Phytophthora infestans avirulence genes; mapping, cloning and diversity in field isolates

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General introduction

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Phytophthora infestans and potato late blight

Late blight, caused by the oomycete plant pathogen *Phytophthora infestans*, is a devastating disease of potato and was responsible for the epidemics that led to the Irish potato famine in 1845. The pathogen *P. infestans* is generally considered to cause disease specifically on leaves, stems and tubers of potato or tomato crops, although natural infection of plants outside the genera *Solanum* has been reported (Erwin and Ribiero, 1996; Becktell et al., 2006). The disease spreads optimally under cool and moist weather conditions. Leaf symptoms appear as pale green water soaked lesions that expand rapidly resulting in total destruction of the plant in a few days. Lesions also appear on the petioles and stems as black, greasy areas. Stem lesions may girdle the stem and kill the foliage above the lesions. From the foliage, the disease spreads to the tubers and causes rotting before or after harvest.

The disease cycle

The disease cycle of *P. infestans* is depicted in Figure 1. The initial inoculum consists of asexual spores called sporangia that are dispersed by wind or in water drops. Infection generally occurs in the foliage and occasionally on stems. When sporangia land on a host leaf surface they either germinate directly or undergo cytoplasmic cleavage to form seven to eight swimming zoospores, depending on environmental conditions. Zoospores are attracted to the host where they halt, retract their flagella and secrete material to form a cell wall. A sporangium or cyst germinates and the germ tube tip differentiates into an appressorium, a spherical or elliptical structure from which a penetration peg emerges to breach the plant cuticle and cell wall. After having passed the epidermis, the hyphae grow mainly intercellularly and form haustorium-like feeding structures that protrude into mesophyll cells. In later stages, the pathogen adopts a necrotrophic lifestyle. In this stage, after three to four days with conditions favorable to the pathogen, hyphae emerge through the stomata and sporangiophores with sporangia are formed which can start a new cycle of infection. Tubers may be infected whenever sporangia are washed from lesions on stems and foliage to the soil. The invasion proceeds into the outer layers of the tuber tissue and the affected areas become firm and dry and somewhat sunken (dry rot). Secondary infections by fungi and bacteria result in wet rot and the tubers will disintegrate. Infected tubers are a common source of inoculum at the beginning of the season.

Sexual reproduction only occurs when a leaf or a stem is invaded by two *P. infestans* strains, each with a different mating type. *P. infestans* is an outcrossing heterothallic species with two known mating types, A1 and A2. When strains with opposite mating type grow in each others' vicinity, specific hormones are produced. The α1 mating hormone is produced by an A1 mating type strain and triggers the formation of oogonia (♀) and antheridia (♂) in an A2 mating type strain (Qi et al., 2005; Harutyunyan et al., 2008). Vice versa, A2 strains produce α2 mating

hormone that induces the formation of ♀ and ♂ gametangia on A1 mating type strains. The nuclei in the gametangia undergo meiosis and via a fertilization tube a male haploid nucleus is deposited in the oogonium, which then develops into a thick walled oospore. The nuclei fuse during maturation of the oospore. When blighted potato plants decompose, numerous oospores are released and these tough structures are able to overwinter in the soil. Germinating oospores can form sporangia which can start infection of potato.

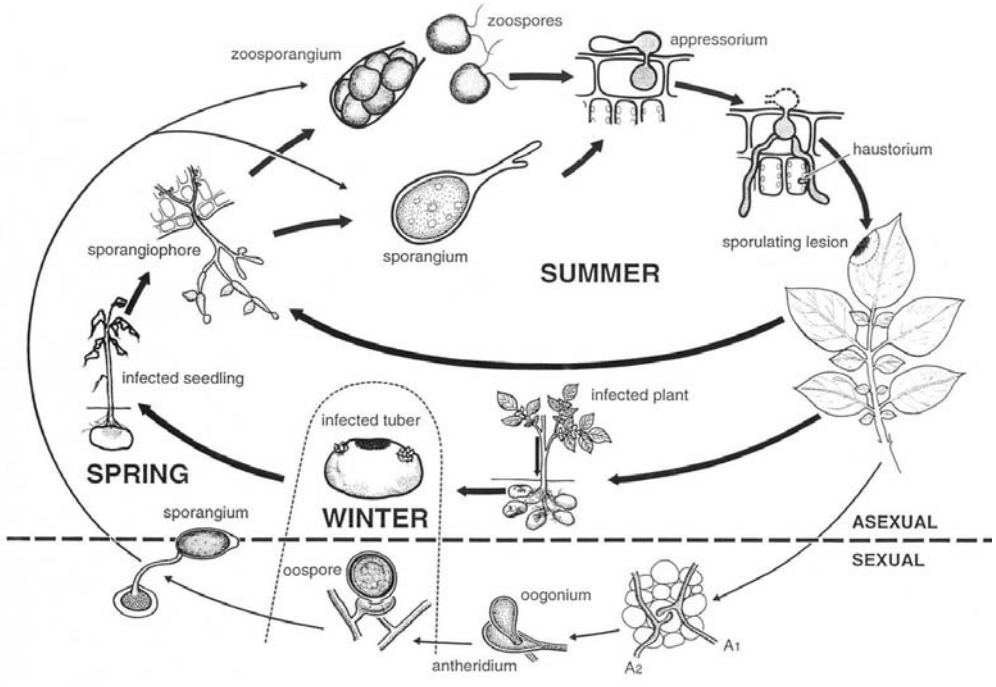

Figure 1. The disease cycle of *Phytophthora infestans*.

Taxonomy of *Phytophthora infestans*

P. infestans belongs to the oomycetes, a diverse group that includes both saprophytes and pathogens of plants, insects, fish, vertebrates, and microbes. Oomycetes are classified as Stramenopiles or Heterokonts which, together with the Alveolates, form the Chromalveolates, one of the five supergroups in the tree of eukaryotes (Figure 2) (Keeling et al., 2005). The supergroup Unikonts includes another major group of plant destroyers, i.e. pathogenic fungi. Although oomycetes and fungi are look-alikes with respect to growth morphology (mycelium) and propagation (via spores), their physiology, biochemistry, and genetics differ. Nevertheless, their weaponry to attack plants is quite similar (Judelson and Blanco, 2005; Latijnhouwers et al., 2003). The distinct evolutionary history of the two groups, however, implies that their pathogenic behavior evolved independently and that convergent evolution has shaped the genomes of these two major groups of plant pathogens.

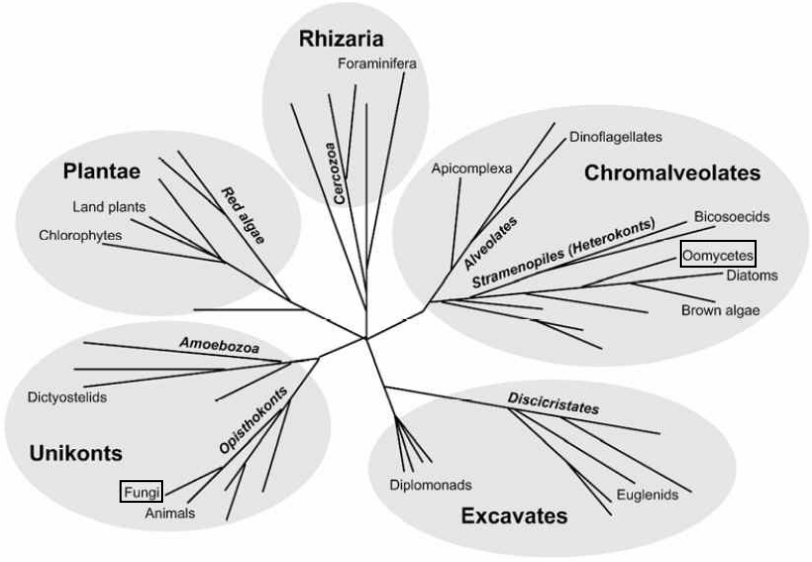

Figure 2. The five eukaryotic supergroups according to Keeling et al. (2005). Note the evolutionary distance between oomycetes and fungi (shown in boxes). Reproduced from Govers en Gijzen (2006).

The impact of potato late blight

Potato is the world's most widely grown tuber crop, and the fourth largest food crop, after wheat, maize, and rice. With a total production of more than 323 million tons in 2005, it contributes greatly to the world's food supply (http://www.fao.org/es/ess/top/commodity.html). Potato is the largest food crop in the Netherlands, occupying 20% of the arable lands. The Netherlands plays a major role in the world trade of potato, especially in seed potatoes. Nearly 80% of seed potato is in the hands of Dutch companies (Huang et al., 2005). China is at present the world's largest potato producer, reaching an annual production of 68 million tons with an average growth rate of 6.7% from between 1980-83 and 2000-03 (Qu et al., 2004).

The first reports of late blight epidemics date from the 1840s when the disease devastated the foliage and tubers of potatoes in nearly all of Europe and the northeastern United States, and set the stage for the disaster recorded in history as the Irish potato famine. Ireland, at that time dependent on potato as the chief staple, lost nearly half of its population; over a million Irish starved to death and many more emigrated (Govers and Latijnhouwers, 2004). Since its first appearance in mid nineteenth century *P. infestans* has spread globally and even today late blight is the greatest threat to potato production worldwide. Loss of yield and quality, and the costs of chemical control of potato late blight have been estimated to be around 3 billion US dollars annually worldwide (Duncan, 1999). In the Netherlands, the direct economical costs of chemical late blight control sum up to approximately 55 million US dollars per year (Davidse et al., 1989). China produces 20% of the total potato production in the world, and it was estimated that the loss of late blight in China is about 600 million US dollars annually (Huang et al., 2005). Despite continuous and tremendous efforts by farmers, breeders, scientists, and crop protection companies, problems with pathogens persist, and potato late blight continues to be one of the

clearest examples of the difficulties plant diseases impose on sustainable food production.

Variation in *P. infestans* populations

The evolutionary potential of a pathogen population is reflected in its population genetic structure which means the amount and distribution of genetic variation within and among populations. This genetic structure is a consequence of interactions among the five forces that affect the evolution of populations. The five forces refer to (i) mutation, (ii) population size and random genetic drift, (iii) gene and genotype flow, (iv) reproduction and mating system, and (v) selection (McDonald and Linde, 2002; Goodwin, 1997). *P. infestans* populations exhibit the highest risk of evolution owing to high mutation rates, large effective populations, a high gene/genotype flow, a mixed reproduction system and an efficient directional selection (McDonald and Linde, 2002). Therefore, understanding and unravelling the genetic structures of *P. infestans* populations is compulsory for predicting the effectiveness of new management practices that aim at controlling potato late blight in a sustainable manner.

A range of phenotypic and genotypic tests has been applied to study *P. infestans* population genetic structure. With the exception of the already diverse populations in the central highlands of Mexico's Toluca Valley (Goodwin et al., 1992), an overall trend of increasing diversity in *P. infestans* populations has been observed in the last two decades in many potato-growing regions of the world (Drenth et al. 1994; Day & Shattock, 1997; Zwankhuizen et al., 2000; Knapova & Gisi, 2002; Cooke et al., 2003; Day et al., 2004; Gotoh et al., 2005; Cooke et al., 2006). The most widely studied phenotypic traits are mating type (Gallegly & Galindo, 1957), virulence (Malcolmson & Black, 1966) and fungicide resistance (Dowley & O'Sullivan, 1981).

Whilst phenotypic traits are important for understanding the selection pressures on *P. infestans* populations, in isolation they do not fulfill many of the criteria of an ideal marker system (Cooke and Lees, 2004). Many different genotypic markers have been used to study *P. infestans* populations, such as analysis of isozymes (Shattock et al., 1986; Sujkowski et al., 1994), mtDNA, RG57 restriction fragment length polymorphism (RFLP) patterns (Goodwin et al., 1994; Gavino and Fry, 2002), amplified fragment length polymorphisms (AFLPs) (Cooke et al., 2003; Flier et al., 2003) and, more recently, simple sequence repeats (SSRs) (Knapova & Gisi, 2002; Lees et al., 2006). Of the methods discussed, SSRs appear to offer the greatest potential across a wide range of applications and the overall aim of the community is to develop SSRs as the universal standard for genotyping *P. infestans* populations worldwide (Cooke et al. abstract 15, The Third International Late Blight Conference, Beijing, China, April, 2008).

In the near future also SNPs (single nucleotide polymorphisms) and sequence analysis may become powerful tools to study *P. infestans* population diversity. The genome sequence of *P. infestans* (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html) is available, and with the rise of the next generation sequencing platforms such as Solexa/Illumina and GS-Flex/454 that make DNA sequencing faster and cheaper, the sequencing of sibling

species and field isolates is within reach. In addition, new developments such as RNAi (Whisson et al., 2005) and effector genomics (Vleeshouwers et al., 2008) aid in speeding up the functional characterization of *P. infestans* genes. This will allow the parallel tracking of neutral and functional markers and hence, will help to identify the forces driving pathogen evolution (Blair et al., 2008).

Oomycete effectors

Effectors are defined as molecules produced by pathogens that manipulate host cell structure and function, thereby facilitating infection (virulence factors or toxins) and/or triggering defense response (avirulence factors or elicitors). The dual function of effectors has been broadly reported in plant-microbial pathosystems, such as bacterial and fungal diseases (Kjemtrup et al., 2000; Lauge and de Wit, 1998). It is thought that oomycetes accomplish parasitic colonization by molecular reprogramming of the host defense circuity, specifically by introducing an array of effectors that functions in the plant apoplast and cytoplasm (Kamoun, 2006). Oomycetes secrete two classes of effectors that target distinct sites in the host plant: apoplastic effectors that are secreted into the plant extracellular space, where they interact with extracellular targets and surface receptors; and cytoplasmic effectors that are translocated inside the plant cell presumably through specialized structures like infection vesicles and haustoria that invaginate inside living host cells (Figure 3) (Kamoun, 2006).

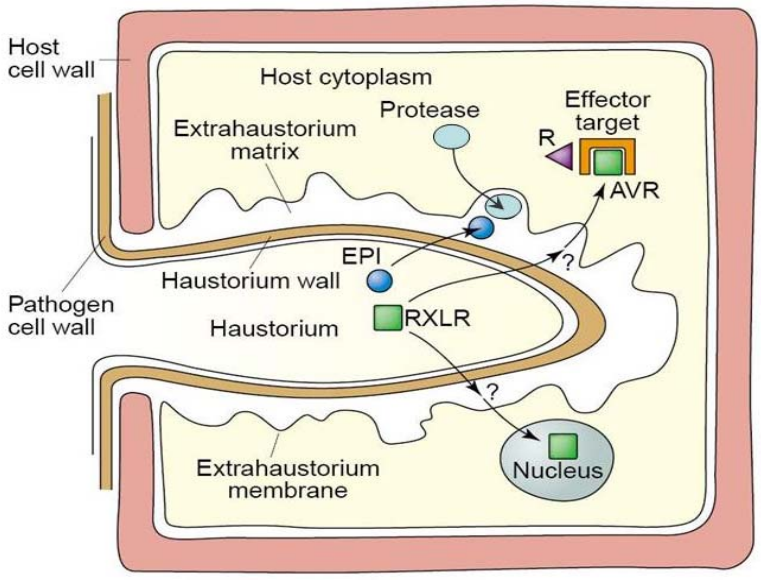

Figure 3. The interaction between a *Phytophthora infestans* haustorium and a potato cell. The potential sites of action of recently identified *P. infestans* effector proteins, including AVR proteins, are shown. Extracellular protease inhibitor (EPI) proteins target extracellular host-defense-associated proteases. RXLR proteins are secreted and potentially translocated to the inside of host cells as has been shown recently for Avr3a (Whisson et al. 2007). It is unknown which host proteins are targeted by the RXLR effectors (effector target) to manipulate host defenses and potentially enabling recognition by 'guarding' R proteins in the cytoplasm. Reproduced from Birch et al. (2006).

Effectors as avirulence factors

Race-specific resistance to *Phytophthora* spp. follows the gene-for-gene model, which implies that *Avr* genes from the pathogen are perceived directly or indirectly by matching resistance (*R*) genes from the plant (Staskawicz et al., 1995). To date, several race-specific *Avr* genes from oomycetes have been cloned (Table 1). The proteins they encode are secreted and share a conserved motif at the N-terminus, named RXLR (Rehmany et al., 2005). Similar to the function of the conserved PEXEL/VTS motif in *Plasmodium* species (Hiller et al., 2004; Marti et al., 2004), the RXLR motif in oomycete effectors plays a role in translocating the proteins into the host cytoplasm (Whisson et al. 2007).

Most of the oomycete *Avr* genes cloned so far result from positional cloning. Shan et al. (2004) used linkage analysis of polymorphic DNA markers and phenotype to identify a locus in *P. sojae* containing two genes, *Avr1b*-1 and *Avr1b*-2. *Avr1b*-1encodes a small secreted protein, and *Avr1b*-2 is a regulatory gene that is required for the accumulation of *Avr1b*-1 mRNA. In some isolates of *P. sojae* virulent on cultivars containing the cognate *Rps1b* resistance gene, *Avr1b*-1 had numerous substitution mutations indicating a strong diversifying selection. Also two *Avr* genes in the downy mildew pathogen ***Hyaloperonospora parasitica***, *ATR13* and $ATR1^{NdWsB}$, were cloned by positional cloning (Allen et al., 2004 and Rehmany et al., 2005). *ATR13* encodes a protein with extreme levels of amino acid polymorphisms, and as in *P. sojae Avr1b*-1 this is indicative of diversifying selection and potentially driven by, or driving, the polymorphism seen in the matching resistance protein RPP13.

Table 1. Overview of oomycete avirulence genes that encode secreted proteins, their cognate resistance genes and the strategies used for cloning

Avirulence gene	Species	*R* gene	Identification strategy	Reference
$ATR1^{NdWsB}$	*H. parasitica*	*RPP1*	Positional cloning	Rehmany et al., 2005
ATR13	*H. parasitica*	*RPP13-Nd*	Suppression subtractive hybridization combined with positional cloning	Allen et al., 2004
Avr1b-1	*P. sojae*	*Rps1b*	Positional cloning	Shan et al., 2004
Avr3a	*P. infestans*	*R3a*	Bioinformatic prediction of secreted and polymorphic proteins coupled with association genetics	Armstrong et al., 2005
ipiO (*Avr-blb1 / Avr-sto1*)	*P. infestans*	*Rpi-blb1* *Rpi-sto1*	Effector genomics screening	Vleeshouwers et al., 2008

To facilitate the positional cloning of *Avr* genes in *P. infestans* van der Lee at al. (1997; 2004) constructed a molecular genetic linkage map using AFLP markers and positioned six *Avr* genes and the mating type locus on the map (van der Lee et al., 2001). So far, one locus that carries three of the six *Avr* genes has been cloned. With the help of AFLP markers linked to this complex *Avr* locus and marker landing on a genomic Bacterial Artificial Chromosome (BAC)

library, a physical map of the *Avr3b-Avr10-Avr11* region was obtained (Whisson et al., 2001). The distance, however, between the marker and the putative *Avr* genes was still relatively large and the problem that arose was the inability to generate large segregating mapping populations. *P. infestans* is heterothallic and the germination efficiency of the oospores is relatively low. Also, the large genome size (240 Mb) reduces the marker density. To complement the positional cloning strategy a suitable transcriptional profiling strategy was adopted. cDNA-AFLP combined with bulked segregant analysis (BSA) resulted in the identification of a few *Avr*-associated transcripts (Guo et al., 2006; **Chapter 3**). In addition, an Affymetrix array (the Syngenta custom designed *Phytophthora* Genechip), that was developed based on a large unigene set of *P. infestans* (Randall et al., 2005; Judelson et al., 2008), was hybridized with RNA isolated from virulent and avirulent strains and this also resulted in a few new *Avr*-associated transcripts. Integration of the AFLP markers, the physical map, the *Avr*-associated cDNA-AFLP markers and the selected Affymetrix array clones resulted in the cloning of the *Avr3b-Avr10-Avr11* locus (Jiang et al., 2006). This locus contains a gene named *Pi3.4* which does not encode a secreted effector but a protein of 1956 amino acids with regulatory domains characteristic for transcription factors. Interestingly, the *Avr3b-Avr10-Avr11* locus shows copy number variation (CNV) with a high copy number in avirulent strains and a single copy in virulent strains. Moreover, the amplification only covers the 3' end of the *Pi3.4* gene. Because the amplified copies are all slightly different it was hypothesized that the amplification generates modular diversity at the *Avr3b-Avr10-Avr11* locus and this could be a novel mechanism for *P. infestans* to quickly adapt to its environment (Jiang et al., 2006). How this putative transcription factor influences the avirulence phenotype remains to be determined. One hypothesis put forward by Jiang et al. (2006) is that it regulates the coordinated expression of at least three genes encoding secreted effectors, namely the cognate effectors of the resistance genes *R3b, R10* and *R11*, respectively.

