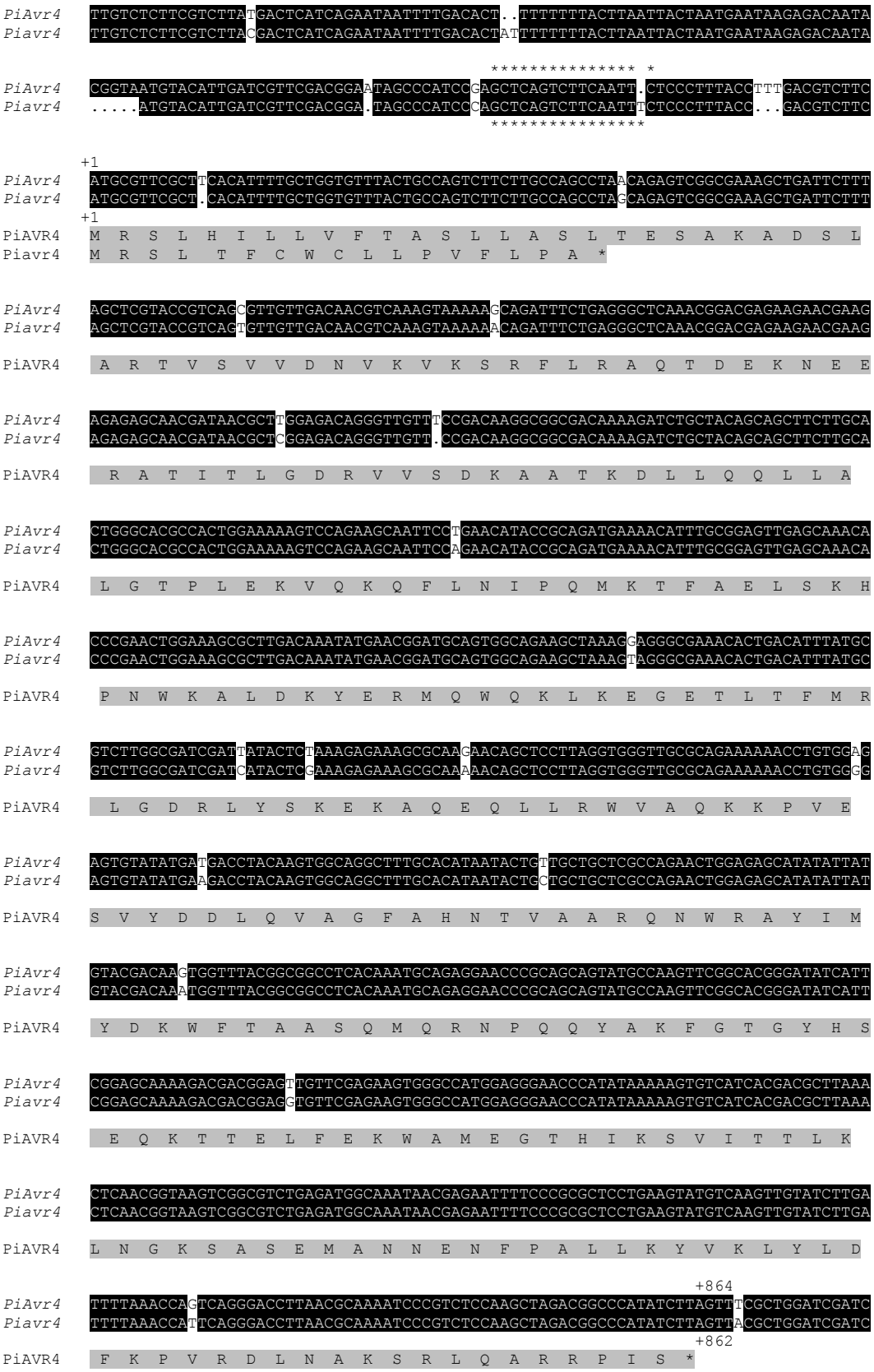
SUPPLEMENTARY MATERIAL

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+1
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+1
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Piavr4 M R S L T F C W C L L P V F L P A *
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Piavr4 AGAGAGCAACGATAACGCTCGGAGACAGGGTTGTTT.CCGACAAGGCGGCGACAAAAGATCTGCTACAGCAGCTTCTTGCA
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+864
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+862
PiAVR4 F K P V R D L N A K S R L Q A R R P I S *

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PiAvr4    GGTATAAGACCGTGACGAAATGCCAAATAACCGTCATTCCGCTTTTACTGCGTCAGTTGCTGTTGTCTCTCCTCCATTCT
Piavr4    GGTATAAGACCGTGACGAAATGCCAAATAACCGTCATTCCGCTTTTACTGCGTCAGTTGCTGTTGTCTCTCCTCCATTCT

PiAvr4    AGGTGGTCGAC   +971
Piavr4    AGGTGGTCGAC   +969
```

Figure S1. DNA sequence alignment of *Piavr*4 and *PiAvr*4 and the deduced amino acid sequences. Identical nucleotides are shaded black. Gray shaded lines show the amino acid sequence of PiAVR4 and the 17 amino acid peptide deduced from *Piavr4*. The position of the 16 nucleotide oomycete core consensus sequence (GCTCATTYYNCAWTTT; Pieterse et al., 1994; McLeod et al., 2004) is indicated by *. Numbers show the position relative to the first nucleotide of the ATG start codon at +1.

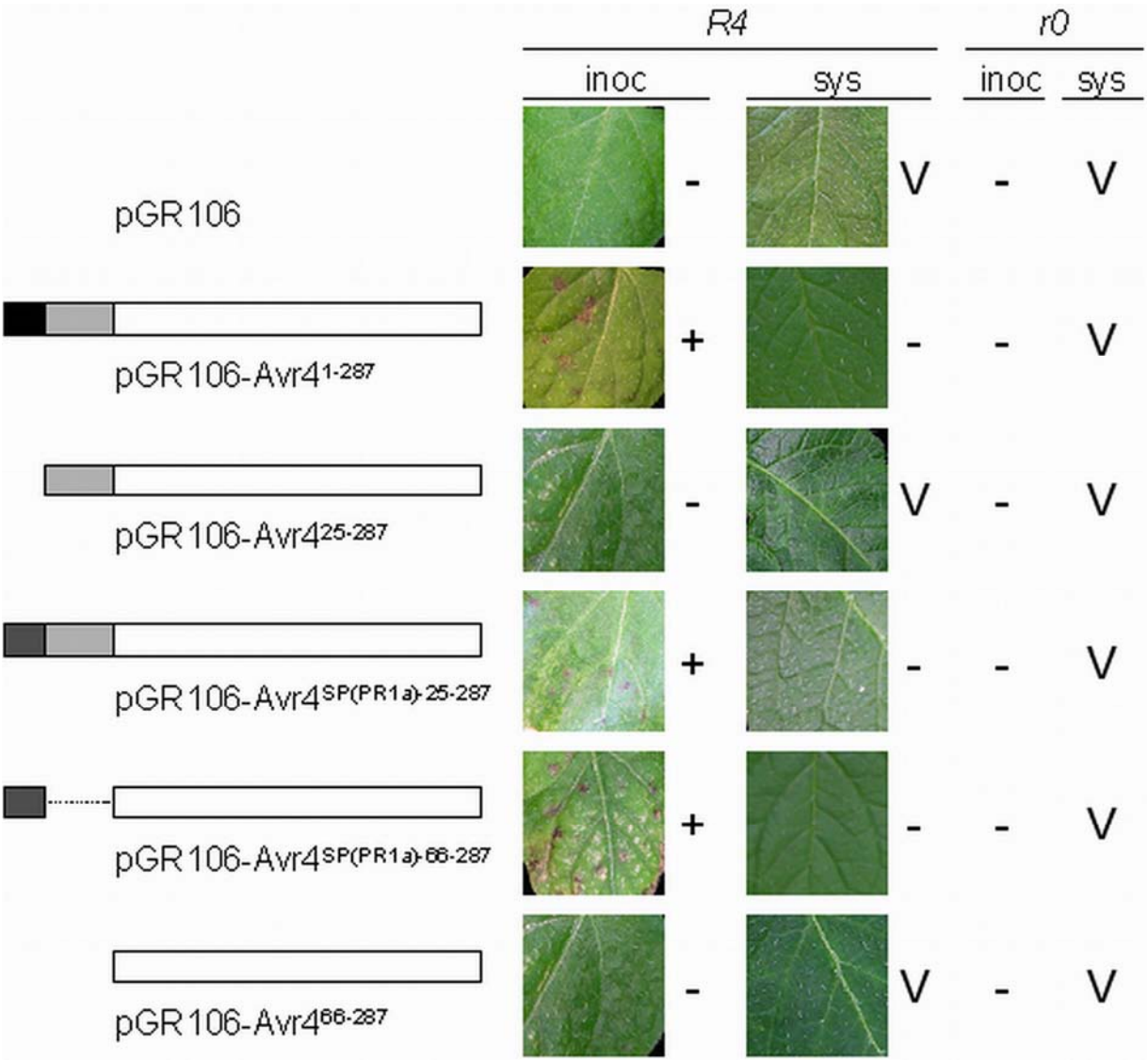
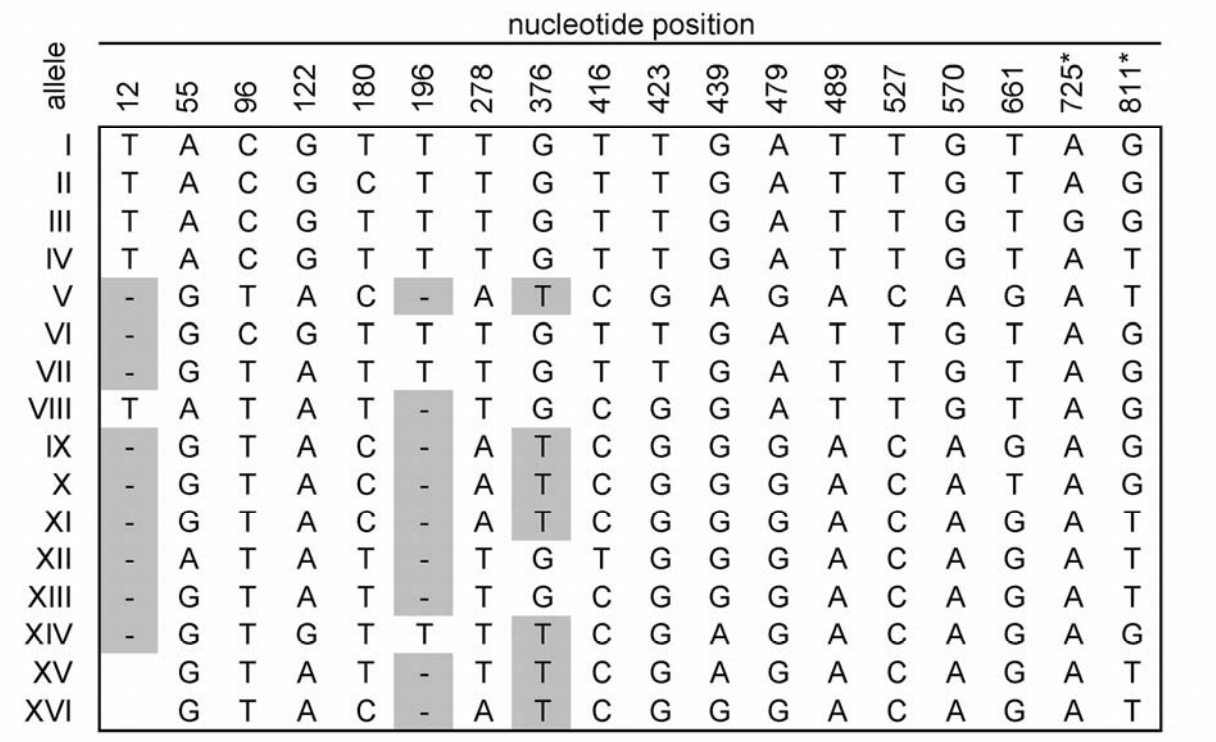


Figure S2. Inoculation of potato line Cebeco4431-5 (*R4*) and cultivar Bintje (*r0*) with PVX particles containing various *Avr4* constructs. PVX inoculation of potato line Cebeco4431-5 (*R4*) and cultivar Bintje (*r0*) with strains carrying pGR106 constructs as indicated. At 5 dpi the primary inoculated leaves (inoc) were scored for the development of necrosis (indicated by +). At 21 dpi systemic virus infection (sys) was scored (indicated by V). In the schematic representation of the constructs the SP of PiAvr4 is shown in black, the SP of PR1a in dark gray, the RXLR-dEER region (aa 25-65) in light gray and the C-terminal region (aa 66 to 287) as an open bar.



allele	nucleotide position																	
	12	55	96	122	180	196	278	376	416	423	439	479	489	527	570	661	725*	811*
I	T	A	C	G	T	T	T	G	T	T	G	A	T	T	G	T	A	G
II	T	A	C	G	C	T	T	G	T	T	G	A	T	T	G	T	A	G
III	T	A	C	G	T	T	T	G	T	T	G	A	T	T	G	T	G	G
IV	T	A	C	G	T	T	T	G	T	T	G	A	T	T	G	T	A	T
V	-	G	T	A	C	-	A	T	C	G	A	G	A	C	A	G	A	T
VI	-	G	C	G	T	T	T	G	T	T	G	A	T	T	G	T	A	G
VII	-	G	T	A	T	T	T	G	T	T	G	A	T	T	G	T	A	G
VIII	T	A	T	A	T	-	T	G	C	G	G	A	T	T	G	T	A	G
IX	-	G	T	A	C	-	A	T	C	G	G	G	A	C	A	G	A	G
X	-	G	T	A	C	-	A	T	C	G	G	G	A	C	A	T	A	G
XI	-	G	T	A	C	-	A	T	C	G	G	G	A	C	A	G	A	T
XII	-	A	T	A	T	-	T	G	T	G	G	G	A	C	A	G	A	T
XIII	-	G	T	A	T	-	T	G	C	G	G	G	A	C	A	G	A	T
XIV	-	G	T	G	T	T	T	T	C	G	A	G	A	C	A	G	A	G
XV		G	T	A	T	-	T	T	C	G	A	G	A	C	A	G	A	T
XVI		G	T	A	C	-	A	T	C	G	G	G	A	C	A	G	A	T

Figure S3. Overview of the nucleotide variation in sixteen identified *PiAvr4* alleles. The positions of the nucleotides that are polymorphic are indicated in the top row. Position 1 is the A of the start codon ATG. A nucleotide deletion is indicated by -. Polymorphisms that give rise to a frameshift mutation or a premature stop codon (ΔT^{12}, ΔT^{196} and $G^{376}T$) are marked by grey boxes. The two nucleotides marked by an asteriks (*) indicate the position where a SNP results in a non-synonymous amino acid substitution in the intact PiAvr4 peptide. Two cells are empty because these alleles were not completely sequenced.

Table S1 Overview of BACs selected by screening the BAC library with AFLP markers (shaded cells in the first block) and by hybridization with a 3'UTR probe of *PiAvr4* (shaded cells in the second block). In columns 2 to 6 in the first block a '+' marks the presence of an *Avr4*-linked AFLP marker on the BAC. The two columns in the last block indicate which BACs carry the avirulent and virulent allele, respectively

	AFLP screening	E+AG/M+AAs288	E+GG/M+ATs366	E+TT/M+GAs176	E+TG/M+ATs238	E+AT/M+AGs608		3'UTR hybridization		Avr4	avr4
BAC01E13	+		+	+	+			+		+	
BAC02E12								+			+
BAC03I07								+			+
BAC03K21								+		+	
BAC03P10								+			+
BAC13G19								+			+
BAC18E06	+					+					
BAC19J18	+			+	+						
BAC22J22	+	+	+					+		+	
BAC27B19								+			+
BAC32C06								+			+
BAC32D01	+	+	+					+		+	
BAC33F18								+		+	
BAC33G14	+	+	+					+		+	
BAC38B05	+		+	+	+	+		+		+	
BAC40K10	+	+	+					+		+	
BAC52D13								+		+	
BAC67P09	+		+					+		+	

Table S2 Primers used for RT-PCR and cloning

Primer	Sequence
PiAvr4F	5'-ATGCGTTCGCTTCACATTTTGCTGG-3'
PiAvr4R	5'-CTAAGATATGGGCCGTCTAGCTTGGAG-3'
PiAvr4seqF	5'-TAACGCTCGGAGACAGGGTT-3'
PiAvr4seqR	5'-AGCGCGGGAAAATTCTCGTT-3'
RTAvr4F	5'-GCTGGTGTTTACTGCCAGTCTTCTTGCCAG-3'
RTAvr4R	5'-CCCACCTAAGGAGCTGTTCTTGCGC-3'
RTActAF	5'-CGGCTCCGGTATGTGCAAGGC-3'
RTActAR	5'-GCGGGCACGTTGAACGTCTC-3'
NotIAvr4F	5'-CAGCGGCCGCATGCGTTCGCTTCACATTTTG-3'
NotIAvr4R	5'-GTGCGGCCGCTAAGATATGGGCCGTCTAGC-3'
NotIAvr4^{-SP}F	5'-CAGCGGCCGCATGGATTCTTTAGCTCGTAC-3'
ClaIPR1aF	5'-CATCGATATGGGATTTGTTCTCTTTTCAC-3'
Pr1a-Avr4F	5'-TTGCCGTGCCGATTCTTTAGCTCGTACCGTC-3'
Pr1a-Avr4R	5'-GCTAAAGAATCGGCACGGCAAGAGTGGG-3'
ClaAvr4$^{66-287}$F	5'-GATCGATATGCGTTCGCTTCACATTTTGC-3'
SP(PR1a)-Avr4$^{66-287}$F	5'-CTCTTGCCGTGCCGACAAGGCGGCG-3'
SP(PR1a)-Avr4$^{66-287}$R	5'-CGCCGCCTTGTCGGCACGGCAAGAG-3'

Table S3 Constructs used for transformation of *Phythophthora infestans*, agroinfection and agroinfiltration

clone	insert	insert size (bp)	vector
pSKA23	BAC01E13 subclone	2889	pBluescript SK$^-$
pSKB5	BAC38B05 subclone	2889	pBluescript SK$^-$
pSKC21	BAC40K10 subclone	2889	pBluescript SK$^-$
pSK7A2	BAC32C06 subclone	3824	pBluescript SK$^-$
pTHA23.9	A23	2889	pTH209SK
pGR106	-	-	pGR106
pGR106-Avr4$^{1-287}$	PiAvr4$^{1-287}$	864	pGR106
pGR106-Avr4$^{25-287}$	PiAvr4$^{25-287}$	792	pGR106
pGR106-Avr4$^{SP(PR1a)-25-287}$	Pr1a signal peptide - PiAvr4$^{25-287}$	882	pGR106
pGR106-PiAvr4$^{66-287}$	PiAvr4$^{66-287}$	669	pGR106
pGR106-Avr4$^{SP(PR1a)-66-287}$	PR1a signal peptide - PiAvr4$^{66-287}$	759	pGR106
pGR106-CRN2	CRN2	1371	pGR106
pGRAB	-	-	pGRAB
pGRAB-Avr4$^{1-287}$	PiAvr4$^{1-287}$	864	pGRAB
pGRAB-Avr4$^{25-287}$	PiAvr4$^{25-287}$	792	pGRAB
pGRAB-CRN2	CRN2	1371	pGRAB

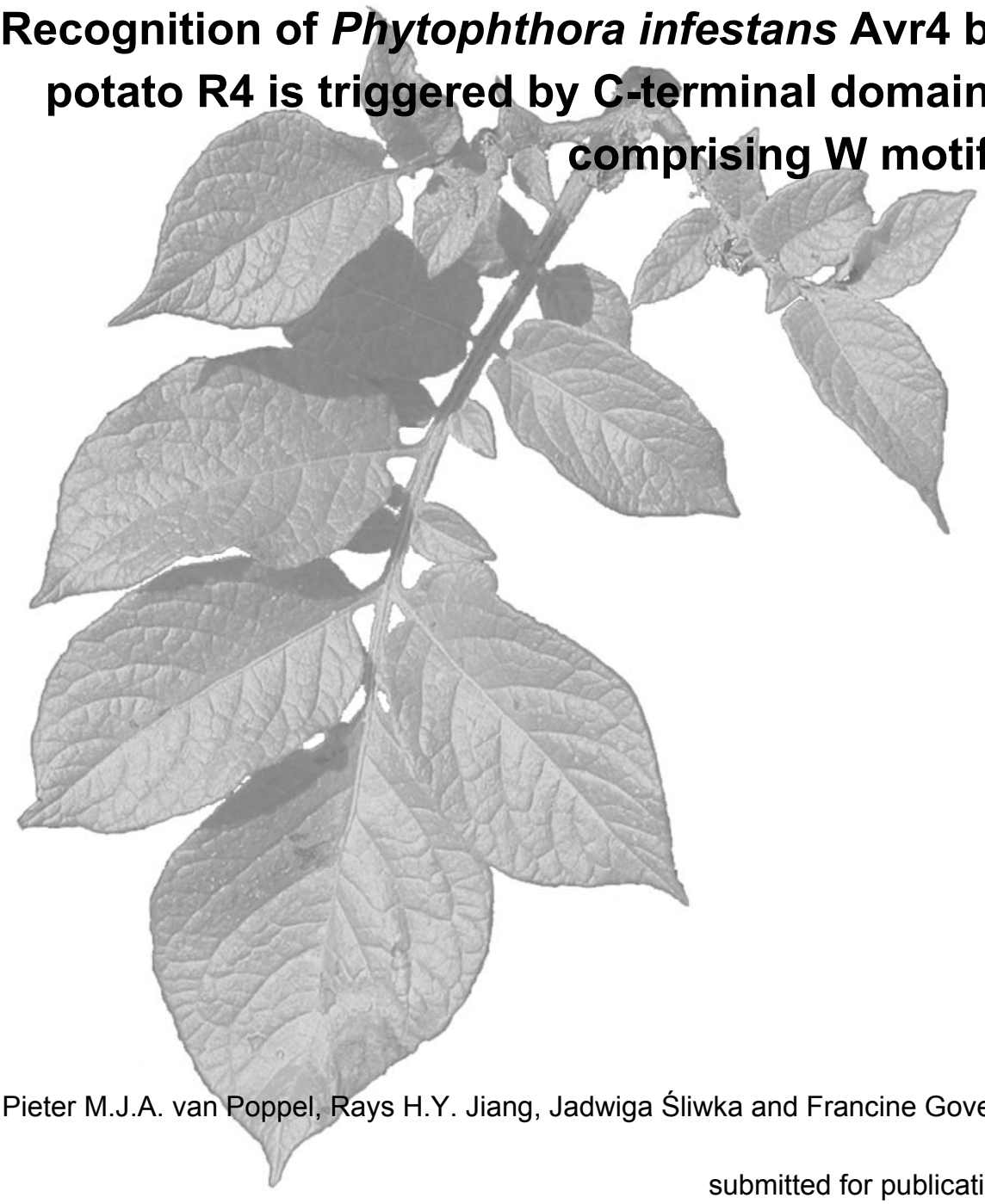
Table S4 Primers and templates used to generate pGR106 and pGRAB constructs by PCR (upper panel) and overlap PCR (lower panel)

Construct	Template	F primer	R primer
pGR106-Avr4$^{1-287}$	PiAvr4	NotIAvr4F	NotIAvr4R
pGR106-Avr4$^{25-287}$	PiAvr4	NotIAvr4^{-SP}F	NotIAvr4R
pGR106-Avr4$^{66-287}$	PiAvr4	ClaAvr4$^{66-287}$F	Pr1a-Avr4R
pGRAB-Avr4$^{1-287}$	PiAvr4	NotIAvr4F	NotIAvr4R
pGRAB-Avr4$^{25-287}$	PiAvr4	NotIAvr4^{-SP}F	NotIAvr4R

Construct	Templates		PR1a primers		Avr4 primers	
pGR106-Avr4$^{SP(PR1a)-25-287}$	PR1a		ClaIPR1aF	Pr1a-Avr4R		
		PiAvr4			Pr1a-Avr4F	NotIAvr4R
	PR1a	PiAvr4	ClaIPR1aF			NotIAvr4R
pGR106-Avr4$^{SP(PR1a)-66-287}$	PR1a		ClaIPR1aF	Pr1a-Avr4R		
		PiAvr4			Pr1a-Avr4F	NotIAvr4R
	PR1a	PiAvr4	ClaIPR1aF			NotIAvr4R

CHAPTER 3

Recognition of *Phytophthora infestans* Avr4 by potato R4 is triggered by C-terminal domains comprising W motifs



Pieter M.J.A. van Poppel, Rays H.Y. Jiang, Jadwiga Śliwka and Francine Govers

submitted for publication

SUMMARY

Oomycete RXLR-dEER effector proteins are rapidly evolving and the selective pressure is targeted predominantly at the C-terminal part of these proteins. The majority of the RXLR-dEER proteins has recognizable motifs of 21-30 amino acids in the C-terminal domain that are named after conserved amino acid residues at fixed positions within the respective motifs. Here we describe that the *Phytophthora infestans* RXLR-dEER protein Avr4 has three W motifs and one Y motif in its C-terminal domain. Agroinfection assays using constructs encoding modified forms of PiAvr4 showed that the region containing the W2 motif in combination with either the W1 or W3 motif triggers a necrotic response in potato plants carrying the resistance gene *R4*. By mining the superfamily of Avirulence Homologs (Avh) deduced from three sequenced *Phytophthora* genomes we identified several Avh proteins as homologs of PiAvr4, six in *P. infestans*, one in *P. ramorum* and seven in *P. sojae*. One very close homolog of *PiAvr4* was cloned from the sibling species, *Phytophthora mirabilis.* This species is not pathogenic on potato but, similar to PiAvr4, PmirAvh4 triggered a necrotic response on potato clones carrying *R4* but not on clones lacking *R4*. Genes encoding RXLR-dEER effectors are often located in regions showing genome rearrangements. Alignment of the genomic region harboring *PiAvr4* with syntenic regions in *P. sojae* and *P. ramorum* revealed that *PiAvr4* is located on a 100 kb indel block and surrounded by transposable elements.

INTRODUCTION

Oomycete plant pathogens are responsible for a large number of devastating diseases on many crop plants and ornamentals (Bouwmeester et al., 2009). Like many other plant pathogens, they secrete a range of effector proteins that facilitate the infection of host plants, for example by suppressing defense responses. One class of secreted effectors comprises the RXLR-dEER proteins that have two conserved amino acid motifs in the N-terminal region, RXLR and dEER. These motifs were first found in a number of proteins encoded by oomycete avirulence (*Avr*) genes that have 'gene-for-gene' interactions with host resistance (*R*) genes (Govers and Gijzen, 2006; Rehmany et al., 2005; **Chapter 2**). More recent studies showed that the domain carrying the RXLR and dEER motifs is required for host-cell targeting of the effector proteins (Whisson et al., 2007; Dou et al., 2008b). Genome mining revealed that each of the sequenced oomycete genomes contains hundreds of genes encoding RXLR-dEER effectors (Whisson et al., 2007; Jiang et al., 2008). The majority of the RXLR-dEER genes seem to be derived from a common ancestor and, because of their homology to known *Avr* genes they are also referred to as avirulence gene homologs or *Avh* genes (Jiang et al., 2008). The three oomycete genomes that have been sequenced and annotated (*Phytophthora ramorum, Phytophthora sojae* and *Phytophthora infestans*) show a high degree of conserved synteny (Jiang et al., 2006a; 2006b). *Avh* genes, however, are often located on indel blocks and in regions showing genome rearrangements (Jiang et al., 2006a; 2008). Typically the *Avh* genes are flanked by transposon-like sequences and this may explain the dispersal of these genes throughout the genome (R.H.Y.J. and M. C. Zody, unpublished data).

As postulated in the zig-zag model (Jones and Dangl, 2006) effectors can evolve to evade host resistance responses. Analyses of the C-terminal domains of RXLR-dEER proteins showed that these effectors are indeed exposed to strong positive selection, leading to fast evolution and diversifying sequences (Win et al., 2007; Jiang et al., 2008). In a recent study the RXLR-dEER effector reservoir in two *Phytophthora* species was analyzed and Hidden Markov Model (HMM) searches were used to identify conserved motifs in the C-terminal region (Jiang et al., 2008). The motifs that were found in over half of all RXLR-dEER proteins, were named W, Y and L after a conserved amino acid residue at a fixed position in the respective motifs. The W (tryptophan), Y (tyrosine) and L (leucine) motifs are 21-30 amino acids in length and can occur in modules in the order W-Y-L. The number of modules and motifs varies in each RXLR-dEER protein. In *P. sojae* and *P. ramorum*, 30% of the RXLR-dEER proteins possess two to eight W-Y-L modules. Others lack recognizable motifs or have only W motifs, or W and Y motifs. The

number of modules correlates with the length of the respective proteins. For example, one RXLR-dEER protein in *P. infestans* carries 11 W-Y-L modules on a total length of 989 amino acids (unpublished data). Of the known oomycete Avr proteins, Avr1b from *P. sojae* has one W and one Y motif, and an additional K motif with several lysine residues (Dou et al., 2008a). Avr3a and IPI-O from *P. infestans* each have a single W motif and no Y or L motifs. The Avr proteins ATR1 and ATR13 from *Hyaloperonospora parasitica* lack W, Y and L motifs although ATR13 carries several repeats in the C-terminal region (Allen et al., 2004). As shown by mutational analyses, both the W and Y motif in Avr1b are involved in governing avirulence of *P. sojae* towards soybean plants carrying the resistance gene *Rps*1b as well as in suppressing BAX-mediated programmed cell death (PCD) (Dou et al., 2008a). The difference between the virulent and avirulent form of the *P. infestans* Avr3a effector is restricted to two amino acids in the C-terminal region, one of which at position 103 is located in the W domain (Armstrong et al., 2005). The avirulent form has $K^{80}I^{103}$, while the virulent form has $E^{80}M^{103}$. The entire 75 amino acid C-terminal region, including the W motif, is required to elicit *R3a*-dependent HR and to suppress INF1-triggered PCD, but a $K^{80}M^{103}$ variant is unable to suppress PCD while it remains avirulent on *R3a* plants (Bos et al., 2006). These results suggest a role in avirulence of the sequences flanking the W motif in Avr3a.

Previously we have identified *PiAvr4*, a *P. infestans* avirulence gene that has a gene-for-gene interaction with the potato resistance gene *R4* (**Chapter 2**). In this study we show that the RXLR-dEER effector PiAvr4 and its close homologs carry W and Y motifs. We used deletion constructs to investigate which part of the C-terminus of PiAvr4 is required for recognition by potato R4 and the role of the W motifs. We also analyzed the region in the *P. infestans* genome that harbors *PiAvr4* and demonstrate that, like many *Avh* genes, *PiAvr4* is located at a synteny breakpoint.

RESULTS

Homologs of *PiAvr4* in *Phytophthora infestans* and other *Phytophthora* species

Previously we have identified several allelic variants of *PiAvr4* in different *P. infestans* isolates and performed genomic Southern blot analysis that suggested the presence of putative *PiAvr4* homologs in *P. infestans* and in sibling species of *P. infestans*, including *Phytophthora phaseoli, Phytophthora andina*, *Phytophthora mirabilis* and *Phytophthora ipomoeae* (**Chapter 2**). To obtain a *PiAvr4* homolog from *P. mirabilis* we used *PiAvr4*-specific primers for PCR amplification and cloned

a homolog from strain PIC99111. *PmirAvh4* encodes a 290 amino acid protein with a high similarity to PiAvr4 (BLASTP E-value = 2 e-137, sequence similarity 89%) and all the characteristics of an RXLR-dEER protein including a signal peptide and an RXLR-dEER domain. Compared to PiAvr4, PmirAvh4 is three amino acids larger. This is due to an insertion of six amino acids and a deletion of three amino acids between the RXLR and dEER motifs in PmirAvh4 (Fig. S1).

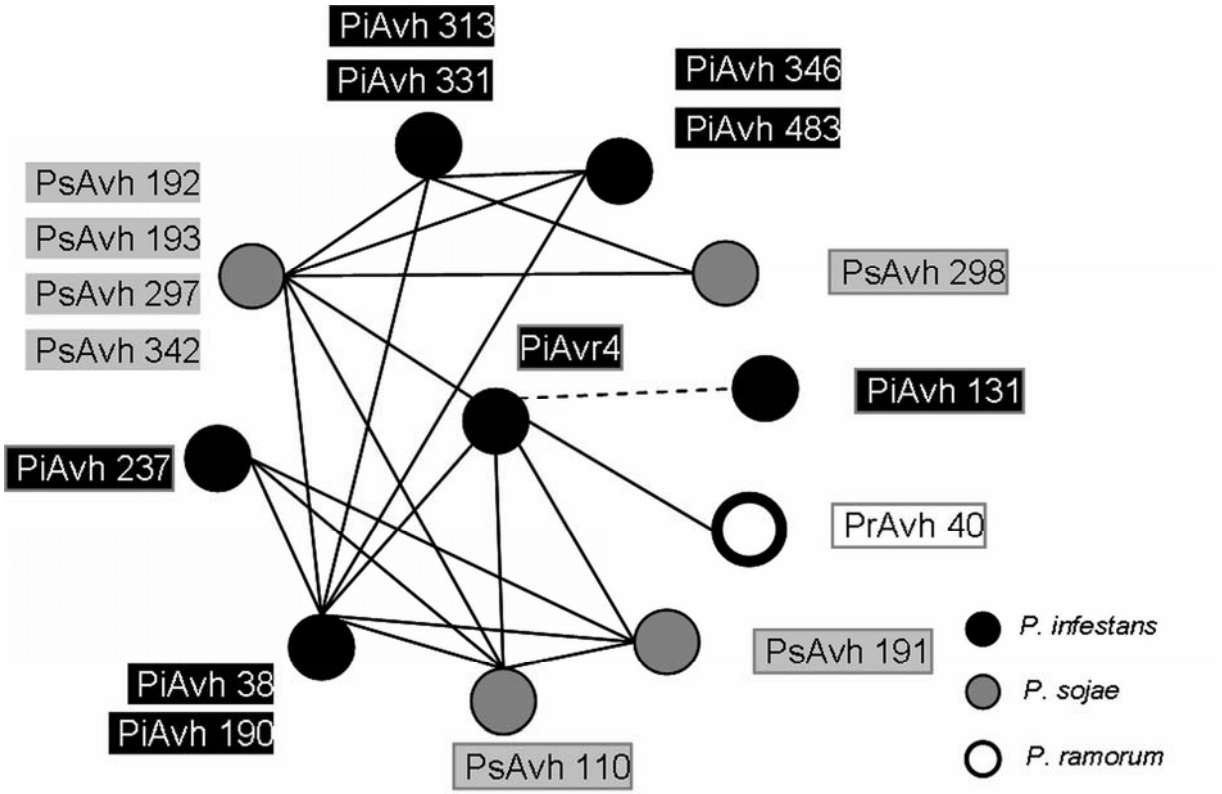


Figure 1. Sequence similarities between PiAvr4 and its homologs. Each protein has been used for BLASTP searches against the entire set of Avh proteins from *P. infestans, P. sojae* and *P. ramorum*. Proteins are represented by dots and labeled next to the dot. Proteins with sequence similarities above 90% are represented by one dot. Different shading of dots and labels is used to distinguish effectors from the different species. A line connecting two proteins represents a BLASTP hit (*E* value < 1e-5). The dotted line indicates a similarity that is restricted to the N-terminus of the two connected proteins.

BLASTN searches of public databases with the *PiAvr4* sequence resulted in a single hit to an expressed sequence tag (EST) of *P. sojae* (AY183415; Qutob et al., 2002; **Chapter 2**). Moreover, ten RXLR-dEER proteins named PsAvh_110, PsAvh_191, PsAvh_192, PsAvh_193, PsAvh_297, PsAvh_342, PiAvh_38, PiAvh_131, PiAvh_190, and PrAvh_40, were identified in a BLASTP search (amino acid sequence identity > 30%) of a pool of *Phytophthora* Avirulence Homologs (Avh), of which PsAvh_110 is the protein corresponding to the previously identified EST. The Avh pool was created by gathering all predicted RXLR-dEER effectors from three *Phytophthora* species: 385 in *P. sojae*, 370 in *P.*

ramorum (Jiang et al., 2008) and 562 in *P. infestans* (RHYJ and B. Haas, unpublished data). By subsequent BLASTP searches using the ten Avr4 homologs six additional Avr4 family members were identified (Fig. 1, Table S1). By the same search criteria, no homologs could be found in the genomes of *Phytophthora capsici* and *H. parasitica*.

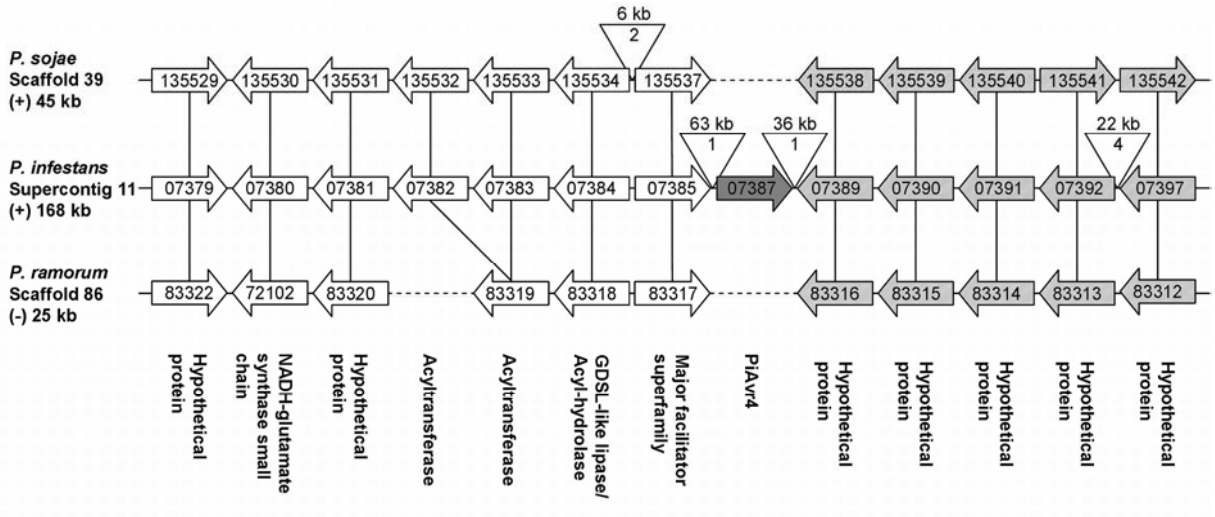


Figure 2. A schematic representation of the genomic region in *P. infestans* that carries *PiAvr4* (gene model 07387) and regions with conserved synteny in *P. sojae* and *P. ramorum*. The sizes of the aligned regions are indicated, as well as the orientation of the regions in the respective scaffolds or contig (+ or -). The orthologous gene models are aligned and the predicted function is indicated. Gene models with a high similarity to transposon sequences are depicted in the inverted triangles in which the number of transposon gene models is indicated. Of the gene cluster located downstream of *PiAvr4* only five of the more than 30 gene models are shown (shaded arrows). The spacing between the gene models is not on scale. Dotted lines are used to connect gene models but do not represent the actual relative distance between gene models.

PiAvr4 in *Phytophthora infestans* is located on a 100 kb indel block

Comparative analyses of sequenced *Phytophthora* genomes revealed that the majority of the *Avh* genes is located on indel blocks that interrupt regions of conserved synteny (Jiang et al., 2006a; 2008). To determine the genomic context of *PiAvr4* we aligned the genomic region surrounding *PiAvr4* to the genome sequences of *P. sojae* and *P. ramorum*. *PiAvr4* is located on supercontig 11 (size 3761 kb; *P. infestans* genome assembly version 1.0) at position 359782-360645. Alignment of a 168 kb region surrounding *PiAvr4* with a 45 kb region on *P. sojae* scaffold 39 (size 598 kb; genome assembly version 1.1) showed a conserved order and orientation of twelve gene models with the exception of one inversion (Fig. 2; Table 1). Similarly, a 25 kb region on *P. ramorum* scaffold 86 (size 203 kb; genome assembly version 1.1) contains eleven of the gene models in the same order. One gene model encoding an acyltransferase is duplicated in *P. sojae* and *P. infestans* but not in *P. ramorum.* The most obvious difference between the three genomes is

the presence of a 100 kb indel block in *P. infestans* that breaks the conserved synteny between the three species. This indel block carries three gene models, one of which is *PiAvr4* while the other two encode transposons. Apart from these two transposon gene models the indel block consists almost entirely of transposon-like sequences. Neither *P. sojae* nor *P. ramorum* carries a gene model for a PiAvr4 homolog or any other Avh protein in the syntenic regions on scaffolds 39 and 86 respectively. In *P. sojae* the closest *PiAvr4* homolog (i.e. *PsAvh_110*) is located on scaffold 102 and in *P. ramorum* (i.e. *PrAvh_40*) on scaffold 100. This analysis shows that *PiAvr4* marks a synteny breakpoint in *P. infestans.* The fact that *PiAvr4* is flanked by many transposon-like sequences, suggests that transposons were involved in the rearrangement of the genomic region carrying *PiAvr4.*

Downstream of *PiAvr4* is a large gene cluster that is conserved between the three species. The more than thirty genes in this cluster are highly similar (with paralog sequence identity of 85%) but have no homology to known genes. In this gene cluster *P. infestans* has an indel block of 22 kb that carries four transposon gene models but no *Avh* genes. In *P. infestans* the closest *PiAvr4* homologs are located on other scaffolds. *PiAvh_38* and *PiAvh_190* sharing over 90% sequence similarity but with less than 50% similarity to *PiAvr4,* are located on scaffolds 9 and 19, respectively, and *Pi_Avh131* with similarity restricted to the N-terminus is located on scaffold 1 (Table S1). *PiAvh_38* and *PiAvh_190* are likely recently duplicated paralogs, one of which is located on a segmental duplication of 3 kb that settled elsewhere in the genome. Often such closely related paralogs are found on the same scaffold as is the case for *PiAvh_331* and *PiAvh_131* that share 91% protein sequence similarity and are 75 kb apart from each other, and the nearly identical genes *PsAvh_192* and *PsAvh_193* that are only 32 kb apart. Remarkably, also the less divergent *PsAvh_110* and *PsAvh_191,* the two closest *PiAvr4* homologs in *P. sojae* and sharing 60% protein sequence similarity, are only 25 kb apart. In contrast to the clustering of anciently duplicated paralogs such as elicitin genes (Jiang et al., 2006b) and sPLD-like genes (Meijer and Govers, 2006), many of the more divergent *Avh* genes are often scattered over the genome (Jiang et al., 2008).

Table 1 Gene models surrounding the *PiAvr4* gene in *Phytophthora infestans*

Model	Start	Stop	+/-[a]	Predicted protein	Ortholog in *P. ramorum*	Ortholog in *P. sojae*
PITG_07379.1	259036	261015	-	Unknown	Pr83322	Ps135529
PITG_07380.1	261154	262848	-	NADH-glutamate synthase small chain	Pr72102	Ps135530
PITG_07381.1	265234	267078	-	Unknown	Pr83320	Ps135531
PITG_07382.1	290966	292351	-	Acyltransferase	Pr83319	Ps135532
PITG_07383.1	293408	293362	-	Acyltransferase	Pr83319	Ps135533
PITG_07384.1	295171	295112	-	GDSL-like Lipase/Acyl-hydrolase	Pr83318	Ps135534
PITG_07385.1	295644	297048	+	Major Facilitator Superfamily	Pr83317	Ps135537
PITG_07386.1	298302	298113	-	Transposon	-[b]	-
PITG_07387.1	359782	360645	+	PiAvr4	-	Ps109418
PITG_07388.1	367654	368802	-	Transposon	-	N/A
PITG_07389.1	396383	397687	-	Unknown	Pr83315	Ps135538
PITG_07390.1	398114	399417	-	Unknown	Pr83314	Ps135539
PITG_07391.1	400069	401375	-	Unknown	Pr83312	Ps135540
PITG_07392.1	402975	402920	-	Unknown	Pr83315	Ps135541
PITG_07393.1	404429	403757	-	Transposon	-	-
PITG_07394.1	406403	408042	+	Transposon	-	-
PITG_07395.1	408697	408282	-	Transposon	-	-
PITG_07396.1	421229	422366	+	Transposon	-	-
PITG_07397.1	425226	426428	+	Unknown	Pr83314	Ps135542

[a] Orientation of the predicted open reading frame.
[b] No ortholog present.

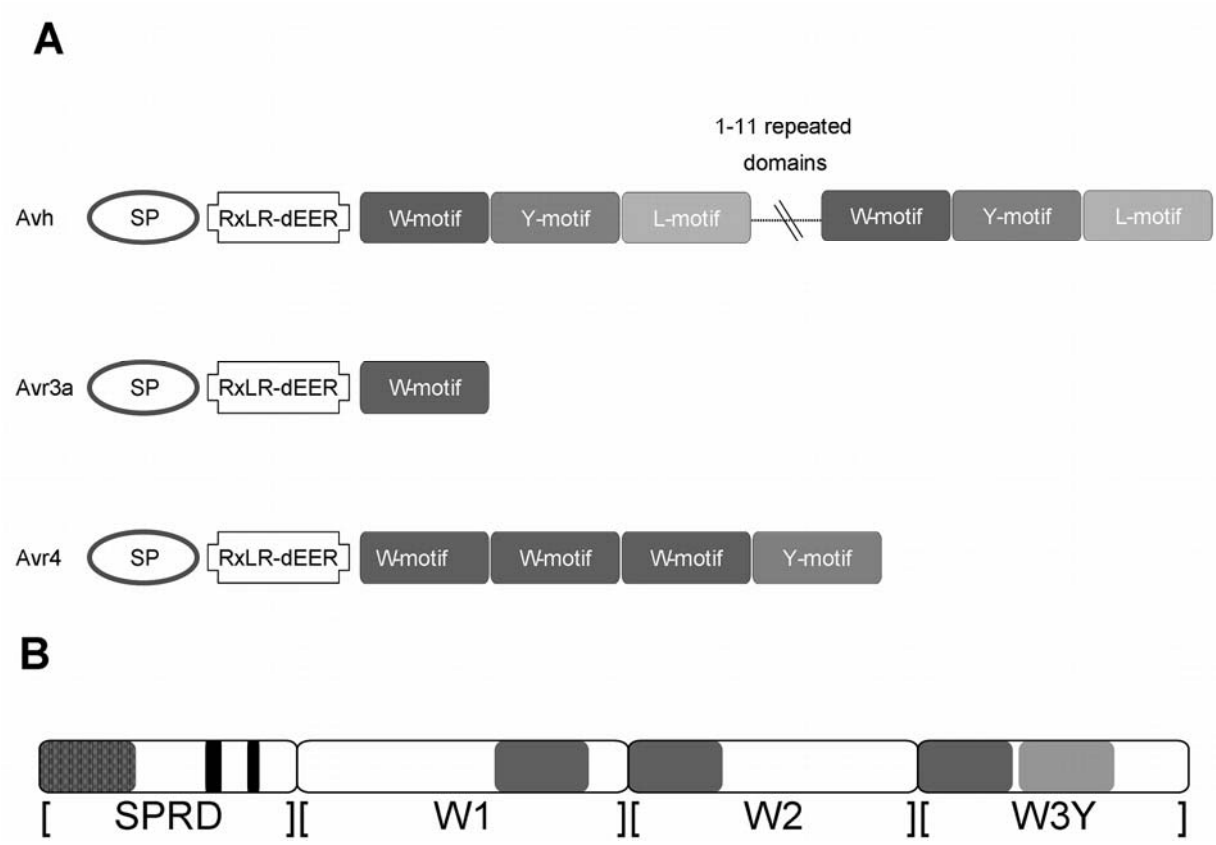


Figure 3. Oomycete Avh proteins have a modular structure. **A.** Avh shows the basic components of an RXLR-dEER protein, with an N-terminal signal peptide (SP), the RXLR-dEER domain and the C-terminus with a variable number of modules that consist of W, Y and L motifs. PiAvr4 carries three W motifs and one Y motif. Avr3a, shown as an example of another *P. infestans* Avr protein, carries only a single W motif. **B.** A schematic representation of the relative positions and sizes of the conserved motifs in PiAvr4 and the building blocks that were used to generate deletion constructs. Building block [SPRD] covers the signal peptide (dashed) and the RXLR and dEER motifs (black). [W1], [W2] and [W3Y] cover the W motifs (dark grey) and the Y motif (light grey).

Conserved motifs in the C-terminus of PiAvr4

To find conserved W, Y and L motifs in the C-terminal domain of PiAvr4 we used the HMMs that were developed by Jiang et al. (2008). Three W motifs, named W1, W2 and W3, and a single Y motif were identified but no L motifs (Fig. 3A; Table 2). The HMM scores of the W motifs in PiAvr4 vary. W2 and W3 have scores of 19.8 and 10.9, respectively, while the score of W1 is 0.6. Also the *P. mirabilis* Avr4 homolog PmirAvh4 carries three W motifs and a single Y motif with a low HMM score for W1. The HMM score of the PmirAvh4 W2 motif is highest with a value of 20.3 while W3 has a value of only 6.7. PsAvh_110, the closest PiAvr4 homolog in *P. sojae*, has two W motifs and a single Y motif (Jiang et al., 2008). Compared to PiAvr4 the W1 motif appears to be absent in PsAvh_110. Motifs W2, W3 and Y and the inter-motif regions are conserved between PiAvr4 and PsAvh_110, although the similarity is low (27% identity). Of the other Avr4 family members three have no

recognizable motifs in the C-terminal domain, including the one that is selected based on similarity in the N-terminus (PiAvh_131); eight contain one W motif and no Y, and four contain two W motifs with or without Y. Apart from PiAvr4, only one family member contains three W motifs (Table S1). The variable number of motifs among homologs suggests that dynamic deletion or duplication of motifs plays a role in diversification of the C-terminal domains of RXLR-dEER effectors.

To further investigate the potential role of the W and Y motifs we analyzed the secondary structure of PiAvr4. In total 12 α-helices, ranging in size from 5 to 41 amino acids, are predicted in the C-terminal region of PiAvr4 (http://www.sbg.bio.ic.ac.uk/phyre). Two of these are amphipathic α-helices with hydrophobic and hydrophilic residues clustered on opposite sites; helix 5 ranges from residue 129 to 154 and covers parts of motifs W1 and W2, while helix 6 spans part of W2 and ranges from residue 159 to 199 (Fig. S2). In *P. sojae* Avr1b three such amphipathic helices are found and Dou et al. (2008a) showed that polymorphic residues are exclusively located in the hydrophilic sites of two helices coinciding with the W motif in Avr1b. Polymorphisms between PiAvr4 and PmirAvh4 do not specifically localize to either hydrophobic or hydrophilic sides of the helices and the secondary structure of both proteins is well conserved. However, part of the amphipathic structure in helix 5 is disrupted by the polymorphisms resulting in a helix in PmirAvh4 that is 12 residues shorter. Also the predicted structures in helices 6, 8 and 9 are slightly different in PmirAvh4 compared to PiAvr4, but the amphipathic structures are not disrupted.

Elicitor activity is confined to restricted regions in the C-terminus of PiAvr4

In a previous study (**Chapter 2**) we expressed *PiAvr4* in *R4* potato plants and determined that the RXLR-dEER domain is not required for elicitor activity of PiAvr4. To determine if changes in any of the 27 amino acids in the C-terminus of PiAvr4 that differ from PmirAvh4 abolish elicitor activity on *R4* plants, we performed an agroinfection assay and transiently expressed *PmirAvh4 in planta*. *R4* potato plants showed a hypersensitive response (HR) at the inoculation site but on Bintje, the potato cultivar lacking any known *R* gene, no response was observed (Fig. 4). Each of the three W domains of PmirAvh4 differ in four amino acids compared to the corresponding W domain in PiAvr4 (Table 2) but apparently these amino acids can be changed without losing elicitor activity on *R4* plants.

Table 2 HMM scores of the various domains and motifs in PiAvr4 and its closest homologs

Region	Effector	Range	Sequence	HMM score
SP	PiAvr4	1-24	MRSLHILLVFTAS·LLASLTESAKA	0.999[a]
	PmirAvh4	1-24	MRSLHILLVITAS·LLASLAVSAEA	1.000[a]
	PsAvh_110	1-20	MRVSS·LLVIAAGFLLAS···SEA	1.000[a]
RXLR-dEER	PiAvr4	42-55	RFLR·····AQTDEKNEER	12.8
	PmirAvh4	42-58	RFLRDGG··TTEAQTD···EER	18
	PsAvh_110	39-55	RFLRTAALETT··RDD···EER	21,0
W1	PiAvr4	115-138	KYERMQWQKLKEGETLTFMRLGDR	0,6
	PmirAvh4	118-141	KYERMQWQKLNEGQTLTYMRVGDR	0.5
	PsAvh_110	-[b]	–	-
W2	PiAvr4	148-171	QLLRWVAQKKPVESVYDDLQVAGF	19,8
	PmirAvh4	151-174	QLLRWVAQKKTVKSVYDDLQIEGF	20.3
	PsAvh_110	74-97	LLNLWYKTGESEASVAAKLGISSV	16,6
W3	PiAvr4	221-244	LFEKWAMEGTHIKSVITTLKLNGK	10,9
	PmirAvh4	224-247	VFEKWAMEGTHIKSVIKTLNLNNK	6.7
	PsAvh_110	138-161	QMARWAVEGKSEAWVAGKLGMSML	3,9
Y	PiAvr4	246-267	ASEMANNENFPALLKYVKLYLD	12,4
	PmirAvh4	249-270	ASEMANNENFPALLKYVKLYLD	12.4
	PsAvh_110	166-187	MKVHRNFKAFDLFLQYQKGVAS	No score

[a] Signal peptide probability by SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/).
[b] W1 region is absent in PsAvh_110.

To investigate which part of the C-terminus of PiAvr4 is responsible for elicitor activity we generated various deletion constructs in a binary PVX vector for transient expression *in planta.* For the design of the constructs the C-terminal region of PiAvr4 was divided in three parts each covering one of the W motifs. The constructs were comprised of the building blocks [SPRD], [W1], [W2] and [W3Y] (Fig. 3B). The control construct, encoding the full length PiAvr4, is thus pGR106[SPRD-W1-W2-W3Y] (Fig.3B; Table S2). Despite several attempts, the cloning of both pGR106[W1] and pGR106[SPRD-W1] was unsuccessful. Since W1 has a low HMM score no further attempts were made.

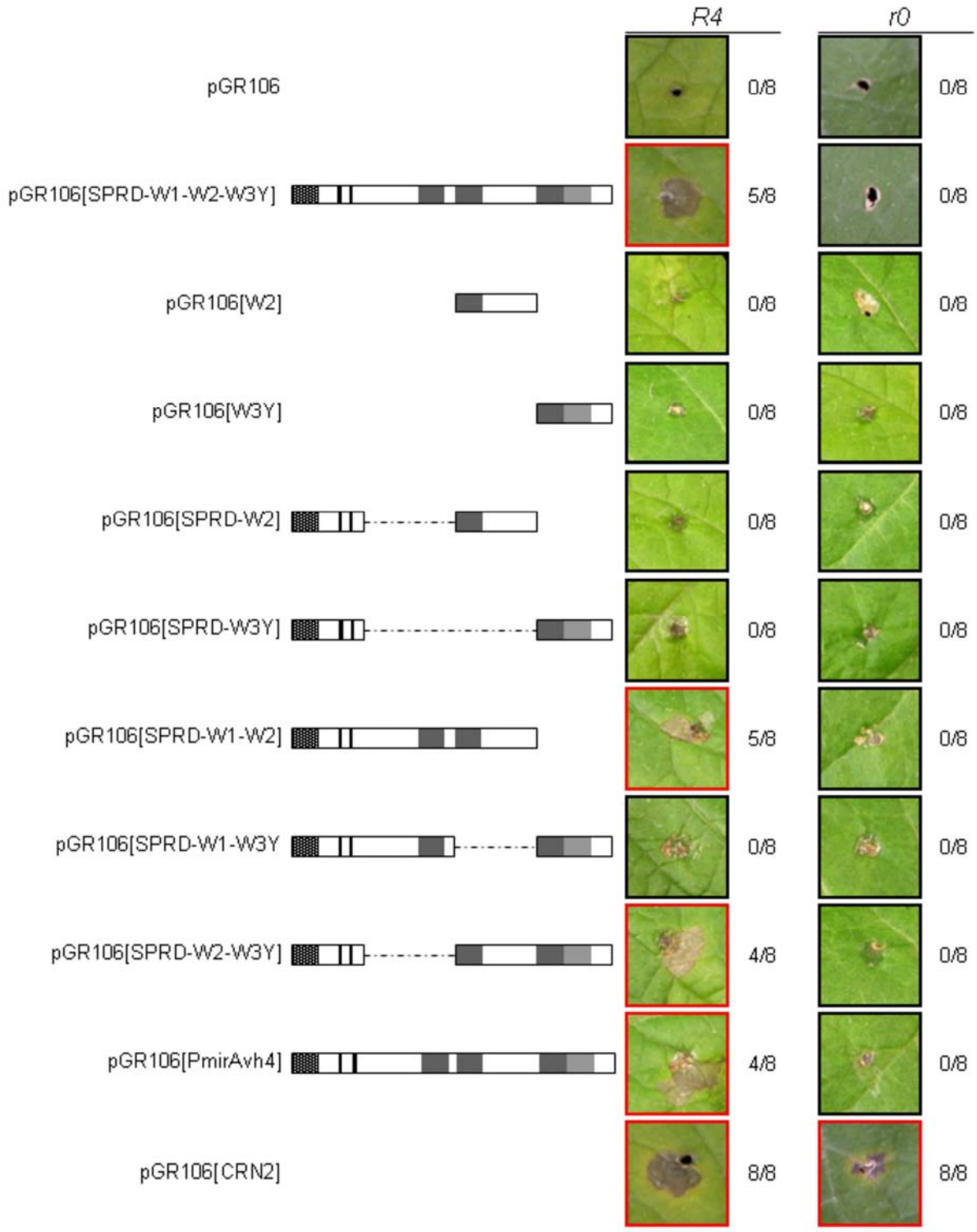


Figure 4. Agroinfection of potato line Cebeco44-31-5 (*R4*) and cultivar Bintje (*r0*) by toothpick inoculation with strains carrying pGR106 constructs as indicated. Dashed lines connect the building blocks that are included in the constructs. The signal peptide is marked as a dashed box, the RXLR-dEER motif in black and the W motifs and Y motif in dark and light grey, respectively. Inoculations that led to a necrotic response are bordered with red lines and those that show no response with black lines. The numbers show the ratio of hypersensitive responses and the total number of toothpick inoculations in a typical experiment. Pictures were taken 18 dpi.

Toothpick inoculation with *Agrobacterium tumefaciens* carrying pGR106[SPRD-W1-W2] and pGR106[SPRD-W2-W3Y] resulted in an HR on *R4* plants within 13 days post inoculation (dpi) whereas no response was visible on pGR106[SPRD-W1-W3Y] or any of the constructs carrying a single [W2] or [W3Y] building block (Fig. 4). None of the strains carrying the empty vector pGR106 showed a necrotic response, and neither did any of the inoculations on Bintje (*r0*). As positive controls we used *A. tumefaciens* strains carrying pGR106-CRN2; CRN2 is a *P. infestans* elicitor that induces general necrosis (Torto et al., 2003). These results show that the region carrying the W2 domain is required but not sufficient for eliciting HR on plants carrying *R4*.

DISCUSSION

A typical oomycete Avr protein consists of a SP, and RXLR-dEER domain and a C-terminal region. The SP is removed when the Avr protein is secreted by the pathogen and, as shown for *P. infestans* Avr3a (Whisson et al., 2007) and *P. sojae* Avr1b (Dou et al., 2008a), the RXLR-dEER domain plays a crucial role in host cell targeting. The C-terminus is the region that determines the avirulence or virulence function of the effector protein; it is responsible for recognition by host R proteins but in the absence of the cognate R protein it can often suppress cell death (Bos et al., 2006; Dou et al., 2008a). It is therefore not surprising that the C-terminal regions of RXLR-dEER effectors are under high positive selection (Win et al., 2007; Jiang et al., 2008). Nevertheless, more than half of these C-terminal regions contain conserved motifs named W, Y and L (Jiang et al., 2008). The fact that these motifs are retained suggests that they are important for the function of RXLR-dEER effectors. When assuming that R proteins recognize conserved regions in Avr proteins, the W, Y and L motifs probably encounter a strong host-driven positive selection. Indeed, most variation in the *P. sojae* effector Avr1b was found in the W and L motif and in a so-called K motif. Moreover, mutations in conserved residues in these motifs abolished elicitor activity, avirulence function and suppression of cell death (Dou et al., 2008a).

Here we have shown that the *P. infestans* RXLR-dEER effector PiAvr4 has a C-terminal region with three W motifs and one Y motif. Also most of the PiAvr4 homologs have W motifs but usually less than three. The closest homolog is PmirAvh4 obtained from the sibling species *P. mirabilis.* PmirAvh4 has the same number of motifs and 89% similarity in the C-terminal region. Sequences identical to *PmirAvh4* were found in two other sibling species, *P. phaseoli* and *P. ipomoeae,* but not in *P. infestans* (unpublished data), thus suggesting that *PmirAvh4* is the

ancestor of *PiAvr4*. *P. mirabilis* is a pathogen of *Mirabilis jalapa*, the four o'clock flower, and not pathogenic on potato (Grünwald and Flier, 2005). To address the question whether PmirAvh4 is recognized by potato R4 we determined its effector activity by transient expression in *R4* plants. In a previous study we showed that agroinfection with *Agrobacterium tumefaciens* strains carrying a full length *PiAvr4* construct in a binary PVX vector resulted in an HR on R4 plants (**Chapter 2**) whereas constructs of *PiAvr4* lacking the SP sequence did not elicit a response on *R4* plants. In agroinfiltration assays the difference between constructs plus and minus SP sequence was less evident but in all cases a full length *PiAvr4* construct triggered necrotic responses specifically on *R4* plants and not on cv. Bintje (*r0).* Since deletion of the RXLR-dEER domain did not change the response we concluded that elicitor activity of PiAvr4 is restricted to the C-terminal region (**Chapter 2**). In this study we cloned the full length *PmirAvh4* gene in the binary PVX vector and found that, similar to PiAvr4, PmirAvh4 is recognized by R4. This shows that potato R4 recognizes an ancestral RXLR-dEER effector and that the 27 amino acids that changed since the divergence of *P. infestans* from its sibling species are not essential determinants for recognition. Apparently, more substantial or more specific mutations are required to evade recognition by potato R4. The finding that all *P. infestans* isolates that are virulent on potato *R4* carry a frameshift mutation in *PiAvr4* (**Chapter 2**) suggests that *P. infestans* uses a rather robust mechanism to get rid of PiAvr4 activity. A more subtle mechanism is a specific mutation as found for *P. infestans Avr3a* and the *Cladosporium fulvum Avr4* gene. For the latter, recognition by the tomato R protein Cf-4 is abolished by a single amino acid change in Avr4 (Joosten et al., 1994). In the case of *Avr3a,* isolates that are virulent on *R3a* plants have an intact ORF at the *Avr3a* locus. This ORF encodes an effector that has two specific point mutations but lacks elicitor activity on *R3a* plants (Armstrong et al., 2005). Similarly, ATR13 in *H. parasitica* has four amino acid residues that determine RPP13-mediated resistance (Allen et al., 2008). Mutations in these residues abolish elicitor activity.