A different strategy to clone *Avr* genes from *P. infestans* was exploited by Amstrong et al. (2005). From an expressed sequence tag (EST) database (Kamoun et al., 1999) they selected ESTs encoding secreted proteins and analysed the polymorphism of the corresponding genes in a set of field isolates with known race structure. By searching for associations of the polymorphisms with the virulence/avirulence phenotype, a so called association genetics approach, they were able to identify *Avr3a,* a gene encoding a protein that triggers *R3a*-dependent cell death (Armstrong et al., 2005). Screening of a large set of field isolates revealed that the difference between an avirulent and a virulent phenotype on *R3a* plants is based on only three amino acid changes in the Avr3a protein, two of which are located in the region that has elicitor function (Bos et al., 2006). *Avr3a* resides in a region of the *P. infestans* genome that is colinear with the locus containing $ATR1^{NdWsB}$ in *H. parasitica*.

When the picture emerged that most of the oomycete *Avr* loci that were cloned by a positional cloning strategy or by association genetics carry genes that encode a secreted effector with a conserved RXLR motif, Vleeshouwers et al. (2008) designed an elegant approach to find

novel *Avr-R* combinations. This approach, dubbed as effector genomics, is based on a transient *in planta* expression method that makes use of binary PVX vectors. Putative effector genes are transiently expressed in plants and subsequently the responses to these effectors are monitored. Torto et al. (2003) developed this system for screening the necrosis inducing activity of a set of secreted *P. infestans* proteins on tobacco and discovered the crinckler and necrosis inducing genes (*crn* genes). The set of putative effector genes that Vleeshouwers et al. (2008) used consisted entirely of RXLR genes. It was mainly composed of *P. infestans* RXLR genes that were mined from ESTs but also included RXLR genes that were previously cloned. One of the latter was *ipiO,* a putative pathogenicity gene identified by Pieterse et al. (1993) in a screening aimed at finding *in planta* induced *P. infestans* genes. Apart from a cell attachment motif RGD, the IPI-O protein had no recognizable features but with the discovery of the RXLR motif IPI-O could be classified as a member of the RXLR effector family. The effector genomics screening on *Solanum* accessions with resistance to late blight revealed that the cognate *R* genes of *ipiO* are *Rpi-blb1* and *Rpi-sto1,* two nearly identical *R* genes in two different *Solanum* species, *S. bulbocasteneum* and *S. stoloniferum,* respectively. In IPI-O the RXLR overlaps with the RGD motif and Gouget et al. (2006) found that IPI-O via its RGD motif can bind to an *Arabidopsis thaliana* lectin receptor kinase LecRK79. LecRK79 may possibly function as an effector target that mediates uptake of RXLR-dEER effectors into the host cell (Bouwmeester et al., personal communication).

Taken together, five of the six oomycete avirulence loci that have been cloned so far encode secreted proteins sharing RXLR motif. Number six is the *Avr3b-Avr10-Avr11* locus that could be involved in regulation of expression of RXLR effector genes. The research described in this thesis and unpublished data from other laboratories and presented at recent conferences, reveals that there are several more oomycete *Avr* genes encoding RXLR effectors and the RXLR motif is now considered as a hallmark of oomycete avirulence genes that have 'gene-for-gene' interactions wth *R* genes.

The RXLR effector superfamily

The discovery of a conserved RXLR motif in oomycete avirulence factors was a stimulus to search for more of this type of proteins by genome mining. Currently, the genome sequence of three *Phytophthora* species is available, *P. ramorum, P. sojae,* and *P. infestans*, and for a fourth species, *P. capsici,* sequencing and assembly is in progress (Tyler et al., 2006; Govers and Gijzen., 2006). Genome mining revelaed a total of 374, 396 and 563 RXLR effectors in *P. ramorum*, *P. sojae* and *P. infestans*, respectively (Jiang et al., 2008; Jiang R.H.Y., personal communication). One of the most apparent characteristics of these effectors is the combination of a rather conserved N-terminus, that carries a signal peptide and the two motifs RXLR and dEER, combined with a highly divergent C-terminus where the actual elicitor domains are located. Remarkably, most RXLR effector genes belong to one family. Despite the extensive sequence divergence they are all related and likely evolved from a common ancestor by rapid duplication

and divergence (Jiang et al., 2008). This RXLR superfamily, that already comprises over 700 members from just two *Phytophthora* species, is one of the most rapid evolving parts of genome. A thorough bioinformatic analysis of the C-terminal regions with MEME searches and HMM screening revealed the existence of three conserved motifs in many of the RXLR effectors that often occur in a repeated fashion (Jiang et al., 2008). They are named W, Y and L motifs after the most conserved amino acid at a certain position. The W-motif, for example, is 25 amino acids long and always has a tryptophan residue at position 5. It occurs in 60% of all superfamily members. In 95% of the cases the W-motif is flanked by a Y- and/or L-motif the length of which is 22 and 33 amino acids, respectively. Many RXLR effectors contain multiple W, Y and L-motifs in a repeated fashion and in part this may contribute to the rapid expansion of the superfamily.

The highly dynamic behaviour of the RXLR superfamily is also supported by the observation that nearly all RXLR genes are located at synteny breakpoints. The conserved synteny that exists between large genomic regions in *P. ramorum* and in *P. sojae* is interrupted at sites where one of the two species harbours an RXLR gene (Jiang et al., 2006; 2008). Maybe diversifying selection exerted by plant hosts contributes to these dynamic features of the RXLR superfamily. It is speculated that many of the RXLR effectors may play an important role in interacting with plant host, especially in virulence, host specificity and pathogenicity.

Resistance proteins recognizing RXLR effectors in *P. infestans*

P. infestans exhibits a gene-for-gene interaction with potato. This implies that avirulence gene products from the pathogen are recognized by the host plant expressing the cognate resistance gene. It is well known that among wild *Solanum* species late blight resistance is a common phenomenon. To date, twenty *R* genes, conferring foliage resistance against potato late blight, have been positioned on a molecular genetic linkage map (Simko et al., 2007). These include *R1*, *R2*, *R3a*, *R3b*, *R5, R6, R7, R8, R9, R10 and R11* from *S. demissum*, *RB*/*Rpi-blb1*, *Rpi-blb2*, *Rpi-blb3 and Rpi-abpt* from *S. bulbocastanum*, R_{ber}/$R_{pi\text{-}be}$ from *S. berthaultii*, *Rpi1* from *S. pinnatisectum*, and *Rpi-moc1* from *S. mochiquense*. Up to now, five late blight *R* genes have been cloned and they all encode CC-NBS-LRR proteins. These five are *R1* (Ballvora et al., 2002), *R3a* (Huang et al., 2005), *RB/Rpi-blb1* (Song et al., 2003; van der Vossen et al., 2003), *Rpi-blb2* (van der Vossen et al., 2005) and *Rpi-sto1* (Vleeshouwers et al., 2008). CC-NBS-LRR proteins reside in the cytoplasm and this is consistent with the idea that RXLR effectors are targeted into the host cell. For two of the five cloned late blight *R* genes the corresponding RXLR effector is known. The pair Avr3a-R3a is the most intensively studied combination (Amstrong et al., 2005; Huang et al. 2005; Bos et al., 2006; Whisson et al. 2007). Also for the combination *Rpi-blb1/Rpi-sto1*, on the one hand, and *ipiO* (*Avr-blb1/Avr-sto1*), on the other hand, there is now solid evidence that they have a gene-for-gene interaction (Vleeshouwers et al., 2008; Bouwmeester K. & Govers F., unpublished). With the finding that *RB/Rpi-blb1*-based resistance is triggered by an effector that belongs to a highly dynamic superfamily, the initial idea that

RB/Rpi-blb1 confers broad spectrum resistance and can block infection by a broad range of isolates should be revisited.

Scope of this thesis

The primary aim of this thesis was to clone and identify race-specific avirulence genes in *P. infestans.* In 2002 when this research project started, there were no publications describing the cloning of oomycete *Avr* genes and the RXLR motif was not yet discovered. There were also no *P. infestans* Avr proteins identified and since previous attempts to purify *P. infestans* Avr proteins were unsuccesful (Alfonso and Govers, 1995), the option to use a reverse genetics approach was not even considered. By taking advantage of existing high density molecular genetic linkage maps of regions carrying *Avr* genes (van der Lee et al. 2001) we adopted a positional cloning strategy. In first instance more *Avr*-associated markers were generated by transcriptional profiling using cDNA-AFLP markers and this led to the identification of transcripts associated with *Avr4* and *Avr3b-Avr10-Avr11* (**Chapter 3**). In addition, SNP markers and more AFLP markers were generated to enrich the marker density on the map and this resulted in AFLP markers more closely linked to *Avr1* than the ones previously identified by van der Lee et al. (2001) (**Chapter 2**). In a later stage, when the hallmarks of oomycete *Avr* genes were uncovered and the genome sequence of *P. infestans* was released, bioinformatic analysis of a selected genomic region was performed and this was instrumental in the search for candidates for race-specific *Avr* genes, in particular for *Avr1* (**Chapter 5**).

Screening of a *P. infestans* BAC library with two *Avr4*-associated transcripts and a set of previously identified *Avr4*-linked AFLP markers (van der Lee et al., 2001) resulted in the cloning of a candidate for *Avr4* (**Chapter 4**). The encoded protein appeared to encode an RXLR effector. To provide solid evidence that we cloned the *Avr4* gene, we transformed race 4 strains with *Avr4* and performed *in planta* expression assays of *Avr4* in *R4* plants. We also analysed the polymorphism of *Avr4* in a variety of field isolates.

To identify candidates for the *P. infestans Avr1* gene we combined a variety of *Avr1*-associated markers for landing on the *P. infestans* genome sequence (**Chapter 5**). In this way we narrowed down a genomic region of 800 kb that most likely carries *Avr1.* All RXLR genes located in this region were extensively analysed using bioinformatics and these candidates can now be tested in functional assays.

To investigate how *Avr* genes and the dynamic RXLR effectors behave in natural populations one has to analyse a variety of field isolates into more depth. To this end we studied the phenotypic and genotypic diversity of *P. infestans* isolates collected in Northern China between 1997 and 2003, especially in Inner Mongolia (**Chapter 6**). The virulence phenotypes in the set of 43 isolates were highly diverse and in sharp contrast with the uniform genotype that was shared by all isolates. It is likely that these isolates have a clonal origin but, nevertheless, are polymorphisms in their *Avr* genes.

The last chapter (**Chapter 7**) highlights the implications of the findings described in the previous chapters with emphasis on *Avr* genes, RXLR-dEER effectors, virulence diversity and durable resistance to *P. infestans*.

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Chapter 2

A linkage map of the oomycete pathogen *Phytophthora infestans* based on AFLP and SNP markers

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A linkage map of the oomycete pathogen *Phytophthora infestans* based on AFLP and SNP markers

Abstract

We generated a molecular genetic linkage map of *Phytophthora infestans* based on two different types of polymorphic DNA markers, Single Nucleotide Polymorphism (SNP) and Amplified Fragment Length Polymorphism (AFLP). In a mapping population of 83 F_1 progeny derived from two Dutch field isolates 398 SNP markers and 233 AFLP markers segregated. Of these 631 markers, 534 markers were positioned on 14 major linkage groups and 5 minor linkage groups spanning a total of 1144 cM and an average distance of 2.14 cM between adjacent markers. The map will be a resource for investigating *P. infestans* genome properties, mutation mapping and positional cloning of candidate genes.

Introduction

Genetic linkage maps are a prerequisite for studying the inheritance of qualitative and quantitative traits and are instrumental for map-based cloning (Morgante and Salamini, 2003). For map-based cloning the gene of interest is positioned on the genetic linkage map and polymorphic DNA markers located in the vicinity of the gene are used as starting point for chromosome walking. This strategy, also referred to as positional cloning, can be used for cloning genes showing a phenotype that is determined by one genetic locus and segregates in a Mendelian fashion in a population.

The subject of our studies is *Phytophthora infestans,* an oomycete plant pathogen that causes late blight and is the number one problem in potato production worldwide. In recent years map-based cloning strategies have been exploited to isolate *P. infestans* genes that play a role in the interaction with the host plant, in particular avirulence (*Avr*) genes that interact in a gene-for-gene manner with resistance (*R*) genes in potato (Jiang et al., 2006; **Chapter 4**; van Poppel et al., 2008). The first molecular genetic linkage map of *P. infestans*, was published in 1997 (van der Lee et al., 1997). The mapping population that was used consisted of F_1 progeny obtained by crossing two Dutch *P. infestans* field isolates, the A1 mating type strain NL80029 and the A2 strain NL88133 (designated as cross 71; Drenth et al., 1995). The map was largely composed of Amplified Fragment Length Polymorphism (AFLP) markers, 183 in total. Linkage analysis of these 183 markers together with seven Restriction Fragment Length Polymorphism (RFLP) markers and the mating type locus resulted in 10 major and 7 minor linkage groups (LG) and covered a total of 827 cM. A major LG is defined as a linkage group composed of markers derived from both parents, whereas a minor LG contains markers from only one parent. Non-Mendelian segregation ratios were found for the mating type locus and for 13 AFLP markers, all of which were located on the same LG as the mating type locus, i.e. LG III (van der Lee et al., 1997). A follow-up study (van der Lee at al., 2004) that included the analysis of over 595 segregating markers in cross 71 and 425 in a second cross (cross 68) revealed a chromosomal translocation spanning the mating type locus on LG III in one of the two parents of cross 71. In *P. infestans* aberrant segregation ratios are not restricted to markers linked to the mating type locus; van der Lee et al. (2004) observed deviations from Mendelian segregation ratios for several markers in cross 71 as well as 68, and Carter et al. (1999) who analysed segregation of RFLP markers and virulence/avirulence phenotypes in yet another F_1 progeny of *P. infestans*, found the same. Trisomic and triploid offspring appeared to be the main cause and this severely hampered linkage analysis in this organism. Currently the most comprehensive linkage map of *P. infestans* is an AFLP map of cross 71 that consists of 13 major LGs and 10 minor LGs with major LGs ranging in length from 11 to 101 cM (van der Lee et al., 2004).

So far, six *P. infestans Avr* genes were positioned on the genetic linkage map of cross 71 (van der Lee et al., 2001; 2004). *Avr1* was located at the very end of LG IV with the closest AFLP marker at a distance of 8 cM, and *Avr2* mapped on LG V with several AFLP markers in

close vicinity. The minor LG A2-a carried *Avr4* and LG VII contained a cluster of three *Avr* genes, *Avr3b, Avr10* and *Avr11. Avr4* as well as a gene located at the *Avr3b-Avr10-Avr11* locus were cloned by exploiting linked AFLP markers and the map based cloning strategy was thus successful (**Chapter 4**; van Poppel et al., 2008; Whisson et al., 2001; Jiang et al., 2006).

The existing map of cross 71 is largely composed of AFLP markers that were generated using *Eco*R1 and *Mse*I as restriction enzymes and all possible primer combinations with two base pairs extensions (i.e. 256). Since van der Lee et al. (2001; 2004) analysed all *Eco*RI/*Mse*I AFLP markers that segregated in cross 71 we had to switch to other markers to saturate the map further. Firstly, we aimed at generating more AFLP markers by selecting another combination of restriction enzymes. The selection was based on *in silico* restriction analyses of a set of 1000 randomly selected *P. infestans* ESTs performed by Dong et al. (2004) and aimed at predicting the best combination of restriction enzymes to be used for cDNA-AFLP. Of five combinations that were analysed, we chose *Pst*I/*Hha*I as a combination that was more likely to generate markers associated with coding regions than *Eco*RI/*Mse*I. Secondly, we analysed the *P. infestans* genome sequence for Single Nucleotide Polymorphisms (SNPs) that could be used a markers. The last decade SNP-based genetic markers have attracted more and more attention for creating dense genetic linkage maps. A SNP is the most basic unit of genetic variation and represents the most common class of DNA-based markers that could be useful in identifying candidate genes of interest (Cho et al., 1999; Rafalski, 2002).

The present study was undertaken with the objective to generate more AFLP markers for enriching the *P. infestans* genetic map and to integrate SNP markers in the map.

Materials and methods

P. infestans mapping population

The *P. infestans* mapping population consisted of F_1 progeny from cross 71. The two parents are NL80029 (A1 mating type) and NL88133 (A2 mating type), field isolates collected in the Netherlands in 1980 and 1988, respectively. The cross 71 F_1 progeny was previously described and characterized by Drenth et al. (1995) and van der Lee et al. (1997) and consists of over 150 individuals. About half of these were derived from oospores generated *in vitro* on V-8 agar and the other half from oospores generated in infected leaves (*in vivo*). In this study the two parental strains and 83 F_1 progeny were used to construct the map. Forty out the 83 were in common with the F_1 progeny that was used previously by van der Lee et al. (2004) to generate the *Eco*RI/*Mse*I AFLP map. Genomic DNA isolation was performed as described previously (van der Lee et al., 1997). From the same DNA sample one aliquot was used to prepare the primary template for AFLP analyses and a second aliquot was sent to the Broad Institute of Harvard and MIT, Cambridge, MA, USA where it entered a SNP analysis pipeline.

AFLP analysis

AFLP analysis was performed according to the protocol described by van der Lee et al. (1997) with some minor modifications. In stead of radioactive labeled primers, fluorescent primers were used and the amplified fragments were separated on a Licor sequencer (LI-COR, Lincoln Nebraska). Twenty seven *Pst*I/*Hha*I primer combinations (PCs) with two selective bases were used that were selected on the basis of the total number of bands and the level of polymorphism observed when comparing the two parents and in four samples consisting of DNA pools from F_1 progeny. These pools were used in a Bulked Segregant Analysis aimed at identifying markers associated with the *Avr1* gene (**Chapter 5**). All AFLP markers were visually scored by the same person. Three types of markers were distinguished; A and B markers that were heterozygous in the A1 parent and the A2 parent, respectively, and H markers heterozygous in both. Markers were coded according to the existing nomenclature (van der Lee et al., 1997), i.e. the type of marker (A, B or H) followed by the primer (H and P) associated with a number representing the selective bases, and the estimated size of the DNA fragment in nucleotides with the prefix 's'. The numbers 11~26 represent AA, AC, AG, AT, CA, CC, CG, CT, GA, GC, GG, GT, TA, TC, TG and TT, respectively. A 10-bp DNA ladder was used to estimate the size of the AFLP fragments.

Selection of SNP markers

To select SNPs in the assembled genome sequence of *P. infestans*, an electronic SNP (eSNP) discovery approach was adopted. The *P. infestans* genome sequence is derived from isolate T30-4 (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans), an F_1 progeny of cross 71. Initially, all regions containing a 48-mer which occurred elsewhere in the genome were masked. Regions where at least two high quality reads produced each of two calls at a position were considered to be SNPs. However, regions which contained evidence of possible misassembly were considered suspect and SNPs in those regions were dropped. Also SNPs which had too many other SNPs in the local region were considered likely to be overcollapsed duplications and were discarded. SNP markers were designated by the type of marker (A, B or H) followed by contig number and base position.