The frameshift mutations in PiAvr4 in virulent isolates always occur at two fixed positions and the proteins encoded by the remaining ORF are either 17 or 92 amino acids in size (**Chapter 2**). The latter covers the RXLR-dEER domain and a small part of the C-terminal domain but this is not sufficient to trigger HR. To determine which part of the C-terminal domain has elicitor activity we tested various deletion constructs. The data show that the region containing the W2 motif is essential but not sufficient; flanking regions, either upstream or downstream, are necessary to elicit an HR. Since the W1 and W3 motifs have lower HMM scores than W2 they may be less important for the recognition itself. Possibly, the flanking regions provide stability to the central region that comprises W2.

In the genomes of *Phytophthora* spp. *Avh* genes are mostly located in regions that show genome rearrangements and mark breakpoints of conserved synteny (Jiang et al., 2008). *PiAvr4* follows this pattern; the genomic region comprising *PiAvr4* has conserved synteny with genomic regions in both *P. sojae* and *P. ramorum* but the conserved synteny is disrupted by *PiAvr4.* The 45 kb region in *P. sojae* and the 25 kb region in *P. ramorum* that match 168 kb in *P. infestans*, lack *Avh* genes. The size differences are a result of, on the one hand, transposon-like sequences that are more abundant in *P. infestans* and dispersed throughout this region, and, on the other hand, a 100 kb indel that carries *PiAvr4* flanked by transposon-derived sequences. Also *P. sojae Avr1b*-1 is located on a 50 kb indel that is absent in the syntenic region in *P. ramorum* (Jiang et al., 2006a). Overall, *Avh* genes in *Phytophthora* are often associated with retroelements, such as transposons. As observed with several of the *PiAvr4* homologs, new paralogs are often clustered, whereas other older paralog members are scattered around the genome. This scattering may prevent homogenization via illegitimate recombination between duplicated genes and may contribute to the rapid divergence of the *Avh* gene family (Jiang et al., 2008).

MATERIALS AND METHODS

Genome sequences
The genomic sequences and gene models of *P. sojae* (version 1.1) and *P. ramorum* (version 1.1) were retrieved from the website of the DOE Joint Genome Institute (http://genome.jgi-psf.org/) and of *P. infestans* (version 1.0) from the website of the Broad Institute (http://www.broad.mit.edu/). The genomic sequences of *P. capsici* and *H. parasitica* were accessed at http://shake.jgi-psf.org/Phyca1 and http://vmd.vbi.vt.edu/, respectively.

Identification of C-terminal motifs by Hidden Markov Models
By using the program HMMER 2.3.2 (15) (http://hmmer.wustl.edu/), three HMMs were built from the RXLR-dEER effectors that carry conserved C-terminal motifs to detect W, Y and L motifs. One HMM was built from the RXLR-dEER motifs, with the variable spacing arbitrarily placed in between. The RXLR-dEER motif is defined as the occurrence of the string RXLR together with the trailing acidic motif (containing more than 10% D or E residues). The HMM building method is very similar to that described by Jiang et al. (2008). To increase the sensitivity of a database search, the model was calibrated by 'hmmcalibrate' to give an empirical *E* value calculation according to the HMM model as suggested by the program instructions. Motif searches were performed with these four HMMs on the total set of RXLR protein sequences from *P. infestans, P. sojae* and *P. ramorum*.

Homolog search
The entire sets of RXLR-dEER effectors of *P. infestans* (562), *P. sojae* (385) and *P. ramorum* (370) were gathered to make an effector pool. PiAvr4 was used to perform BLASTP against the effector pool and to identify direct homologs. Similar BLASTP searches

were performed with these direct homologs to discover more RXLR-dEER family members and a few additional, most similar homologs were included in the Avr4 family.

Ortholog search
The location of *PiAvr4* was determined by a BLASTN search against the complete *P. infestans* genome sequence. Gene models surrounding *PiAvr4* were selected and used for a BLAST search of the complete genome sequences of *P. sojae* and *P. ramorum.* Gene models which had the best reciprocal BLAST hit were assigned as orthologs. Because transposon derived sequences rarely have homologs in syntenic regions and because of the repetitive nature of transposon sequences, these gene models were excluded from analysis.

Binary PVX constructs and agroinfection
For expressing *PiAvr4* in *R4* and *r0* potato plants, Cebeco44-31-5 and cv. Bintje respectively, binary PVX constructs were made in the vector pGR106 (Jones et al., 1999). BAC subclone pSKA23 (**Chapter 2**) was used as template to amplify specific parts of the *PiAvr4* gene. Primers ClaIAvr4F and NotIAvr4R were used to amplify a *PiAvr4* homolog from *P. mirabilis* (isolate PIC99111) (Flier et al, 2002). The primers that were used contained appropriate restriction sites or sequences for overlap PCR (listed in Table S3). For cloning purposes a *Cla*I restriction site was incorporated in the forward primer of the construct and a *Not*I site in the reverse primer. The obtained amplicons were digested by the appropriate enzymes and cloned into pGR106. The binary PVX constructs were than transformed to *Agrobacterium tumefaciens* strain GV3101 for agroinfection and strain AGL1 for agroinfiltration assays.

Agroinfection assays on potato were performed as described previously (Vleeshouwers et al., 2006). Briefly *A. tumefaciens* strains containing the binary PVX constructs were grown for 2 days on LBman agar medium. Toothpicks were used to transfer bacteria to the leaves and to pierce the leaf creating wound tissue. Responses were monitored for up to 4 weeks post inoculation.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

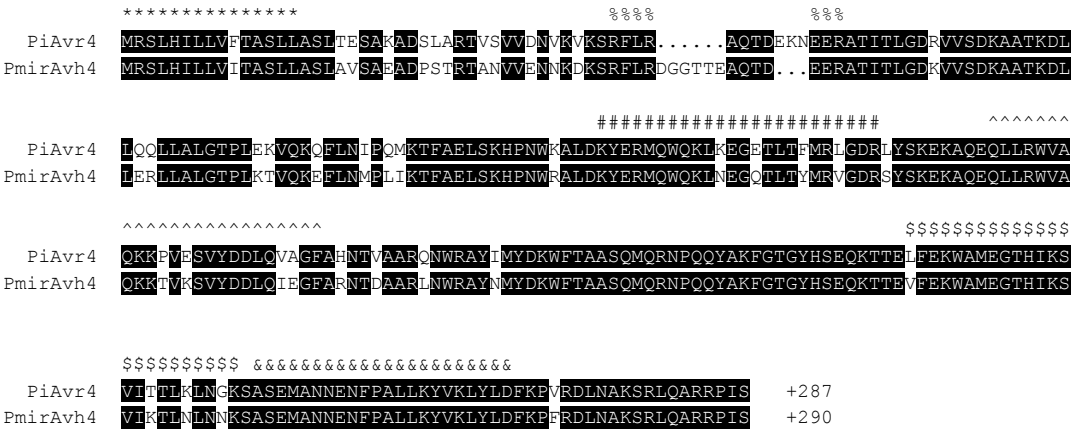


Figure S1. Alignment of PiAvr4 and PmirAvh4. Identical amino acids are shaded black. The positions of the predicted signal peptides (*), RXLR-dEER domains (%) and the C-terminal motifs W1 (#), W2 (^), W3 ($) and Y (&) are marked.

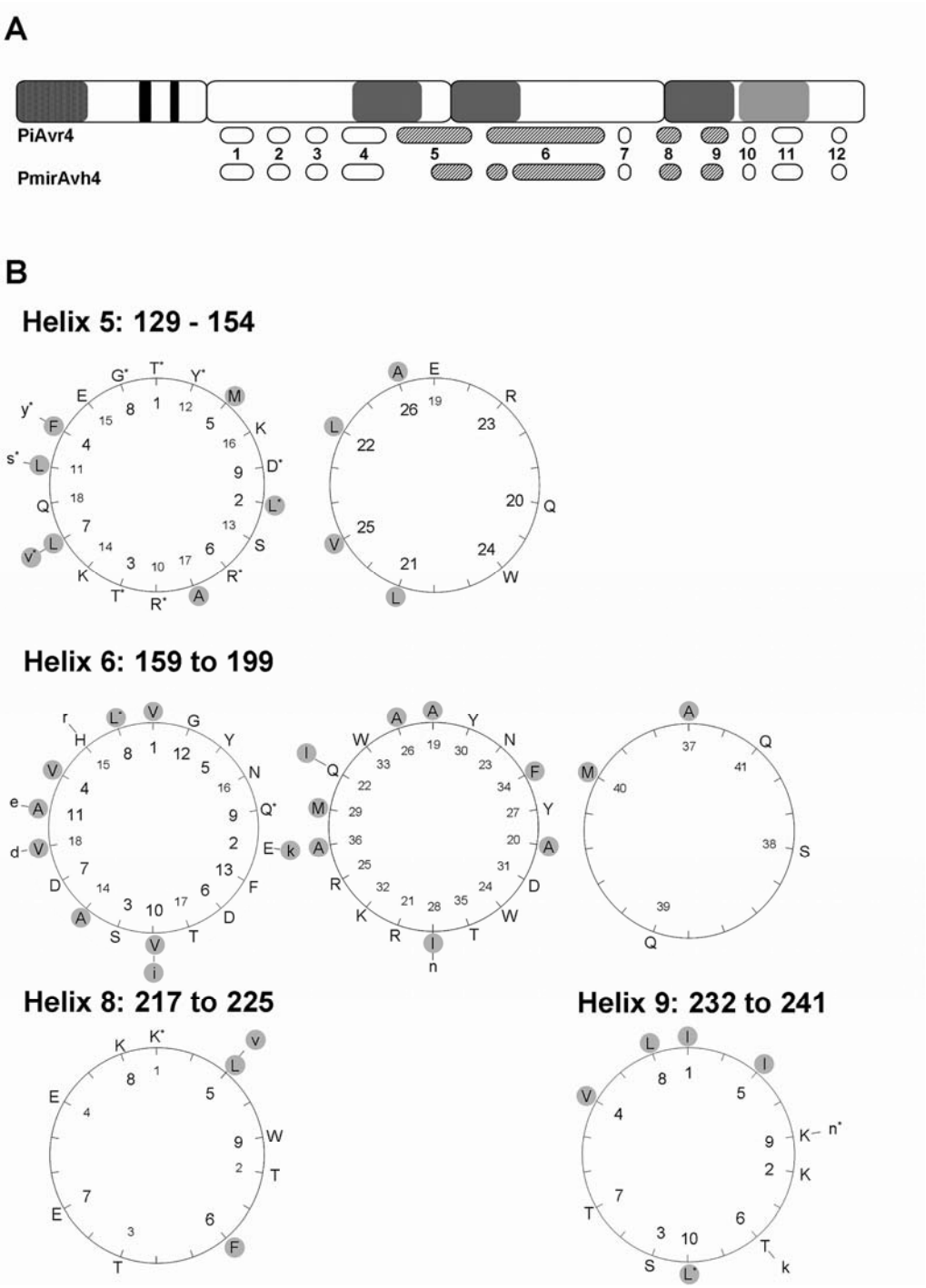


Figure S2. Predicted α-helices in PiAvr4 and helical wheel projections of the amphipathic helices. **A.** The upper bar represents PiAvr4 and PmirAvh4 with the signal peptide (dashed), the RXLR-dEER motif (black) and the W and Y motifs (dark and light grey, respectively). The lower bars represent the 12 predicted α-helices in the C-terminal region. Helices 5, 6, 8 and 9 are shown as hatched bars because they contain amino acids that are polymorphic between PiAvr4 and PmirAvh4. **B.** Helical wheel projections of α-helices 5, 6, 8 and 9. Numbers in the circles indicate the position of the amino acid residues within the α-helix. The amino acids in PiAvr4 are shown in capitals, while polymorphic residues in PmirAvh4 are shown in lower case in the outer circle. Grey dots indicate hydrophobic amino acid residues. Residues marked by an asterisk (*) are not part of the predicted α-helices in PmirAvh4.

Table S1 Sizes of Avr4 family members and occurrence of motifs in their C-terminal domains

Effector ID	Coordinates[a]	ID	Length[b]	C-terminal motifs
PiAvr4	supercont1.11:359782-360645	RXLR139	287	WWWY
PiAvh_38	supercont1.9:1498553-1499149	RXLR340	198	WWY
PiAvh_131	supercont1.1:4150993-4150508	RXLR395	161	-[c]
PiAvh_190	supercont1.19:466087-465491	RXLR490	198	WWY
PiAvh_237	supercont1.11:708113-707613	RXLR292	166	W
PiAvh_313	supercont1.66:335110-334793	RXLR216	105	-[c]
PiAvh_331	supercont1.66:260069-259623	RXLR202	148	-[c]
PiAvh_346	supercont1.250:71114-70476	RXLR180	211	W
PiAvh_483	supercont1.128:196022-196657	RXLR48	211	W
PrAvh_40	scaffold_100:31365-30646	97235	240	WYWW
PsAvh_110	scaffold_102:157518-156946	159077	191	WWY
PsAvh_191	scaffold_102:183635-183183	159147	205	WW
PsAvh_192	scaffold_17:724606-724178	159148	143	W
PsAvh_193	scaffold_17:757039-756611	159149	143	W
PsAvh_297	scaffold_1621:534-106	159253	143	W
PsAvh_298	scaffold_169:16669-16103	159254	170	W
PsAvh_342	scaffold_499:285-713	159298	143	W

[a] Coordinates refer to versions 1.1 of the *P. sojae* and *P. ramorum* genome (www.jgi.doe.gov and vmd.vbi.vt.edu) and version 1.0 of the *P. infestans* genome sequence (http://www.broad.mit.edu/).
[b] The length is in amino acids and includes the signal peptide.
[c] - no recognizable C-terminal motif.

Table S2 Constructs used in this study

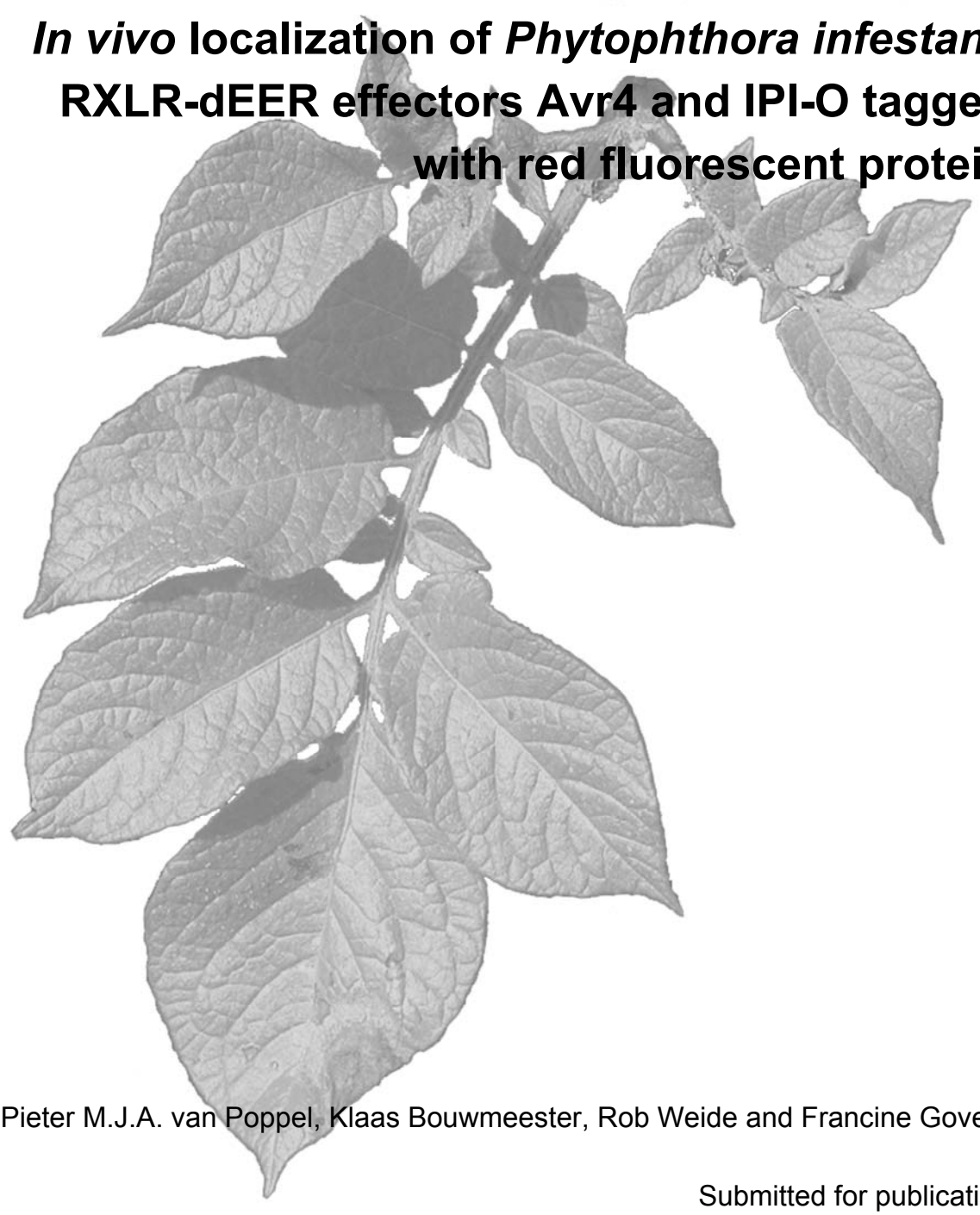
plasmid name	insert	nucleotides	amino acids
pGR106	-	-	-
pGR106[SPRD-W1-W2-W3Y]	PiAvr4	1-864	1-287
pGR106[W2]	W2	429-588	148-220
pGR106[W3]	W3	589-864	221-287
pGR106[SPRD-W2]	SP-RXLR-W2	1-195/429-588	1-65/148-220
pGR106[SPRD-W3Y]	SP-RXLR-W3	1-195/589-864	1-65/221-287
pGR106[SPRD-W1-W2]	SP-RXLR-W1-W2	1-588	1-220
pGR106[SPRD-W1-W3Y]	SP-RXLR-W1-W3	1-428/589-864	1-147/221-287
pGR106[SPRD-W2-W3Y]	SP-RXLR-W2-W3	1-195/429-864	1-65/148-287
pGR106[PmirAvh4]	PmirAvh4	1-873	1-290
pGR106[CRN2]	Crn2	1-1371	1-456

Table S3 PCR primers used for cloning

Primer	Sequence
ClaIAvr4F	5'-CCATCGATATGCGTTCGCTTCACATTTTGC-3'
ClaIAvr4W2F	5'-GCATCGATATGCAGCTCCTTAGGTGG-3'
ClaIAvr4W3F	5'-GCATCGATATGTTGTTCGAGAAGTGG-3'
Avr4SPRXLR-W2R	5'-CCACCTAAGGAGCTGAACAACCCTGTCTCC-3'
Avr4SPRXLR-W2F	5'-GGAGACAGGGTTGTTCAGCTCCTTAGGTGG-3'
Avr4SPRXLR-W3F	5'-GGAGACAGGGTTGTTTTGTTCGAGAAGTGG-3'
Avr4SPRXLR-W3R	5'-CCACTTCTCGAACAAAACAACCCTGTCTCC-3'
Avr4W1-W3F	5'-GAGAAAGCGCAAGAATTGTTCGAGAAGTGG-3'
Avr4W1-W3R	5'-CCACTTCTCGAACAATTCTTGCGCTTTCTC-3'
NotIAvr4W1R	5'-GCGCGGCCGCCTATTCTTGCGCTTTCTC-3'
NotIAvr4W2R	5'-GCGCGGCCGCCTACTCCGTCGTCTTTTGC-3'
NotIAvr4R	5'-GTGCGGCCGCTAAGATATGGGCCGTCTAGC-3'

CHAPTER 4

In vivo localization of *Phytophthora infestans* RXLR-dEER effectors Avr4 and IPI-O tagged with red fluorescent protein



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Submitted for publication

SUMMARY

Avr4 and IPI-O, effectors of the potato late blight pathogen *Phytophthora infestans,* belong to a superfamily of proteins sharing N-terminal RXLR-dEER domains. These domains mediate the translocation of proteins into the host cell. We investigated the *in vivo* targeting of Avr4 and IPI-O in *P. infestans* transformants expressing either Avr4 or IPI-O fused to monomeric red fluorescent protein (mRFP). Fluorescence microscopy showed that fluorescence in young hyphae of a free living, non-sporulating colony accumulates in vesicles that are evenly distributed in these hyphae. In germinating cysts, however, the tips of the germ tubes and the appressoria showed mRFP fluorescence, and during infection of etiolated potato plantlets localized fluorescence was visible at the haustorial neck. Haustoria are highly specialized infection structures that are in close contact with the plant cell and have a putative role in delivering effector proteins into the host cell. The novel experimental set-up, in which etiolated *in vitro* grown potato plantlets are inoculated with *P. infestans,* has the advantage that there is no autofluorescence of chlorophyll that masks the mRFP fluorescence, and disturbs microscopic analysis. The lack of chlorophyll does not seem to interfere with infection; zoospores are capable to encyst and to germinate, and the etiolated tissues are readily colonized by *P. infestans*.

INTRODUCTION

Any biotrophic pathogen will produce effectors to facilitate infection of a host plant and to manipulate the host metabolism (Kamoun, 2007). The functions of these effectors include the transcriptional activation of developmental reprogramming (Kay et al., 2007), suppression of enzymes such as apoplastic proteases (Tian et al., 2005), protection of the pathogen against host defenses (van Esse et al., 2007) and suppression or induction of cell death (Bos et al., 2006; Kanneganti et al., 2006; Dou et al., 2008a). Some of the effectors function extracellularly and remain in the plant apoplast after secretion (Rep, 2005). Other effector proteins function inside the plant cell and therefore the pathogen or the host has to facilitate transport of these effectors into the plant cell. Several plant pathogenic bacteria, including *Pseudomonas* and *Xanthomonas* spp., use the type III secretion system to transport effectors into the host (Alfano and Collmer, 2004). As yet, a comparable host cell targeting system has not been described for fungal and oomycete plant pathogens (Ellis et al., 2006). Unlike fungal effectors, oomycete effectors share a conserved N-terminal host cell targeting domain, which harbors the RXLR and dEER motifs (Rehmany et al., 2005). The putative role of these N-terminal motifs in host cell targeting was deduced from a similar motif ($RXLX^{E}/_{Q}$) in effectors of the malaria parasite *Plasmodium falciparum* named PEXEL/VTS (Charpian and Przyborski, 2008). There is now ample evidence that the RXLR-dEER domain is indeed responsible for targeting effectors into the host cytoplasm (Whisson et al., 2007; Dou et al., 2008b; Govers and Bouwmeester, 2008).

The oomycete *Phytophthora infestans* is the causal agent of potato late blight. For this pathogen zoospores are important asexual propagules that are spread by wind and rain. When zoospores land on leaves or stems of a host plant they encyst; the cysts then germinate and form appressoria from which penetration pegs emerge that enter epidermal cells. Inside the epidermis an infection vesicle is formed which serves as a starting point for further growth of hyphae invading the extracellular spaces of the mesophyll. These intercellular hyphae form digit-like structures, named haustoria, which penetrate mesophyll cells for feeding. Under optimal conditions sporangiophores will appear on the leaf surface from which new zoospores or sporangia are released (Erwin and Ribeiro, 1996).

The *P. infestans* effectors Avr4 (**Chapter 2**) and IPI-O (Pieterse et al., 1994) are members of the RXLR-dEER effector family. The genes encoding these effectors are expressed in germinating cysts and in early stages of the *P. infestans*-potato interaction, and involved in gene-for-gene interactions with potato *R* genes (**Chapter 2**; Vleeshouwers et al., 2008). Moreover, IPI-O can bind to an Arabidopsis lectin receptor kinase that has a transmembrane domain and likely

spans the plant cell membrane, suggesting targeting of IPI-O to the plant cell membrane (Gouget et al., 2006). The aim of this study was to determine the subcellular location of Avr4 and IPI-O in different developmental stages of *P. infestans* and during infection of potato. To this end we transformed *P. infestans* with chimeric constructs carrying the effector genes *PiAvr4* and *ipiO1* fused to a sequence encoding a monomeric red fluorescent protein (*mRFP*; Campbell et al., 2002), and used fluorescence microscopy to visualize effector localization. The analysis revealed that in pre-infection stages Avr4 and IPI-O are both targeted to the tips of germ tubes and to appressoria whereas in infected tissues the two effectors accumulate in haustoria.

RESULTS

Phytophthora infestans transformants expressing *mRFP, Avr4-mRFP* and *ipiO-mRFP*

To obtain *P. infestans* strains that produce mRFP tagged effector proteins we transformed *P. infestans* with constructs based on the plasmid pTORmRFP4 (Whisson et al., 2007) carrying the open reading frame (ORF) of either *PiAvr4* or *ipiO1*. The stop codons of the *PiAvr4* and *ipiO* ORFs were removed to create a continuous ORF with the *mRFP* gene that is present in pTORmRFP4 downstream of the multiple cloning site (Fig. 1). As recipient strain for transformation we used T35-3, a sibling of strain T30-4 (Drenth et al., 1995). Of the initial colonies that appeared upon transformation 13 putative Avr4-mRFP transformants, six IPI-O-mRFP transformants and five mRFP transformants were still able to grow upon transfer to fresh selective medium. Of these transformants, five Avr4-mRFP transformants, three IPI-O-mRFP transformants and one mRFP transformant were selected for bioassays (Table 1).

For these transformants the presence and expression of the transgenes was confirmed by PCR and RT-PCR respectively. All the selected transformants were able to infect leaves of the susceptible potato cultivar Bintje. To test whether the PiAvr4-mRFP chimeric effector protein is able to elicit a hypersensitive response (HR) on *R4* potato we inoculated the Avr4-mRFP transformants on the *R4* differential potato clone Cebeco44-31-5. Contrary to transformants carrying a *PiAvr4* transgene that are avirulent on *R4* potato (**Chapter 2**) none of the Avr4-mRFP transformants showed gain of avirulence on R4 potato. In other words, the Avr4-mRFP transformants remained virulent on *R4* plants like the recipient strain. The recipient strain T35-3 carries *ipiO* alleles or variants that confer avirulence on potato clones carrying *Rpi-blb1* or *Rpi-sto1* (Vleeshouwers et al., 2008), and

therefore it was not possible to determine whether or not IPI-O-mRFP behaved as a functional avirulence protein.

Table 1 *Phytophthora infestans* transformants used in this study

Transformant	Recipient strain	Insert	Transgene	mRFP mRNA
TR2.1	T35-3	pTORmRFP4-IPIO	+	+
TR12.3	T35-3	pTORmRFP4-IPIO	+	+
TR22.1	T35-3	pTORmRFP4-IPIO	+	+
TR4.2	T35-3	pTORmRFP4-Avr4	+	+
TR14.3	T35-3	pTORmRFP4-Avr4	+	+
TR24.4	T35-3	pTORmRFP4-Avr4	+	+
TR24.6	T35-3	pTORmRFP4-Avr4	+	+
TR24.8	T35-3	pTORmRFP4-Avr4	+	+
211.2b1	T35-3	pTORmRFP4	+	+

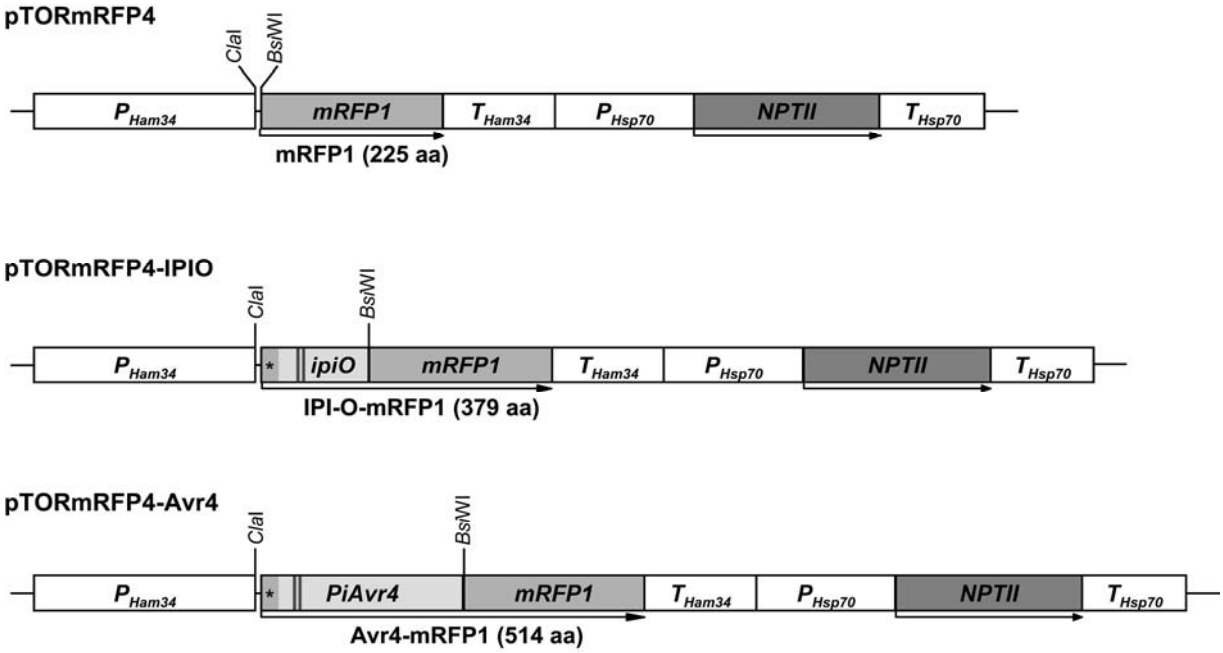

Figure 1. A schematic representation of the constructs used for transformation of *P. infestans* strain T35-3. The expression cassette consists of the *Bremia lactucae Ham34* promoter, a *Cla*I/*Sbf*I/*Bsi*WI multiple cloning site, the *mRFP* open reading frame and a *Ham34* terminator. Both the location of the predicted signal peptide (*) and the RXLR-dEER motifs (||) in the ORFs of *PiAvr4* and *ipiO* are indicated. The antibiotic selection cassette consists of the *Bremia lactucae Hsp70* promoter, an *NPTII* gene for geneticin resistance and an *Hsp70* terminator. The sizes and positions of the different components in the constructs are on scale.