SNP analysis

SNP genotyping of the parental strains and F_1 progeny of cross 71 was performed at the Broad Institute of Harvard and MIT, Cambridge, MA, USA and the data were kindly provided by Michael C. Zody (Zody et al., unpublished data). SNPs were genotyped by Sequenom iPlex (for details see www.sequenom.com) on a 96-well plate and in pools of up to 24 SNPs. Each parental strain of cross 71 was spotted twice on the plate to give extra opportunities to collect a parental genotype. The T30-4 DNA used for the sequencing libraries was also spotted once as a positive control. The remaining 91 samples were offspring from cross 71.

Mating type determination

The mating type of the F_1 progeny was determined by checking for the presence of oospores when co-cultured with tester strains of known mating type (**Chapter 6**; van der Lee et al., 1997).

Linkage analysis and map construction

Linkage analysis was performed using JoinMap version 3.0 software (van Ooijen and Voorrips, 2001). Markers were tested against the expected segregation ratio using a χ^2 goodness-of-fit and the *P*-value was recorded. Distorted markers were used for linkage analysis unless they affected the order of neighboring markers. To guarantee the reliability of mapping, we adopted the quality criteria that van der Lee et al. (2004) used to process the mapping data. Initially two separate parental maps were constructed. The A map was composed from a data set containing A and H markers and the B map from a data set containing B and H markers. A and B maps were used as a fixed framework for the integrated map containing A, B and H markers. Linkage groups were determined using a minimum LOD threshold of 3.0 and maps were constructed using the Kosambi mapping function with the following JoinMap parameter settings: Rec = 0.4, LOD = 1.0, Jump = 5. A third round of ordering whereby problematic markers were forced onto a map was not employed; these markers were discarded. Maps were drawn using MapChart software (Voorrips, 2001).

Results

In the cross 71 mapping population a total 631 markers were scored, of which 233 were AFLP markers and 398 SNP markers. All three marker types, A, B and H, were found: 253 A markers, 248 B markers and 130 H markers (Table 1). Analysis of the genotype frequencies of the 233 AFLP markers revealed that 38 of these (16%) showed a significant segregation distortion ($P < 0.01$, χ^2 test), 58% of which were H markers. Of the 631 markers, 13 were scored on less than 45 progeny and these were discarded from linkage analysis. Thus, linkage analysis was performed with 618 markers on 83 progeny. Twenty-nine markers remained unlinked to at least two other markers. From the remaining 589 markers we generated a genetic linkage map that comprised 19 LGs, including 14 major LGs and 5 minor LGs, and covered 1144 cM (Figure 1; Table 2). The major LGs were numbered 1 to 14. The minor LGs consisting of A markers A1-1 to A1-3 and those consisting of B markers A2-1 and A2-2. Of the 589 markers, 534 (91%) could be positioned on a LG. The 55 that could not be placed either affected the order of their neighboring markers or caused an excessive increase in the size of the LG. Of the 55, 49 with a high χ^2 jump (>5) are listed at the bottom of the LG to which they belong based on linkage analysis. The remaining 6 were rejected based on quality criteria. The mating type locus AMAT was placed manually on LG5 at a position based on the direct distance to the closest markers.

Table 1. Numbers and types of markers segregating in F_1 progeny of cross 71.

Marker type	Heterozygous state present in			
	NL80029 ab x aa[1]	NL88133 aa x ab[1]	NL80029 and NL88133 ab x ab[2]	
	A marker	B marker	H marker	Total
AFLP	86	93	54	233
SNP	167	155	76	398
Total	253	248	130	631

[1] Expected egregation ratio 1:1 (ab:aa); [2] Expected segregation ratio 1:2:1 (aa:ab:bb)

The LGs ranged in size from 7 (A1-2) to 94 (LG5) cM. The average distance between markers was 2.14 cM and no gap larger than 20 cM was found. Of the five minor LGs three (A1-2, A1-3 and A2-1) are relatively small in size from 7 to 37 cM. Linkage groups A1-1, A1-2 and A2-1showed strong distorted segregation ratio as indicated in Table 2. In contrast to minor LGs, the major LGs have a high marker density with an average interval of 1.98 cM. However, some LGs, such as LG4, or regions, such as the top of LG5 or the bottom of LG9, are low in the marker density. Almost all major LGs except LG4 and LG8 contain clusters of SNP markers originating from either one parent or both parents. Especially LG1, LG5, LG9 and LG10 have clusters with high marker density. Some markers which were positioned on eight major linkage groups (LG1, LG2, LG4, LG5, LG7, LG9, LG11 and LG12) displayed strong aberrations in the segregation ratio (Table 2).

Discussion

In the present study, we constructed a molecular-genetic linkage map of *P. infestans* using a new set of markers, SNP markers that were selected from the *P. infestans* genome sequences and AFLP markers generated with a new combination of restriction enzymes, i.e. *Pst*I and *Hha*I. The map contains 534 markers and spans 1144 cM divided over 14 major LGs and 5 minor LGs with an average distance of 2.14 cM between adjacent markers. Our initial aim was to integrate the new markers in the existing high density map that is largely based on *Eco*RI/*Mse*I AFLP markers (van der Lee et al., 2004). Unfortunately, this attempt failed. Only 40 of the 83 F_1 progeny that were genotyped in this study were present in the dataset that van der Lee et al. (2004) used and therefore the quality criteria for constructing an integrated map were not met. To distinguish the map obtained in the present study from the other maps we used Arabic numbers for the LGs as opposed to the Roman numbers used by van der Lee et al. (1997; 2001; 2004).

In the present map we positioned the mating type locus (AMAT) on LG5. The AMAT showed significant deviation ($P<0.005$) from the expected segregation ratio and could not be grouped according to the quality criteria. Therefore the mapping was done manually. The linkage

to other markers is not very strong and the distance to the closest marker is 5 cM on both sides. Since van der Lee et al. (1997; 2004) positioned the mating type locus on LG III it is likely that LG5 is actually LG III or that LG III and LG5 partially overlap. The difficulties encountered by van der Lee et al. (2004) in integrating the A and the B map for LG III were due to the fact that on this LG six H markers that were strongly linked to B markers were not linked to any of the A markers on LG III. Therefore they presented two maps for LG III, a and b, and suggested the occurrence of a chromosomal translocation at the mating type locus. In the present study six out of 18 A markers on LG5 showed abberant segregation ratios, four of which were positioned on the most distal part of LG5. Although our dataset supported integration of the A and B map it is obvious that the distorted segregation ratio for the mating type locus in this study and previous studies (van der Lee et al., 1997; 2001) warrant caution. Hopefully, the attempts by others (Randall et al., 2003) to clone of the mating type locus will soon be successful. This will reveal the differences at the DNA level and gene level between an A1 and an A2 strain and may explain the abberrant segregation ratios. Also the mechanisms underlying homothallism and hetereothallism in the genus *Phytophthora* can then be addressed as well as the synthesis or perception of the mating hormones. Recent advances in that direction are the purification and identification of natural mating hormone α1 from *Phytophthora nicotianae* (Qi et al., 2005) and the subsequent synthesis of artificial mating hormone α1. The synthetic hormone is biologically active and can induce oospore formation in different *Phythophthora* species including *P. infestans* (Harutyunyan et al., 2008).

In this study, we found two AFLP markers (AP23H19s130 and APH22 H23s161) co-segregating with the *Avr1* locus (**Chapter 5**). Previously, van de Lee et al. (2001) positioned *Avr1* at the most distal part of LG IV. In line with this, the two *Avr1*-linked markers found in this study also map on the most distal part of a linkage group, in this case LG12. Hence, LG12 is overlapping with LG IV. By combining the *Avr1*-linked AFLP markers with other *Avr1* -associated markers for landing on the genome sequence we could identify one supercontig that should carry *Avr1*. All oomycete *Avr* genes identified to date belong to the RXLR-dEER superfamily (Govers and Gijzen, 2006). The 800 Kb region that is delineated by the markers contains a limited number of RXLR-dEER genes and hence, these are the most likely candidates for *Avr1* (**Chapter 5**).

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Table 2. Linkage groups of *Phytophthora infestans* and statistics on the map construction.

Linkage group	Number of markers				LOD group[1]	Positioned[2] A+B+H		Rejected[3]				Segregation ratio[4]		Length	Average spacing	Largest interval
	Total	A	B	H	A+B+H	1	2	Quality	A+B+H	A	B	A	B	(cM)	(cM)	(cM)
LG1	56	9	30	17	6.5	49	51	0	5	0	1		*******	69	1.35	7
LG2	38	18	12	8	6.5	23	28	1	9	7	1	******		54	1.93	13
LG3	28	4	17	7	8.0	24	26	0	2	0	1			40	1.54	7
LG4	34	19	11	4	6.0	27	28	0	6	6	0	****		86	3.07	18
LG5	53	19	25	9	5.5	46	51	0	2	1	1	*****		94	1.84	10
LG6	28	9	16	3	5.0	24	24	0	4	3	1			52	2.17	11
LG7	31	10	9	12	4.5	26	27	0	4	2	1	*******		62	2.30	13
LG8	20	6	9	5	4.5	17	18	0	2	2	0			71	3.94	13
LG9	53	41	4	8	4.5	48	48	0	5	2	2		****	89	1.85	17
LG10	44	8	9	27	4.5	41	44	0	0	0	0			86	1.95	12
LG11	40	9	27	4	4.0	39	39	0	1	1	0		*******	74	1.90	10
LG12	39	28	8	3	4.0	30	35	4	0	0	0	****		48	1.37	9
LG13	32	17	11	4	6.5	28	31	0	1	0	1			63	2.03	8
LG14	35	14	16	5	3.5	31	31	0	4	0	4			62	2.00	11
A1-1	20	18	0	2	7.5	17	17	1	2	2	0	*****	n.a.	71	4.18	10
A1-2	4	4	0	0	7.5	4	4	0	0	0	0	****	n.a.	7	1.75	5
A1-3	4	3	0	1	7.0	4	4	0	0	0	0			11	2.75	5
A2-1	11	0	8	3	7.5	10	10	0	1	0	1	n.a.	*******	37	3.70	11
A2-2	19	0	19	0	4.0	18	18	0	1	0	1	n.a.		68	3.78	10
Total	**589**	**242**	**225**	**122**	**n.a.**	**506**	**534**	**6**	**49**	**26**	**15**	**n.a.**	**n.a.**	**1144**	**2.14**	**n.a.**

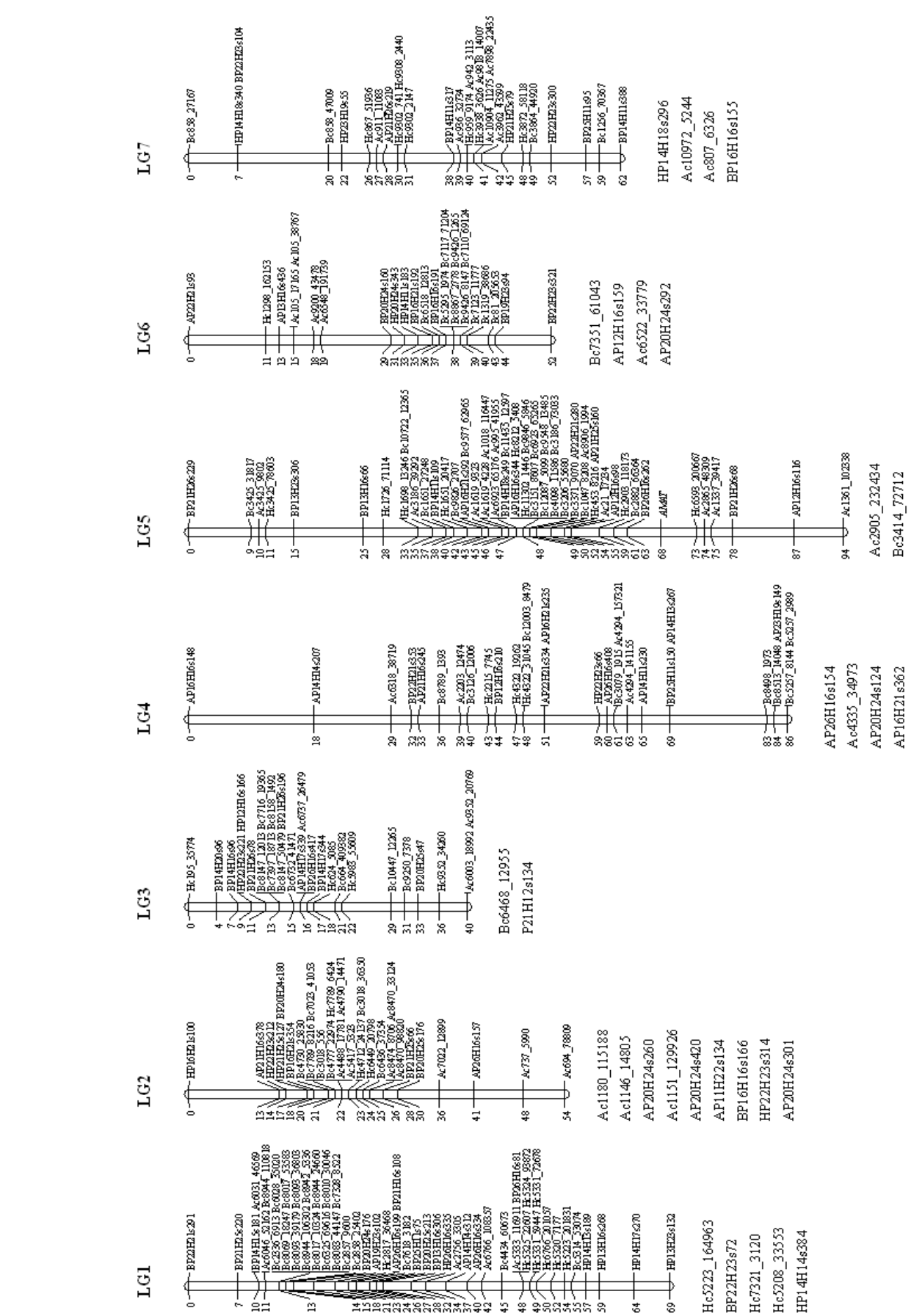
[1] LOD value that groups markers A, B and H in the LG of the integrated map

[2] Number of markers positioned in the first (1) and second (2) round of JoinMap 3.0

[3] Number of rejected markers using a restriction of a χ^2 jump of 5 or that affected the order of neighbors in the group

[4] Maxium deviation from the expected segregation ratio.

*** $P < 0.01$, **** $P < 0.005$, ***** $P < 0.001$, ****** $P < 0.0005$, ******* $P < 0.0001$. n.a. not applicable.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LG2
LG3
LG4
LG5
LG6
LG7
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BP22H23s72
Hc7321_3120
Hc5208_33553
HP14H14s384
Ac1151_129926
AP20H24s420
AP11H22s134
BP16H16s166
HP22H23s314
AP20H24s301
Ac1180_115188
Ac1146_14805
AP20H24s260
Bc6468_12955
P21H12s134
AP26H16s154
Ac4335_34973
AP20H24s124
AP16H21s362
AP26H16s276
AP21H16s56
Ac2905_232434
Bc3414_72712
Bc7351_61043
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Ac6522_33779
AP20H24s292
HP14H18s296
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Ac8D7_6326
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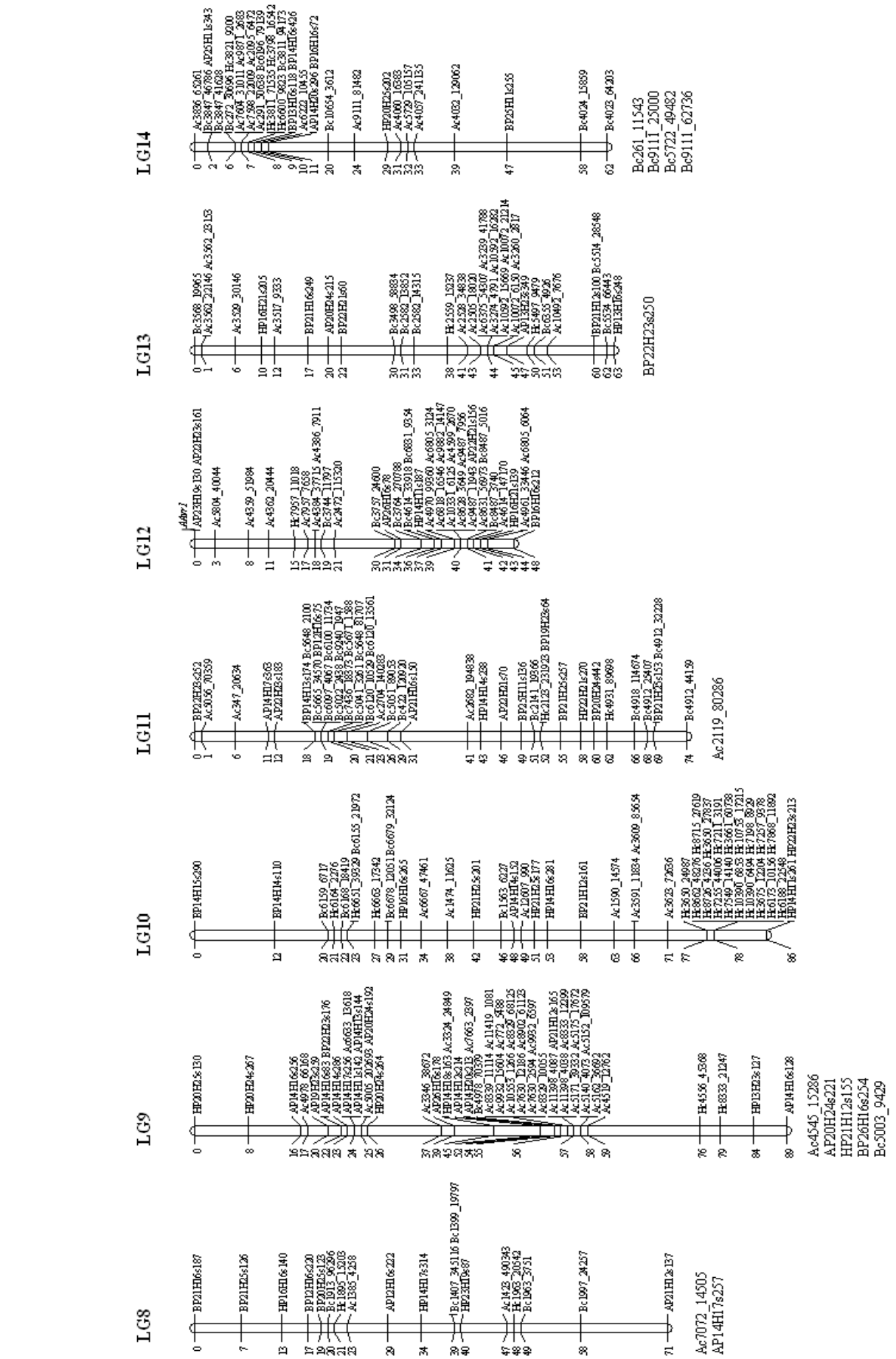
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LG9
LG10
LG11
LG12
LG13
LG14
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AP20H24s221
HP21H12s155
BP26H16s254
Bc5003_9429
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Bc261_11543
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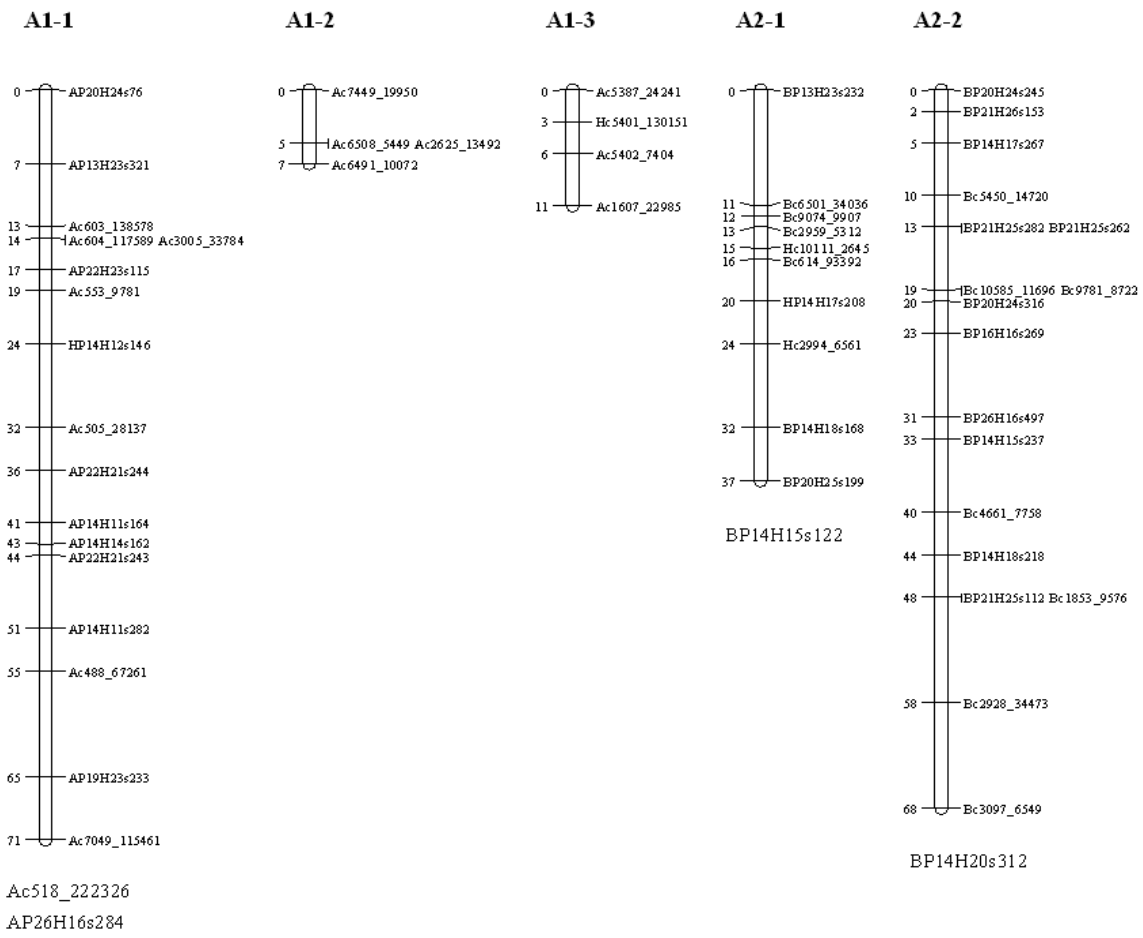

Figure 1. *Phytophthora infestans* linkage map based on 534 AFLP and SNP markers that segregated in 83 progeny of cross 71. The map is composed of 14 major LGs with A, B and H markers and five minor LGs with either A markers (A1-1 to A1-3) or B markers (A2-1 to A2-2), with or without H markers. Markers are indicated on the right and cumulative distances on the left in centimorgans. The mating type locus on LG5 and *Avr1* locus on LG12 were positioned manually. Markers that were rejected by a χ^2 jump>5 are listed below each linkage group.

Chapter 3

A cDNA-AFLP based strategy to identify transcripts associated with avirulence in *Phytophthora infestans*

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A cDNA-AFLP based strategy to identify transcripts associated with avirulence in *Phytophthora infestans*

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Abstract

Expression profiling using cDNA-AFLP is commonly used to display the transcriptome of a specific tissue or developmental stage. Here cDNA-AFLP was used to identify transcripts in a segregating F_1 population of *Phytophthora infestans,* the oomycete pathogen that causes late blight. To find transcripts derived from putative avirulence (*Avr)* genes germinated cyst cDNA from F_1 progeny with defined avirulence phenotypes was pooled and used in a Bulked Segregant Analysis (BSA). Over 30.000 transcript derived fragments (TDFs) were screened resulting in 99 *Avr*-associated TDFs as well as TDFs with opposite pattern. With 142 TDF sequences homology searches and database mining was carried out. cDNA-AFLP analysis on individual F_1 progeny revealed 100% co-segregation of four TDFs with particular AVR phenotypes and this was confirmed by RT-PCR. Two match the same *P. infestans* EST with unknown sequence and this is a likely candidate for *Avr4.* The other two are associated with the *Avr3b-Avr10-Avr11* locus. This combined cDNA-AFLP/BSA strategy is an efficient approach to identify *Avr*-associated transcriptome markers that can complement positional cloning.

Introduction

Many plant-pathogen interactions are governed by specific interactions between pathogen avirulence (*Avr*) genes and corresponding plant resistance (*R*) genes. An interaction where a corresponding pair of *R* gene and *Avr* gene is present and expressed, results in incompatibility and the plant is resistant. When one of the two is inactive or absent, the interaction is compatible and the plant susceptible. This cross talk between host and pathogen was assembled in the gene-for-gene model by Flor (1942), who extracted the concept from his work on the interactions between flax and flax rust. Since the early nineties numerous *R* genes from model plant or crop species have been identified and cloned (Young, 2000; Dangl and Jones, 2001) and, in parallel, many *Avr* genes mainly from fungi and bacteria (White et al., 2000; Luderer and Joosten, 2001; van't Slot and Knogge, 2002). The availability of both a cloned *R* gene and its corresponding cloned *Avr* gene offers exciting opportunities to elucidate the gene-for-gene interaction at the molecular and cellular level. In recent years the guard model has won ground particularly by studies on a few model pathosystems such as the interactions between Arabidopsis or tomato and the bacterial speck pathogen *Pseudomonas syringae,* and tomato and the leaf mold fungus *Cladosporium fulvum* (Innes, 2004; Rooney et al., 2005). In this model R proteins and pathogen effectors (i.e., AVR proteins) are part of a larger dynamic complex. The pathogen effectors target host cell proteins in order to suppress defense responses or elicit susceptible responses. R proteins evolved as a counter-defense and function to monitor the effector targets.

The subject of our studies is *Phytophthora infestans,* the notorious Irish potato famine pathogen and the causal agent of late blight (Govers and Latijnhouwers, 2004). *Phytophthora* species resemble fungi morphologically but in the tree of life they are classified as oomycetes, a unique group of eukaryotes that evolved independently from fungi. Oomycetes include significant pathogens of insects and animals and they are responsible for a wide variety of destructive plant diseases. All *Phytophthora* species (more than 65), and the majority of the *Pythium* species are plant pathogens, and also all downy mildew diseases and white rusts are caused by oomycetes (Agrios, 1997). Oomycetes not only look like fungi, they also behave like fungi and use the same weaponry to attack plants (Latijnhouwers et al., 2003). Similarly, the R proteins that plants use to defeat oomycetes have the same architecture as R proteins that stop fungal invasions (Ballvora et al., 2002; van der Vossen et al., 2003; Gao et al., 2005; Huang et al., 2005) and many oomycete-plant interactions follow the gene-for-gene model. Genetic analyses on host and pathogen have demonstrated that this model also suits the potato-*P. infestans* pathosystem (van der Lee et al., 2001).

Unlike R proteins, the pathogens' AVR proteins or effectors are highly divergent (Luderer and Joosten, 2001; van't Slot and Knogge, 2002). Many of the fungal *Avr* genes were cloned by reverse-genetics using purified elicitor preparations as starting material. For genetically more tractable fungi, like for example *Magnaporthe grisea,* positional cloning appeared to be a suitable approach, and for cloning bacterial *Avr* genes classical bacterial genetics such as genetic

complementation proved to be very efficient (van den Ackerveken and Bonas, 1997; Collmer, 1998). In the case of *Phytophthora* however, *Avr* gene cloning has lagged behind (Tyler, 2001; Tyler, 2002). Because of the (hemi-)biotrophic nature of many oomycete-plant interactions purifying elicitors is difficult and, in our hands attempts to identify race specific elicitors from *P. infestans* were unsuccessful (Alfonso and Govers, 1995). Therefore reverse genetics is not an option. Moreover low DNA transformation efficiencies and relatively large genome sizes hamper complementation or gene tagging approaches. A more suitable approach is positional cloning and recently three oomycete *Avr* genes have been identified starting off with this approach: *Avr1b*-1 from *Phytophthora sojae* (Shan et al., 2004), and *ATR13* and *ATR1*^{NDWsB} from the Arabidopsis downy mildew pathogen *Hyaloperonospora parasitica* (Allen et al., 2004; Rehmany et al., 2005). These two species are homothallic and the number of inbred progeny that was generated was sufficient to obtain recombinants in the *Avr* regions and to identify closely linked markers.

For cloning *Avr* genes in *P. infestans* we also adopted a positional cloning approach and generated high-density maps of chromosomal regions carrying *Avr* genes (van der Lee et al., 2001). In addition a BAC library of a strain carrying six dominant *Avr* genes and suitable for marker landing, is available (Whisson et al., 2001). However, *P. infestans* is heterothallic and the problem we face is the inability to generate large segregating mapping populations. Also the relatively large genome size (245 Mb) reduces the marker density and even with high-density linkage maps (van der Lee et al., 2004) we were not able to generate enough markers for efficient landing. To complement the positional cloning strategy we aimed at generating transcriptome markers. In this study we combined a cDNA-AFLP based strategy with Bulked Segregant Analysis (BSA) to identify *Avr*-associated transcripts. cDNA-AFLP is a relatively simple method to obtain a genome-wide display of differentially expressed genes and it has already been successfully used for gene discovery in *P. infestans* (Avrova et al., 2003; Dong et al., 2004). Many of the known *Avr* genes show a relatively high expression or a stage specific expression in pre-infection stages and therefore we used germinating cysts as starting material for RNA isolation. cDNA-AFLP patterns obtained from pools of strains with identical AVR phenotypes revealed a high number of putative *Avr*-associated transcript derived fragments (TDFs) for each of the four *Avr* loci that were targeted. Subsequently, segregation of the *Avr*-associated TDFs in an F_1 mapping population was analyzed resulting in transcriptome markers for two *Avr* loci.

Materials and methods

P. infestans strains and mapping population

The *P. infestans* strains used in this study are two Dutch field isolates of opposite mating type (80029; A1 and 88133; A2) and 18 F_1-progeny (designated as cross 71). The cross 71 mapping population was previously described and characterized by Drenth et al (1995) and van der Lee et al. (1997). The nomenclature of genes, gene clusters and phenotypes is according to van der Lee

et al. (2001) with one exception; *Avr3* now has the suffix 'b' to indicate that this avirulence gene elicits resistance on plants carrying resistance gene *R3b* and not *R3a* (Huang et al., 2004). Consequently, an avirulent and virulent phenotype on *R3b* plants is indicated by AVR3b and avr3b, respectively.

P. infestans culture conditions

P. infestans strains were routinely grown at 18 °C in the dark on rye agar medium supplemented with 2% sucrose (RSA) (Caten and Jinks, 1968). To obtain germinating cysts for RNA isolation, sporulating mycelium grown on RSA was flooded with ice-cold water and incubated at 4 °C. At this temperature sporangia release the zoospores into the water. After 4 hours incubation the zoospore suspension was filtered through a 10-µm nylon mesh to remove sporangia and mycelial fragments. Cysts were obtained by vigorous shaking of the zoospore suspension for 2 min. To allow germination the cyst suspension was incubated at 18 °C for at least 2 hours. The germination rate and germ tube length were checked with regular time intervals. When more than half of the cysts were germinated and the length of their germ tubes was 4-6 times the diameter of the cysts the tissue was collected by centrifugation (5 min at 3000 g), frozen in liquid nitrogen and stored at -80 °C.

cDNA-AFLP analysis

RNA isolation, cDNA synthesis and cDNA-AFLP analysis were performed as described previously for *P. infestans* by Dong et al. (2004). Total RNA from germinated cysts was isolated using Trizol (Gibco-BRL) according to the manufacturer's instructions and subsequently purified using phenol-chloroform extraction. Poly A^+ RNA was isolated from 100 µg total RNA with the QIAGEN Oligotex mRNA kit. cDNA was synthesized using oligodT (12-18) and Superscript II reverse transcriptase (Gibco-BRL). The primary template for cDNA-AFLP was prepared in a one-step restriction-ligation reaction in which adapters were ligated to *Apo*I/*Taq*I digested cDNA fragments. The quality of each primary template was checked by performing a PCR on the diluted primary template using primers matching the adapters and by analyzing the PCR products on agarose gel. Based on the intensity on gel the quantity was estimated. Pre-amplification was performed in 25 cycles using primers corresponding to the *Apo*I and *Taq*I adapters without extension (A and T primers as in Dong et al., 2004). The diluted pre-amplification products were used as template for the selective amplification with two selective base extensions at the 3'-end of the primers (A+2 and T+2 primers). The A+2 primers were either labeled by phosphorylating the 5'-end with [γ-^{32}P]ATP for detection of the cDNA-AFLP fragments by autoradiography, or with IRD700 or IRD800 for fluorescence detection using LI-COR Global IR^2 systems. For analysis of the cDNA-AFLP fragments by silver staining the primers were not labeled. Separation of the cDNA-AFLP fragments was performed on 4 to 6% denaturating polyacrylamide gels as described by van der Lee et al. (1997).

Bulked segregant analysis

Bulked segregant analysis (BSA) was performed essentially following the procedure described by Michelmore *et al.* (1991). Ten F_1 progeny of the cross 71 mapping population were selected and divided over four pools consisting of 2 or 3 F_1 progeny with identical or nearly identical avirulence phenotypes (Table 1). Each phenotype is represented by 4-6 F_1 progeny divided over two pools. From the six avirulence genes that segregate in cross 71 *Avr3* (renamed *Avr3b*), *Avr10* and *Avr* 11 are closely linked (van der Lee et al., 2001) and in this study we consider *Avr3b-Avr10-Avr11* as one locus. Primary templates of the 2 or 3 F_1 progeny that made up one pool were mixed in equal amounts (based on the quantity and quality check described above) and served as template for the pre-amplification. In the selective amplification all 256 *Apo*I+2 / *Taq*I+2 primer combinations were used. In Fig. 1B, 1C, 1D and 1E the expected patterns for each of the pools are shown.

Isolation, cloning and sequencing of TDFs

The cDNA-AFLP fragments (i.e. TDFs) of interest were excised from gels using a razor blade. The gel slices were rehydrated in 100 µl of water and incubated at 70 °C for 15 min. The eluted fragment was reamplified with the primers with the same two base pair extension as used in the cDNA-AFLP analysis. PCR products were purified using QIAquick PCR purification kit (Qiagen, Hilden) and cloned into pGEM-T Easy (Promega, Madison, WI, USA). Recombinant clones were sequenced by BaseClear (Leiden, The Netherlands) or Shanghai Biotech (Shanghai, China).

Table 1. Composition of BSA pools for selecting *Avr*-associated TDFs.

		Phenotypes on differentials containing resistance gene*					
Pool	Strain	*R1*	*R3b*	*R10*	*R11*	*R4*	*R2*
1	re11-16	AVR	avr	avr	avr	AVR	AVR
	T15-1	AVR	avr	avr	avr	AVR	AVR
	T30-2	AVR	avr	avr	avr	AVR	AVR
2	D12-2	avr	avr	avr	avr	avr	AVR
	D12-23	avr	avr	avr	avr	avr	AVR
	T35-3	avr	avr	avr	avr	avr	avr
3	D12-17	AVR	AVR	AVR	AVR	avr	avr
	T15-9	AVR	AVR	AVR	AVR	avr	avr
4	T20-2	avr	AVR	AVR	AVR	AVR	AVR
	E12-3	avr	AVR	AVR	AVR	AVR	avr

DNA sequence analysis and bioinformatics

Sequences were analysed in Vector NTI 8. For BLAST searches we used the NCBI BLAST program and the Standalone-BLAST Version 2.2.3 (Altschul et al., 1997). The *P. infestans* EST databases are accessible at http://www.pfgd.org and http://staff.vbi.vt.edu/estap (Kamoun et al., 1999; Randall et al., 2005). The genomic sequences and annotated protein sequences of *P. sojae*

and *P. ramorum* were obtained from the website of the DOE Joint Genome Institute (http://www.jgi.doe.gov/genomes). TDF sequences were searched against GenBank and EST databases by BLASTX and BLASTN, respectively. A GenBank hit was considered to be a homologue if the BLASTX *E* value is less than 1e-3. A TDF was considered to be represented by an EST if the BLASTN identity is equal to or larger than 99%. RT-PCR primers were designed based on the cloned TDF sequence or the EST sequence if the TDF has a corresponding EST. Primer lengths were between 18 bp to 25 bp with melting temperatures higher than 55 ^{o}C in all cases. The primer sequences are available from the authors upon request.

RT-PCR analysis

To remove genomic DNA from RNA preparations, 10 μg total RNA was treated with 4 units RQ1 RNase-free DNase (Promega, Madison, WI) at 37 °C for 1 h. The removal of all DNA was verified in a PCR reaction under the same conditions as those used for the RT-PCR reaction, except that the cDNA synthesis step was omitted. The first-strand cDNA was synthesized using oligo(dT16) and Superscript II reverse transcriptase for 30 min at 40 °C (Gibco-BRL). Sequence-specific primers were used in the subsequent PCR with cDNA as template with 30 cycles (30 s at 94 °C, 30 s at 56-60 °C and 60 s at 72 °C).

Nomenclature of TDFs

The cDNA-AFLP fragments and the clones containing the fragments are named TDF followed by a number that refers to the *Avr* gene for which, according the BSA pattern, the TDF was a candidate. This *Avr*-associated number is then followed by a period and a random clone number. For *Avr3b-Avr10-Avr11* the *Avr*-associated number is 3. In cases where an ‘x’ is added as suffix the TDF showed an opposite pattern in the BSA. Occasionally, an ‘s’ is added at the very end to indicate that the TDF was selected in the BSA analysis on silver stained gels.

Results and discussion

BSA for selecting transcripts associated with avirulence

BSA was initially developed as a method for rapidly identifying polymorphic DNA markers linked to any specific gene or genomic region (Michelmore et al., 1991). Two bulked DNA samples are generated from a segregating population from a single cross. Each pool, or bulk, contains individuals that are identical for a particular trait or genomic region but arbitrary at all unlinked regions. The two bulks are therefore genetically dissimilar in the selected region but seemingly heterozygous at all other regions. Previously, the *P. infestans* cross 71 mapping population was succesfully used for BSA to identify AFLP markers linked to six *Avr* genes segregating in cross 71 (van der Lee et al., 2001). In the present study we used the same cross 71 mapping population, a similar pool design and the same pool sizes for a BSA approach aimed at selecting transcripts derived from *Avr* genes. Instead of DNA, cDNA of different individuals

from the cross was pooled. The phenotypes of the strains that constitute the four BSA pools are listed in Table 1. Anticipating that *Avr* genes are expressed just prior to infection we used RNA isolated from germinating cysts as starting material.

It is logical to combine a BSA approach with an efficient genome-wide transcriptional profiling method. Recently, Dong et al. (2004) described an optimized cDNA-AFLP protocol for *P. infestans* that was based on *in silico* cDNA-AFLP fingerprinting of a large set of *P. infestans* ESTs. The primer combination *Apo*I / *Taq*I and selective amplification using primers with two base extensions resulted in clear transcription profiles that were easy to score. Fig. 1A shows a section of a typical autoradiograph with cDNA-AFLP patterns obtained from the four BSA pools with four primer combinations following the protocol of Dong et al. (2004). All 256 *Apo*I+2 / *Taq*I+2 primer combinations were used to generate radioactive TDFs that were visualized by autoradiography. A subset of the primer combinations was used to generate unlabeled TDFs and those were visualized by silver staining. Over 30,000 TDFs ranging in size from 40-600 bp were analyzed. Overall the patterns obtained with autoradiography and silver staining were comparable but remarkably some TDFs that were detected with the radioactive primer were not visible as a band on silver stained gels and, *vice versa*, some clear bands on silver stained gels were absent on autoradiographs.