Transformants carrying chimeric *mRFP* constructs show localized fluorescence in infectious stages

Both *PiAvr4* and *ipiO1* encode RXLR-dEER effector proteins, which are secreted by *P. infestans* and presumably targeted into the host cell. The earliest infectious stage of *Phytophthora* is the germinating cyst, which is formed upon landing of a zoospore on a leaf surface (Hardham, 2001). In all transformants fluorescence was visible (Fig. 2A, D and G), but not in the non-transformed recipient strain T35-3. The fluorescence was not homogenous, but appeared to be concentrated in spheres that moved through the cytoplasm (Fig. 2J). Fluorescence was not observed outside the mycelium. By comparing mycelium of different ages it appeared that the intense fluorescence was only visible in young hyphae (Fig. 3). Germinating cysts of Avr4-mRFP and IPI-O-mRFP transformants showed specific localization of fluorescence in the tip of the germ tube (Fig. 2K). It should be noted, however, that the cytoplasm has moved to the tip of the germ tube and that the cyst itself is devoid of cytoplasm.

In the early stages of infection, encystment and germination is followed by the formation of an appressorium. An appressorium appears like a hyphal swelling at the end of the germ tube and facilitates attachment to and penetration of the host tissue (Fig. 2B, E and H). Formation of appressoria requires a hydrophobic surface, such as the cuticle of a leaf (Hardham, 2001). As substitute for potato leaves we used polypropylene foil as a transparent surface to which germinating cysts can attach and form appressoria *in vitro* (Latijnhouwers et al., 2004). We observed that fluorescence in Avr4-mRFP and IPI-O-mRFP transformants was concentrated mainly in the appressoria (Fig. 2E and H). Occasionally fluorescence was observed in the germ tube, but the intensity in germ tubes was always lower than that observed in appressoria. Further development of germinating cysts was halted due to shortage of nutrients as they were germinated in water. Zoospores derived from the mRFP transformant did show fluorescence in appressoria (Fig. 2B); however fluorescence was less intense and more diffused throughout the cytoplasm than the fluorescence observed in the Avr4-mRFP and IPI-O-mRFP transformants.

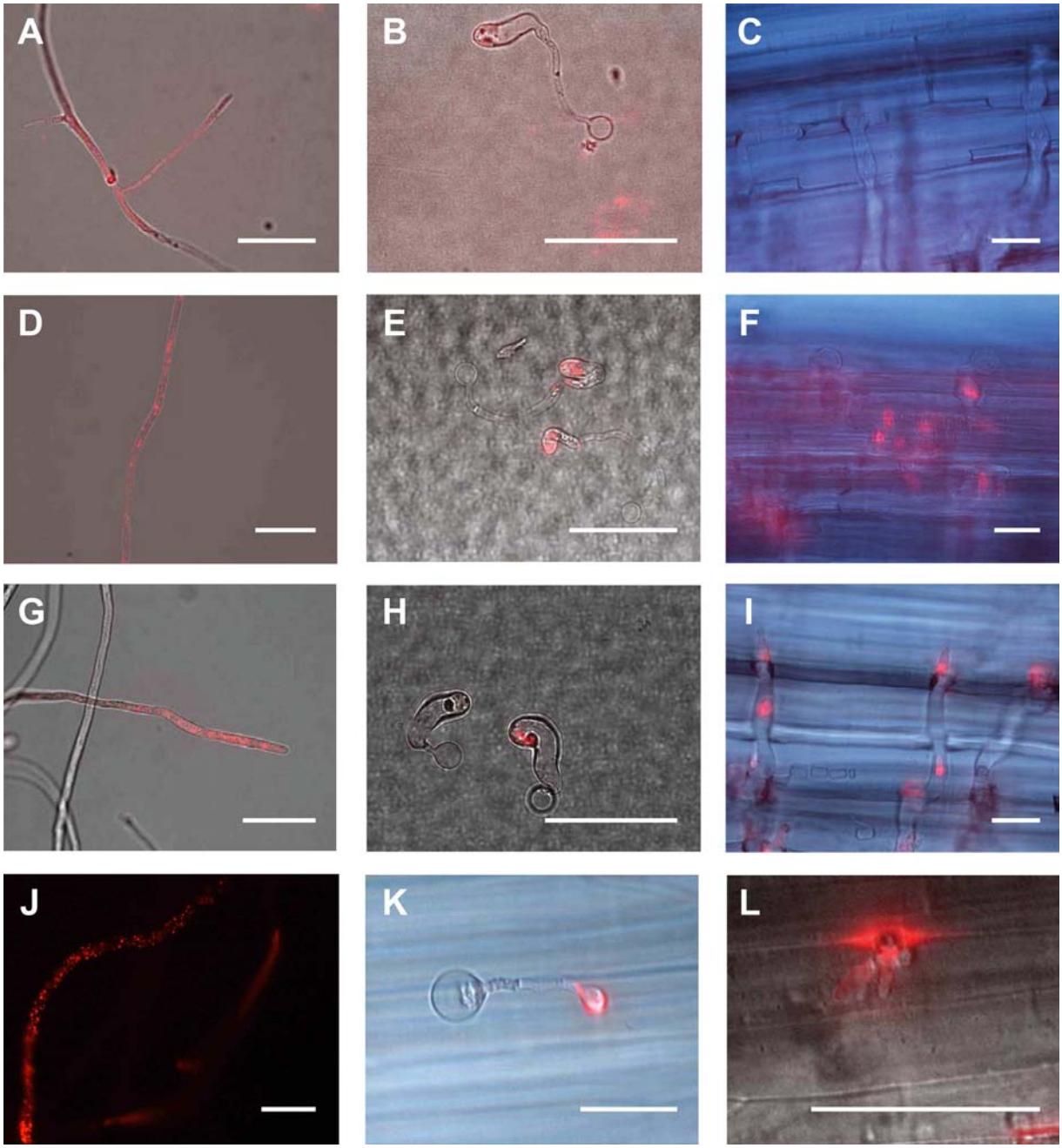

Figure 2. Localized fluorescence observed in *P. infestans* mRFP transformant 211.2b1 (**A**, **B** and **C**), IPI-O-mRFP transformant TR2.1 (**D**, **E** and **F**) and Avr4-mRFP transformant TR4.2 (**G**, **H**, **I**, **J**, **K** and **L**). Localized fluorescence observed in mycelium (**A**, **D**, **G** and **J**), appressoria (**B**, **E** and **H**), a germinating cyst (**K**) and in hyphal tips invading etiolated potato plantlets (**C**, **F**, **I**). mRFP fluorescence in a haustorium of Avr4-mRFP transformant TR4.2 during infection of etiolated potato plantlets (**L**). Pictures consist of a brightfield layer and a red fluorescence layer, except **J** that only shows the red fluorescence layer. The size bars represent 20 µm.

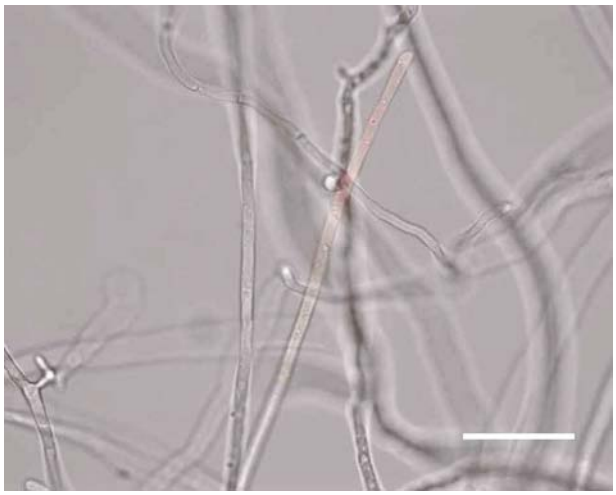

Figure 3. mRFP fluorescence observed in mycelium of the *P. infestans* Avr4-mRFP transformant TR4.2. Mycelium was grown in liquid clarified rye sucrose medium. The picture consists of a brightfield layer and a red fluorescence layer. A young hyphen in the center is surrounded by older hyphae.

Fluorescence during potato infection is localized in haustoria

In our laboratory we usually perform *P. infestans* infection assays on detached potato leaves (Vleeshouwers et al., 1999). Chlorophyll in leaf chloroplasts is red fluorescent with an emission peak at 680 nm (Berg and Beachy, 2008). This autofluorescence interferes with fluorescence of mRFP. Moreover, the low transparency of the potato leaf tissue hampers non-confocal light microscopy. To avoid these interferences we used etiolated *in vitro* grown potato plantlets as tissue for infection (Fig. 4). Previous studies have shown that *in vitro* grown potato plantlets are reliable for *P. infestans* infection assays (Huang et al., 2005b). Here we inoculated the stems of detached etiolated potato plantlets with *P. infestans* zoospores and this resulted in growing lesions, high infection efficiency and hyphae clearly visible in the plant tissue. At 24 hours post-inoculation all tested transformants had formed macroscopic lesions on the etiolated plantlets. The mRFP transformant showed hardly any fluorescence and this fluorescence was randomly distributed (Fig. 2C). In contrast, in both the Avr4-mRFP and the IPI-O-mRFP transformants the red fluorescence accumulated on sites where haustoria emerged (Fig. 2F, I and L). Fluorescence was localized specifically at the haustorial neck and not at the tip of the haustoria. Although these digit-like structures penetrate the plant cell and are in close contact with host tissue, fluorescence was neither found in the extrahaustorial space nor in the infected potato cells.

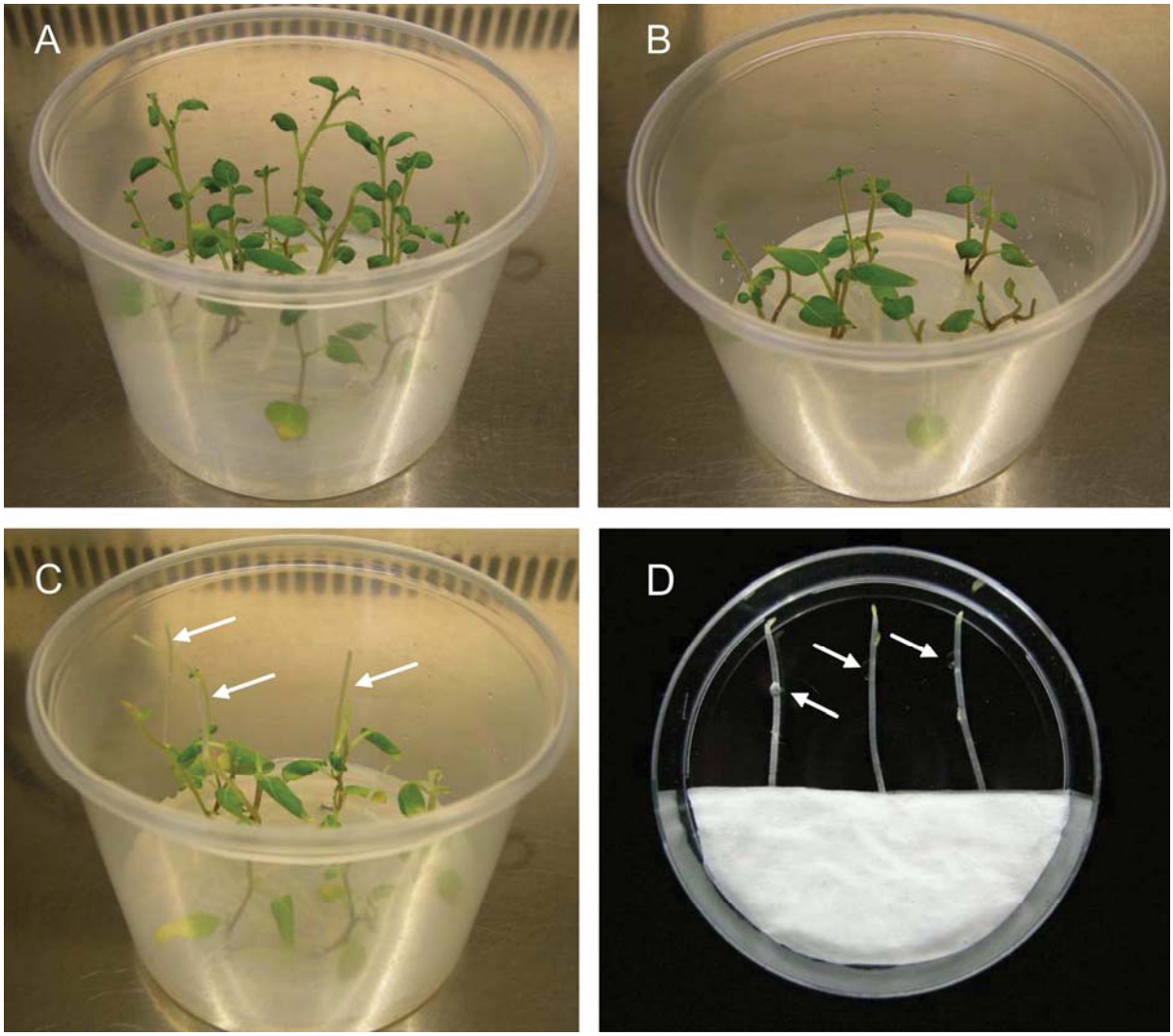

Figure 4. *In vitro* growth of etiolated stems of potato plantlets. The plantlets are grown in transparent jars (**A**) and decapitated (**B**). To obtain etiolated shoots the jars are placed in the dark for one week (**C**). Then the etiolated stems are cut, transferred to a Petri dish and inoculated with *P. infestans* zoospores (**D**). The inoculation sites are marked by white arrows.

DISCUSSION

Fluorescent tags are convenient tools for *in vivo* localization studies (Berg and Beachy, 2008) and have previously been used to obtain fluorescent *Phytophthora* strains (Bottin et al., 1999; van West et al., 1999c). In this study we used the fluorophore mRFP (Campbell et al., 2002) for *in vivo* detection of *P. infestans* effector proteins Avr4 and IPI-O. Fusion proteins of mRFP with either Avr4 or IPI-O were localized specifically in pre-infection stages such as the tips of germ tubes, in appressoria, and in haustoria during infection. In the haustoria formed by both IPI-O-mRFP and Avr4-mRFP transformants, mRFP fluorescence was localized at the

base, in the haustorial neck, and not in the tip of the haustoria. Similar fluorescence patterns were observed in haustoria formed by *P. infestans* transformants carrying the RXLR-dEER effector Avr3a fused to mRFP (Whisson et al., 2007). This suggests that the RXLR-dEER effectors accumulate at the haustorial base and this could be the site where RXLR-dEER effectors are released into the extrahaustorial membrane and from there targeted into host cells.

Previous studies showed that both *PiAvr4* and *ipiO1* are highly expressed prior to and early during infection (**Chapter 2**; van West et al., 1998). The promoter that is used to drive the expression of the fusion proteins in the transformants is constitutive and presumably active in free living mycelium. Indeed, in young hyphae we observed fluorescence: the Avr4-mRFP and IPI-O-mRFP fusion proteins were localized in cytoplasmic spheres. These spheres were absent in the control transformant suggesting that the signal peptide targets the fusion proteins into vesicles. However, release of the vesicle content into the extracellular space or into the apoplast of the infected plantlets was not observed. This is in line with the studies by Whisson et al. (2007) who were also unable to detect extracellular fluorescence with Avr3a-mRFP transformants.

If the fusion proteins are secreted from the mycelium they either diffuse very quickly, resulting in low local concentrations of fluorescent proteins, or they are unstable. The fact that the proteins accumulate in appressoria and in the haustorial neck suggests some kind of docking mechanism that guides RXLR-dEER effectors to a particular location where the pathogen is in close contact with host tissue. This is likely the site where effectors are released and translocated into the host cell. It is, however, questionable whether the fusion proteins are secreted. The mRFP tag substantially increases the sizes of the effector proteins, from 287 amino acids to 514 for Avr4 and from 152 to 379 for IPI-O, and this may prevent secretion and targeting of the effectors. The C-terminal mRFP tag could also block plant-mediated uptake by changing the tertiary structure of the effector or obstructing host cell targeting domains. The suggestion that the mRFP tag disturbs proper targeting is supported by the observation that the Avr4-mRFP transformants remained virulent on potato plants carrying *R4.* Previously, we demonstrated that *P. infestans* race 4 transformants carrying a *PiAvr4* transgene under control of its native promoter became avirulent on *R4* plants (**Chapter 2**). A similar gain of avirulence was reported for Avr3a transformants on *R3a* potatoes (Whisson et al., 2007), but Avr3a-mRFP transformants remained virulent (Stephen C. Whisson, personal communication).

This lack of complementation is not necessarily due to improper targeting. It can also be due to confirmation changes caused by the C-terminal mRFP tag that disrupt the effector region of Avr4 and thus prevent recognition by *R4*. As

shown for *P. infestans* Avr3a and Avr4, *P. sojae* Avr1b and *H. parasitica* ATR13 the domains in RXLR-dEER proteins that are recognized by the cognate R proteins are located in the C-terminal part (Bos et al., 2006; **Chapter 2**; Dou et al., 2008a, Allen et al., 2008). However, the activity of the TMV-elicitor p50 was not affected by a C-terminal fluorescent tag (Burch-Smith et al., 2007). Both p50 and the cognate resistance protein N were produced as cytoplasmic proteins and did not have to cross any membranes in order to interact. To distinguish between improper targeting and disruption of effector activity one could use a biolistics approach to express the Avr4-mRFP construct in *R4* potato plants and monitor HR responses.

In this study we used the stems of etiolated potato plantlets for microscopic studies of the *P. infestans*-potato interaction (Fig. 4). In nature many *Phytophthora* species infect stems or roots, but typical *P. infestans* infections are usually found on leaves. Occasionally infections also occur on the stem of the host plant. In this study we demonstrated that *P. infestans* zoospores are capable of infecting etiolated stems at high infection efficiency. The strong reduction of chloroplasts in the etiolated plantlets decreases the autofluorescence otherwise caused by chlorophyll. The etiolated stems are also more transparent which allows the microscopic observation of infection structures below the epidermis. Since this *in vitro* system requires only limited space in a growth chamber it is very suitable for a quick screening of transformants. The plantlets are grown in a sterile environment and can be used multiple times for growing etiolated stems. Altogether, the system presented here accommodates a quick and relatively inexpensive way for microscopic studies of *P. infestans*-potato interactions.

MATERIALS AND METHODS

Phytophthora infestans growth conditions and inoculum preparation

Zoospores of *P. infestans* were obtained as described in **Chapter 2**. Released zoospores were transferred to glass flasks and encysted by shaking and were subsequently allowed to germinate in water for 2-4 hours at room temperature. To obtain appressoria, cysts were incubated overnight at room temperature on polypropylene foil (Plastibrand catalogue number 759705, Sigma-Aldrich, St. Louis, MO, USA; Latijnhouwers et al., 2004) in Petri dishes with wet paper towels to provide a moist environment. Young mycelium was obtained from sporangia (Latijnhouwers and Govers, 2003) and grown for 3 days at 18˚C in liquid rye sucrose medium.

Nucleic acid manipulations

DNA was isolated using a microprep buffer (0.2 M Tris, pH=8.5, 0.25 M NaCl, 25 mM EDTA, 2 % SDS) that was added to frozen mycelium in 2.2 mL microcentrifuge tubes. Samples were homogenized with glass beads (Ø 3 mm) using a FastPrep instrument (Qbiogene, Carlsbad, CA, USA). A phenol/chloroform extraction was performed, followed by an RNase

treatment and DNA precipitation. RNA isolation was performed as described previously (**Chapter 2**).

Table 2 Primers used for cloning

Primer	Sequence
ClaI-PiAvr4-F	5'-CCATCGATATGCGTTCGCTTCACATTTTGC-3'
BSiWI-PiAvr4-R	5'-CGCGTACGAGATATGGGCCGTC-3'
ClaI-IpiO-F	5'-CCATCGATATGCGTTCGCTCCTGTTGACCG-3'
BsiWI-IpiO-R	5'-CGCGTACGGCTAGGGCCAACGTTT-3'
RFP-F	5'-GCAGGCGTACGATGGCCTCC-3'
RFP-R	5'-TCGAACTCGTGGCCGTTCAC-3'

DNA transformation of *Phytophthora infestans*

For transformation we used plasmids based on pTORmRFP4 (Fig. 1; Whisson et al., 2007). pTORmRFP4 carries a geneticin resistance gene under control of a *HAM* promoter and a cloning site followed by an *mRFP* sequence (Campbell et al., 2002). The plasmids used for transformation are pTORmRFP4, pTORmRFP4-Avr4 and pTORmRFP4-IPIO (Fig. 1; Table 1). PCR primers were used to generate different constructs with a 5' *Cla*I and a 3' *Bsi*WI (Table 2) and these constructs were ligated into the pTORmRFP4 plasmid. The fragments lack the 3' stop codon and were cloned into pTORmRFP4.

P. infestans isolate T35-3 was stably transformed using the PEG protoplast transformation protocol as described previously (**Chapter 2**). After transformation protoplasts were resuspended in pea broth with 0.75 % agar, supplemented with 3 µg mL^{-1} geneticin (G418) and 500 µL aliquots were poured in 24-wells plates. Two and four days after transformation two additional layers of 700 µL pea broth agar supplemented with 3 µg mL^{-1} G418 and 0.75 and 1.5 % agar respectively were added. Transformants appeared 5-14 days after the second overlay was added. Expression of the *mRFP* transcript was confirmed by RT-PCR using the SuperScript III One-Step RT-PCR System (Invitrogen). The virulence phenotypes of the transformants were analyzed on detached leaves as described previously (**Chapter 2**).

***In vitro* plant growth conditions and infection assays**

In this study potato cv. Bintje (*r0*) was used for infection assays. Plants were maintained *in vitro* in climate chambers (16 hour photoperiod) at 20°C on MS30 medium. To obtain etiolated plantlets, *in vitro* plants were decapitated after which the lower parts were allowed to form new shoots in the dark. After one week etiolated plantlets of approximately 4 cm long were grown (Fig. 4). Infection assays on *in vitro* plantlets were adapted from Huang et al. (2005b). Several 10 µL droplets of a zoospore suspension with 50 zoospores $µL^{-1}$ were spot inoculated on stems of detached etiolated plantlets (Fig. 4D). The plantlets were placed in Petri dishes with wet paper towels to provide a moist environment. Lesions developed overnight in the dark at 18°C.

Microscopy

Microscopic analysis was performed using a Nikon Eclipse 90i epifluorescence microscope (Nikon, Badhoevedorp, The Netherlands). To visualize fluorescence of mRFP (561 nm excitation; emission 600-630 nm) the microscope was equipped with a TRITC filter cube (EX540/25, DM 565, BA 605/55). The NIS-Elements software package was used to analyze and merge digital pictures.

ACKNOWLEDGEMENTS

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CHAPTER 5

Differential recognition of *Phytophthora infestans* races in *R4* potato clones

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submitted for publication

SUMMARY

Introgression breeding has resulted in several potato clones that are resistant to late blight, a devastating plant disease caused by the oomycete *Phytophthora infestans*. The traditional differential set consists of potato clones with eleven late blight resistance specificities, referred to as *R1* to *R11*. With the exception of the *R4* locus, all the resistance loci in these clones have been genetically mapped or positioned in *R* gene clusters. In this study, we show that potato clones that are defined to carry *R4* do not necessarily recognize the same *P. infestans* strains. Field isolates appeared to be avirulent on either the *R4* differential developed by Mastenbroek or the one developed by Black, but not on both. Previously, we identified the avirulence gene *PiAvr4*, which is a member of the RXLR effector family. *In planta* expression of *PiAvr4* revealed that recognition of PiAvr4 is strictly confined to the Mastenbroek *R4* differential. Segregation of the trait in two independent F1 progenies showed that late blight resistance in this differential is determined by a single dominant gene, now referred to as $R4^{Ma}$.

INTRODUCTION

The genetic basis for plant resistance is explained by the gene-for-gene model, first postulated by Harold H. Flor (Flor 1942). This model states that a single resistance (*R*) gene product in the plant is required for recognition of a single protein, that is encoded by a pathogen avirulence (*Avr*) gene. The recognition of such an Avr factor results in the activation of plant defenses that culminate in a hypersensitive response (HR), local cell death and the halt of pathogen growth. Introgression breeding has been used to introduce disease resistance in several crop species. In potato this has resulted in clones and cultivars that are resistant to different races of the late blight pathogen *Phytophthora infestans* (de Bary, 1876; Erwin and Ribeiro, 1996). In total, eleven different recognition specificities were identified and this was the basis for a differential set of potato clones, named *R1* to *R11,* which is used worldwide to determine virulence in the pathogen population (Black et al., 1953; Malcolmson and Black 1966; Malcolmson 1969; Cooke and Lees, 2004).

In the last decade, several *R* genes that confer late blight resistance have been mapped in potato and some have been cloned (Gebhardt and Valkonen 2001; Simko et al., 2007). The *R1* gene was positioned on chromosome V in the same genetic region as the Potato Virus X (PVX) resistance genes *NB* and *Rx2* (De Jong et al., 1997), *R2* co-localizes with the late blight *R* genes *Rpi-blb3* and *R2-like* on chromosome IV (Park et al., 2005). Chromosome XI harbors a region that contains a cluster of late blight *R* genes. Initially, El-Kharbotly et al. (1996a) mapped a cluster of three *R* genes (*R3, R6* and *R7)* on this chromosome and later *R10* and *R11* were mapped in the same cluster (Bradshaw et al., 2006). Furthermore, Huang (2005) showed that also *R5*, *R8*, *and R9* could be located in this cluster either as separate loci (paralogs) or as allelic versions (orthologs) of the *R3* locus. This major late blight resistance complex may even carry more *R* genes or allelic versions than currently known. In fact, one new *R* gene has already been identified at this locus (Huang et al., 2004). When an F1 mapping population that was predicted to show segregation for *R3* was tested with *P. infestans* isolates that were known to be virulent on one of the parents and avirulent on the other, the progeny appeared to give differential responses to these isolates. This suggested the presence of two different *R3* genes and indeed, high density linkage mapping of the *R3* region revealed two distinct *R3* genes (Huang et al., 2004). For both *R3a* and *R3b* the cognate avirulence loci in *P. infestans* have been identified. One carries the effector gene *Avr3a* (Armstrong et al., 2005) and the other encodes a putative transcription factor that governs avirulence on *R3b*, *R10* and *R11* potato clones (Jiang et al., 2006c). From the original *R1-R11* differential set defined by Black and Mastenbroek three *R* genes have been cloned, *R1, R2* and *R3a,* and all

encode CC-NBS-LRR proteins (Ballvora et al., 2002; Lokossou et al., 2008; Huang et al., 2005a). Of the eleven *R* loci, differential clone *R4* is the only one for which the putative genomic position is unknown (El-Kharbotly et al., 1996b).

The work on the *R3* gene showed that a single potato differential might contain more specificities than originally postulated. As it turns out *R3a* appears to originate from the differential set that was developed in Scotland by Black (cv. Pentland Ace) whereas the *R3b* gene originates from the differential set that was developed by Mastenbroek in the Netherlands as clone Cebeco46-42-1. As for *R3*, two independent potato clones were presented for *R4* (Black et al., 1953). The series developed by Mastenbroek comprises clone Cebeco44-31-5 as *R4* differential whereas clone 1563 c (14) was described as equivalent in the series developed by Black (Fig. 1). In addition, several clones carrying *R4* have been developed including the breeding clones Cebeco46-174-30 (*R1R4*) (Fig. 1) and Cebeco44-14-2 (*R2R4*) and the commercial cultivars Avondale, Epoka, Gelda, Greta, Isola, Pentland Squire and Tylva. In commercial potato crops, however, *R4* carrying cultivars have not been used extensively.

Previously we cloned the *P. infestans* avirulence gene *PiAvr4* and demonstrated that introduction of this gene into race 4 *P. infestans* strains confers avirulence on potato clones carrying *R4* (**Chapter 2**). Similar to other oomycete *Avr* genes *PiAvr4* encodes a RXLR-dEER protein and is member of a large family that comprises over 550 genes in *P. infestans* and around 370 in *Phytophthora sojae* and *Phytophthora ramorum* (Whisson et al., 2007; Jiang et al., 2008). They share the host cell targeting motif RXLR-dEER in the N-terminus but are highly diverse in the C- terminus (Jiang et al., 2008; Bouwmeester et al., 2009). In this study we compared the two *R4* differentials and investigated how they respond to inoculation with *P. infestans* isolates that carry a dominant avirulent allele of *PiAvr4* (and thus have an AVR4 phenotype), or isolates that have only virulent alleles (*Piavr4*; avr4 phenotype). Since the isolates were recognized by either the Mastenbroek differential or the Black differential our data show that potato clones defined to carry *R4* have a differential recognition of *PiAvr4.* Moreover, we show that recognition of *PiAvr4* is strictly confined to potato clones carrying the *R4* locus that was introgressed by Mastenbroek.

RESULTS

The clones Ma-R4 and Bl-R4 recognize different *Phytophthora infestans* isolates

To determine whether the *R4* clones that are included in the differential set of Mastenbroek (Cebeco44-31-5; hereafter named Ma-R4; Table 1) and Black (1563 c (14); hereafter named Bl-R4) recognize the same *P. infestans* isolates we performed a series of infection assays on detached leaves. In these assays the cultivars Isola (*R4*), Bintje (*r0*) and Katahdin (*r0*) were included. We tested 14 *P. infestans* field isolates and found that seven of these were avirulent on cultivar Isola and clone Ma-R4 (Table 2). The resistance in both, Ma-R4 and Isola, is derived from *Solanum demissum*, but we were unable to trace whether the *S. demissum* accession in their pedigree is the same (Fig. 1). Surprisingly the response of clone Bl-R4 differed from that of clone Ma-R4. Of the tested isolates 13 were virulent on Bl-R4 whereas only one, i.e. SC96.21.1.1, was avirulent on this differential. Since isolate SC96.21.1.1 is virulent on Isola and Ma-R4 (Fig. 2a), these data show that the determinants of resistance in clone Bl-R4 differ from the ones in clone Ma-R4 and cultivar Isola.

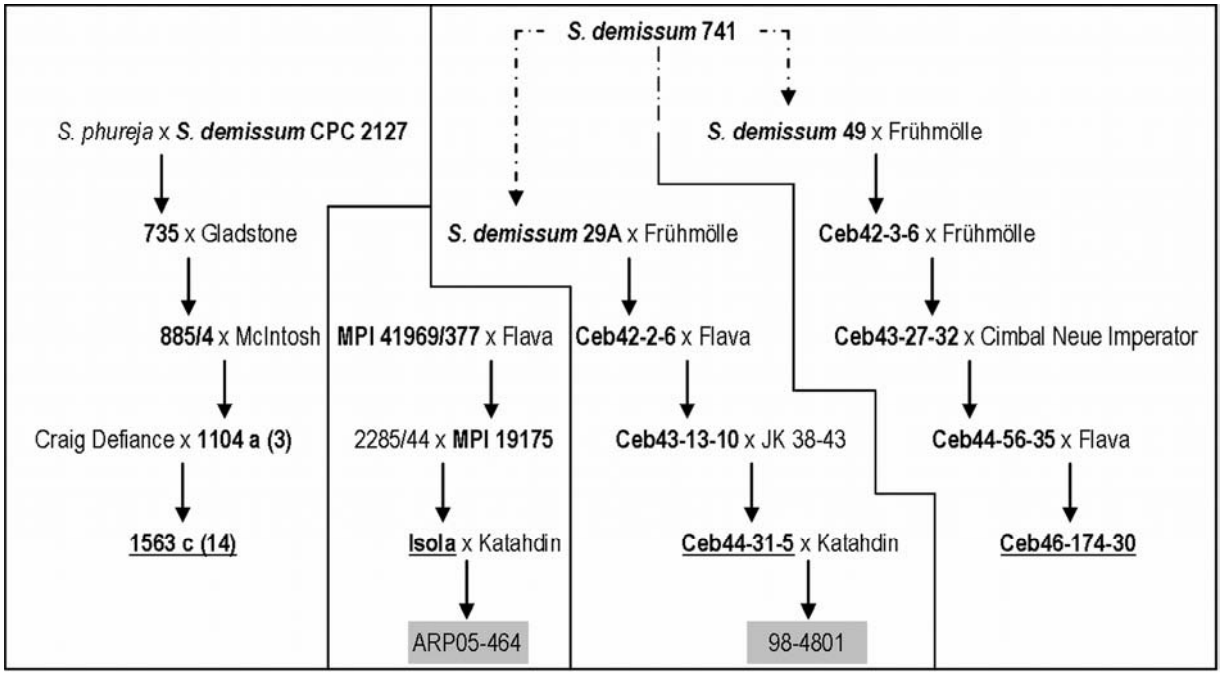

Figure 1. The pedigrees of potato clones and cultivars used in this study (underlined). From left to right: clone Bl-R4 (1563 c (14)), cultivar Isola, clone Ma-R4 (Ceb44-31-5) and clone Ma-R1R4 (Ceb46-174-30). ARP05-464 and 98-4801 (shaded) are segregating F1 populations. Clones depicted in bold carry the *R4* resistance phenotype.

Table 1 Potato clones and cultivars used in this study

Clone / Cultivar	Differential	Year	Origin	Parent (♀)	Parent (♂)	Before this study	After this study
Cebeco44-31-5	Ma-R4	1944	NL	Cebeco43-13-10	JK 38-43	*R4*	$R4^{Ma}$
Isola		1958	GER	2285/44	MPI_19175	*R4*	$R4^{Ma}$
1563 c (14)	Bl-R4		UK	Craig Defiance	1104 a (3)	*R4*	$R4^{Bl}$
Cebeco43-154-5	Ma-R1	1943	NL	Eigenheimer	Erika	*R1*	$R1^{Ma}$
Cebeco46-174-30	Ma-R1R4	1946	NL	Cebeco44-56-35	Flava	*R1R4*	$R1R4^{Ma}$
Katahdin		1932	USA	USDA 40568	USDA 24642	*r0*	*r0*
Bintje		1910	NL	Munstersen	Jaune d'or	*r0*	*r0*

The AVR4 phenotype in cross 71 is confined to clone Ma-R4

In a previous study the map position of *PiAvr4* in *P. infestans* was determined by making use of a mapping population that showed a 1:1 segregation of the AVR4 phenotype (Van der Lee et al., 1997). The mapping population consisted of F1 progeny of cross 71 and the AVR4 phenotyping was performed on clone Ma-R4. Here we repeated the AVR4 phenotyping of eight offspring of cross 71 on clone Ma-R4 and, in addition, determined their phenotype on cultivar Isola and clone Bl-R4. In the cross 71 progeny the segregation of the AVR4 phenotype on Isola and Ma-R4 was identical, further confirming that Ma-R4 and Isola contain the same *R4* specificity (Table 3). However, the parents of cross 71, NL80029 and NL88133, and all tested progeny appeared to be virulent on clone Bl-R4 (Table 3) hence demonstrating that clone Bl-R4 lacks resistance to the parents and the progeny of cross 71.

Table 2 Compatible (C) and incompatible (I) interactions of *Phytophthora infestans* isolates with different potato clones and cultivars

Isolate	Clone Ma-R4	Cultivar Isola	Clone Bl-R4	Cultivar Bintje
NL80029	C	C	C	C
NL88133	I	I	C	C
Ger8601	I	I	C	C
US87000	I	I	C	C
NL68308	C	C	C	C
NL85026	I	I	C	C
NL88069	C	C	C	C
NL89094	C	C	C	C
NL89148-27	I	I	C	C
NL89148-9	I	I	C	C
NL91001	I	I	C	C
NL99018	C	C	C	C
PIC99180	C	C	C	C
SC96.21.1.1	C	C	I	C

Clones Ma-R1R4 and Ma-R4 have the same *R4* specificity

The differential set developed by Mastenbroek also contains an *R1R4* potato clone (Cebeco46-174-30, hereafter named Ma-R1R4). This clone, however, has a pedigree different from clone Ma-R4 (Fig. 1). In order to verify that Ma-R4 and MaR1R4 have the same recognition specificity towards PiAvr4, infection assays were performed on clone Ma-R1R4 and on Cebeco43-154-5, the clone that carries *R1* (clone Ma-R1). Of the ten isolates tested, only two behaved different on clone Ma-R1R4 as compared to clone Ma-R4 (Table 3). Since these two isolates, NL88133 and T20-2, both have an AVR1 phenotype it is likely that the presence of

R1 in clone Ma-R1R4 is responsible for the difference in behavior. These results strongly suggest that clone Ma-R1R4 and clone Ma-R4 posses the same *R4* specificity.

Table 3 Compatible (C) and incompatible (I) interactions between parental lines and F1 progeny of *Phytophthora infestans* cross 71 and different potato clones and cultivars

Isolate	Clone Ma-R4	Cultivar Isola	Clone Bl-R4	Cultivar Bintje	Clone Ma-R1	Clone Ma-R1R4
NL80029	C	C	C	C	I	I
NL88133	I	I	C	C	C	I
D12-2	C	C	C	C	C	C
D12-17	C	C	n.d.[a]	C	I	I
D12-23	C	C	C	C	C	C
T20-2	I	I	C	C	C	I
T30-2	I	I	C	C	I	I
T30-4	I	I	C	C	I	I
T35-3	C	C	C	C	C	C
re11-16	I	I	C	C	I	I

[a] Not determined.

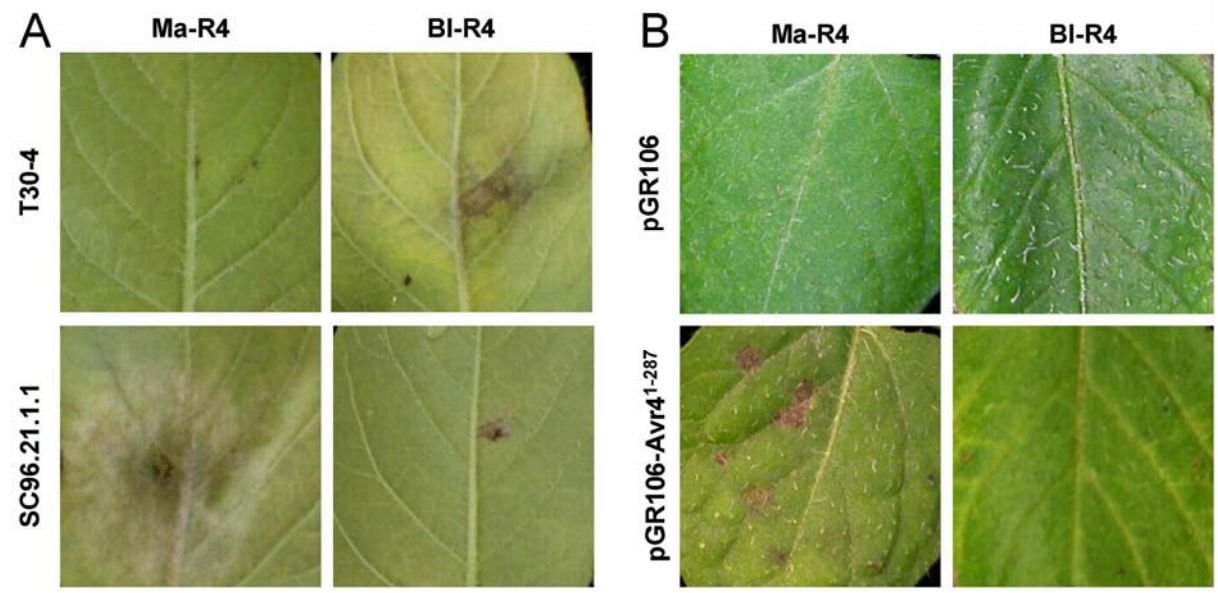

Figure 2. Bioassays demonstrate the different recognition specificities of clones Ma-R4 and Bl-R4 towards *PiAvr4*. **A.** Interactions of clones Ma-R4 and Bl-R4 with *P. infestans* isolates T30-4 containing *PiAvr4* and SC96.21.1.1 lacking *PiAvr4*. Photographs were taken 5 days post inoculation. **B.** Reponses of Ma-R4 and Bl-R4 after inoculation with wild type PVX (pGR106) and recombinant PVX expressing *PiAvr4* (pGR106-Avr4$^{1-287}$). Photographs were taken 5 days post inoculation.

In planta expression of *PiAvr4* on *R4* clones results in differential responses

Recently, we have cloned the avirulence gene *PiAvr4* from isolate T30-4, an F1 progeny of cross 71, and have demonstrated in transient *in planta* expression

assays that this gene encodes an effector that elicits HR on clone Ma-R4 (**Chapter 2**). The observation that clones Ma-R4 and Bl-R4 respond differently towards certain *P. infestans* isolates prompted us to determine the responses towards PiAvr4 on the two potato clones. To test this we expressed *PiAvr4 in planta* using Potato Virus X (PVX) as expression vector. Wild type and recombinant PVX particles were collected from agroinfected *N. clevelandii* plants and used to inoculate the two potato clones. Wild type PVX particles derived from the empty pGR106 vector caused no necrotic response on either Ma-R4 or Bl-R4 clones. Inoculation of clone Ma-R4 with recombinant PVX particles derived from pGR106-PiAvr4$^{1-287}$ caused necrotic lesions on the inoculated leaves, 5 days post inoculation. In contrast, no necrosis was observed on inoculated leaves of clone Bl-R4 (Fig. 2b). These results demonstrate that the RXLR effector encoded by *PiAvr4* is not recognized by clone Bl-R4 and confirm its recognition by clone Ma-R4.

Resistance of clone Ma-R4 is determined by a single dominant locus

In order to determine the genetic basis of the resistance phenotype in clone Ma-R4 we tested how late blight resistance segregates in two independent F1 populations, 98-4801 and ARP05-464, that have clone Ma-R4 and Isola, respectively, as resistant parent (Fig. 1). Two *P. infestans* isolates, IPO-0 and T30-4, were used for inoculation. Both have the AVR4 phenotype and carry a full length dominant allele of *PiAvr4.* As a control we used avr4 isolates that have frameshift mutations in *PiAvr4* and, as expected, all parents and progeny were susceptible to these isolates. A total of 67 progeny of cross 98-4801 was tested; 34 were resistant and 33 susceptible (Table 4). Of the second cross ARP05-464, 60 progeny were tested; 28 were resistant, 28 susceptible while for 4 progeny the phenotype was difficult to score (Table 3). As control we used avr4 isolates that have frameshift mutations in *PiAvr4* and, as expected, all parents and progeny were susceptible to these isolates.

The observation that the segregation ratios in both populations did not differ significantly from the expected 1:1 ratio shows that the resistance phenotype is determined by a single dominant locus. To discriminate the two tentative *R4* genes we refer to the *R* gene in clone Ma-R4 as *R4*^{Ma}.

Table 4 Segregation of late blight resistance in F1 progeny of two potato crosses

Cross	Parents		Progeny			χ^{2} [c]	*P* [c]
	♀	♂	Observed[a]	Expected[a]	N[b]		
98-4801	Ceb44-31-5	Katahdin	34:33	1:1	67(67)	0,015	0,903
ARP05-464	Isola	Katahdin	28:28	1:1	56(60)	0,000	1,000

[a] Ratio resistance to susceptible.
[b] Number of progeny categorized; in parentheses, total number of progeny tested.
[c] The χ^2 and the corresponding *P* value were calculated to test the probability that the data fit an expected ratio of 1:1 for segregation of a single *R* gene.

DISCUSSION

In this study we demonstrated that two potato clones that were thought to carry the same late blight resistance locus recognize different strains of *P. infestans*. Several isolates that were avirulent on clone Ma-R4 appeared to be virulent on clone Bl-R4, whereas one strain that is avirulent on Bl-R4 could infect Ma-R4. This difference in recognition specificity was also demonstrated by the observation that only one of the two clones, Ma-R4, gave a necrotic response when challenged with the effector protein PiAvr4. Clone Bl-R4 gave no response and this is in line with the observation that isolates that carry the dominant allele of *PiAvr4* can colonize clone Bl-R4. Analysis of a F1 population with clone Ma-R4 as the female parent demonstrated that the resistance in Ma-R4 is determined by a single gene, i.e. $R4^{Ma}$, thus pinpointing *PiAvr4* and $R4^{Ma}$ as pairs in a gene-for-gene interaction.

In 1953, Black et al. (1953) proposed a standardized international nomenclature for resistant potato clones and the corresponding *P. infestans* races. In order to compare their results Black and Mastenbroek exchanged potato clones and tested the resistance response to different *P. infestans* races. The actual isolates, however, were not exchanged and it is not documented which isolates were used for these resistance tests. The first differential set that they defined consisted of the differentials *R1*, *R2*, *R3* and *R4.* Later, seven more were added, which resulted in the current *R1-R11* set (Malcolmson and Black 1966; Malcolmson 1969).

To explain why clone Ma-R4 and clone Bl-R4 are resistant to different *P. infestans* races, we traced the origin of the late blight resistance trait that was introgressed into these clones. In both clones the resistance originates from *S. demissum* but the accessions used are different. In contrast, clone Ma-R4 and clone Ma-R1R4, seem to share a common ancestor (Fig. 1). The *S. demissum* genotypes 29A and 49 were originally imported for potato breeding in Indonesia (Mastenbroek, 1952; Toxopeus, 1964). It is likely that genotypes 29A and 49 were two individuals derived from accession 741 but this is not documented. Since Ma-R4 and Ma-R1R4 are both resistant to *P. infestans* isolates that have a dominant *PiAvr4* allele, it is conceivable that $R4^{Ma}$ originates from *S. demissum* 741. We were unable to trace the relationship between *S. demissum* 741 and clone MPI 41969/377, the resistant ancestor of cultivar Isola. Our data, however, clearly show that clone Ma-R4 and Isola have an identical recognition specificity, and therefore it is likely that the Mastenbroek clones and the MPI 41969/377 clone carry the same *R4* gene possibly originating from the same ancestor, or highly homologous *R4* genes (orthologs) from different ancestors.

The observations that there are two different *R4* specificities resemble those of Huang et al. (2004) with respect to the *R3* recognition specificity. The *R3* differential developed by Mastenbroek (Cebeco46-42-1) and the one developed by Black (cv. Pentland Ace) have also different specificities towards *P. infestans* races. The *R3a* gene in cv. Pentland Ace is derived from *S. demissum* CPC 2127, the same accession that gave rise to clone Bl-R4. The *R3b* gene in Cebeco46-42-1 originated from *S. demissum* 49, the clone that also carries $R4^{Ma}$ (Huang, 2005). Despite their different origin, *R3a* and *R3b* map on the same chromosome (XI) and are tightly linked (Huang et al., 2004). Whether or not $R4^{Ma}$ is linked to the resistance locus in Bl-R4 and where $R4^{Ma}$ is located, has yet to be determined. A previous attempt by El-Kharbotly et al. (1996b) to map *R4* by analyzing the progeny of a cross with clone Ma-R4 as resistant parent was unsuccessful. They found an excess of resistant progeny and a deviation of the 1:1 Mendelian segregation, suggesting involvement of a second locus. In contrast, we have found that $R4^{Ma}$ segregates as a single dominant locus indicating that a single gene in clone Ma-R4 is responsible for recognition of PiAvr4. To avoid that other gene-for-gene interactions obscure the resistance phenotype conferred by the *R* gene that is targeted, it might be more efficient to screen segregating populations for response to one defined effector than scoring resistance phenotypes by inoculation with pathogen races (Vleeshouwers et al., 2008). Since the *PiAvr4* gene has been cloned (**Chapter 2**) one can now use *in planta* expression of *PiAvr4* to monitor segregation of $R4^{Ma}$ and this may accelerate mapping and cloning of $R4^{Ma}$ gene.

Studies describing race structures of *P. infestans* populations have shown that isolates virulent on R4 plants occur more frequent than avirulent ones (Flier and Turkensteen 1999; Hermansen et al., 2000; Lebreton et al., 1998). However, often these studies do not mention which potato clones have been used for the bioassays and it is therefore impossible to conclude which Avr factor is less prominent, the one recognized by Ma-R4 or the one recognized by Bl-R4. Our findings stress the importance of using well documented biological materials, for research. Just mentioning which pathogen race is used or which *R* differential is not sufficient. Screening of our own culture collection revealed several isolates that carry a dominant allele of *PiAvr4* and infection assays confirmed that these isolates are avirulent on clone Ma-R4 (**Chapter 2**). In contrast, we could not easily trace an isolate that was incompatible with clone Bl-R4; based on literature searches and personal communications we obtained just one isolate that was collected in Scotland in 1996 (SC96.21.1.1.) and was compatible with Ma-R4. The latter is consistent with the fact that the two *PiAvr4* alleles in SC96.21.1.1 have frameshift mutations that give rise to truncated PiAvr4 proteins (P.J.M.A. van Poppel and F.

Govers, unpublished data). Isolates avirulent on both clones, Ma-R4 and Bl-R4, were not found.

The finding that the R4 differentials have different recognition specificities may also explain contradicting data with respect to inheritance studies of *Avr4*. Al-Kherb et al. (1995) analyzed several F1 progenies of *P. infestans* isolates that differed in virulence spectrum with to aim to define the genetic determinants of virulence and avirulence in *P. infestans.* All parental strains and the majority of the progeny were virulent on the Bl-R4 clone. The authors considered the appearance of a few avirulent progeny as an indication that avirulence is dominant but further evidence for this was lacking. Because they used only clone Bl-R4 as R4 differential these analyses were not informative for the *Avr* gene determining avirulence on $R4^{Ma}$. In earlier studies Spielman et al. (1989; 1990) described F1 populations that segregated for virulence on clone PI 203900, which is identical to Cebeco44-31-5, the Ma-R4 clone. Data from one cross suggested that virulence towards Ma-R4 was dominant, but the fit for this model was very poor. In another cross avirulence was found to be dominant but there was a divergence from a 1:1 segregation that pointed to the involvement of two genes. In the cross 71 mapping population, that was the basis for the positional cloning of *PiAvr4*, avirulence on clone Ma-R4 segregated as a single dominant locus (van der Lee et al., 2001).

In recent years several oomycete *Avr* genes have been cloned, mainly by positional cloning. Nearly all these *Avr* genes, including *PiAvr4,* encode RXLR-dEER effector proteins (Armstrong et al., 2005, Allen et al., 2004; Rehmany et al., 2005; **Chapter 2**). Also R proteins that can stop invasion of oomycete pathogens follow a common theme. They all belong to the class of NBS-LRR proteins and reside inside the host cell (Ballvora et al., 2002; Huang et al., 2005a; van der Vossen et al., 2003; Wroblewski et al., 2007). As yet, we have no clue about the molecular nature of the $R4^{Ma}$ gene, nor about the Avr-R pair that underlies the resistance in the Bl-R4 clone (i.e., the putative $R4^{Bl}$ and $PiAvr4^{Bl}$). Given the fact that PiAvr4 is an RXLR-dEER protein it is very likely that $R4^{Ma}$ turns out to be an NBS-LRR gene. Indeed, by exploiting NBS-LRR profiling (van der Linden et al., 2004) we have identified an $R4^{Ma}$-associated marker (**Chapter 6**). When anticipating that the cognate *Avr* gene $PiAvr4^{Bl}$ that triggers resistance in Bl-R4 is an RXLR-dEER effector one could use an effector genomics approach to identify this *Avr* gene. *Phytophthora* species have hundreds of different RXLR-dEER effectors (Jiang et al., 2008; Whisson et al., 2007). A subset has been cloned in *in planta* expression vectors and high throughput screening for effector activity on a wide range of *Solanum* species has already resulted in the identification of novel Avr-R pairs (Vleeshouwers et al., 2008). Including the Bl-R4 clone in these screenings could lead to the identification of $PiAvr4^{Bl}$.

Since we lack crucial information on the breeding clones and isolates that were used by Black et al. (1953), it is difficult to reconstruct why they could not discriminate between Ma-R4 and Bl-R4. From the pathogen side we can think of a hypothesis that is based on co-regulation of expression of RXLR-dEER genes. Jiang et al. (2006c) described the cloning of one avirulence locus in *P. infestans* that does not encode an RXLR-dEER effector. Instead, this locus harbors a gene named *Pi3.4* that encodes a protein reminiscent of a transcription factor. This *Pi3.4* locus determines avirulence towards at least three *R* genes, *R3b*, *R10* and *R11,* and the current hypothesis is that Pi3.4 regulates transcription of a subset of RXLR-dEER genes including the ones that trigger recognition in *R3b*, *R10* and *R11* potato clones. A similar situation could exist for *PiAvr4* and *PiAvr4*^{BI}: co-regulation of these two effector genes by one transcription factor. To fit this hypothesis the isolates used by Black and Mastenbroek should have had dominant alleles of the two effector genes that then conferred avirulence on both the Ma-R4 and the Bl-R4 clone. In the broad set of field isolates that we tested in a previous study (**Chapter 2**) this is not the case; the virulent phenotype was always due to frameshift mutations in both alleles resulting in truncated proteins that are no longer recognized by the cognate *R* protein. However, it can not be excluded that transcriptional regulation of *PiAvr4* plays an additional role especially when considering the fact that the positional cloning was partly based on transcriptome markers (**Chapter 2**).

MATERIALS AND METHODS

Potato clones and crosses

Potato clones and cultivars used in this study are described in Table 1. The pedigrees of potato clones were derived from the potato pedigree database (van Berloo et al., 2007). Plantlets were maintained *in vitro* on Murashige and Skoog (MS) medium with 30 g per liter sucrose (MS30) and 0.8% agar and grown at 20°C, 16 hours light / 8 hours dark for 4 weeks. Then they were transferred to potting soil and maintained in the greenhouse (21°C, 16 hours light / 19°C, 8 hours dark).

The F1 population 98-4801 was generated in 1998 at the Laboratory of Plant Breeding of Wageningen University. The female parent is the tetraploid clone Cebeco44-31-5, which is the *R4* differential from the Mastenbroek set. The tetraploid cultivar Katahdin, which is susceptible to late blight, was used as the male parent. F1 population ARP05-464 was generated in 2006 from a cross between cultivar Isola as female parent and Katahdin as male parent. Cultivar Isola is described as carrying resistance tot race 4 of *P. infestans* (Joosten, 1991).

Virulence assays and resistance screening

Phytophthora infestans isolates used in this study are listed in Tables 2 and 3. According to the nomenclature described by van der Lee et al. (2001) isolates virulent or avirulent on *R4*

plants have the avr4 or AVR4 phenotype, respectively. For detached leaf assays, leaflets of plants grown for 4 weeks in potting soil were inoculated at the abaxial side with zoospores. Zoospores were harvested from 10 day old *P. infestans* cultures that were grown on rye sucrose agar medium (Caten and Jinks 1968). To obtain zoospores, mycelium was flooded with cold water and placed at 4°C for 3 hours. The concentration of the zoospores was adjusted to $1x10^5$ zoospores mL^{-1}. On the abaxial side of each leaflet, 4 drops containing 1000 zoospores (10 µL) were placed. Infection efficiency and lesion growth rate was monitored for 5 days and measured on days 3, 4 and 5 post inoculation.

Binary PVX constructs and *in planta* expression assays
Binary PVX constructs carrying wild type PVX (pGR106) or recombinant PVX (pGR106-Avr4$^{1-287}$) were maintained in *Agrobacterium tumefaciens* strain GV3101 (**Chapter 2**). *A. tumefaciens* strains carrying either pGR106 or pGR106-Avr4$^{1-287}$ were used for agroinfection of *Nicotiana clevelandii*. Bacteria were transferred and wound tissue was created on the lower leaves of two weeks old plants by toothpick inoculation. After approximately three weeks the leaves that showed systemic mosaic symptoms were cut and homogenized in 50 mM potassium phosphate buffer, pH 7.0. The homogenate containing the PVX particles was used as inoculum to infect leaves of potato plants that were grown for one week in potting soil. Responses to wild type PVX and recombinant PVX expressing *PiAvr4* were monitored up to 5 days post inoculation.

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CHAPTER 6

A marker for the late blight resistance gene *R4*^{Ma} selected by NBS profiling

Pieter M.J.A. van Poppel and Francine Govers

SUMMARY

Plant resistance (*R*) genes govern race-specific resistance to a wide range of pathogens. The largest class of *R* genes, that includes all known *R* genes acting against plant pathogenic oomycetes, encodes NBS-LRR type proteins. In potato, the map positions of several major *R* genes against the late blight pathogen *Phytophthora infestans* have been determined but not the position of the *R* gene *R4*. Segregation ratios of resistance to *P. infestans* strains carrying *PiAvr4* in two independent potato F1 populations suggested that $R4^{Ma}$ resistance is determined by a single dominant locus. Here nucleotide binding site (NBS) profiling in combination with bulked segregant analysis (BSA) was used to generate $R4^{Ma}$ associated genetic markers. In the BSA several candidate markers were found, one of which co-segregated with resistance mediated by $R4^{Ma}$ in individual offspring. DNA sequencing of this marker revealed high similarity with the *Rx1*/*Gpa2* family and hence, $R4^{Ma}$ could be a member of this large gene family and might be located in one of the *Rx1*/*Gpa2* clusters.

INTRODUCTION

To withstand infection of oomycetes and other pathogens, plants posses a large number of resistance (*R*) genes. Effectors produced by certain pathogen species or even by specific isolates of one pathogen species are recognized by R proteins and this recognition can lead to resistance (Flor, 1971). So far five major groups of R proteins have been described that all have different domain architectures (Dangl and Jones, 2001, Van Ooijen et al., 2007). The largest group of *R* genes encodes proteins with nucleotide binding site and leucine rich repeat (NBS-LRR) domains. This group is further subdivided into the CC-NBS-LRR and TIR-NBS-LRR superfamilies, with either a coiled coil domain (CC) or a Toll/Interleukin1 receptor (TIR) domain at the N-terminus (McHale et al., 2006). Several structural and functional domains are conserved within the NBS-LRR superfamily of proteins. The C-terminal LRR domain is supposed to be responsible for the recognition specificity of the R protein. The NBS region of the protein is involved in ATP binding and hydrolysis. This region, known as NB-ARC, carries several highly conserved domains, such as the P loop, the kinase-2 motif and the GLPL (GxP) motif (Takken et al., 2006).

Late blight, one of the most important diseases on cultivated potato (*Solanum tuberosum*), is caused by the oomycete *Phytophthora infestans* (de Bary, 1876; Govers and Latijnhouwers, 2004). Oomycete plant pathogens possess large reservoirs of highly diverse effectors that share a conserved RXLR-dEER motif in the N terminus (Jiang et al., 2008). For several of these effectors it was shown that they function as avirulence (Avr) factors that are recognized by NBS-LRR proteins in a gene-for-gene manner. Examples are *P. infestans* Avr3a and potato R3a (Armstrong et al., 2005; Huang et al., 2005a), *H. parasitica* ATR1 and Arabidopsis RPP1 (Rehmany et al., 2005; Botella et al., 1998) and *H. parasitica* ATR13 and Arabidopsis RPP13 (Allen et al., 2004; Bittner-Eddy et al., 2000). To date, *R* genes acting against oomycete plant pathogens have been identified in a variety of plant species and they all encode NBS-LRR proteins (Ballvora et al., 2002; Bhattacharyya et al., 2005; Bittner-Eddy et al., 2000; Botella et al., 1998; Gao et al., 2005; Huang et al., 2005a; McDowell et al., 1998; Parker et al., 1997; Sandhu et al., 2004; Shen et al., 2002; Slusarenko and Schlaich, 2003; Song et al., 2003; van der Vossen et al., 2003; van der Vossen et al., 2005; Wroblewski et al., 2007).

Previously we identified an RXLR-dEER effector in *P. infestans* that is specifically recognized by potato plants carrying $R4^{Ma}$ and hence, named the effector PiAvr4 (**Chapter 2**). $R4^{Ma}$ is one of the 11 *R* genes that were introgressed from *Solanum demissum* into cultivated potato (Black et al., 1953; Malcolmson and Black, 1966; Mastenbroek, 1953). The $R4^{Ma}$ gene has not yet been cloned, and

even its map position is unknown (El-Kharbotly, 1996b; **Chapter 5**). The goal of this study was to identify markers that would facilitate the cloning of *R4*^{Ma}. NBS profiling is a strategy that makes use of highly conserved domains in the NBS regions of R proteins to identify markers for *R* genes and resistance gene homologs (RGHs) (van der Linden et al., 2004). Here we describe the use of NBS profiling in combination with bulked segregant analysis (BSA) (Michelmore et al., 1991) for the identification of candidate markers for the potato late blight *R* gene *R4*^{Ma}.

RESULTS

Bulked segregant analysis on F1 population ARP 05-464 that segregates for *R4*^{Ma}

In a previous study we described that the potato cultivar Isola carries the same late blight resistance specificity as clone Ma-R4. We therefore designated the *R* gene responsible for resistance in Ma-R4 and Isola as *R4*^{Ma}. A cross of Isola with Katahdin, a cultivar without any known late blight resistance, resulted in an F1 progeny that shows a 1:1 segregation of *R4*^{Ma} (**Chapter 5**). For BSA, two pools of genomic DNA of 10 individual progeny and DNA from the parental lines were used for NBS profiling (Table 1; 2) using five restriction enzymes and five NBS-specific primers (Table 3). For each enzyme/primer combination 122-219 fragments were visible on the autoradiographs, 22-87 of which were polymorphic. In this way 29 fragments were identified that segregate with *R4*^{Ma}. They were present in the pool derived from resistant progeny and in the resistant parent but absent in the pool derived from susceptible progeny and in the susceptible parent. Seven fragments were found to segregate with the susceptible phenotype, since they were only present in susceptible plants. Several enzyme/primer combinations produced multiple segregating fragments (Table 4; Fig. 1).