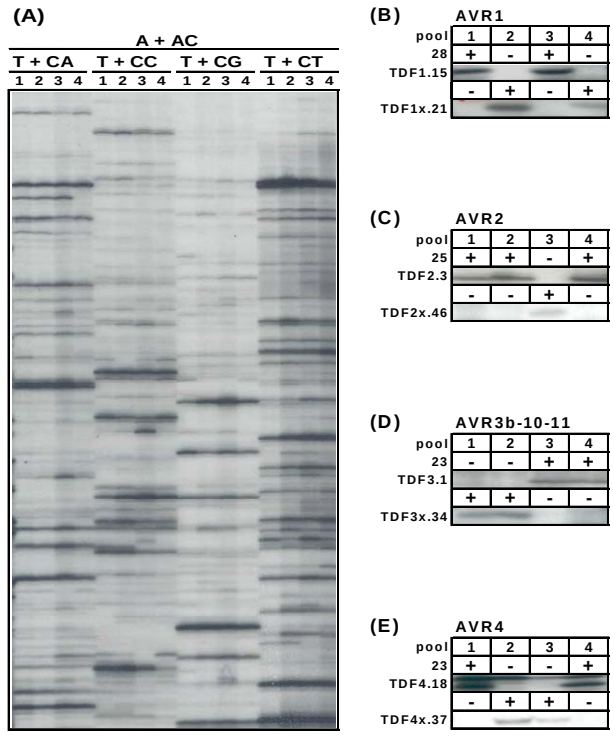

Fig. 1. **cDNA-AFLP analysis**. (A) Section of autoradiograph showing cDNA-AFLP fingerprints in four BSA pools generated with the indicated primer combinations. For the composition of pools 1, 2, 3, and 4 see Table 1. In panels (B), (C), (D) and (E) the second row shows the number of *Avr*-associated TDF candidates found in this study and the expected cDNA-AFLP patterns in the four pools for TDFs associated with AVR1, AVR2, AVR3b-AVR10-AVR11 and AVR4 phenotypes, respectively. The third row shows examples of candidate TDFs with the expected pattern. The fourth row shows the expected opposite pattern and the fifth row examples. In (B) TDF1.25 was obtained with primer combination A+TG/T+CG and TDF1x.21 with A+GA/T+GT. In (C) TDF2.3 with A+AG/T+TT and TDF2x.46 with A+TG/T+GG. In (D) TDF3.1 with A+AG/T+AC and TDF3x.34 with A+GT/T+CC. In (E) TDF4.18 with A+TG/T+GG. and TDF4x.37 with A+GT/T+TA.

TDFs present in avirulent but not in virulent strains are expected to show up in two pools (or three in the case of *Avr2*) but not in the others. In fact, the pool design included internal controls, for example, an *Avr1* specific transcript should only be present in pool 1 and pool 3

whereas an *Avr4* specific transcript should be present in pool 1 and pool 4 but not in pool 2 nor pool 3. For each of the three *Avr* genes and the *Avr3b-Avr10-Avr11* locus 23 or more TDFs that behaved according to the predicted patterns were detected. In total 99 such *Avr*-associated TDFs were found, some of which were only visible by silver staining. In all cases TDFs with opposite pattern were also found. Examples are shown in Fig. 1B, 1C, 1D and 1E. Although the observed BSA patterns suggest that the TDFs represent genes that are specifically expressed in either avirulent or virulent strains one should bear in mind that also polymorphisms in the *Apo*I or *Taq*I recognition site or in the two base pair extensions may result in differential cDNA-AFLP patterns.

Segregation of *Avr*-associated TDFs in cross 71

From previous studies in which the segregation of the avirulence phenotypes in cross 71 was analyzed, it was evident that the AVR1, AVR2 and AVR4 phenotypes behave as single dominant traits (Alfonso and Govers, 1995; van der Lee et al., 2001). AVR3b, AVR10 and AVR11 are also dominant but the genes are closely linked (van der Lee et al., 2001). The *Avr3b-Avr10-Avr11* locus might harbour three independent genes but it can not be excluded that the locus contains a single gene that either controls other loci conferring avirulence on *R3b*, *R10* and *R11* plants or that interacts with an uncharacterized R gene shared by *R3b*, *R10* and *R11* plants. Many of the known avirulence factors from plant pathogens are effector proteins that are present in avirulent strains but absent, unstable or mutated in virulent strains (Westerink et al., 2004). Hence, the *Avr*-associated TDFs that were identified in the BSA may all represent candidate *Avr* genes. However, we hypothesize that from each set only TDFs derived from one transcript (or possibly three in the case of *Avr3b-Avr10-Avr11*) can represent the real *Avr* gene. To make a further selection, we performed fluorescent and silver stained cDNA-AFLP analyses on the two parental lines of cross 71 and 18 F_1 progeny, and screened for presence or absence of TDFs. For 25 *Avr*-associated TDFs there was segregation in the F_1 progeny, 8 of which were associated with *Avr1*, 8 with *Avr2*, 4 with *Avr3b-Avr10-Avr11* and 5 with *Avr4*. Representative patterns are shown in Fig. 2. Two of the 25 were not polymorphic in the parental lines and are thus unlikely candidates to represent an *Avr* gene. However, for four of the 25 *Avr*-associated TDFs the presence/absence pattern matched exactly with the avirulence phenotypes of the two parental lines and the 18 F_1 progeny making them ideal transcriptome markers representing an *Avr* gene. Two are associated with the *Avr3b-Avr10-Avr11* locus (TDF3.1 and TDF3.4), and two with *Avr4* (TDF4.1s and TDF4.2s) (Fig. 2).

None of the *Avr1* and *Avr2* candidates cosegregated with avirulence and it is therefore unlikely that these TDFs are derived from *Avr1* or *Avr2*. Nevertheless, based on the segregation patterns of the remaining 19 TDFs we anticipate that some of them are linked to the *Avr* locus (data not shown). If the polymorphism represents a DNA polymorphism they could be used as markers for fine mapping the *Avr* regions. Alternatively, they could be used for the construction of a transcriptome map (Brugmans et al., 2002).

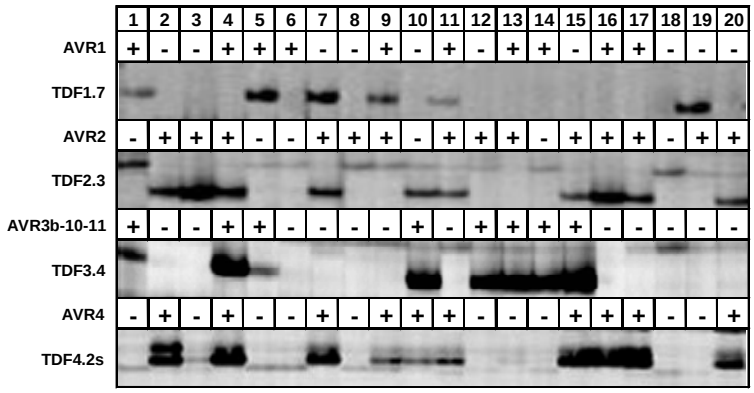

Fig. 2. cDNA-AFLP patterns showing the segregation of four Avr-associated TDFs in 18 F1 progeny of cross 71 (lanes 3-20). Lane 1 and 2 show the cDNA-AFLP patterns obtained from the two parental isolates 80029 and 88133, respectively. The avirulence and virulence phenotypes of parents and progeny are indicated by + and -, respectively. TDF1.7 was obtained with primer combination A+AT/T+GA, TDF2.3 with A+AG/T+TT, TDF3.4 with A+AA/T+AG and TDF4.2s with A+TC/T+TC.

TDF cloning and sequencing

To enable further analysis of the TDFs we cloned the majority of the 99 *Avr*-associated TDFs and a number of TDFs with opposite pattern. TDFs were excised from gel, re-amplified, cloned and sequenced. Based on the size of the clone insert and the presence or absence of the expected two-base primer extension in the sequence, it was concluded that 142 TDFs were successfully cloned. Overall, the success rate of cloning was over 94%. GenBank accession numbers of the cloned TDFs and AFLP codes showing the primer extensions and fragment size, are listed in Table 2. The TDF nucleotide sequences were used to design primers for RT-PCR analysis and for similarity searches in various databases (Table 3).

RT-PCR expression analysis of *Avr*-associated TDFs

In parallel to the segregation analysis of the TDFs in cross 71, expression of cloned TDFs was analyzed by RT-PCR. For 42 TDFs suitable primers were designed and for 38, RT-PCR products were obtained. The primer design was based on the TDF sequence itself or on the sequence of a matching *P. infestans* EST with a sequence similarity higher than 99%. The majority of the RT-PCR products could be visualized on agarose gels but for several the small size of the RT-PCR product or the occurrence of multiple bands with size differences of only a few base pairs required an electrophoresis system with a higher resolution (i.e. polyacrylamide gels). Table 3 lists the 38 TDFs including the amplicon sizes. The RT-PCR analysis included the two parental strains of cross 71 and 9 F_1 progeny. Of the 38 TDFs four showed an RT-PCR expression pattern that perfectly matched the avirulence phenotypes in parents and F_1 progeny, and these are the same four TDFs that matched in the segregation analysis based on cDNA-AFLP patterns: TDF3.1, TDF3.4, TDF4.1s and TDF4.2s (Table 3). In the avirulent parent and progeny the RT-PCR product was present and in the virulent parent and progeny it was

absent. Since both RT-PCR and cDNA-AFLP give this black and white pattern it is very likely that the difference is caused by presence versus absence of mRNA and not by polymorphisms in the sequences. Hence, the genes corresponding to these TDFs seem to be regulated at the transcriptional level.

Table 2. Primer extensions, fragment sizes and GenBank accession numbers of 142 cloned TDFs.

TDF	AFLP code	Accession number	TDF	AFLP code	Accession number	TDF	AFLP code	Accession number
1.1	A+AA/T+AAs169	DW010060	2.21	A+TG/T+GAs399	DW010177	3x.29	A+GG/T+GGs193	DW010132
1.2	A+AA/T+CTs232	DW010070	2.22	A+TT/T+CGs314	DW010178	3x.30	A+GG/T+GGs120	DW010133
1.3	A+AA/T+GGs345	DW010173	2.23	A+TT/T+CGs312	DW010179	3x.33	A+GT/T+AGs178	DW010134
1.5	A+AT/T+ACs234	DW010078	2.1s	A+CC/T+TAs210	DW010093	3x.34	A+GT/T+CCs137	DW010135
1.6	A+AT/T+CTs71	DW010079	2.2s	A+GG/T+GCs220	DW010095	3x.35	A+GT/T+GTs381	DW010188
1.7	A+AT/T+GAs233	DW010080	2x.8	A+CC/T+CCs93	DW010103	3x.42	A+TA/T+GTs303	DW010189
1.8a	A+AT/T+TTs72	DW010081	2x.39	A+TA/T+ACs346	DW010181	3x.43	A+TA/T+TTs77	DW010137
1.8b	A+AT/T+TTs72	DW010081	2x.46	A+TG/T+GGs254	DW010102	3x.45	A+TA/T+GTs112	DW010138
1.9	A+CA/T+AGs362	DW010174	3.1	A+AG/T+ACs153	DW010104	3x.47	A+TG/T+TGs257	DW010139
1.10	A+CA/T+GGs360	DW010172	3.2	A+AG/T+ACs104	DW010114	3x.48	A+TG/T+AGs227	DW010140
1.11	A+CC/T+CGs152	DW010061	3.3	A+AG/T+TTs242	DW010116	3x.51	A+TT/T+GTs291	DW010190
1.12	A+CC/T+TAs65	DW010062	3.4	A+AA/T+AGs156	DW010117	3x.52	A+TT/T+CCs218	DW010142
1.13	A+TC/T+ACs219	DW010063	3.6	A+AT/T+GAs160	DW010118	4.1	A+AA/T+AGs233	DW010146
1.14	A+TC/T+ACs212	DW010064	3.7	A+AT/T+TCs315	DW010184	4.2	A+AA/T+GGs105	DW010155
1.15	A+TC/T+GTs179	DW010065	3.8	A+AT/T+TCs252	DW010119	4.3	A+AC/T+AGs139	DW010158
1.16	A+CT/T+ATs222	DW010066	3.9	A+AT/T+TCs108	DW010120	4.4	A+AC/T+CGs183	DW010159
1.17	A+CT/T+ATs222	DW010066	3.10	A+GC/T+CAs147	DW010105	4.5	A+AC/T+GTs69	DW010160
1.18	A+CT/T+CTs240	DW010067	3.11	A+GC/T+GAs250	DW010106	4.7	A+AT/T+AAs232	DW010161
1.19	A+GC/T+AGs225	DW010068	3.12	A+GC/T+GTs265	DW010107	4.8	A+TC/T+AAs123	DW010162
1.20	A+GG/T+TAs124	DW010071	3.13	A+GC/T+TAs156	DW010108	4.9	A+CT/T+CGs96	DW010163
1.21	A+GG/T+TTs161	DW010072	3.14	A+GA/T+CAs281	DW010109	4.10	A+GC/T+CCs114	DW010147
1.22	A+GT/T+CAs268	DW010073	3.15	A+GG/T+CCs164	DW010110	4.11	A+GA/T+AGs503	DW010191
1.23	A+TA/T+TGs132	DW010074	3.16	A+GG/T+CCs84	DW010111	4.12	A+GA/T+CGs238	DW010148
1.24	A+TA/T+GTs110	DW010075	3.17	A+GG/T+CCs84	DW010111	4.13	A+GG/T+AGs85	DW010149
1.25	A+TG/T+CGs109	DW010076	3.19	A+GT/T+TAs168	DW010112	4.14	A+GT/T+AGs296	DW010192
1.1s	A+TA/T+GAs150	DW010069	3.20	A+TT/T+AAs347	DW010182	4.15	A+TA/T+GTs354	DW010193
1.2s	A+TC/T+CAs200	DW010077	3.21	A+TT/T+GCs293	DW010183	4.16	A+TG/T+AAs240	DW010150
1x.15	A+GC/T+CTs169	DW010082	3.22	A+TT/T+TTs110	DW010115	4.17	A+TG/T+AGs117	DW010151
1x.21	A+GA/T+GTs137	DW010083	3.1s	A+TA/T+AGs90	DW010113	4.18	A+TG/T+GGs116	DW010152
2.1	A+AG/T+GCs155	DW010084	3x.2	A+AG/T+ATs93	DW010126	4.19	A+TT/T+CAs95	DW010153
2.3	A+AG/T+TTs137	DW010096	3x.4	A+AC/T+ACs257	DW010136	4.20	A+TT/T+GGs117	DW010156
2.4	A+AA/T+AAs251	DW010097	3x.5	A+AT/T+GAs158	DW010141	4.1s	A+TC/T+GAs125	DW010154
2.5	A+AC/T+CCs117	DW010098	3x.6	A+CA/T+ACs163	DW010143	4.2s	A+TC/T+TCs144	DW010157
2.6	A+AC/T+GTs152	DW010099	3x.7	A+CA/T+CCs130	DW010144	4.3s	A+GT/T+TTs180	DW010198
2.7	A+AC/T+TGs183	DW010100	3x.9	A+TC/T+GGs164	DW010145	4x.3	A+AC/T+TTs74	DW010167
2.8	A+AT/T+CTs319	DW010180	3x.10	A+CG/T+AAs169	DW010121	4x.10	A+TC/T+TCs133	DW010164
2.9	A+CA/T+CCs117	DW010101	3x.11	A+CG/T+GAs215	DW010122	4x.12	A+CG/T+TAs196	DW010165
2.10	A+CC/T+GCs101	DW010085	3x.13	A+GC/T+ATs172	DW010123	4x.18	A+GC/T+TTs483	DW010194
2.11	A+TC/T+GCs97	DW010086	3x.14	A+GC/T+CCs333	DW010185	4x.20	A+GA/T+GCs187	DW010166
2.12	A+TC/T+GGs72	DW010087	3x.16	A+GC/T+GCs216	DW010124	4x.31	A+GT/T+ACs364	DW010195
2.13	A+TC/T+ACs97	DW010088	3x.17	A+GC/T+GTs270	DW010125	4x.32	A+GT/T+ACs375	DW010196
2.14	A+CT/T+ATs144	DW010089	3x.19	A+GA/T+CAs400	DW010186	4x.37	A+GT/T+TAs361	DW010197
2.15	A+CT/T+CCs113	DW010090	3x.22	A+GA/T+TCs215	DW010127	4x.38	A+GT/T+TGs216	DW010168
2.16	A+CG/T+GAs412	DW010175	3x.23	A+GG/T+ATs151	DW010128	4x.40	A+TA/T+CTs286	DW010169
2.17	A+CG/T+GGs401	DW010176	3x.24	A+GG/T+CAs167	DW010129	4x.49	A+TT/T+TAs258	DW010170
2.18	A+GA/T+GAs238	DW010091	3x.25	A+GG/T+GGs352	DW010187	4x.50	A+TT/T+TGs206	DW010171
2.19	A+GT/T+ATs274	DW010092	3x.27	A+GG/T+GGs236	DW010130			
2.20	A+TA/T+CGs168	DW010094	3x.28	A+GG/T+CAs218	DW010131			

Several of the other 34 TDFs showed differential RT-PCR patterns but there was no association with the avirulence phenotypes. A substantial number, however, showed no differential expression at all. Again none of the *Avr*-associated TDFs tested by RT-PCR appeared to be a candidate for *Avr1* or *Avr2*.

Table 3. RT-PCR analysis and sequence similarity of 38 *P. infestans* TDFs

TDF	*P. infestans* EST hit [a]	amplicon size (bp) [b]	RT-PCR [c]	*P. sojae* homologue [d]	*P. ramorum* homologue [d]	SwissProt hit of *P. sojae* homologue	BLAST identity (%)	*E*-value
1.1		117	-	pro135357	pro75828	RB38_HUMAN (P57729) Ras-related protein Rab-38	40	3.00E-33
1.2		190	-	pro135623	pro71960	RDPO_SCHPO (Q05654) Retrotransposable element Tf2 155 kDa protein	29	1.00E-99
1.3	CON_001_13933	304*	-	pro135300	pro75790	PTPJ_HUMAN (Q12913) Protein-tyrosine phosphatase	36	1.00E-36
1.5		202	-					
1.6		38	-					
1.7	CON_003_04202	390*	-	pro140341	pro83108			
1.14		90	-	pro143752	pro87069	RDPO_SCHPO (Q05654) Retrotransposable element Tf2 155 kDa protein	27	2.00E-66
1.22	CON_016_07340	400*	-	pro125097	pro83808	SYM_ARATH (Q9SVN5) Probable methionyl-tRNA synthetase	37	9.00E-21
2.3	CON_001_16821	191*	-	pro129917	pro73127	VTL2_MOUSE (O89116) Vesicle transport v-SNARE protein Vti1-like 2	29	4.00E-21
2.7	CON_001_30638	472*	-	pro109725	pro87143	AQP3_HUMAN (Q92482) Aquaporin 3	43	1.00E-34
2.11		59	-					
2.13		59	-					
2.15	CON_001_14541	380*	-	pro131502	pro84862	GTT2_HUMAN (P30712) Glutathione S-transferase theta 2	33	1.00E-20
3.1		54	yes					
3.3		148	-	pro108156	pro39196	ENGA_RICPR (Q9ZCP6) Probable GTP-binding protein engA	24	1.00E-03
3.4		115	yes	pro133266	pro80794	MYH3_CHICK (P02565) Myosin heavy chain, fast skeletal muscle	22	2.00E-07
3.7		282	-	pro131930	pro74150	TRHY_SHEEP (P22793) Trichohyalin	18	1.00E-11
3.8		172	-					
3.9		62	-					
3.16		52	-					
3.19	CON_002_01106	377*	-	pro133266	pro80794	MYH3_CHICK (P02565) Myosin heavy chain, fast skeletal muscle	22	2.00E-07
3.20	CON_010_06936	490*	-	pro137091	pro85962	NSB1_HUMAN (P82970) Nucleosomal binding protein 1	23	3.00E-04
3x.7		94	-		pro80914			
3x.11		182	-	pro143645	pro80057	MYSJ_DICDI (P54697) Myosin IJ heavy chain	33	1.00E-114

3x.22		182	-	pro131604	pro80644	DSPP_HUMAN (Q9NZW4) Dentin sialophosphoprotein precursor	17	7.00E-09
3x.33	CON_001_10962	236*	-	pro131005	pro85669			
4.1		191	-	pro131094	pro86402	BFR1_SCHPO (P41820) Brefeldin A resistance protein	20	6.00E-17
4.2		67	-					
4.10		80	-	pro138207	pro81288			
4.13		42	-					
4.14	CON_001_33999	271*	-	pro140951	pro72858	CATL_DROME (Q95029) Cathepsin L precursor	40	5.00E-58
4.18		71	-					
4.19	CON_001_29569	452*	-	pro131364	pro73340			
4.20		75	-	pro134550	pro74902	CSK_CHICK (P41239) Tyrosine-protein kinase	28	4.00E-18
4.1s	CON_001_33634	186*	yes	pro109418	pro83335			
4.2s	CON_001_33634	186*	yes	pro109418	pro83335			
4x.3		41	-					
4x.50	CON_014_07231	473*	-	pro138318	pro82098			

[a] *P. infestans* EST hits with *E* value < 1e-50 and identity > 99% are listed..
[b] RT-PCR amplicon size was calculated based on TDF or EST sequence information; * indicates that the primers were designed on the EST sequence.
[c] 'yes' indicates that the RT-PCR polymorphism correlates with the AVR phenotypes of the parents and 9 F_1 progeny; - indicates no polymorphism or no correlation with the AVR phenotypes.
[d] *P. sojae* and *P. ramorum* whole genome sequences and the gene annotation at the JGI website (http://www.jgi.doe.gov/genomes) were used for analysis. Genes with BLAST *E* value less than 1e-3 were considered homologues.