Table1 Potato clones and cultivars used in this study

Clone	Known *R* genes
Cebeco44-31-5	*R4*^{Ma}
Isola	*R4*^{Ma}
1563 c (14)	*R4*^{Bl}
Cebeco43-154-5	*R1*^{Ma}
Cebeco46-174-30	*R1*^{Ma}*R4*^{Ma}
Katahdin	*r0*

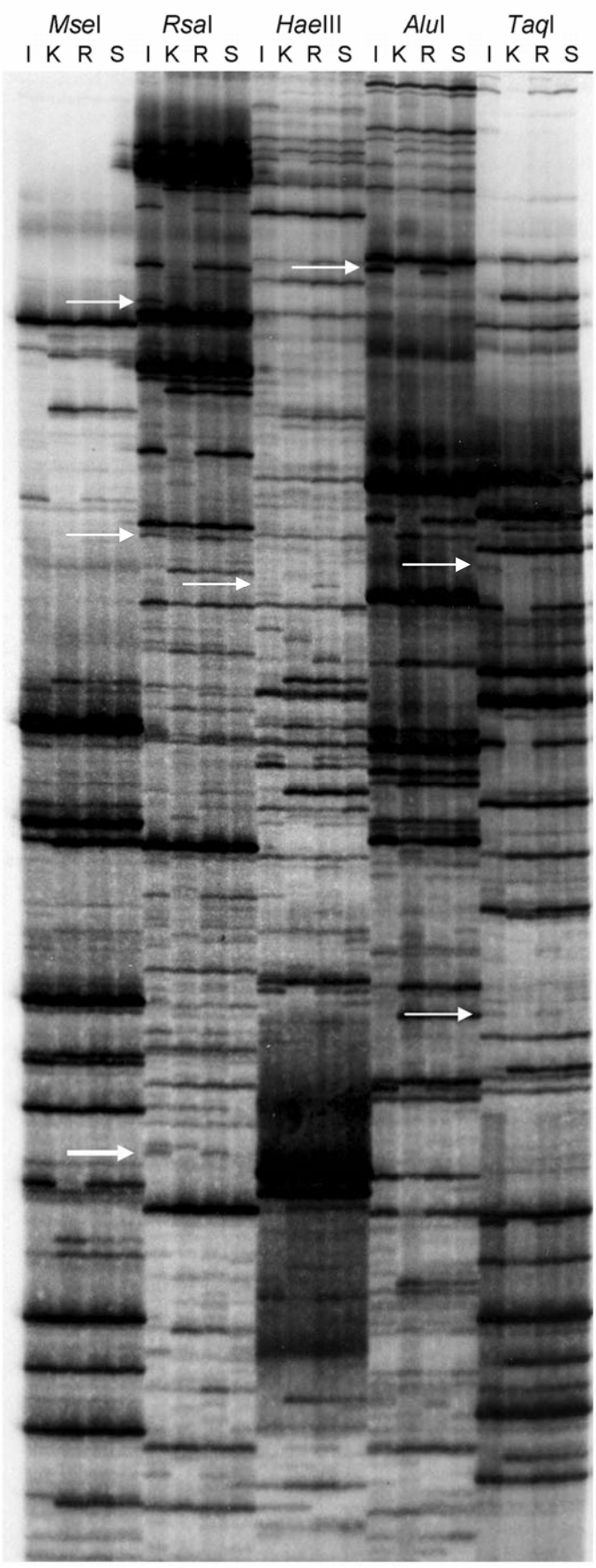

Figure 1. NBS profiles obtained with primer NBS9 on DNA of the cultivars Isola (I) and Katahdin (K) and on DNA bulks of resistant (R) and susceptible (S) F1 progeny of these two cultivars. The five restriction enzyme treatments that were used are indicated. The white arrows indicate the positions of fragments that segregate in the BSA. The arrow pointing to fragment 9R_3_237 is in bold.

Segregation of *R4*^{Ma} markers in ARP05-464

In the next step we selected a subset of eight enzyme/primer combinations to test the individual progeny of cross ARP 05-464 (Table 4). The selection was based on intensity and size of the fragments that were obtained in the BSA screening. In the 20 progeny that were included in the BSA pools (Table 2), five fragments showed co-segregation with the R4^{Ma} phenotype. These were further tested on all available F1 progeny and the *R4* differentials Cebeco44-31-5 (Ma-R4) 1563 c (14) (Bl-R4) and Cebeco46-174-30 (Ma-R1R4) (see **Chapter 5** for more details on the differentials). Three enzyme/primer combinations, *Alu*I/NBS5a+6, *Alu*I/NBS9 and *Rsa*I/NBS9, were needed to visualize the five fragments (Tables 4 and S1). None of the fragments obtained with *Alu*I/NBS5a+6 and *Alu*I/NBS9 appeared to be a marker for *R4*^{Ma} since there was no co-segregation with the phenotype. However, one of the three fragments obtained with *Rsa*I/NBS9 did co-segregate and was designated as marker 9R_3_237. The 237 bp fragment was absent in all susceptible progeny and present in most but not all the resistant progeny (Fig. 2).

Table 2 Composition of BSA pools used for NBS-profiling

	Pool R	**Pool S**
1	ARP05-464-3	ARP05-464-7
2	ARP05-464-10	ARP05-464-13
3	ARP05-464-15	ARP05-464-14
4	ARP05-464-17	ARP05-464-19
5	ARP05-464-29	ARP05-464-23
6	ARP05-464-31	ARP05-464-26
7	ARP05-464-33	ARP05-464-30
8	ARP05-464-39	ARP05-464-38
9	ARP05-464-44	ARP05-464-45
10	ARP05-464-48	ARP05-464-53

Table 3 Primers used for NBS-profiling

Primer	**Sequence**
NBS1	5'-GCIARWGTWGTYTTICCYRAICC-3'
NBS2	5'-GTWGTYTTICCYRAICCISSCAT-3'
NBS3	5'-GTWGTYTTICCYRAICCISSCATICC-3'
NBS5a+6	5'-YYTKRTHGTMITKGATGAYRTITGG-3'
NBS9	5'-TGTGGAGGRTTACCTCTAGC-3'
Adapter	5'-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3'

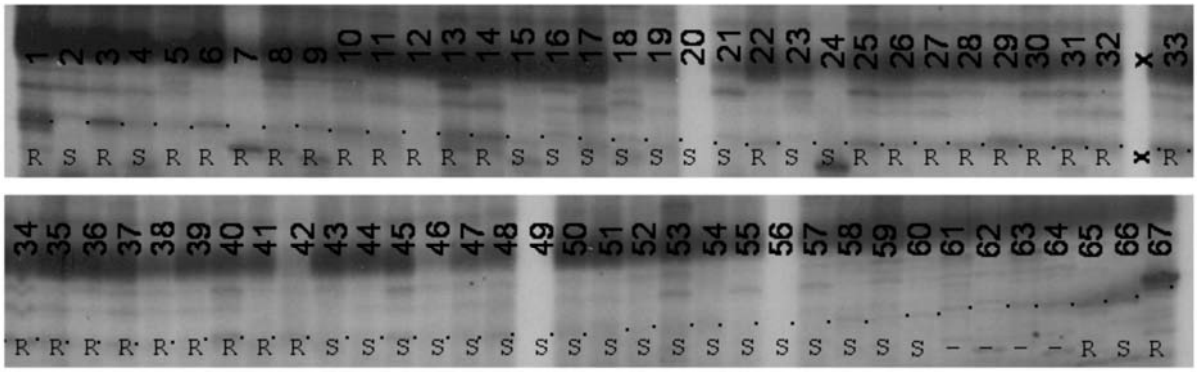

Figure 2. Detail of the NBS profile of marker 9R_3_237 (dots) that was obtained with primer/enzyme combination NBS9/*Rsa*I. The lane numbers correspond to the sample numbers in Table S1. R and S refer to the phenotypes of the corresponding plants and bulks, i.e., resistant and susceptible, respectively. In the lane marked by X (between lanes 32 and 33), there was no sample applied.

The DNA sequence of the candidate marker has homology to known *R* genes

Marker 9R_3_237 that co-segregates with the $R4^{Ma}$ phenotype was cloned and sequenced. Sequence analysis did not reveal a continuous open reading frame (ORF) in any of the six frames. BLASTN searches against public databases revealed that marker 9R_3_237 has homology to the potato resistance gene *Gpa2* (van der Vossen et al., 2000). *Gpa2* confers resistance to the potato cyst nematode *Globodera pallida* and encodes a CC-NBS-LRR protein. Because of the similarity between 9R_3_237 and *Gpa2* we decided to make a multiple sequence alignment with known homologs of *Gpa2* and its close relative *Rx1,* the potato virus X resistance gene (Bendahmane et al., 1999). Several of the *Rx1/Gpa2* homologs have been cloned and sequenced (Butterbach, 2007) and many have been mapped to chromosomes V and XII (De Jong et al., 1997; Bendahmane et al., 1997; Bakker et al., 2003). Sequence comparison of 9R_3_237 and 75 *Rx1/Gpa2* homologs (Butterbach, 2007) revealed close homology of the $R4^{Ma}$ marker to several RGHs with the closest homology to FRN_RGH6 (Fig. 3), an RGH from *Solanum fernandezianum* that has an alternative stop codon. Alignment at the amino acid level revealed that two stop codons, caused by point mutations, disrupt the open reading frame of marker 9R_3_237, thus suggesting that the marker is derived from a pseudogene (Fig. 3B). None of the sequences present in this dataset is identical to marker 9R_3_237 (Table 5). The *Rsa*I restriction site that was initially used to generate an adapter ligation site for NBS profiling is present in marker 9R_3_237, but not in any of the 75 RGHs.

A BLASTN search against more than 300 sequenced potato BAC clones revealed that BAC clones RH135M18-6 and R036DXR00904 carry a sequence that is highly homologous (1.00e-109) to marker 9R_3_237 (Fig. 3; Table 5). These two BACs were both mapped on chromosome XII, one of the two chromosomes

that contain several *Rx1/Gpa2* homologs (De Jong et al., 1997; Bendahmane et al., 1997; Bakker et al., 2003). The BAC sequences have homology to RGHs, but do not contain full length open reading frames capable of producing a complete R protein. They also lack the *Rsa*I restriction site that was found in 9R_3_237.

Table 4 Candidate markers identified with NBS profiling using DNA of the parents and progeny of population ARP05-464 as template

Primer[a]	Total number of fragments	Polymorphic fragments	Candidate markers after BSA [a]		Candidate markers after testing 20 F1 progeny	Candidate markers after testing 67 potato lines
			+-+-	-+-+		
NBS1	946	286	3 (0)	2 (0)	-	-
NBS2	928	329	8 (6)	1 (0)	1	-
NBS3	942	206	2 (0)	3 (0)	-	-
NBS5a+6	748	247	5 (3)	1 (1)	1	-
NBS9	790	309	11 (8)	0 (0)	3	1
Total	3408	1091	29 (17)	7 (1)	5	1

[a] Each primer was combined with 5 restriction enzymes: *Mse*I, *Rsa*I, *Hae*III, *Alu*I and *Taq*I.
[b] Number of candidate markers that were selected for NBS profiling on 20 F1 progeny is in brackets.

Table 5 *R4*^{Ma}-associated fragments obtained by NBS profiling

Dataset	Hit	Score	E-value
Potato BAC sequences [a]	RH135M18-6	198	1e-109
	R036DXR00904	198	1e-109
Rx1/Gpa2 homologues [b]	FRN_RGH6	180	2e-047
GenBank	gnl|ti|1798992943	224	6e-058

[a] Obtained from CBSG-I project P3 (www.CBSG.nl).
[b] Butterbach, 2007.

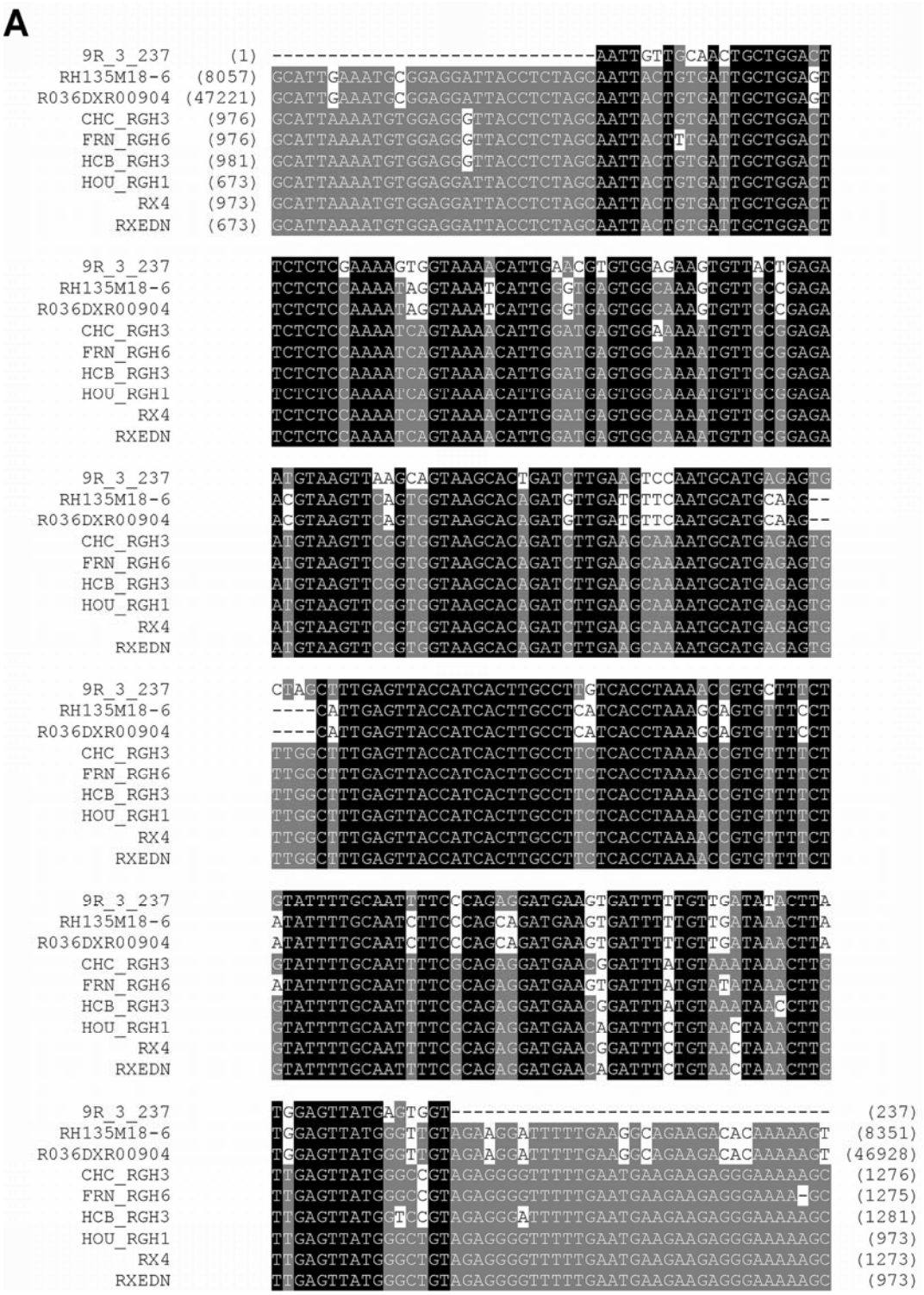

Figure 3. Sequence alignment of marker 9R_3_237 with partial sequences of BACs RH135M18-6 and R036DXR00904 and six *Rx1/Gpa2* homologs that were described by Butterbach (2007). **A.** shows an alignment of nucleotide sequences. The nucleotide positions within the respective sequences are indicated within brackets. **B.** shows an alignment of the protein sequences based on the partial sequences in **A.** The amino acid position within the predicted open reading frames are indicated within brackets. Predicted stop codons are indicated by a *.

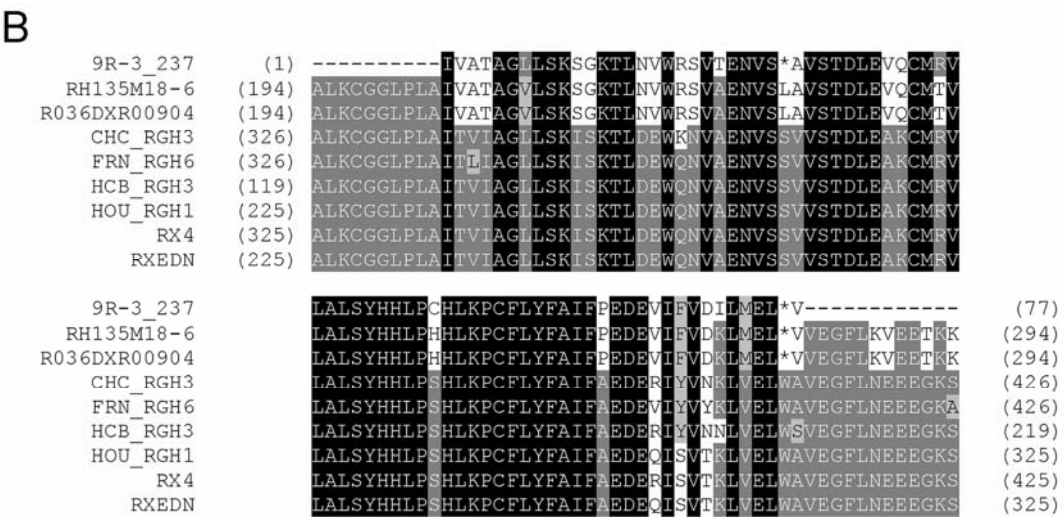

Figure 3. Continued from previous page

DISCUSSION

In this study we set out to identify markers for the potato *R4*^{Ma} gene by NBS profiling. In a previous study we showed that *R4*^{Ma} is involved in a gene-for-gene interaction with the *P. infestans Avr* gene *PiAvr4* and segregates in the F1 population ARP 05-464 (**Chapter 5**). Here we identified the NBS marker 9R_3_237 that co-segregates with *R4*^{Ma} mediated resistance.

With the cloning of the *P. infestans* avirulence gene *PiAvr4* (**Chapter 2**) we became interested in the properties of the cognate resistance gene *R4*^{Ma}. Only a few cultivars carrying *R4* have been used as commercial cultivars, including Avondale, Epoka, Gelda, Greta, Isola, Pentland Squire and Tylva (**Chapter 5**). Still race 4 strains are predominant in field populations (Flier and Turkensteen 1999; Hermansen et al., 2000; Lebreton et al., 1998) and have supposedly lost the ability to produce the Avr4 effector. As described in **Chapter 2**, virulent isolates produce a truncated version of PiAvr4 but remain pathogenic on *r0* potato plants. Hence, it is unlikely that *R4*^{Ma} is a suitable source for durable resistance to late blight and the interest in exploiting the *R4*^{Ma} gene for practical applications is expected to be limited. Our incentive to focus on cloning *R4*^{Ma} is driven by our interest in more fundamental questions concerning R-Avr pair and other protein-protein interactions in the resistosome complex.

In an earlier attempt to identify *R4*^{Ma} El-Kharbotly et al. (1996b) used a cross between the *R4*^{Ma} differential Cebeco44-31-5 and a susceptible potato clone; however, they were not able to position *R4*^{Ma} on the genetic map (El-Kharbotly et al., 1996b). The *R4*^{Ma}-mediated resistance did not segregate in a Mendelian

fashion and a second locus was proposed to be involved in the R4^{Ma} phenotype. Here we chose the biased approach of NBS profiling, a PCR based strategy that generates markers derived from *R* genes and RGHs. NBS profiling has been successful in identifying putative *R* genes in crop species such as potato (van der Linden et al., 2004), apple (Calenge et al., 2005), durum wheat (Mantovani et al., 2006) and lettuce (Syed et al., 2006). NBS profiling primers are designed on conserved sequences in the NBS region of NBS-LRR genes and the markers obtained trough NBS profiling are therefore highly enriched for RGH derived fragments. Since NBS-LRR proteins form the largest class of plant resistance proteins (Takken et al., 2006) and since all known *R* genes that confer resistance to oomycetes encode NBS-LRR proteins, a biased approach to identify *R4*^{Ma} seemed validated.

The *R4*^{Ma} NBS marker 9R_3_237 shows high sequence similarity to members of the *Rx1/Gpa2* gene family, but lacks a continuous ORF. Point mutations result in stop codons disrupting the ORF. Possibly, 9R_3_237 is a marker that co-segregates with *R4*^{Ma}. Co-segregation of resistance and a NBS marker that is not derived from an intact *R* gene is not surprising since *R* genes and RGHs often occur in clusters (Gebhardt and Valkonen, 2001). Two clusters of *Rx1/Gpa2* homologs have been described in potato, one on chromosome V and the other on chromosome XII (De Jong et al., 1997; Bendahmane et al., 1997). Interestingly, two BAC sequences that have been mapped to chromosome XII carry sequences that are highly similar the marker 9R_3_237. Previously, two quantitative trait loci (QTLs) for *P. infestans* resistance were identified on chromosome XII, but none of the potato plants used in those studies carried known *R* genes, such as *R4*$^{Ma.}$(Oberhagemann et al., 1999; Ghislain et al., 2001). Although we have, as yet, no indications that *R4*^{Ma} is located in a cluster of late blight *R* genes the situation on chromosome XII could resemble the situation on chromosome IV where a QTL for late blight resistance coincides with a region of RGHs. A QTL locus that was introgressed from *Solanum microdontum* was mapped in the same region as the NBS-LRR genes $R_{Pi\text{-}blb3}$, *R2*, $R_{2\text{-}like}$, and $R_{Pi\text{-}abpt}$ and the authors tentatively assigned the QTL to an NBS-LRR gene that they named $R_{Pi\text{-}mcd1}$ (Tan et al., 2008). In theory, the identified late blight QTLs on chromosome XII could be allelic versions of *R4*^{Ma}, but this awaits further investigation.

Like most resistance genes from solanaceous species *R4*^{Ma} apparently could belong to the CC-NBS-LRR gene family. Also the late blight *R* genes *R1*, *R3a* and *Rpi-blb1* from potato belong to this family of NBS-LRR genes (Van Ooijen et al., 2007). As yet, none of the cloned *R* genes that confer resistance to late blight belong to the *Rx1*/*Gpa2* family. However, the highly similar CC-NBS-LRR

genes *Rx1* and *Gpa2* confer resistance to two highly dissimilar pathogens, the potato virus X (PVX) and the cyst knot nematode *G. pallida*. Thus *R4*^{Ma} could fit in the model that close *R* gene homologs confer resistance to a wide range of pathogens.

In conclusion, we have presented the identification of an NBS marker, 9R_3_237, linked to *R4*^{Ma}. A dense molecular-genetic linkage map, with many markers linked to *R* genes, is available for potato (van Os et al., 2006; https://cbsgdbase.wur.nl/UHD/) and homologous sequences of 9R_3_237 were mapped to chromosome XII. Integrating these data can facilitate the future cloning of the *R4*^{Ma} late blight resistance gene.

MATERIALS AND METHODS

Plant Material

The potato clones and cultivars used in this study are listed in Table 1. Potato clones Cebeco44-31-5 (*R4*) and Cebeco46-174-30 (*R1R4*) are included in the Mastenbroek differential set while clone 1563 c (14) is the *R4* differential of the Black set (Black et al., 1953). The F1 population ARP05-464, derived from a cross between cultivars Isola (*R4*) and Katahdin (*r0*), segregates for the R4^{Ma} phenotype and was described previously (Table 2; **Chapter 5**).

DNA isolation

Genomic DNA was isolated from 200 mg of the young top leaves of mature potato plants. The DNA isolation protocol was adapted from the procedure described by Fulton et al. (1995). Leaf samples were grinded in 2.2 mL microcentrifuge tubes, with 5 glass beads (Ø 3 mm) and 750 µL microprep buffer containing sodium bisulfite using a FastPrep instrument (Qbiogene, Carlsbad, CA, USA). A phenol/chloroform extraction was performed, followed by an RNase treatment and DNA precipitation.

Bulked segregant analysis

Genomic DNA samples were isolated from either ten resistant or ten susceptible F1 progenies of cross ARP05-464 and were pooled for BSA (Table 2). Each pool contains 40 ng genomic DNA of each individual F1 progeny, resulting in a total of 400 ng. The profiles of these DNA pools were compared to the profiles of parental cultivars Isola and Katahdin. Candidate markers, that were obtained using BSA, were screened on individual F1 progeny.

NBS profiling

NBS profiling was performed as described previously (van der Linden et al., 2004). Briefly 400 ng genomic DNA was digested by different restriction enzymes (*Mse*I, *Rsa*I, *Hae*III, *Alu*I and *Taq*I, respectively), followed by adapter ligation. The obtained DNA fragments were subsequently PCR-amplified using a set of primers of which one matches the adapter sequence, while the other primer has a NBS-region specific sequence. The five restriction enzymes and five NBS-specific primers that were used are listed in Table 3. The obtained PCR products were re-amplified using ^{33}P-labeled NBS-specific primers for radioactive detection. PCR products were than separated on a 6% polyacrylamide gel and an X-ray film was used to visualize the radioactive labeled fragments.

Isolation of segregating fragments
To isolate the putative *R4*^{Ma} markers, the fragments for these markers were excised from gels. To indicate the position of the markers the gels were covered with the developed autoradiograph. Fragments were individually excised from gel and the DNA was eluted in water by heating for 15 minutes at 70°C. Fragments were than re-amplified using the appropriate primers, cloned into pGEM-T easy (Promega, Madison, WI, USA) and sequenced.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIAL

Table S1 Results for NBS profiling on potato cultivars, clones and population ARP05-464

	Clone	R4 phenotype[a]	*NBS9*/Rsa*I 1*	*NBS9*/Rsa*I 3*	*NBS5a+6*/Alu*I*	*NBS9*/Alu*I*
1	Isola	R	+	+	+	+
2	Katahdin	S	-	-	-	-
3	pool R	R	+	+	+	+
4	pool S	S	-	-	-	-
5	ARP05-464-3	R	+	-	+	-
6	ARP05-464-10	R	+	+	+	+
7	ARP05-464-15	R	+	-	+	+
8	ARP05-464-18	R	-	+	-	-
9	ARP05-464-29	R	+	+	+	-
10	ARP05-464-31	R	-	+	+	+
11	ARP05-464-33	R	-	-	+	-
12	ARP05-464-39	R	-	-	-	+
13	ARP05-464-44	R	-	+	-	+
14	ARP05-464-48	R	-	+	-	-
15	ARP05-464-7	S	-	-	-	-
16	ARP05-464-13	S	-	-	+	-
17	ARP05-464-14	S	-	-	-	+
18	ARP05-464-19	S	-	-	-	-
19	ARP05-464-23	S	-	-	-	-
20	ARP05-464-26	S	-	-	-	-
21	ARP05-464-30	S	-	-	-	-
22	ARP05-464-38	S	-	+	-	-
23	ARP05-464-45	S	-	-	-	-
24	ARP05-464-53	S	+	-	-	-
25	ARP05-464-1	R	-	-	+	+
26	ARP05-464-2	R	-	+	-	-
27	ARP05-464-4	R	-	+	-	-
28	ARP05-464-8	R	-	+	-	-
29	ARP05-464-12	R	+	+	-	-
30	ARP05-464-16	R	+	+	+	+
31	ARP05-464-17	R	-	+	-	-
32	ARP05-464-25	R	-	+	-	-
33	ARP05-464-27	R	+	+	+	-
34	ARP05-464-28	R	-	+	-	+
35	ARP05-464-32	R	-	+	-	+
36	ARP05-464-35	R	-	+	-	+
37	ARP05-464-36	R	+	+	+	-
38	ARP05-464-37	R	+	-	-	-
39	ARP05-464-41	R	+	-	-	+
40	ARP05-464-42	R	-	+	+	-
41	ARP05-464-47	R	-	-	-	-
42	ARP05-464-52	R	-	+	-	+
43	ARP05-464-5	S	-	-	-	+
44	ARP05-464-6	S	-	-	-	+
45	ARP05-464-11	S	-	-	-	-
46	ARP05-464-20	S	-	-	+	-
47	ARP05-464-21	S	-	-	+	+
48	ARP05-464-22	S	-	-	+	-
49	ARP05-464-24	S	-	-	+	-
50	ARP05-464-34	S	-	-	+	-
51	ARP05-464-40	S	-	-	-	+
52	ARP05-464-43	S	-	-	-	+
53	ARP05-464-46	S	-	-	+	+
54	ARP05-464-49	S	-	-	-	-
55	ARP05-464-50	S	-	-	-	-
56	ARP05-464-54	S	-	-	-	-
57	ARP05-464-55	S	-	-	-	-
58	ARP05-464-57	S	-	-	-	+
59	ARP05-464-58	S	-	-	-	+
60	ARP05-464-59	S	-	-	-	-
61	ARP05-464-9	I	-	+	-	-
62	ARP05-464-51	I	+	-	-	+
63	ARP05-464-56	I	-	+	-	+
64	ARP05-464-60	I	-	+	-	+
65	Cebeco44-31-5	R	-	+	+	-
66	1563 c (14)	S	+	-	-	-
67	Cebeco46-174-30	R	+	+	+	-

[a] R resistant; S susceptible; I intermediate phenotype.

CHAPTER 7

General discussion

Parts of this chapter are published in

Klaas Bouwmeester*, Pieter M.J.A. van Poppel* and Francine Govers (2009) **Genome biology cracks enigmas of oomycete plant pathogens**. In: Annual Plant Reviews Vol. 34, Molecular aspects of plant disease resistance. pp. 102–133 (ed. J. Parker) Willey-Blackwell, Oxford, UK.

* Equal contribution

The discovery of the RXLR-dEER class of effector proteins, to which also the *Phytophthora infestans* avirulence (Avr) protein PiAvr4 belongs (**Chapter 2**), was a major breakthrough in the field of molecular oomycete biology (Govers and Gijzen, 2006). RXLR-dEER effectors are highly diverse and are thought to play important roles during plant infection. This discussion is devoted to these and other types of secreted proteins of oomycetes and the potential role that RXLR-dEER effectors play in oomycete-plant interactions.

THE *PHYTOPHTHORA* SECRETOME

Plant pathogens secrete numerous effector molecules that fulfill important roles in mediating pathogenesis and scouting for targets in the host to deploy their action. The secretome is defined as the sum of all proteins secreted by an organism. Effectors function at the forefront to establish adhesion, penetration of host tissue, and degradation of cell walls. Moreover, effectors interact with plant cell components at the cell wall, plasma membrane or in the cytoplasm thereby reprogramming the host cell to accommodate the needs of the pathogen. For biotrophic pathogens the fine-tuning of this reprogramming is very important. They probably have to make compromises: the host cell has to stay alive and should support the pathogen in its urge to survive. Hence, the pathogen has to be able to suppress basal defense responses (PAMP-triggered immunity or PTI) triggered by pathogen compounds known as pathogen associated molecular patterns (PAMPs) (Jones and Dangl, 2006). Once these barriers are overcome, the pathogen may face another barrier, i.e. the effector-triggered immunity (ETI). ETI, however, is only triggered when the plant possesses *R* genes that monitor specific effectors or effector targets. Figure 1 gives a simplified view of the interplay between plant and pathogen in a compatible and incompatible interaction.