Sequence similarity of TDFs with *Phytophthora* sequences and known sequences

Sequence similarity to known sequences may help in assigning a function to the genes from which the TDFs are derived. All TDF sequences were compared by BLAST algorithm to the NCBI GenBank and *P. infestans* EST databases with an *E*-value cutoff of 1e-03. Of the 142 TDFs 56% had no match at all. A small percentage (16 %) had a match in GenBank and 39 % had high sequence similarity to *P. infestans* ESTs (Fig. 3A). The *P. infestans* EST database comprises over 75.000 ESTs obtained from cDNA libraries representing a broad range of growth conditions, stress responses, and developmental stages (Randall et al., 2005). It is likely that more TDFs have matching cDNA clones in the EST libraries but because many of the ESTs are only partially sequenced, matching cDNA clones may not always be recognizable. On the other hand cDNA-AFLP is a very sensitive method and is able to detect very low abundance mRNA that may not be present in the EST database.

The 142 TDFs were also BLASTed against the fully sequenced genomes of *P. sojae* and *P. ramorum*. Over one third had no homologues in *P. sojae* and one third had homologues with a similarity higher than 80% (Fig. 3B). The homologues in the *P. sojae* proteome that were assigned to represent the TDFs were subsequently BLASTed against the SwissProt database. A wide range of hits was found, such as proteins that function as phosphatase or kinase but also an ABC transporter, a water channel protein, and molecular motor proteins. As expected, there are also TDFs that do have a match in the *P. sojae* proteome but no hit in SwissProt. Table 3 shows the results for 38 of the 142 cloned TDFs.

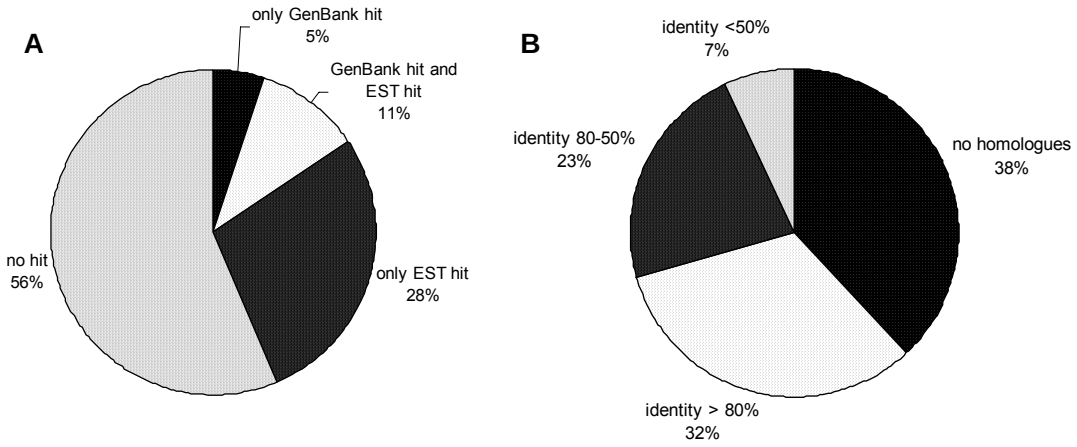

Fig. 3. Percentages of 142 cloned *P. infestans* TDFs with (A) sequence homology in GenBank and *P. infestans* EST databases and (B) homologues in *P. sojae*. Homologues were counted if the BLASTX *E* value was less than 1e-3. In the GenBank sequences the *P. infestans* ESTs deposited in GenBank were not included.

TDFs represented by *P. infestans* ESTs and TDFs with *P. sojae* homologues with a variety of putative functions were taken for further data mining and bioinformatics analysis such as *in silico* expression analysis, signal peptide prediction and gene copy number of the homologues in *P. sojae* and *P. ramorum* (Table 4). In the *P. infestans* EST database (Randall et al., 2005) we analyzed the distribution of ESTs representing the TDFs over the various libraries and, based on these numbers, we predicted stage specific expression patterns and expression levels. For example, for TDF3x.34 many ESTs are found in the germinating cysts library and zoospore library but none in a mycelium library. This indicates that the TDF3x.34 gene is specifically expressed at a relatively high level in zoospores and germinating cysts. In contrast, for TDF2.7 there is only one EST in the database, which indicates that this aquaporin-like gene is transcribed at a low level in wall-less zoospores. Of the 55 TDFs for which we found a matching *P. infestans* EST, only 16 have ESTs in germinating cyst stages. Our screening strategy did not include a stage specific selection but since we used germinating cysts as starting material one would expect to find matching ESTs in that stage. This is true for only one third of the TDFs confirming that cDNA-AFLP is a very sensitive method that can reveal very low abundance transcripts.

Table 4. Analysis of *Avr*-associated TDFs using data mining and bioinformatics.

TDF	*P. infestans* EST hit [a]	protein size [b]	signal peptide [c]	Cys spacing pattern [d]	transcripts in *P. infestans* EST database [e]	*P. sojae* homologue [f]	SwissProt BLAST hit of *P. sojae* homologue	putative function	phylogenetic distribution [g]	genes in *P. sojae* [h]	genes in *P. ramorum* [h]
1.7	CON_003_04202	nd	-		ZO(1) SP(1) MY(1)	pro140341		unknown	only in *Phytophthora*	1	1
2.3	CON_001_16821	nd	-		MY(1)	pro129917	VTL2_MOUSE (O89116)	vesicle transporter	other species	1	1
2.7	CON_001_30638	nd	-		ZO(1)	pro109725	AQP3_HUMAN (Q92482	water channel	other species	>10	>10
3.4		nd	-		-	pro133266	MYH3_CHICK (P02565)	cyto-skeleton related	other species	1	1
3x.34	CON_020_07430	159	SP	C-n20-C-n9-C-n8-C	ZO(13) CY(7)SP(1)	pro138143		unknown secreted protein	only in *Phytophthora*	8	3
4.1s	CON_001_33634	>150		none	MY(1)	pro109418		unknown secreted protein	only in *Phytophthora*	2	1
4.1		nd	-		-	pro131094	BFR1_SCHPO (P41820)	ABC transporter	other species	>10	>10
4.20		nd	-		-	pro134550	CSK_CHICK (P41239)	kinase	other species	1	1
4x.49	CON_011_07076	300	SP	C-n28-C-n3-C-n10-C-n17-C-n23-C-n103-C	MY(8)	pro144423		unknown secreted protein	only in *Phytophthora*	>10	>10

[a] *P. infestans* EST hits with *E* value < 1e-50 and identity > 99% are listed.

[b] nd indicates that the protein sequence is incomplete.

[c] SP indicates that a signal peptide is predicted at the N-terminus by the program SignalP (Nielsen et al., 1997; Nielsen and Krogh, 1998).

[d] The proteins with signal peptide were used for cysteine spacing analysis.

[e] The tissue types from which the EST libraries are derived are zoospores (ZO), germinated cysts (CY), sporangia (SP) and mycelia (MY). The numbers in brackets indicate the number of ESTs present in the various libraries (Randall et al., 2005).

[f] *P. sojae* whole genome sequences and the gene annotation at the JGI website (http://www.jgi.doe.gov/genomes) were used for analysis. Genes with BLAST *E* value less than 1e-3 were considered homologues.

[g] Homologues in species other than *Phytophthora* were considered as homologues when the BLAST *E* value was less than 1e-3 and the similarity >30%.

[h] *P. sojae* and *P. ramorum* whole genome sequences and gene annotation at the JGI website (http://www.jgi.doe.gov/genomes) were used for analysis. Genes with BLAST similarity higher than 50% were considered to be members of the same gene family. Numbers indicate the size of the family.

Many of the fungal and oomycete elicitors identified to date are small secreted proteins with an even number of cysteine residues that usually form disulfide bridges to stabilize the protein (van't Slot and Knogge, 2002). Another feature typical for oomycete elicitors is the RXLR motif, a motif shared by four oomycete avirulence factors that lack cysteines (Allen et al., 2004; Shan et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005). For the *Avr*-associated TDFs the presence of a signal peptide combined with a particular cysteine signature or an RXLR motif can be indicative for elicitor function. Two proteins representing TDF3x.34 and TDF4x.49 are predicted to be secreted by the program SignalPv2.0 (Nielsen et al., 1997; Nielsen and Krogh, 1998) and are also rich in cysteine residues. The protein represented by TDF4.1s has a homologue in *P. sojae* that is a secreted protein so we anticipate that the full length TDF4.1s protein also has a signal peptide (Table 4). These three proteins have no homology with any known protein but, interestingly, the *P. sojae* homologue of the TDF4.1s protein has an RXLR motif making TDF4.1s a promising candidate for an *Avr* gene.

With the exception of one *Phytophthora* elicitor, i.e. NIP1 (Fellbrich et al., 2002; Qutob et

al., 2002), all oomycete elicitors and avirulence factors identified to date are unique for oomycetes: there are no homologous in organisms other than oomycetes. This is true for elicitins (Jiang et al., 2006) and the glycoprotein elicitor (gpe) containing pep13 (Sacks et al., 1995; Brunner et al., 2002), two protein families which are ubiquitous in the *Phytophthora* genus and have elicitor activity on a large variety of plant species. This is also true for the four ecotype- or cultivar-specific oomycete avirulence factors with the RXLR motif (Allen et al., 2004; Shan et al., 2004; Armstrong et al., 2005; Rehmany et al., 2005). In contrast to elicitin genes and *gpe* genes, however, none of these four avirulence genes belongs to a conserved gene family. Apart from the conserved RXLR motif they all show high sequence divergence with their homologues in other species and this may be a hallmark for host- or cultivar-specific avirulence genes. To evaluate the likelihood that the TDFs are derived from *Avr* genes we analyzed the phylogenetic distribution and we investigated whether the cloned TDFs belong to a gene family. Of the 88 TDFs that have homologues in *P. sojae* and *P. ramorum,* 25 seem to be unique for *Phytophthora.* Noticeably, the two secreted proteins listed in Table 4 only occur in *Phytophthora.* The homologues of TDF 4x.49 form a large gene family with over 10 members in both, *P. sojae* and *P. ramorum*, and those of TDF3x.34 appear to form a larger family in *P. sojae* than in *P. ramorum*. TDF4.1s has only two weak homologues (BLAST identity < 40%) in *P. sojae* and one weak homologue in *P. ramorum*, which suggests that this gene is of high sequence divergence among *Phytophthora* species.

Conclusions

In this study we demonstrate that combining a bulked segregant analysis strategy with a highly efficient transcriptional profiling method can be very effective in selecting *Avr*-associated transcripts. We focused on four *Avr* genes and for two of these we found TDFs that fulfill all criteria that make the TDF a likely *Avr* candidate. First of all, the TDFs occurred in germinating cysts, a preinfection stage in which an *Avr* gene is most likely to be expressed. Secondly, the TDFs were present in pools consisting of strains having an AVR phenotype but were absent in pools consisting of virulent strains (avr phenotype). Thirdly, segregation of the TDFs in F_1 progeny correlated entirely with segregation of the AVR/avr phenotypes and, fourthly, RT-PCR confirmed the *Avr*-associated segregation in the F_1 progeny.

The two TDFs that were assigned as candidates for *Avr4,* TDF4.1s and TDF4.2s, appear to match to the same *P. infestans* EST contig but the deduced protein is an unknown protein. Since the homologues in *P. sojae* and *P. ramorum* are very divergent the protein seems to be unique for *P. infestans.* The *P. sojae* homologue though, has all the hallmarks of the family of RXLR proteins: a signal peptide, an RXRL motif and high sequence divergence with the other family members. All four oomycete *Avr* genes identified so far, *P. sojae Avr1b*-1 (Shan et al., 2004), *P. infestans Avr3a* (Armstrong et al., 2005) and the two *Hyaloperonospora parasitica* ecotype-specific *Avr* genes, *ATR13* and *ATR1* (Allen et al., 2004; Rehmany et al., 2005), belong

to this RXLR super family and sequencing of the full-length gene represented by TDF4.1s and TDF4.2s showed that this gene is also an RXLR family member (**Chapter 4**). Hence the TDF4.1/TDF4.2 gene is a likely candidate for *Avr4*. Functional characterization is in progress.

The two TDFs that associate with the *Avr3b-Avr10-Avr11* locus, TDF3.1 and TDF3.4 are more mysterious. They fulfill all selection criteria but there are no matching *P. infestans* ESTs, and only TDF3.4 has an obvious homologue in the *P. sojae* proteome. These TDFs have recently been used as probes and markers to zoom in on the *Avr3b-Avr10-Avr11* locus and physical mapping showed that *Avr3b-Avr10-Avr11*-linked AFLP markers and the TDFs are located on the same BAC contig (R.H.Y.J., Rob Weide and F.G., unpublished).

For *Avr1* and *Avr2* no candidates were recovered. Previously we used the same mapping population and a similar pooling strategy to identify AFLP markers (van der Lee et al., 2001). Also in that study the selection for *Avr4*-and *Avr3b-Avr10-Avr11*-linked markers was much more successful. For *Avr2* this could be explained by the fact that it was not included in the BSA, only random markers were selected. In the present study the pooling for *Avr2* was not optimal which may have caused a lower efficiency. For *Avr1,* however, it is unclear why the screening was unsuccessful. In both studies the BSA screening resulted in the highest number of candidates for *Avr1* but just one AFLP marker (van der Lee et al., 2001) and none of the TDFs passed the next, more stringent selection steps.

Previous studies in *P. infestans* have demonstrated that cDNA-AFLP is a powerful technique that complements other expression profiling approaches such as EST sequencing (Avrova et al., 2003; Dong et al., 2004). Here we showed that cDNA-AFLP can be combined with BSA to find transcripts associated with particular phenotypes. Since the *Avr*-linked genetic markers and the *Avr*-associated TDFs were generated from the same mapping population we can now integrate the various gene discovery approaches to identify *P. infestans Avr* genes.

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Chapter 4

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

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

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Sequence data have been submitted to GenBank under accession numbers EF672354 and EF672355.

Abstract

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., 2005). 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., 2006).

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 F_1 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; **Chapter 3**). 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 F_1 progeny of a mapping population (cross 71) (Guo et al. 2006; **Chapter 3**). 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.

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 F_1 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.

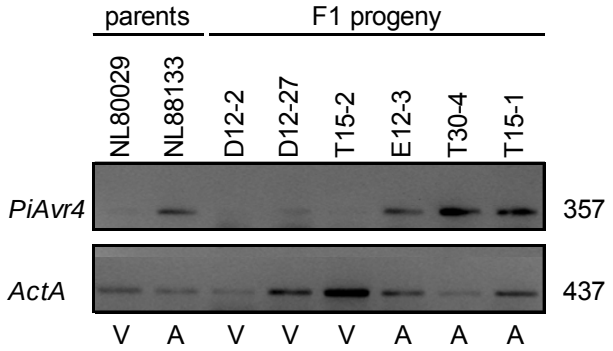

Fig. 1. Expression of a putative *PiAvr4* gene in the parental isolates NL80029 and NL88133, and seven F1 progeny. RT-PCR products obtained with primers specific for PH051G10 (upper panel) and the actin A gene *Act*A (lower panel), and total RNA isolated from germinated cysts as template. Amplicon sizes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

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

Previously, we used a mapping population of 76 F_1 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 F_1 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.

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).

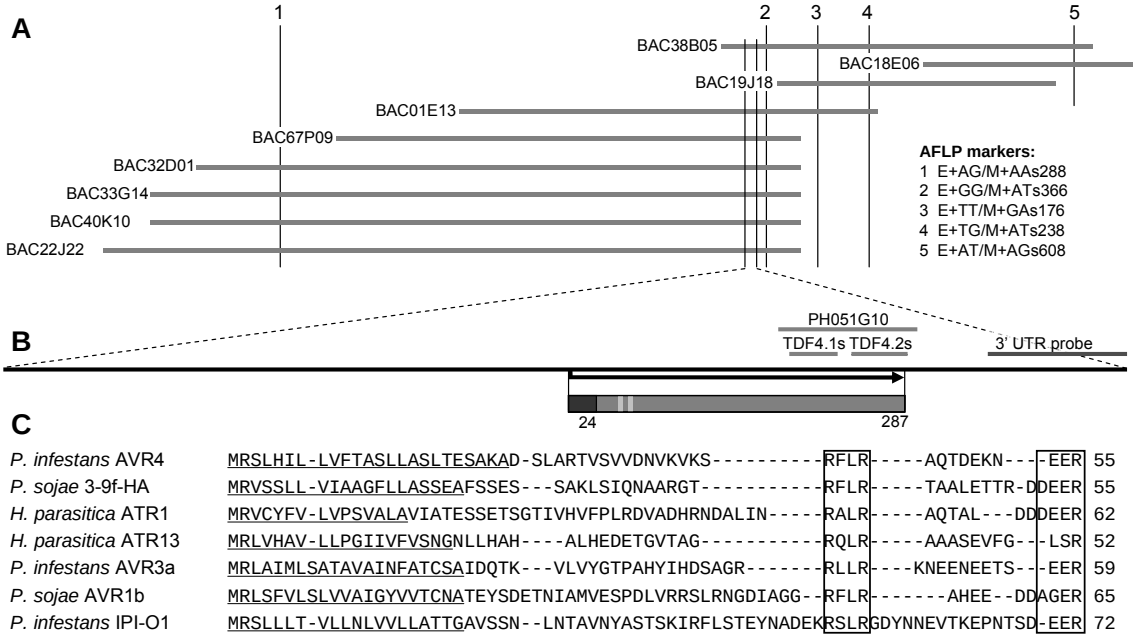

Fig. 2. **A.** BAC contig spanning the *Avr4* locus. The nine BACs were selected by screening the BAC library with *Avr4*-linked AFLP markers, indicated by 1-5. The sizes of the BACs are estimated and not drawn on scale. **B.** The 2890 bp *Bam*HI fragment carrying *PiAvr4*. The open reading frame of 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*, *Phytophthora sojae* 3-9f-HA (accession AY183415), *Hyaloperonspora parasitica* ATR1 (AY842877), *H. parasitica* ATR13 (AY785306), *P. infestans* AVR3a (AJ893357), *P. sojae* AVR1b-1 (AY426744), *P. infestans* IPI-O1 (L23939). Sequences were manually aligned. The RXLR and dEER motifs are boxed and the predicted SPs are underlined.

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.

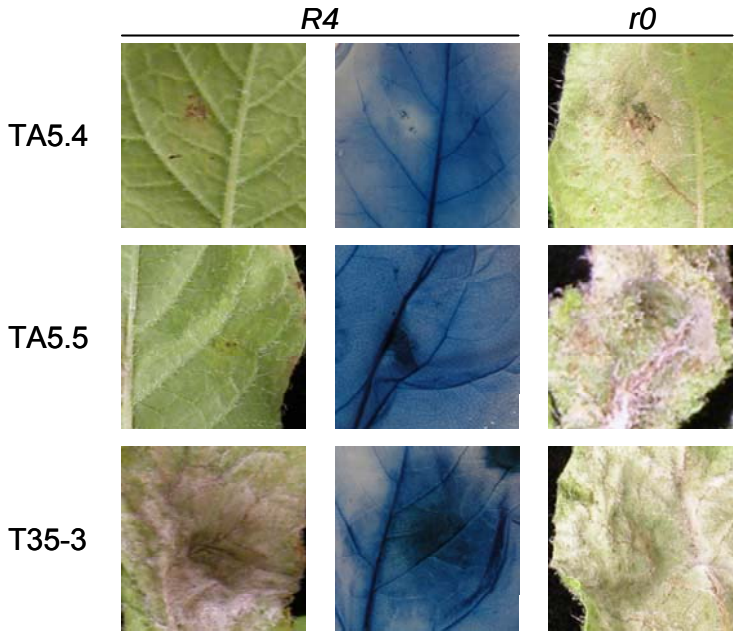

Fig. 3. Virulence phenotypes of recipient strain T35-3 and transformants complemented with *PiAvr4* (TA5.4 and TA5.5) on leaves of potato cultivar Bintje (*r0*) and Cebeco44-31-5 (*R4*). The middle column shows the *R4* leaves displayed in the left column after trypan blue staining. Leaves were spot-inoculated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

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 F_1 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 breeding line 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*.

Table 1. Infection efficiency (IE) and lesion growth rate (LGR) of *Phytophthora infestans* donor and recipient strains (F_1 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 the *PiAvr4* sequence.

[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. 6).

[e] IPO-0 contains virulent allele V and avirulent allele II (Fig.6).

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$^{1\text{-}287}$ resulted in an HR on *R4* plants within 13 days post-inoculation (dpi) whereas Bintje (*r0*) and the *R3a* potato breeding line 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*.

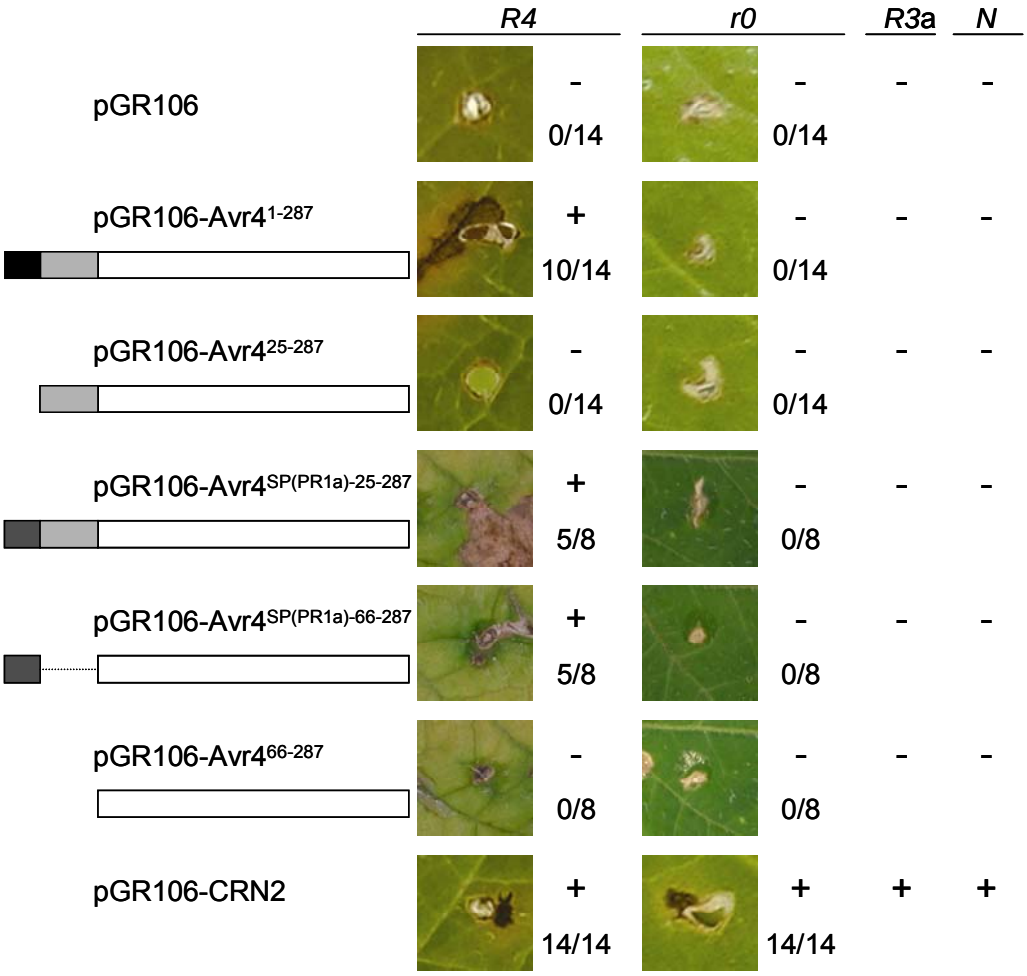

Fig. 4. Agroinfection of potato line Cebeco44-31-5 (*R4*) and cultivar Bintje (*r0*) by toothpick inoculation with strains carrying pGR106 constructs as indicated. + indicates a necrotic response and - indicates no visual response. The numbers show the ratio of '+' responses and the total number of toothpick inoculations in a typical experiment. Pictures were taken 18 dpi. Controls 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, is a universal elicitor that is used as positive control.

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-Avr4SP$^{(PR1a)-25-287}$ 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).

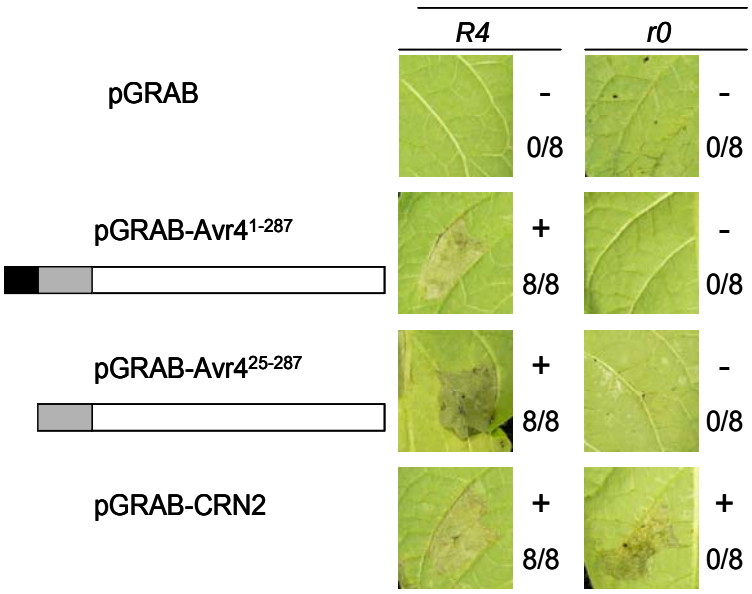

Fig. 5. Agroinfiltration of potato line Cebeco44-31-5 (*R4*) and cultivar Bintje (*r0*) with *A. tumefaciens* strains carrying pGRAB constructs as indicated. + indicates a necrotic response and - indicates no visual response. The numbers show the ratio of '+' responses and the total number of agroinfiltrations in a typical experiment. Pictures were taken 3 dpi. In the schematic representation of the constructs the SP of PiAvr4 is shown in black, 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 pGRAB-CRN2, is a universal elicitor used as positive control.

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$^{1-287}$, but there was no response upon inoculation with particles from pGR106-Avr4$^{25-287}$ 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$^{25-287}$ 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. 5). 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. 5). 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$^{1\text{-}287}$) or the construct with the PR1a SP (pGR106-Avr4$^{SP(PR1a)\text{-}25\text{-}287}$) 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$^{SP(PR1a)\text{-}66\text{-}287}$ resulted in a strong necrotic response specifically on *R4* plants. This response was comparable to the response with the full length construct pGR106-Avr4$^{1\text{-}287}$ or pGR106-Avr4$^{SP(PR1a)\text{-}25\text{-}287}$ (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, imply another re-uptake mechanism that is unknown, or indicate that some PiAVR4 is mis-targeted during secretion, and is retained in the cell.

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.

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 were detected.

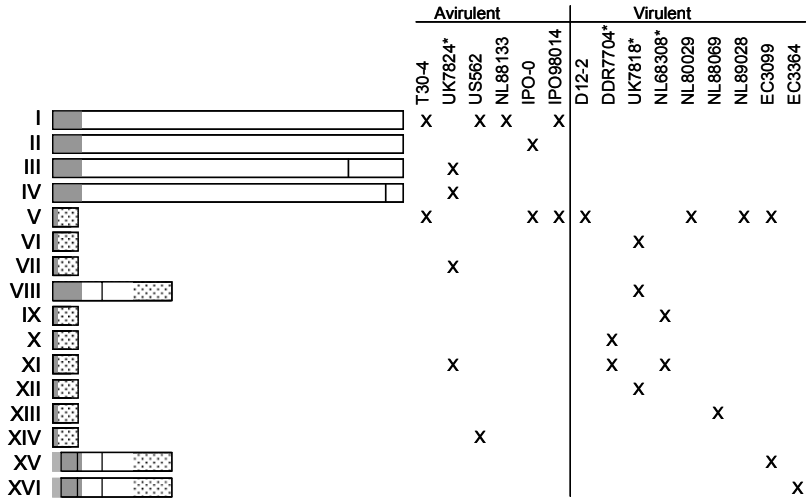

Fig. 6. *PiAvr4* alleles identified in *P. infestans* field isolates. Shown are the proteins encoded by sixteen different alleles numbered I-XVI of which the polymorphisms at the DNA level are provided as Supplementary Information (Figure S2). PiAvr4 encoded by allele I is shown as a bar with the signal 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.

PiAvr4 polymorphism in *P. 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 *Avr4* 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 *Avr4* 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. 6). 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 *Avr4* 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; **Chapter 3**) 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 *S. 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 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. 2008) 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. 2007) 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* AVRA10, 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 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* gene was indeed an *Avr* gene, they infiltrated Avr1b-1 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. (2006) 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. 2008). 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 F_1 progeny were described previously (Drenth et al., 1995; Guo et al., 2006; **Chapter 3**; Jiang et al., 2006; van der Lee et al., 1997). 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 glass beads (Ø 3 mm) were added to frozen mycelium and the mixture was grinded in a FastPrep® instrument (Qbiogene, Carlsbad, CA). Three phenol/chloroform extractions were performed, followed by an RNAse treatment and DNA precipitation. For RNA isolation, glass beads and TRIzol (Molecular Research Center, Inc.) 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) 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).

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., 2006). 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. DNA sequences 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* (FLUKA) (5 mg ml^{-1}) and cellulase from *Trichoderma reesei* (Sigma®) (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 breeding line 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 breeding line SW8540-025 (*R3a*) (Huang et al., 2005) 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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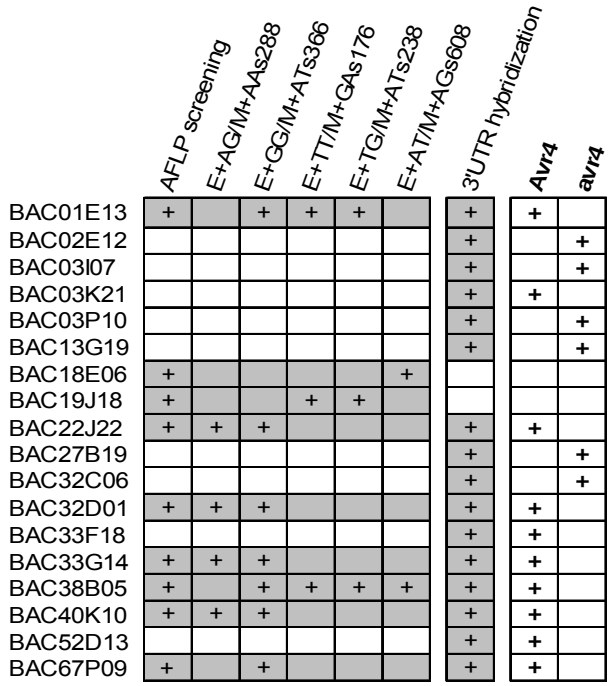
Supplementary Research Data

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 *P. infestans*, agroinfection and agroinfiltration.

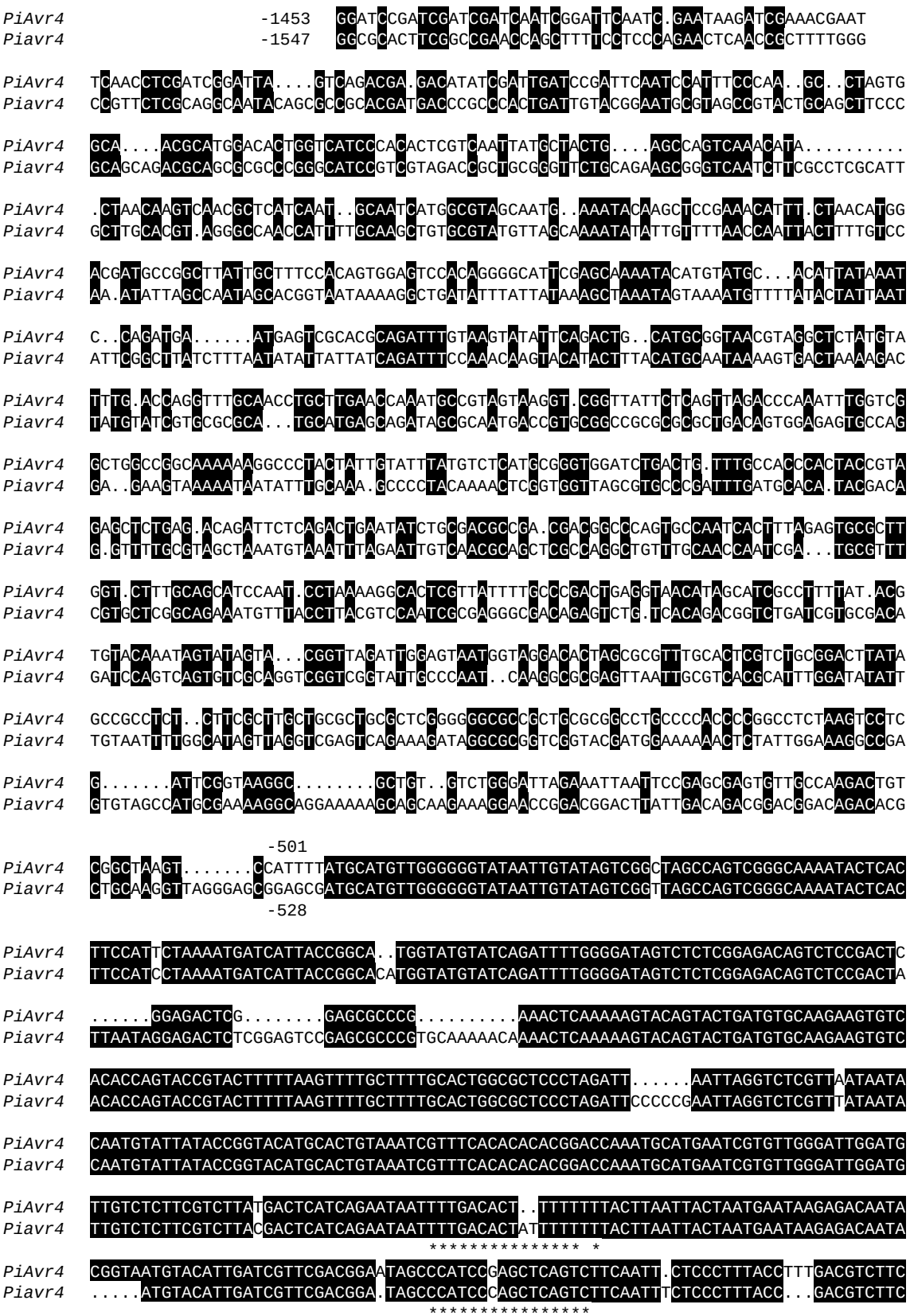
clone	insert	insert size	vector
pSKA23	BAC01E13 subclone	2889 bp	pBluescript SK$^-$
pSKB5	BAC38B05 subclone	2889 bp	pBluescript SK$^-$
pSKC21	BAC40K10 subclone	2889 bp	pBluescript SK$^-$
pSK7A2	BAC32C06 subclone	3824 bp	pBluescript SK$^-$
pTHA23.9	A23	2889 bp	pTH209SK
pGR106	-	0 bp	pGR106
pGR106-Avr4$^{1-287}$	PiAvr4$^{1-287}$	864 bp	pGR106
pGR106-Avr4$^{25-287}$	PiAvr4$^{25-287}$	792 bp	pGR106
pGR106-Avr4$^{SP(PR1a)-25-287}$	Pr1a signal peptide - PiAvr4$^{25-287}$	882 bp	pGR106
pGR106-PiAvr4$^{66-287}$	PiAvr4$^{66-287}$	669 bp	pGR106
pGR106-Avr4$^{SP(PR1a)-66-287}$	PR1a signal peptide - PiAvr4$^{66-287}$	759 bp	pGR106
pGR106-CRN2	CRN2	1371 bp	pGR106
pGRAB	-	0 bp	pGRAB
pGRAB-Avr4$^{1-287}$	PiAvr4$^{1-287}$	864 bp	pGRAB
pGRAB-Avr4$^{25-287}$	PiAvr4$^{25-287}$	792 bp	pGRAB
pGRAB-CRN2	CRN2	1371 bp	pGRAB

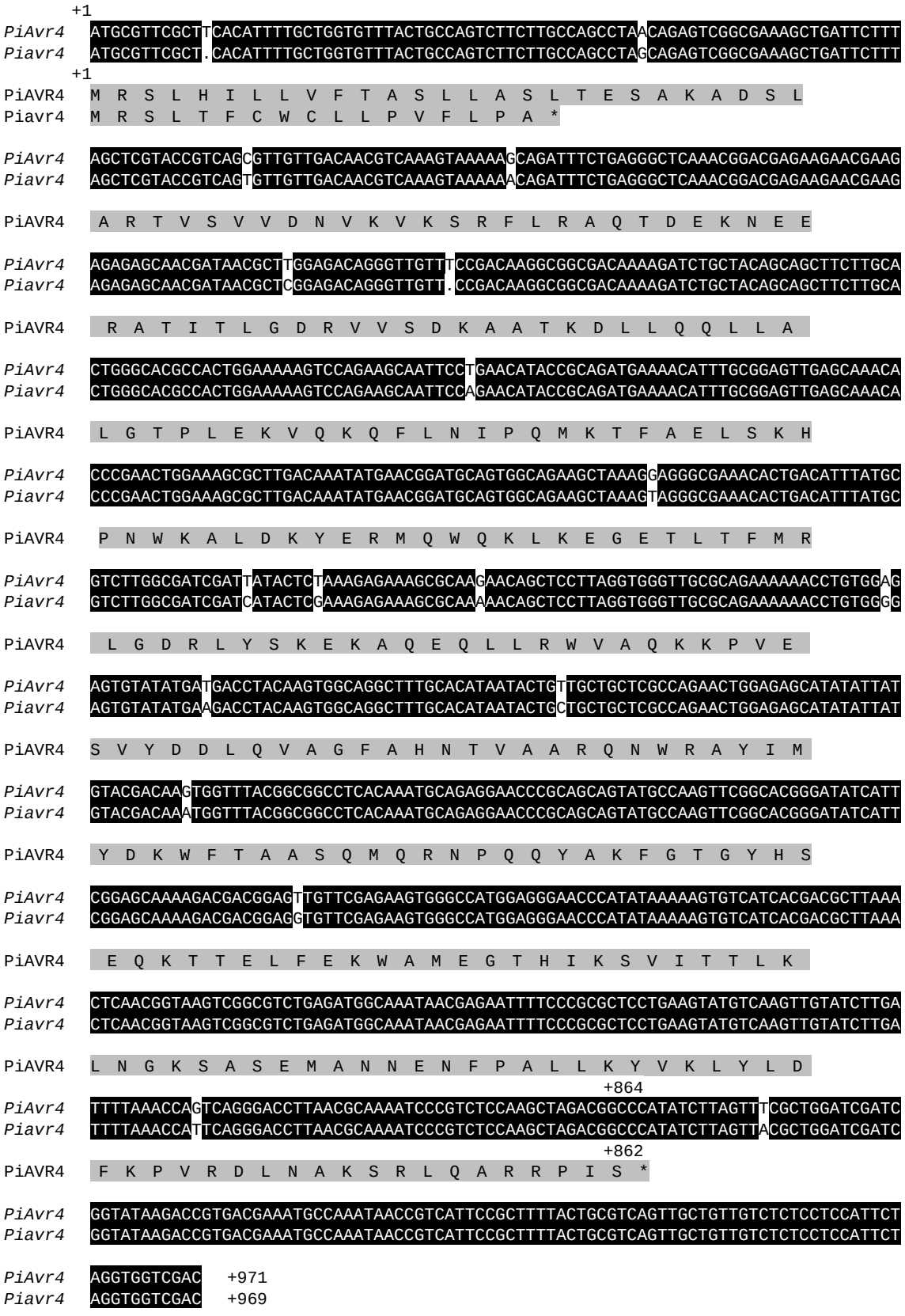
Table S4. Primers and templates used to generate pGR106 and pGRAB constructs by PCR (upper panel) and overlap PCR (lower panel).