Genome mining expeditions focused on the secretome of *Phytophthora* resulted in numbers ranging from 1188 secreted proteins in *P. ramorum* to 1975 in *P. infestans* with *P. sojae* (1464) as intermediate (Jiang, 2006; Tyler et al., 2006; R.H.Y. Jiang, personal communication). The secretome of the diatom *Thalassiosira pseudonana,* the closest relative of oomycetes that is sequenced and does not have a pathogenic lifestyle, is much smaller and lacks many of the enzymes found in *Phytophthora* (Tyler et al., 2006). More than 80% of the genes encoding secreted proteins (defined as *spe* genes) belong to gene families and they often occur in clusters. The percentage of *spe* orthologs is lower than among non-*spe* genes indicating that the secretome evolved at a faster pace than the average genome. This supports the idea that many of the *spe* genes are involved in molecular ‘arms races’. They undergo rapid changes that are driven by diversifying

selection. Within the secretome, however, individual families appear to have evolved at different rates. NPPs (Necrosis inducing Protein *Phytophthora*) and elicitins are highly conserved and this could be due to selection pressure exerted on certain domains, for example to maintain enzymatic functions. In contrast, the RXLR-dEER proteins as well as some families containing proteins composed of repeats are highly divergent (Jiang, 2006).

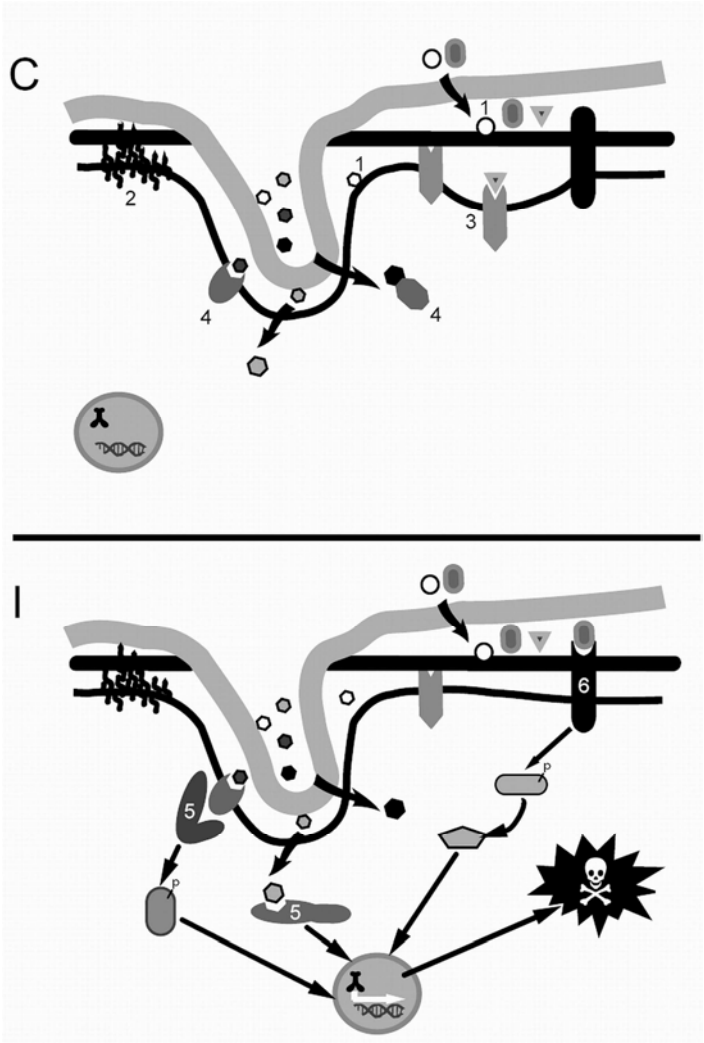

Figure 1. During a host-pathogen interaction effectors ( ) are secreted by the pathogen and directed towards their targets. These targets can be either membrane-spanning or cytoplasmic proteins, or structural molecules such as cell wall components. Effectors are needed to establish a compatible interaction (C) and are active in different steps of the infection process, for example, attachment(1), breakdown of the cell wall by enzymes(2), relaxation of the cell membrane(3) or alteration of host metabolism(4). During an incompatible interaction (I) the actions of effectors are monitored by a warning system in the host. When an effector or its modified target is detected by an R protein(5), or by a receptor-like kinase (RLK)(6) signaling cascades are initiated leading to transcriptional changes and activation of defense responses, and in many cases resulting in cell death. The figures are simplified showing only the cross-talks discussed in this chapter. The system is too complex to show all steps known to be involved in host-pathogen interactions.

Table 1 An overview of known oomycete avirulence genes

Effector gene	Origin	*R* gene	Type of *R* gene	W and Y domains	Protein variants	Positive selection	Types of mutations affecting Avr phenotype
HpATR1^{NdWsB} [a]	*H. parasitica*	*RPP1*	n.d.	-	8	yes	SNPs, indels
HpATR13 [b]	*H. parasitica*	*RPP13-Nd*	CC-NB-LRR [k]	-	6	yes	SNPs, indels
PsAvr1a [c]	*P. sojae*	*Rps1a*	n.d.	W	n.d.		n.d.
***PsAvr1b*-1** [d]	*P. sojae*	*Rps1b*	n.d.	WY	4	yes	Transcript regulation, SNPs
PsAvr3a [e]	*P. sojae*	*Rps3a*	n.d.	-	n.d.		n.d.
PiIpiO [f]	*P. infestans*	*Rpi-blb1*, *Rpi-sto1*	CC-NB-LRR [l]	W	10	yes	SNPs
PiAvr1 [g]	*P. infestans*	*R1*	CC-NB-LRR [m]	WW	5		SNPs
PiAvr2 [h]	*P. infestans*	*R2*	CC-NB-LRR [n]				
PiAvr3a [i]	*P. infestans*	*R3a*	CC-NB-LRR [o]	W	2		SNPs
PiAvr4 [j]	*P. infestans*	*R4*	n.d.	WWWY	6		Frameshifts

[a] Rehmany et al., 2005; [b] Allen et al., 2004; [c] Qutob et al., 2007; [d] Shan et al., 2004; [e] Tedman-Jones et al., 2007
[f] Vleeshouwers et al., 2008; [g] Guo, 2008; [h] Paul Birch, personal communication; [i] Armstrong et al., 2005
[j] **Chapter 2**; [k] Bittner-Eddy et al., 2000; [l] van der Vossen et al., 2003; [m] Ballvora et al., 2002; [n] Lokossou et al., 2008
[o] Huang et al., 2005a; n.d., not determined.

The number of RXLR-dEER effectors found in each of the sequenced genomes is astonishing. Jiang et al. (2008) reported 396 different RXLR-dEER proteins in *P. sojae* and 374 in *P. ramorum*. With the same mining strategy of reiterated BLAST searches and Hidden Markov Models (HMM) around 563 members were found in *P. infestans* (R.H.Y. Jiang, personal communication). Lately, several publications have listed different numbers and this is likely due the different algorithms that are used by the different groups (Lamour et al., 2007; Whisson et al., 2007; Win et al., 2007; Jiang et al., 2008). Dispute about the best mining strategy continues but there is no doubt that the family is extremely large and diverse. The N-terminal part with the signal peptide and the RXLR-dEER motif is conserved but in the C-terminal regions there is little similarity. Avr function and diversity are two characteristics that underscore the role of RXLR-dEER effectors in host specificity. Another characteristic is the distribution of RXLR-dEER genes over the genome. The high level of conserved synteny between *Phytophthora* species over large segments of the genome is disrupted by indel blocks and nearly all RXLR-dEER genes are located in these blocks (Jiang et al., 2006a; Jiang et al., 2008, **Chapter 3**). The genomes are highly dynamic and full of transposons and it is remarkable that RXLR-dEER genes are more often flanked by transposon-like sequences than other genes (R.H.Y. Jiang and M.C. Zody, personal communication). Also the genomic position of *PiAvr4* is flanked by approximately 100 kb of transposon sequences (**Chapter 3**). Apparently transposition is one of the factors that contributed to the rapid evolution of these large effector families.

The role of the RXLR-dEER motif

At the time of discovery the function of the RXLR-dEER motif was a mystery but shortly after the 2004 *Phytophthora* annotation jamboree Hiller et al. (2004) and Marti et al. (2004) reported the existence of a conserved motif, named PEXEL/VTS, in secreted effectors of the Alveolate parasite *Plasmodium falciparum*, the major causal agent of human malaria. RXLR-dEER resembles the PEXEL/VTS motif, both in sequence and location in the protein (Fig. 2A). The PEXEL/VTS motif was shown to be involved in host cell-targeting of effectors. Via this system, the parasite delivers a secretome consisting of an estimated 400 proteins into the cytoplasm of erythrocytes, presumably to carry out virulence and host remodeling functions (Hiller et al., 2004; Marti et al., 2004). This led to the hypothesis that RXLR-dEER effectors are also targeted into host cells. Bhattacharjee et al. (2006) strengthened this hypothesis by showing that the RXLR motif of *P. infestans* Avr3a, and not a mutated motif, can functionally complement a PEXEL/VTS motif in *Plasmodium.* More recently, Whisson et al. (2007) were able to demonstrate that *P. infestans* transformants carrying an *Avr3a* transgene with a

mutated RXLR-dEER motif can not restore the Avr phenotype of a race 3 strain, adding support to the idea that Avr3a has to reach the cytoplasm to be recognized by the intracellular CC-NB-LRR protein R3a. They also transformed *P. infestans* with reporter constructs consisting of GUS fused to wild type and mutated forms of the RXLR-dEER motif and monitored the intracellular location of GUS after infection. GUS is not stable in the apoplast and indeed only in the presence of the wild type motif the infected cells stained blue. Similar results were obtained by Dou et al. (2008b) with Avr1b from *P. sojae.* These experiments strongly support the notion that the RXLR-dEER motif indeed can function as a host cell-targeting signal.

Targeting RXLR-dEER proteins towards the host cell

The information that is now available on host cell-targeting of RXLR-dEER and PEXEL/VTS effector proteins enables to propose a model on the transport of RXLR-dEER effectors from the pathogen cytoplasm into the host cell and towards their intracellular target (Fig. 3). The presence of an N-terminal signal peptide sequence indicates that RXLR-dEER and PEXEL/VTS effectors are cotranslationally translocated into the extracellular space via the endoplasmic reticulum (ER), Golgi apparatus and secretory vesicles (Fig. 3A) (Hiller et al., 2004; Marti et al., 2004; Win et al., 2007). The only known exception is the secreted *P. falciparum* erythrocyte membrane protein (PfEMP-1) that lacks the N-terminal signal peptide but carries a C-terminal transmembrane domain (Hiller et al., 2004; Marti et al., 2004). All secreted oomycete effectors that have been identified up to date carry an N-terminal signal peptide. Signal peptides are found in almost all effectors of pathogenic microbes; however the effectors AVRa10 and AVRk1 of the powdery mildew fungus *Blumeria graminis* f. sp. *hordei* do not require a signal peptide for translocation into the host cytoplasm (Ridout et al., 2006).

An intracellular transport mechanism, e.g. the ER, targets RXLR-dEER effector proteins specifically towards infection structures like appressoria and haustoria (Fig. 3A) (**Chapter 4**, Whisson et al., 2007). Ectopic expression of the RXLR-dEER effectors PiAvr4 and IPI-O in non-sporulating hyphae showed that these proteins, which carried an mRFP tag at the C-terminus, are not targeted to specific cellular locations, but are distributed throughout the mycelium in subcellular spherical structures (**Chapter 4**). The identity of these spherical structures is unknown, but they likely are organelles such as ER-to-Golgi vesicles, Golgi cisternae or lysosomes that are destined for breakdown or recycling (Fig. 3B).

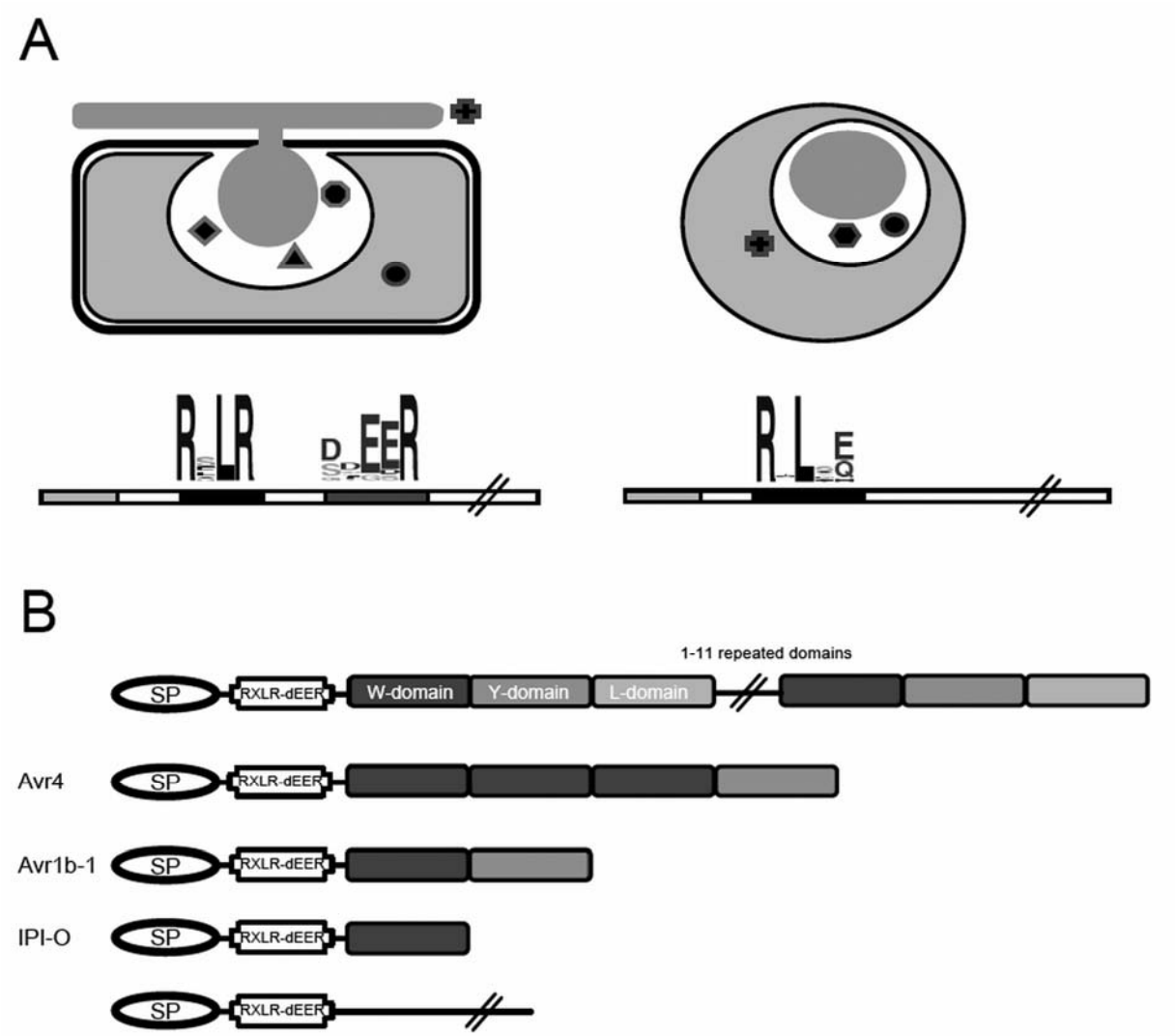

Figure 2. A. Infection strategies of plant pathogenic oomycetes and the malaria parasite *Plasmodium* show several similarities. Biotrophic oomycetes penetrate plant cells by forming haustoria (left). *Plasmodium* is localized inside erythrocytes within parasitophorous vacuoles (PV) (right). Both the haustorium and the PV are surrounded by a host derived membrane. The pathogens secrete effectors into the apoplast or extrahaustorial matrix (oomycetes), or the PV (*Plasmodium*). Effectors equipped with a host cell targeting motif can enter host cells. The host cell targeting motifs, RXLR-dEER in oomycetes and PEXEL/VTS in *Plasmodium*, are located N-terminal and adjacent to the signal peptide (marked in light grey). The dEER motif is conserved in most but not all oomycete RXLR effectors. The sequence logos show the amino acid conservation in a random set of effectors. The C-terminus in these effectors is highly diverse in both sequence and length.
B. In many of the RXLR-dEER effectors the C-terminus carries a variable number of domains that may occur in a repeated fashion (top row) (Jiang et al., 2008). Three domains are defined and named W, Y and L after the most conserved amino acid in that particular domain. Different combinations of domains are found. In a subset of the effectors the domains are lacking (last row). The rows in between show the domain composition of three RXLR-dEER effectors and illustrate the possible variations in the C-terminus. PiAvr4 and IPI-O from *P. infestans* and Avr1b-1 from *P. sojae* are recognized as Avr proteins in plants carrying the cognate *R* genes.

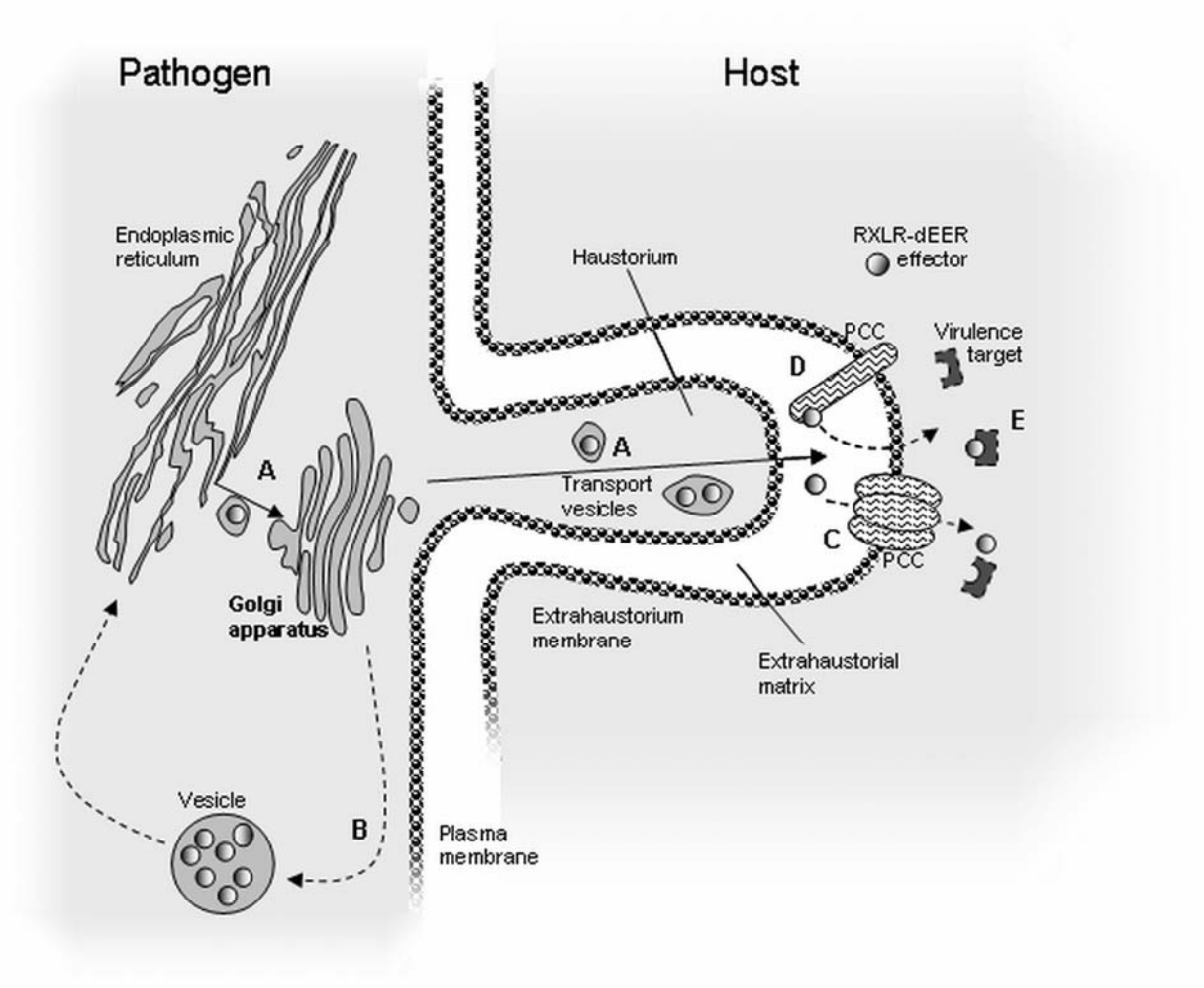

Figure 3. A model for the transport mechanism of oomycete RXLR-dEER effectors towards a host cell. Produced effectors are cotranslationally translocated to the endoplasmic reticulum and are subsequently transported through the Golgi apparatus and secreted via transport vesicles (**A**). In the absence of infection structures, e.g. haustoria and appressoria, vesicles are not targeted for secretion and accumulate in cytoplasmic vesicles (**B**). Once secreted into the extrahaustorium matrix, a hypothetical protein-conducting channel (PCC) that consists of host derived components mediates transport across the extrahaustorium membrane (**C** and **D**). Once in the cytoplasm the effectors can modulate their virulence targets (**E**). Putative transport pathways are indicated by dotted lines.

The oomycete RXLR-dEER motif has both sequence and functional homology to the PEXEL/VTS motif in secreted proteins of the malaria parasite *P. falciparum.* RXLR-dEER can functionally replace the PEXEL motif which was demonstrated by transport of a fluorescent protein fused to the Avr3a RXLR-dEER from *P. falciparum* into an erythrocyte (Bhattacharjee et al., 2006). A hypothetical protein-conducting channel (PCC) was proposed in the parasitophorous vacuole membrane to transport PEXEL proteins from the parasitophorous vacuole into the erythrocyte (Charpian and Przyborski, 2008). An analogous channel can be imagined in the extrahaustorium membrane, the membrane that RXLR-dEER

proteins have to cross to reach the plant cell cytoplasm (Fig. 3C). To what level the hypothetical PCCs in oomycete-plant interactions and *Plasmodium*-human interactions show similarities remains to be determined. The *Plasmodium*-human PCC probably has conserved elements with the oomycete-plant PCC to allow the Avr3a RXLR-dEER motif to function as a *Plasmodium* host cell-targeting motif. Studies on the *P. sojae* effector Avr1b have shown that this RXLR-dEER effector does not require other pathogen derived machinery to target effectors into soybean cytoplasm (Dou et al., 2008b) indicating that the PCC might be completely host derived. Furthermore, IPI-O, the effector that is recognized in the host cytoplasm by an NBS-LRR type R protein (Vleeshouwers et al., 2008) has an RGD motif that overlaps with the RXLR motif. Interestingly, the RGD in IPI-O binds to an Arabidopsis lectin receptor kinase (LecRK79) (Gouget et al., 2006) and this LecRK could be an effector target, but also a PCC that mediates uptake of RXLR-dEER effectors into the host cell. LecRK79 plays a role in the cell wall-plasma membrane adhesions and IPI-O was shown to disrupt these adhesions in Arabidopsis (Senchou et al., 2004). Consistent with the idea that RXLR-dEER effectors have virulence functions, overexpression of *ipiO* in Arabidopsis Col-0 leads to gain of susceptibility to *P. brassicae* strains that cannot infect wild type Col-0 plants (K. Bouwmeester and F. Govers, unpublished results).

Diversifying selection in RXLR-dEER effectors

Another shared feature of the RXLR-dEER effectors in oomycetes and PEXEL/VTS effectors in Plasmodium parasites is positive selection resulting in the coding sequences that are highly divergent (Hiller et al., 2004; Marti et al., 2004; Jiang et al., 2008). Many of the PEXEL/VTS proteins are known antigens that interact with the human adaptive immune system. All of the known oomycete Avr proteins, which are recognized by plant R proteins, belong to the RXLR-dEER effector family. Both the PEXEL/VTS and RXLR-dEER gene families need to adapt to evade recognition by the host immune system, which leads to a high selective pressure and hence the observed sequence divergence. Indeed, the C-terminal domains of several oomycete Avr proteins are under diversifying selection (Table 1). Bos et al. (2006) showed that recognition of Avr3a by R3a is based on the last 75 amino acids of the avirulent variant of Avr3a, Avr3a^{KI}, and that the N-terminal region is dispensable for recognition. The C-terminal part also suppresses HR induced by elicitin INF1 in *N. benthamiana* suggesting that the presence of Avr3a^{KI} confers a selective advantage to the pathogen when infecting a susceptible host. The virulent variant, Avr3a^{EM}, is not recognized by R3a and is, surprisingly, unable to suppress INF1 induced HR. As yet, however, there is no clue about the molecular or biochemical function of the RXLR-dEER effectors, and in the C-

termini of the investigated effectors no catalytic domains or ProSite motifs can be distinguished. Interestingly, motif searches and HMM screening revealed four conserved domains (W, Y, L and K) that are repeated (Jiang et al., 2008; Dou et al., 2008a; **Chapter 3**). W, the most prominent domain, consists of 25 amino acids and occurs in 60% of all RXLR-dEER members. Up to 11 repeats were found (Fig. 2B) and the number correlates with the length of RXLR-dEER proteins. The different numbers of repeats may, at least partly, explain the divergence between paralogs and the conservation of these motifs suggests that they have an essential role in the function of these motifs. Indeed, Dou et al. (2008a) showed that Avr1b mutants, carrying mutations in the K, W and Y motifs, lose both the ability to trigger Rps1b-dependent HR and the ability to suppress BAX-induced programmed cell death (PCD). Mutations in Avr1b that change the structure of the conserved motifs, but that retain the hydrophilic residues, cause loss of function phenotypes. These results imply that the overall structure of these motifs is required for both triggering HR and suppressing PCD. A deletion of the W2 motif of PiAvr4 also caused loss of recognition in *R4*^{Ma} potato plants, while the deletion of either W1 or W3 did not have any effect (**Chapter 3**). Possibly the tertiary structure of a combination of two PiAvr4 W motifs is required for triggering *R4*^{Ma}-dependent HR. In a subset of the RXLR-dEER effectors no W, Y or L domains can be distinguished; an example is ATR13 of *H. parasitica* (Table 1). Instead ATR13 has a heptad repeat region and a direct repeat region but the relevance of these repeats is not clear (Allen et al., 2004). CRN proteins have a highly conserved motif LXLFLAK and this motif was found to overlap with the RXLR motif of 13 RXLR proteins in *H. parasitica*. RXLR proteins with a CRN domain are absent in *Phytophthora*, suggesting that these proteins have recently evolved (Win et al., 2007). When these effectors end up in the host cell, they presumably utilize their C-terminal regions to modulate the host cell machinery. The high diversity of the C-terminus of RXLR-dEER proteins is consistent with the notion that this part is the effector domain that is active in the cell. The genes coding for IPI-O, ATR1, ATR13 and to a lesser extend Avr1b are under diversifying selection (K. Bouwmeester, personal communication; Rehmany et al., 2005; Allen et al., 2004 and Shan et al., 2004), while only limited numbers of alleles have been described for the RXLR-dEER effector Avr3a (Armstrong et al., 2005) which points to purifying selection at the *Avr3a* locus. Different from the other oomycete *Avr* genes, the sequence variations observed in the effector gene *PiAvr4* mostly lead to the abolishment of PiAvr4 production (**Chapter 2**).

The interaction between an R protein and its cognate Avr protein can be either direct or indirect. As yet, no studies on the physical interaction between oomycete Avr proteins and their respective R proteins have been reported. However the interactions for several other Avr-R protein combinations have been

described which can give clues on the type of interactions in oomycete-plant interaction. In case of a direct interaction the resistance (R) protein is able to bind to a pathogen effector protein and this binding will initiate defense responses. An indirect interaction is described by the “guard hypothesis” which states that the R protein will guard an effector virulence target (Dangl and Jones, 2001; Innes, 2004). During infection an effector protein modifies its virulence target and this modification will activate defense responses via the R protein. An effector protein that directly interacts with an R protein can contain distinct effector and avirulence domains and will therefore evolve to evade recognition without losing its effector domain. As a result, the genes that code for such effector proteins will predominantly be subjected to diversifying selection (Rohmer et al., 2004; Jones and Dangl, 2006; Ellis et al., 2007b). The oomycete effector IPI-O, that interacts with LecRK79 (Gouget et al., 2006), but also ATR1, ATR13 and Avr1b-1 appear to be under diversifying selection and therefore seem to be involved in a direct interaction with their respective R proteins (Dou et al., 2008a). In contrast effector proteins that are recognized indirectly by their cognate R protein will predominately evolve to retain the virulence function on the target protein and will therefore be under a purifying selection. The *P. infestans* Avr factor Avr3a could interact indirectly with R3a (Armstrong et al., 2005; Dou et al., 2008a). Alternatively these effectors can avoid recognition by the cognate R protein by incorporating mutations that lead to non-functional proteins, as seen in PiAvr4 (**Chapter 2**; Jones and Dangl 2006; Rohmer et al. 2004). An indirect interaction between PiAvr4 and $R4^{Ma}$ would fit the observation that $R4^{Ma}$ potato plants respond to *in planta* expression of *PmirAvh4* (**Chapter 3**). To fit a model of indirect recognition, both PiAvr4 and PmirAvh4 interact with a host protein (which should than be conserved across *Mirabilis jalapa* and *Solanum tuberosum*) that is guarded in *R4* potato plants.

POTATO RESISTANCE GENES AGAINST *PHYTOPHTHORA INFESTANS*

Gene-for-gene resistance against oomycetes has been described in several plant species. The standard potato differential set describes 11 individual *R* genes against *P. infestans* (Black et al., 1953; Malcolmson and Black 1966; Malcolmson 1969). Similar sets of *R* genes are also described in soybean against *P. sojae* (Polzin et al., 1994; Kasuga et al., 1997) and in Arabidopsis against the downy mildew pathogen *H. parasitica* (Crute et al., 1994). Typically these *R* genes are located on complex loci that mediate resistance against different races and species of pathogens. The potato *R3* locus appeared to contain two distinct genes, *R3a* and *R3b* (Huang et al., 2005a), that govern resistance against two different *Avr*

genes (Armstrong et al., 2005; Jiang et al., 2006c). Similarly two different resistance phenotypes were found in *R4* potato plants (**Chapter 5**). The genetic position of the *R4* genes, $R4^{Ma}$ and $R4^{Bl}$, remains to be determined and as yet there is no evidence for linkage of these two genes.

A genetic marker that was developed through NBS profiling and cosegregates with $R4^{Ma}$ resistance has homology to members of the *Rx*/*Gpa2* gene family (**Chapter 6**). As yet, two genes in this family, and several of their homologs, that have been functionally analyzed confer resistance to either Potato Virus X (*Rx*) or the root cyst nematode *Globodera pallida* (*Gpa2*). None of the cloned *R* genes against oomycetes belong to this gene family of NBS-LRR genes. However, more than 75 Rx/Gpa2 homologs have been identified in potato and since some of these genes are able to govern resistance to unrelated pathogens such as viruses and nematodes it is conceivable that some of them may also govern resistance to *P. infestans.* The $R4^{Ma}$ NBS-marker can now be used together with the available genetic and molecular potato resources (BAC libraries, high-density linkage map and RGH sequence data) to identify $R4^{Ma}$.

FUTURE PERSPECTIVES

The extensive molecular toolbox that is available for oomycete research has facilitated the research that has been described in this thesis. The cloning of *PiAvr4* was possible through the available BAC library and genetic map of *P. infestans.* The further characterization of this *Avr* gene was possible through stable DNA transformation and (transient) heterologous expression studies. The oomycete genome sequences and bioinformatic tools have revealed much of the genomic organization of the *PiAvr4* region and the modular organization of the PiAvr4 effector protein.

The most striking feature of PiAvr4 is the presence of the RXLR-dEER host cell targeting motif. Oomycete researchers are beginning to understand more and more of the mechanism by which RXLR-dEER effectors enter host cells and manipulate the host (Whisson et al., 2007; Dou et al., 2008b). However, still many questions on these effector proteins remain unanswered. The mechanisms by which these effectors are able to cross host barriers and apparently hijack host transport systems are still unknown. The first evidence is now accumulating that these effectors are true pathogenicity factors (Sohn et al., 2007; Rentel et al., 2008), and it will be interesting to unravel the role of the conserved C-terminal motifs in pathogenicity and their interaction with virulence targets and R proteins.

Introgression breeding has been successful to create several pathogen resistant crop species. Examples are resistance in wheat against *Puccinia graminis* f. sp. *Tritici*, in cabbage against *Fusarium oxysporum* f. sp. *conglutinans*, in tobacco against Tobacco mosaic virus and in tomato against *Cladosporium fulvum* (Parlevliet, 2002). However, durable resistance against late blight in potato has not been achieved despite numerous efforts in more than 50 years (Black et al., 1953, Wastie, 1991). The current insight into the effectome of *P. infestans* explains how this pathogen is able to quickly adapt to newly introduced *R* genes. Many of the RXLR-dEER effector proteins might be functionally redundant. When new resistant cultivars are introduced selection will favor isolates that lack the corresponding *Avr* gene but simultaneously retain all the other (>550) effector genes.

The interaction between an effector protein and the cognate R protein can be used to gain more insight in the strategies that oomycetes use to infect their respective hosts. Presumably all the (RXLR-dEER) effectors of *P. infestans* use similar mechanisms to enter the host cell and to perform their pathogenicity function. A comparison of the interactions between the different Avr and R proteins will give insight into these conserved mechanics. So far, only a limited number of Avr-R combinations have been cloned (Table 1) and there are indeed several conserved features between these combinations; all the Avr proteins are RXLR-dEER proteins and the cognate R proteins are all NBS-LRR proteins. However, different types of sequence variations (adaptive versus purifying) have been found in the different *Avr* genes, suggesting different types of Avr-R interactions. So far, *PiAvr4* is the only known oomycete *Avr* gene that escapes *R* gene triggered resistance through a frameshift mutation. The *P. infestans* genome sequence carries several other RXLR-dEER pseudo-genes. Whether these pseudo-genes are also disabled *Avr* genes remains to be determined. Future efforts should focus at identifying the host proteins that interact with PiAvr4, such as the target that potentially mediates extracellular recognition and the R protein that determines the resistance against AVR4 isolates. Moreover to get more insight in the intrinsic function of PiAvr4 in pathogenicity its activity as suppressor of defense responses could be investigated.

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SUMMARY

The potato late blight disease that is caused by the oomycete pathogen *Phytophthora infestans* is a major threat for potato crops worldwide. In recent years research on oomycete plant pathogens was boosted by the availability of novel genomic tools and resources for several oomycete genera, such as *Phytophthora*, *Hyaloperonospora*, *Pythium* and *Aphanomyces*. This has led to the identification of genes involved in diverse biological processes such as sporulation, mating, signaling and pathogenesis.

One of the approaches that breeders use to obtain late blight resistant potato cultivars is the introgression of resistance traits from wild *Solanum* species into the cultivated potato *Solanum tuberosum*. The pathogen, however, is able to circumvent this resistance; it is often lost shortly after introduction of new cultivars. To better understand the mechanisms underlying this loss of resistance it is of utmost importance to gain insight into the characteristics of the cognate avirulence (*Avr*) genes of the pathogen. According to the gene-for-gene model *Avr* genes encode effectors that trigger resistance responses mediated by resistance (*R*) genes. **This thesis** first describes the identification of a *P. infestans Avr* gene, in particular the elicitor activity of the encoded effector protein, the domain structure of the effector and its putative sub-cellular localization. In the second part the recognition specificity of the corresponding *R* gene and the identification of a marker linked to this *R* gene are described.

Chapter 1 summarizes the advances in oomycete genomics in recent years and the tremendous progress that has been made in gene discovery in oomycete plant pathogens. It describes the different oomycete species that have been studied in more detail and assesses which species are suitable model species for research on oomycete-plant interactions.

The identification of the *P. infestans* avirulence gene *PiAvr4* is presented in **Chapter 2**. *PiAvr4,* which encodes an RXLR-dEER effector protein, was isolated by positional cloning. AFLP markers were used for landing on BACs and cDNA-AFLP markers pinpointed the gene of interest. Transformation of race 4 strains with *PiAvr4* resulted in transformants that are avirulent on the *R4* differential of the Mastenbroek differential set (clone Ma-R4). Moreover, *in planta* expression of *PiAvr4* resulted in a necrotic response on clone Ma-R4 but not on plants lacking *R4* such as Bintje. All together this proves that *PiAvr4* is the avirulence gene that corresponds to the *R* gene present in clone Ma-R4. In many identified avirulence proteins one or a few amino acid changes in the protein abolish avirulence function. In case of *PiAvr4,* race 4 strains have frame shift mutations in the open

reading frame, resulting in a truncated protein that is not functional as avirulence factor.

Effectors within the RXLR-dEER family are rapidly evolving. The selective pressure is targeted predominantly on the C-terminal region of these proteins. Despite this selective pressure the majority of these proteins carry motifs that can be distinguished using Hidden Markov Models searches. They are named W, Y and L motifs after the conserved tryptophan (W), tyrosine (Y) and leucine (L) residues, respectively. As described in **Chapter 3** PiAvr4 carries three W motifs and a single Y motif. The motifs together with their flanking regions were tested for activity on Ma-R4 plants. Agroinfection of constructs carrying the W2 motif in combination with either the W1 or W3 motif resulted in a necrotic response. Moreover, we showed that the *PiAvr4* homolog *PmirAvh4*, isolated from *Phytophthora mirabilis* was also able to elicit a necrotic response on the Ma-R4 potato clone.

For several *Phytophthora* RXLR-dEER effectors it was demonstrated that these proteins are targeted into the host cell and that the RXLR-dEER motif is required for translocation. In **Chapter 4** we investigated whether PiAvr4 and IPI-O, like other RXLR-dEER effectors, are also targeted into the host cell. A race 4 *P. infestans* isolate was transformed with constructs encoding either PiAvr4 or IPI-O fused to a monomeric red fluorescent protein (mRFP) at the C-terminus. Fluorescence microscopy of these transformants showed no specific mRFP fluorescence in free living, non-sporulating mycelium. However, in germinating cysts, the tips of germ tubes and appressoria showed mRFP fluorescence, and during infection of etiolated potato plantlets localized fluorescence was visible at the haustorial neck. Haustoria are highly specialized infection and feeding structures that are in close contact with the plant cell and have a putative role in delivering effector proteins into the host cell. In order to monitor the development of the infection a novel experimental set-up was developed. In this method etiolated *in vitro* grown potato plantlets are inoculated with *P. infestans,* which has the advantage that there is no autofluorescence of chlorophyll that masks the mRFP fluorescence and thus disturbs the microscopic analysis in green plant tissues. The lack of chlorophyll does not seem to interfere with infection; zoospores are capable to encyst and to germinate, and the etiolated tissues are readily colonized by *P. infestans*.

The recognition specificity of *R4* potato differentials is described in **Chapter 5**. Initially two different potato clones were developed as *R4* differentials; The Mastenbroek differential set, developed in the Netherlands, contains the clone Cebeco44-31-5 (designated as Ma-R4) and the Black differential set, developed in Scotland, contains clone 1563 c (14) (designated as Bl-R4). Virulence assays

using several wild type *P. infestans* strains revealed that the Bl-R4 clone is susceptible to all isolates that are avirulent on clone Ma-R4. Only one single isolate was found to be avirulent on clone Bl-R4, but virulent on Ma-R4. Moreover, in transient expression assays with binary PVX constructs carrying *PiAvr4*, the Ma-R4 clone but not the Bl-R4 clone responded with an HR. Similar to the *R3* locus two different recognition specificities seem to exist for *R4*. The *R3a* and *R3b* genes are located on one locus but whether this is the case for the two *R4* genes (named $R4^{Ma}$ and $R4^{Bl}$, respectively) remains to be determined. Resistance to *P. infestans* strains carrying *PiAvr4* segregates in an 1:1 ratio in two independent potato F1 populations suggesting that $R4^{Ma}$ resistance is determined by a single dominant locus.

More in depth studies on the recognition of PiAvr4 by its cognate R protein are hampered by the fact that the resistance gene $R4^{Ma}$ has not yet been identified. In **Chapter 6** nucleotide binding site (NBS) profiling was used to generate $R4^{Ma}$-associated markers. NBS profiling is a biased approach based on PCR amplification of conserved NBS motifs in *R* genes and *R* gene homologs. In a bulked segregant analysis, DNA of resistant and susceptible F1 progeny was pooled and used as template for NBS profiling. Several candidate markers were found but eventually one amplified fragment was found to co-segregate with resistance mediated by $R4^{Ma}$. DNA sequencing of this fragment revealed high similarity to BAC sequences that are mapped to potato chromosome 12. Moreover, the $R4^{Ma}$ marker is homologous to members of the *Rx*/*Gpa2* gene family.

Chapter 7 focuses on the secreted effectors of plant pathogenic oomycetes, with special attention to RXLR-dEER effectors, and the role of these proteins in pathogenesis. The RXLR-dEER effector family is rapidly evolving and comprises all secreted oomycete avirulence proteins that are identified up till now. There is now ample evidence that oomycetes utilize the RXLR-dEER domain to deposit effectors inside host cells. Furthermore, this chapter discusses the experimental results described in this thesis in the light of present knowledge on gene-for-gene interactions, effector recognition and late blight resistance.

SAMENVATTING

De aardappelziekte, die wordt veroorzaakt door de oömyceet *Phytophthora infestans*, is wereldwijd een van de grootste problemen in de aardappelteelt. In de afgelopen jaren zijn nieuwe instrumenten en genomische databanken ontwikkeld voor verschillende oömycete plantpathogenen, zoals voor verschillende soorten binnen de genera *Phytophthora*, *Hyaloperonospora*, *Pythium* en *Aphanomyces*. Dit was een enorme stimulans voor onderzoek aan oömyceten en heeft geleid tot de identificatie van genen die betrokken zijn bij diverse biologische processen, zoals voortplanting, signaaloverdracht en pathogenese.

Een van de methoden die aardappelveredelaars gebruiken voor het verkrijgen van rassen die resistent zijn tegen *Phytophthora infestans* is het inkruisen van resistentie uit wilde aardappelsoorten in de gecultiveerde aardappel, *Solanum tuberosum*. De ziekteverwekker is echter vaak in staat deze resistentie te doorbreken met als gevolg dat snel na de invoering van nieuwe cultivars de resistentie verdwenen is. Om te weten welke mechanismen ten grondslag liggen aan dit verlies van resistentie is het van groot belang om inzicht te krijgen in effectoren die het pathogeen produceert. Volgens het gen-om-gen model coderen avirulentie (*Avr*) genen voor effectoren die afweerreacties in de plant activeren. Zo'n afweerreactie treedt alleen op als er een passend resistentie-eiwit is en de plant dus beschikt over het juiste resistentie (*R*) gen. **Dit proefschrift** beschrijft de identificatie van een *P. infestans Avr*-gen, in het bijzonder de avirulentie en effector activiteit, de domeinstructuur en de subcellulaire lokalisatie van het Avr eiwit. Daarnaast beschrijft dit proefschrift de specificiteit van het bijbehorende aardappel *R* gen en de identificatie van een genetische merker die gekoppeld is aan dit *R* gen.

Hoofdstuk 1 geeft een overzicht van de vooruitgang in de genomica van oömyceten in de afgelopen jaren en de enorme vooruitgang die is geboekt bij de ontdekking van genen in plantpathogene oömyceten. Het beschrijft de verschillende soorten oömyceten die in meer detail zijn onderzocht en inventariseert welke soorten geschikt zijn om te dienen als model voor onderzoek aan oömyceet-plant interacties.

De identificatie van het *P. infestans* avirulentiegen *PiAvr4* is beschreven in **Hoofdstuk 2**. *PiAvr4*, een gen dat codeert voor een RXLR-dEER effector eiwit, werd geïsoleerd met behulp van AFLP merkers waarmee BAC klonen werden geselecteerd, en cDNA-AFLP merkers voor identificatie van kandidaat genen. Transformatie van fysio 4 *P. infestans* isolaten met *PiAvr4* resulteerde in transformanten die avirulent waren op de *R4* aardappel kloon die onderdeel uitmaakt van de Mastenbroek differentiële set (kloon Ma-R4). Bovendien,

resulteerde *in planta* expressie van *PiAvr4* in een overgevoeligheidsreactie op kloon Ma-R4, maar niet op *r0* aardappelplanten zoals Bintje. Hiermee is bewezen dat *PiAvr4* het avirulentiegen is dat een gen-om-gen interactie heeft met het *R4* gen in de aardappel kloon Ma-R4. In andere geïdentificeerde avirulentie eiwitten zijn één of enkele aminozuur veranderingen in het eiwit vaak al voldoende om de avirulentiefunctie te verliezen. In het geval van *PiAvr4*, hebben fysio 4 isolaten mutaties die een verschuiving geven in het open leesraam, hetgeen resulteert in een klein eiwit dat niet functioneel is als avirulentiefactor.

Genen die behoren tot de RXLR-dEER effectoren familie, zoals *PiAvr4,* evolueren zeer snel. De selectieve druk is voornamelijk gericht op het deel dat codeert voor de C-terminus. Ondanks deze selectieve druk heeft de meerderheid van de RXLR-dEER eiwitten herkenbare C-terminale motieven genaamd W, Y en L en vernoemd naar aminozuren tryptofaan (W), tyrosine (Y) en leucine (L) die op geconserveerde plaatsen in deze motieven voorkomen. Zoals beschreven in **Hoofdstuk 3** heeft de effector PiAvr4 drie W motieven en een enkel Y motief. Deze verschillende motieven werden, ingebed in de omliggende regio's, getest voor avirulentieactiviteit op Ma-R4 planten. Agroinfectie met constructen die een W2 motief in combinatie met hetzij een W1 of W3 motief bevatten resulteerde in een overgevoeligheidsreactie op de Ma-R4 kloon en niet op Bintje. Daarnaast werd aangetoond dat het *PmirAvh4* gen uit *Phytophthora mirabilis,* dat homoloog is aan *PiAvr4*, ook in staat is zo'n overgevoeligheidsreactie te veroorzaken op een Ma-R4 aardappel kloon.

Van een aantal *Phytophthora* RXLR-dEER effectoren is aangetoond dat ze gericht in de cellen van de gastheerplant afgeleverd worden en dat het RXLR-dEER motief vereist is voor deze translocatie. In **Hoofdstuk 4** is onderzocht of ook Avr4 getransporteerd wordt naar de gastheercel. Een fysio 4 *P. infestans* stam werd hiertoe getransformeerd met een construct dat codeert voor PiAvr4 met aan de C-terminus een rood fluorescerend eiwit (mRFP). Met behulp van fluorescentie microscopie vonden we in deze transformanten specifieke mRFP fluorescentie in het topje van de kiembuis van kiemende cysten. Tijdens infectie van geëtioleerde *in vitro* aardappelplantjes bleek fluorescentie gelokaliseerd te zijn aan de basis van haustoria. Haustoria zijn zeer gespecialiseerde voedingsstructuren die nauw verbonden zijn met de plantencel en die mogelijk een rol hebben in het uitscheiden van effectoren naar de gastheercel. Om de ontwikkeling van de infectie microscopisch te kunnen volgen werd gebruikt gemaakt van geëtioleerde *in vitro* geteelde aardappelplantjes die werden geïnoculeerd met *P. infestans* zoösporen. Deze nieuwe experimentele opzet heeft als voordeel dat de autofluorescentie van chlorofyl, die de fluorescentie van mRFP kan overschaduwen en dus de microscopische analyse in groen plantenweefsel verstoort, niet aanwezig is. Het

gebrek aan chlorofyl bleek de infectie niet te verstoren; zoosporen vormden cysten en ontkiemden, en mycelium was in staat de geëtioleerde weefsels te penetreren.

De specificiteit van *R4* aardappelklonen voor *P. infestans* fysio's is beschreven in **Hoofdstuk 5**. In het verleden zijn twee verschillende aardappelklonen ontwikkeld als R4 differentiële kloon. De differentiële set ontwikkeld in Nederland door Mastenbroek bevat kloon Cebeco-44-31-5 (Ma-R4) en de differentiële set ontwikkeld door Black in Schotland bevat kloon 1563 c (14) (Bl-R4). Virulentietoetsen met verschillende *P. infestans* isolaten toonden aan dat de Bl-R4 kloon gevoelig is voor alle isolaten die avirulent zijn op kloon Ma-R4. Slechts één enkel isolaat bleek avirulent te zijn op kloon Bl-R4, maar virulent op Ma-R4. Bovendien, vertoonde de Ma-R4 kloon, maar niet de Bl-R4 kloon, een overgevoeligheidsreactie na een transiënte expressietoets met binaire PVX constructen die *PiAvr4* tot expressie brachten. Vergelijkbaar met *R3* lijkt *R4* niet een eenduidig, enkel *R* gen te zijn. De genen *R3a* en *R3b* liggen op één locus, maar of dit ook het geval is voor de twee *R4* genen (respectievelijk benoemd als $R4^{Ma}$ en $R4^{Bl}$) moet nog worden onderzocht. Analyse van twee onafhankelijke aardappelpopulaties toonde aan dat R4 resistentie tegen avirulente *P. infestans* isolaten 1:1 uitsplitst in beide F1 populaties and dit suggereert dat $R4^{Ma}$ resistentie wordt bepaald door een enkel dominant allel.

Meer diepgaande studies naar de herkenning van Avr4 door het corresponderende R eiwit worden belemmerd door het feit dat het $R4^{Ma}$ resistentiegen nog niet geïsoleerd is. In **Hoofdstuk 6** wordt beschreven dat "Nucleotide Binding Site (NBS) profiling" gebruikt werd voor het genereren van genetische $R4^{Ma}$ merkers. NBS profiling is gebaseerd op PCR amplificatie van geconserveerd NBS motieven in *R* genen en *R* gen homologen. Voor een "Bulked Segregant Analysis" (BSA), werd het DNA van respectievelijk resistente en gevoelige nakomelingen uit een F1 populatie gebundeld en gebruikt als startmateriaal voor NBS profiling. In de BSA werden verscheidene kandidaat-merkers gevonden. Vervolgens werden alle beschikbare nakomelingen getest en werd één enkel fragment gevonden dat met de $R4^{Ma}$ weerstand overerft. De DNA sequentie van dit fragment bleek grote gelijkenis te vertonen met BAC sequenties die op aardappelchromosoom XII zijn gekarteerd. Bovendien heeft de $R4^{Ma}$ merker homologie met leden van de *Rx1*/*Gpa2* genfamilie.

Hoofdstuk 7 geeft een overzicht van het secretoom van oömyceten, in het bijzonder de klasse van RXLR-dEER effectoren die bestaat uit snel evoluerende eiwitten waartoe alle tot nu toe geïdentificeerde *Phytophthora* avirulentie-eiwitten behoren. Recentelijk is aangetoond dat het RXLR-dEER motief zorgt voor de translocatie van effectoren naar de gastheercel. Dit hoofdstuk legt ook een verband tussen de experimentele resultaten die zijn beschreven in dit proefschrift

en de huidige kennis over gen-om-gen interacties, RXLR-dEER effectoren en de herkenning van deze effectoren, en resistentie tegen de aardappelziekte.

DANKWOORD

Ondanks dat mijn tijd op het Fyto lab al weer even achter me ligt, sluit ik bij deze toch echt mijn AIO-periode af. Vooraf wist ik dat een promotieonderzoek niet altijd makkelijk zou zijn, zo ik had al genoeg verhalen gehoord over de beruchte “AIO-dip”. Inderdaad, er zijn tegenslagen geweest maar die gevreesde AIO-dip, die is er nooit gekomen. Ik vond het namelijk altijd leuk om mijn proeven te doen, artikelen te lezen, presentaties maken en zelfs het schrijven ben ik uiteindelijk ook gaan waarderen.

Francine, dankzij jou heb ik mijn promotieonderzoek kunnen doen aan *Phytophthora infestans*, een “beest” waarvan ik als student nog dacht dat het slechts een onbeduidende schimmel was. Ondertussen weet ik natuurlijk wel beter en roep tegen iedereen die het maar horen wil dat het toch echt een oömyceet is. Je hebt een heel intensieve maar vooral betrokken manier van begeleiden. Dat was vooral het afgelopen jaar duidelijk toen we heel wat weekenden besteed hebben om aan manuscripten te werken (Ad nog bedankt voor de koffie!). Jouw fanatisme en enthousiasme voor wetenschappelijk onderzoek (en voor oömyceten in het bijzonder) hebben een belangrijke rol gehad bij de totstandkoming van dit proefschrift. Bedankt voor alles!

Pierre, jij was altijd de promotor die goed de vinger aan de pols hield en regelmatig eens kwam vragen: “En hoe staat het er voor?”. Met name na werkbesprekingen hadden we de kans om mijn werk eens goed onder de loep te nemen en te bediscussiëren, wat veel nuttige suggesties en ideeën voor mijn onderzoek opleverde. Ook ben ik dankbaar voor alle manuscripten die je altijd snel en zorgvuldig nakeek.

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Rays, jij was werkelijk onmisbaar bij het beantwoorden van alle vragen die ik had over bioinformatica en natuurlijk vooral over RXLR-dEERs. De vele discussies over allerhande wetenschappelijke onderwerpen en dan met name de evolutietheorie waren zeer leerzaam. En waarschijnlijk ben ik niet de enige die iets

aan jouw adviezen heeft gehad, want volgens de NRC heeft Cees Dekker het geloof in Intelligent Design ondertussen opgegeven.

Een flinke bijdrage aan het werk beschreven in dit proefschrift is geleverd door Menno en Maartje. Al jullie werk heeft uiteindelijk twee mooie verslagen en veel interessante data opgeleverd. Jadwiga, thank you for cloning and testing all those constructs in dozens of different plasmids but also for your excellent company, especially during our trips to the greenhouse. Ook wil ik alle studenten en gastmedewerkers die de afgelopen jaren op het Pinf lab hebben gewerkt bedanken. Het lab van fytopathologie kan natuurlijk nooit draaien zonder de steun van Ali en Hans, ik ben erg blij dat jullie altijd klaar stonden voor hulp bij allerhande papierwerk en bestellingen.

Verschillende mensen buiten het lab van fytopathologie hebben mij bijgestaan met waardevolle informatie, data, inzichten en assistentie. Daarom bedankt Dirkjan voor alle aardappels en je speurwerk naar de herkomst van verschillende aardappelcultivars, Sjefke en Mariëlle voor de kruisingen, Ben en Betty voor de hulp bij NBS profiling, Patrick en Aska voor alle sequenties, Theo voor de SNP analyses en Edwin voor de waardevolle adviezen. Ook wil ik alle medewerkers van Unifarm, en met name Bert en Henk bedanken voor de voortreffelijke zorg die mijn aardappelplanten hebben gekregen (zodat ik ze weer kon infecteren).

Gelukkig is een groot deel van het leven van een fytopatholoog niet alleen gericht op wetenschap en labwerk, maar ook op andere aspecten van de “academische vorming”. De legendarische gesprekken aan de koffietafel, die voor een “leek” absoluut niet te volgen waren, zal ik niet zomaar vergeten! Ook buiten werktijd hadden we regelmatig tijd voor allerlei leuke evenementen zoals borrels, kerstdiners en natuurlijk een Sinterklaasdiner. Ook de labweekenden die verspreid over het land plaatsvonden waren altijd heel gezellig, al heb ik er wel eens een kater aan overgehouden... Heel veel lol hebben we gehad op dinsdagavonden als we in wisselende samenstelling met Leve de Koningin! (maar ook als Pinf, Fyto1, de DCSS of de Epjes) in café Tuck onze kennis van trivia konden testen. Hoera! Hoera! Hoera!

Klaas en Peter, of het nou was tijdens het organiseren van een labuitje, het bezoeken van concerten en buitenlandse steden, het zoeken naar oude LP’s op rommelmarkten, het afluisteren van diezelfde LP’s of het borrelen in “Onder de Linden” op vrijdagmiddag, ik heb ontzettend veel lol met jullie gehad de afgelopen jaren.

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het lab bezig was of als ik in het weekend toch nog even wat werk moest doen. Hopelijk kan ik je binnenkort ook helpen met het nakijken van jouw proefschrift.

Met deze zinnen kom ik aan het einde van mijn tijd als promovendus en zoals jullie hebben kunnen lezen heb ik veel plezier beleefd aan deze periode. Toch is het wel een fijn gevoel om vanaf nu weer tijd te hebben voor andere dingen dan mijn promotie.

CURRICULUM VITAE

Petrus Marinus Johannes Abraham (Pieter) van Poppel is geboren te Breda op 8 september 1980 en behaalde in 1998 zijn vwo diploma aan de KSE te Etten-Leur. In datzelfde jaar begon hij aan de studie biologie aan de toenmalige Landbouwuniversiteit Wageningen. Gedurende zijn studie koos hij voor de specialisatie cellulaire biologie. Tijdens zijn hoofdvak bij het Laboratorium voor Virologie heeft hij onder de begeleiding van Dr. ir. Marcel Prins en Prof. dr. Rob Goldbach onderzoek gedaan aan gene silencing in tabak. Vervolgens heeft hij tijdens zijn bijvak onderzoek gedaan naar genexpressie in apomictische planten bij de business unit Bioscience van Plant Research International onder begeleiding van Dr. Kim Boutilier en Dr. ir. Mark Aarts. Voor zijn stage heeft hij gewerkt aan virale expressie vectoren aan de Universiteit van Kaapstad onder begeleiding van Prof. Ed Rybicki. In 2003 studeerde Pieter af aan de Wageningen Universiteit en begon zijn promotie onderzoek bij het Laboratorium voor Fytopathologie. Onder begeleiding van Prof. dr. ir. Francine Govers en Prof. dr. ir. Pierre de Wit werkte hij binnen het CBSG project “Elicitor-receptor research towards identifying elicitors and novel *R*-genes in *Solanum* germplasm”. De resultaten van dit onderzoek zijn beschreven in dit proefschrift. Sinds mei 2008 is hij werkzaam als moleculair veredelaar van tomaten bij De Ruiter Seeds in Bergschenhoek.

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: P.M.J.A. van Poppel
Date: 4 February 2009
Group: Wageningen University, Laboratory of Phytopathology

1) Start-up phase	date
► **First presentation of your project**	
Elicitor-receptor research in the *Phytophthora infestans*-potato interaction	Apr 16, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Genome biology cracks enigmas of oomycete plant pathogens. Annual Plant Reviews 34, 2008	Oct 2008
► **MSc courses**	
Plant-Microbe Interactions, PHP-30306	Mar-Apr 2004
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD Student Day, Vrije Universiteit Amsterdam (NL)	Jun 03, 2004
EPS PhD Student Day, Radboud University Nijmegen (NL)	Jun 02, 2005
EPS PhD Student Day, Wageningen University (NL)	Sep 19, 2006
EPS PhD Student Day, Wageningen University (NL)	Sep 13, 2007
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Wageningen (NL)	Dec 12, 2003
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht (NL)	Sep 17, 2004
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents' and WCS dag, Amsterdam (NL)	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
WCS day 2004, Utrecht (NL)	Jan 22, 2004
Congres Nederlandse Biotechnologische Vereniging, Ede (NL)	Mar 11-12, 2004
Gewasbeschermingsdag KNPV, Wageningen (NL)	Mar 24, 2004
NWO-ALW EPS meeting, Lunteren (NL)	Apr 05-06, 2004
NWO-ALW EPS meeting, Lunteren (NL)	Apr 04-05, 2005
WCS dag 2006, Utrecht (NL)	Jan 19, 2006
NWO-ALW EPS meeting, Lunteren (NL)	Apr 03-04, 2006
NWO-ALW EPS meeting, Lunteren (NL)	Apr 02-03, 2007
► **Seminars (series), workshops and symposia**	
CBSG meetings, twice a year	2003-2008
CBSG resistance meeting, Wageningen (NL)	Jun 10, 2004
Phytophthora Umbrella plan meeting, Wageningen (NL)	Oct 06, 2005
Eucablight TG2 pathogen workshop, Wageningen (NL)	Dec 07-09, 2005
Seminar: Christiane Gebhardt	Oct 30, 2003
Seminar: Barbara Baker	Jun 02, 2004
Seminar: Jean Ristaino	Nov 02, 2004
Seminar: Matteo Garbelotto	Apr 13, 2005
Seminar: Sophien Kamoun	Oct 05, 2005
Seminar: Khaoula Belhaj	jan 30, 2006
Seminar: Jim Beynon	Feb 09, 2006
Seminar: Nick Talbot	May 03, 2006
Seminar: André Drenth	Oct 10, 2007
► **Seminar plus**	
► **International symposia and congresses**	
5th Annual meeting Oomycete Molecular Genetics Research Network, Wageningen (NL)	May 05-06, 2006
6th Annual meeting Oomycete Molecular Genetics Research Network, Asilomar (USA)	Mar 18-20, 2007
24th Fungal Genetics Conference, Asilomar (USA)	Mar 20-25, 2007
► **Presentations**	
Lecture, Department of Plant Pathology, North Dakota State University, Fargo (USA)	Nov 04, 2005
Oral presentation, WCS dag 2006, Utrecht (NL)	Jan 19, 2005
Oral presentation, 5th Annual meeting Oomycete Molecular Genetics Research Network, Wageningen (NL)	May 05, 2006
Oral presentation, 6th Annual meeting Oomycete Molecular Genetics Research Network, Asilomar (USA)	Mar 19, 2007
Poster presentation, 24th Fungal Genetics Conference, Asilomar (USA)	Mar 23, 2007
Oral presentation, NWO-ALW EPS meeting, Lunteren (NL)	Apr 03, 2007
► **IAB interview**	
Interview with Prof.dr. Michel Dron	Sep 08, 2006
► **Excursions**	
Field visit, field trial for screening late blight resistance of wild *Solanum* species, Wageningen (NL)	Jul 14, 2005
Subtotal Scientific Exposure	*18.1 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
Summerschool 'Signaling in Plant development and Plant Defence'	Jun 19-21, 2006
► **Journal club**	
Literature study group Phytopathology	2003-2006
► **Individual research training**	
Subtotal In-Depth Studies	*3.9 credits**

4) Personal development	date
► **Skill training courses**	
Scientific writing c1 level	Feb-Apr 2006
► **Organisation of PhD students day, course or conference**	
Organisation of the 5th Annual meeting Oomycete Molecular Genetics Research Network, Wageningen (NL)	May 04, 2006
Organisation of the annual lab outing, Laboratory of Phytopathology	Jun 11, 2004
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**39,1**

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

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

The work in this thesis was performed at the Laboratory of Phytopathology, Binnenhaven 5, 6709 PD Wageningen, the Netherlands, and within the graduate school for Experimental Plant Sciences (EPS).

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