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

Fig. 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.




+1
PiAvr4 ATGCGTTCGCTTCACATTTTGCTGGTGTTTACTGCCAGTCTTCTTGCCAGCCTAACAGAGTCGGCGAAAGCTGATTCTTT
Piavr4 ATGCGTTCGCT.CACATTTTGCTGGTGTTTACTGCCAGTCTTCTTGCCAGCCTAGCAGAGTCGGCGAAAGCTGATTCTTT
+1
PiAVR4 M R S L H I L L V F T A S L L A S L T E S A K A D S L
Piavr4 M R S L T F C W C L L P V F L P A *
PiAvr4 AGCTCGTACCGTCAGCGTTGTTGACAACGTCAAAGTAAAAAGCAGATTTCTGAGGGCTCAAACGGACGAGAAGAACGAAG
Piavr4 AGCTCGTACCGTCAGTGTTGTTGACAACGTCAAAGTAAAAAACAGATTTCTGAGGGCTCAAACGGACGAGAAGAACGAAG
PiAVR4 A R T V S V V D N V K V K S R F L R A Q T D E K N E E
PiAvr4 AGAGAGCAACGATAACGCTTGGAGACAGGGTTGTTTCCGACAAGGCGGCGACAAAAGATCTGCTACAGCAGCTTCTTGCA
Piavr4 AGAGAGCAACGATAACGCTCGGAGACAGGGTTGTT.CCGACAAGGCGGCGACAAAAGATCTGCTACAGCAGCTTCTTGCA
PiAVR4 R A T I T L G D R V V S D K A A T K D L L Q Q L L A
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Piavr4 CTGGGCACGCCACTGGAAAAAGTCCAGAAGCAATTCCAGAACATACCGCAGATGAAAACATTTGCGGAGTTGAGCAAACA
PiAVR4 L G T P L E K V Q K Q F L N I P Q M K T F A E L S K H
PiAvr4 CCCGAACTGGAAAGCGCTTGACAAATATGAACGGATGCAGTGGCAGAAGCTAAAGGAGGGCGAAACACTGACATTTATGC
Piavr4 CCCGAACTGGAAAGCGCTTGACAAATATGAACGGATGCAGTGGCAGAAGCTAAAGTAGGGCGAAACACTGACATTTATGC
PiAVR4 P N W K A L D K Y E R M Q W Q K L K E G E T L T F M R
PiAvr4 GTCTTGGCGATCGATTATACTCTAAAGAGAAAGCGCAAGAACAGCTCCTTAGGTGGGTTGCGCAGAAAAAACCTGTGGAG
Piavr4 GTCTTGGCGATCGATCATACTCGAAAGAGAAAGCGCAAAAACAGCTCCTTAGGTGGGTTGCGCAGAAAAAACCTGTGGGG
PiAVR4 L G D R L Y S K E K A Q E Q L L R W V A Q K K P V E
PiAvr4 AGTGTATATGATGACCTACAAGTGGCAGGCTTTGCACATAATACTGTTGCTGCTCGCCAGAACTGGAGAGCATATATTAT
Piavr4 AGTGTATATGAAGACCTACAAGTGGCAGGCTTTGCACATAATACTGCTGCTGCTCGCCAGAACTGGAGAGCATATATTAT
PiAVR4 S V Y D D L Q V A G F A H N T V A A R Q N W R A Y I M
PiAvr4 GTACGACAAGTGGTTTACGGCGGCCTCACAAATGCAGAGGAACCCGCAGCAGTATGCCAAGTTCGGCACGGGATATCATT
Piavr4 GTACGACAAATGGTTTACGGCGGCCTCACAAATGCAGAGGAACCCGCAGCAGTATGCCAAGTTCGGCACGGGATATCATT
PiAVR4 Y D K W F T A A S Q M Q R N P Q Q Y A K F G T G Y H S
PiAvr4 CGGAGCAAAAGACGACGGAGTTGTTCGAGAAGTGGGCCATGGAGGGAACCCATATAAAAAGTGTCATCACGACGCTTAAA
Piavr4 CGGAGCAAAAGACGACGGAGGTGTTCGAGAAGTGGGCCATGGAGGGAACCCATATAAAAAGTGTCATCACGACGCTTAAA
PiAVR4 E Q K T T E L F E K W A M E G T H I K S V I T T L K
PiAvr4 CTCAACGGTAAGTCGGCGTCTGAGATGGCAAATAACGAGAATTTTCCCGCGCTCCTGAAGTATGTCAAGTTGTATCTTGA
Piavr4 CTCAACGGTAAGTCGGCGTCTGAGATGGCAAATAACGAGAATTTTCCCGCGCTCCTGAAGTATGTCAAGTTGTATCTTGA
PiAVR4 L N G K S A S E M A N N E N F P A L L K Y V K L Y L D
+864
PiAvr4 TTTTAAACCACGTCAGGGACCTTAACGCAAAATCCCGTCTCCAAGCTAGACGGCCCATATCTTAGTTTCGCTGGATCGATC
Piavr4 TTTTAAACCATTCAGGGACCTTAACGCAAAATCCCGTCTCCAAGCTAGACGGCCCATATCTTAGTTACGCTGGATCGATC
+862
PiAVR4 F K P V R D L N A K S R L Q A R R P I S *
PiAvr4 GGTATAAGACCGTGACGAAATGCCAAATAACCGTCATTCCGCTTTTACTGCGTCAGTTGCTGTTGTCTCTCCTCCATTCT
Piavr4 GGTATAAGACCGTGACGAAATGCCAAATAACCGTCATTCCGCTTTTACTGCGTCAGTTGCTGTTGTCTCTCCTCCATTCT
PiAvr4 AGGTGGTCGAC +971
Piavr4 AGGTGGTCGAC +969

Fig. 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.

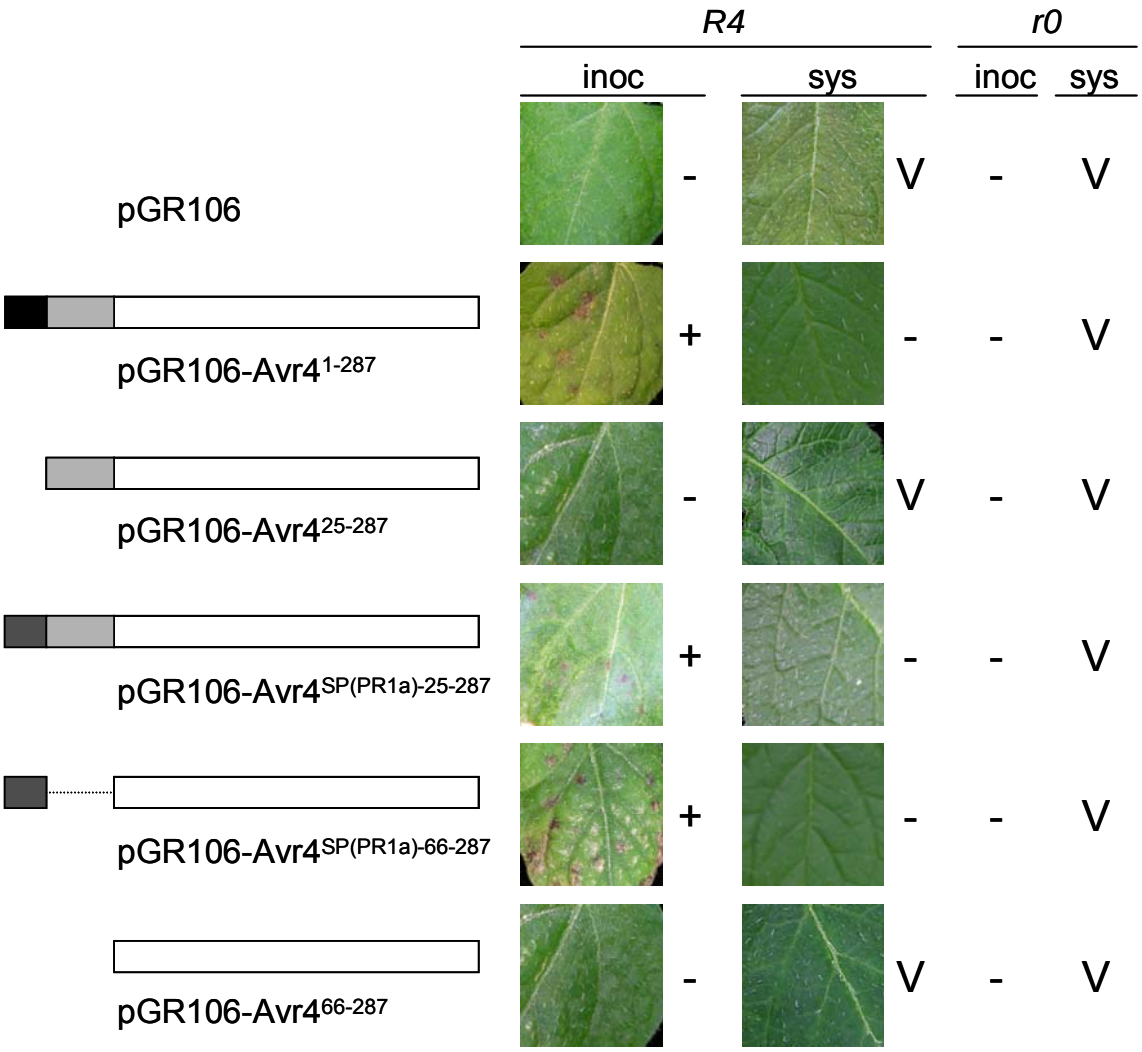

Fig. S3. Overview of the nucleotide variation in sixteen identified *PiAvr4* alleles. The positions of the nucleotides that are polymorphic are indicated in the top row. Position 1 is the A of the start codon ATG. A nucleotide deletion is indicated by -. Polymorphisms that give rise to a frameshift mutation 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.

	nucleotide position																	
allele	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

Chapter 5

Landing of *Avr1*-associated markers on the *Phytophthora infestans* genome narrows down a 800 kb genomic interval that carries seven RXLR effector genes

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Landing of *Avr1*-associated markers on the *Phytophthora infestans* genome narrows down a 800 kb genomic interval that carries seven RXLR effector genes

Abstract

With the aim to clone the avirulence gene *Avr1* in the potato late blight pathogen *Phytophthora infestans*, we used several independent strategies to find markers associated with the target gene. Linkage mapping combined with Bulked Segregant Analysis (BSA) resulted in four *Avr1*-linked AFLP markers, one of which was used for screening a genomic BAC library. Affymetrix® array expression profiling combined with BSA resulted in three array clones showing *Avr1*-associated expression profiles. By anchoring the markers on the *P. infestans* genome sequence we could delineate a region of 800 kb on supercontig 51 containing several of the *Avr1*-associated markers, i.e. one BAC end sequence, one AFLP marker and two array clones. BLAST searches and HMM screening of that region revealed seven RXLR effector genes that are likely candidates for *Avr1*. These seven were further characterized based on bioinformatic predictions such as Hidden Markov Model scores of the RXLR motif and surrounding regions, and the occurrence and numbers of W, Y and L motifs in the C-terminal region. Combining just a few *Avr1*-associated markers directly with the genome sequence and a RXLR motif search diminished the need for chromosome walking and allowed us to swiftly identify a limited number of candidate genes. Functional screening of the candidates using in planta expression assays is in progress.

Introduction

Potato late blight, caused by the oomycete pathogen *Phytophthora infestans*, is the most devastating disease in potato worldwide. So far attempts to introduce durable late blight resistance into potato cultivars by breeding have been largely unsuccessful and therefore, control still heavily relies on chemical crop protection. In recent years insight into the mechanisms that govern disease resistance and susceptibility in plants has increased rapidly and many resistance (*R)* genes and defense related genes have been identified. Also strategies that pathogens use to suppress defense or to circumvent recognition by the host are gradually being unravelled. Whole genome sequencing of plant pathogens has already resulted in many fascinating discoveries, in particular in the case of oomycetes (Govers and Gijzen, 2006; Bouwmeester et al., 2008). One example is the finding that *Phytophthora* species have very large families of proteins that share a conserved motif named RXLR-dEER. This was first reported by Tyler et al. (2006) for *Phytophthora sojae* and *Phytophthora ramorum* and more recently, Whisson et al. (2007) and Jiang et al. (R.H.Y. Jiang, personal communication) made an inventory of an even larger RXLR family in *P. infestans* (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans). These RXLR superfamilies appear to be very rapidly evolving families (Jiang et al., 2008), the conserved N-terminal domain that comprises a signal peptide signature and the RXLR-dEER domain is combined with a highly variable C terminal domain than shows extensive sequence divergence. Nevertheless, the majority of the RXLR family members are all related and likely evolved from a common ancestor by rapid duplication and divergence. More than half contain conserved motifs in the C-terminal domain termed W, Y and L. These motifs are usually arranged as a module that can be repeated up to eight times (Jiang et al. 2008).

At about the same time that the large RXLR families were discovered the first few oomycete avirulence (*Avr*) genes were cloned, including *Avr1b*-1 from *P. sojae* (Shan et al., 2004), $ATR1^{NdWsB}$ (Rehmany et al., 2005) and *ATR13* (Allen et al., 2004) from the downy mildew *Hyaloperonospora parasitica,* and *Avr3a* (Armstrong et al., 2005) from *P. infestans.* Similar to fungal *Avr* genes, all oomycete *Avr* genes encode different proteins but unlike fungal Avr proteins all oomycete Avr proteins share the conserved RXLR domain (Kamoun, 2006). According to the gene-for-gene hypothesis *Avr* genes encode avirulence factors that are directly or indirectly recognized by proteins encoded by resistance (*R*) genes. This recognition triggers a hypersensitive response (HR) and pathogen growth is arrested. In the absence of the cognate *R* gene the Avr factor may function as an effector that contributes to the virulence of the pathogen, for example by suppressing basal defense responses inside host cells. It is therefore anticipated that many of the effectors are targeted to the host cell. Recently Whisson et al. (2007) proved that indeed the RXLR motif in Avr3a functions as a targeting signal to translocate this effector into host cells. Moreover, Avr3a as well as *P. sojae* Avr1b have been shown to suppress cell death responses elicited by the necrosis inducing proteins INF1 and BAX, respectively (Bos et al., 2006; Dou et al., 2008).

The cognate *R* genes that recognize oomycete RXLR proteins as avirulence factors and that have been cloned to date, all encode R proteins of the CC-NB-LRR class (coiled coil - nucleotide binding - leucine rich repeat). These include five late blight resistance genes, *R1* (Ballvora et al., 2002), *R3a* (Huang et al., 2005), *RB* or *Rpi-blb1* (Song et al., 2003; van der Vossen et al., 2003), *Rpi-blb2* (van der Vossen et al., 2005) and *Rpi-sto1* (Vleeshouwers et al., 2008). Although *R1* was the first late blight *R* gene to be cloned (Ballvora et al., 2002) *Avr1* has not yet to be isolated. *R1* is located within a hot spot for pathogen resistance on chromosome V of potato (Ballvora et al., 2002). Studies on the authentic *R1* resistance gene cluster in the hexaploid *Solanum demissum,* the species used for introgressing late blight resistance into potato, revealed high divergence among three haplotypes and a dynamic evolution with respect to gene copy numbers and sequence exchange between paralogs (Kuang et al., 2005). By anticipating that *Avr1* is an RXLR effector that belongs to the highly dynamic and rapidly evolving RXLR superfamily, the *R1-Avr1* pair could be an ideal model to study co-evolution between potato and *P. infestans*.

Previous work in our laboratory involved the identification of polymorphic DNA markers linked to *P. infestans Avr* genes with the aim to use these markers to initiate map-based cloning (van der Lee et al., 2001). For two *Avr* loci, this approach was successful and resulted in the cloning of the RXLR effector gene *PiAvr4* (van Poppel et al., 2008; **Chapter 4**) and the identification of *Pi3.4*, a gene located at the *Avr3b-Avr10-Avr11* locus and encoding a putative transcription factor that shows copy number variation associated with the phenotype (Jiang et al., 2006). For *Avr1* and *Avr2* the map positions were determined but the markers were too far away to start chromosome walking. We therefore initiated a new search for *Avr1*-linked markers by making use of alternative restriction enzymes for AFLP fingerprinting and Bulked Segregant Analysis (BSA) and obtained two new markers. To circumvent the search for more recombinants and more closely linked genetic markers, and to avoid a laborious map-based cloning procedure, we took advantage of the *P. infestans* genome sequence. We collected the sequences of all the potential *Avr1*-associated markers that we had gathered over the years and scanned the genome sequence for matches. These included AFLP markers from linkage mapping (van der Lee et al., 2001; van der Lee et al., 2004; **Chapter 2**), BAC-end sequences from BACs selected by marker landing (Whisson et al., 2001) and transcriptome markers obtained by cDNA-AFLP (Guo et al., 2006; **Chapter 3**) and Affymetrix® array hybridizations (Jiang et al., 2006). In this way we could narrow the genomic interval covering the *Avr1* locus to an 800 kb region. By assuming that the protein encoded by *Avr1* belongs to the RXLR-dEER superfamily we reduced the number of candidate *Avr1* genes to seven.

Materials and methods

P. infestans strains, and nomenclature of phenotypes and markers

The *P. infestans* strains used in this study are the A1 mating type isolate NL80029, the A2 mating type isolate NL88133 and F_1 progeny of these two strains, designated as cross 71.

Characterization and phenotypic analyis of the cross 71 mapping population was described previously (Drenth et al., 1995; van der Lee et al., 1997; van der Lee et al., 2001). F_1 progeny strain T30-4 was used to construct a BAC library (Whisson et al., 2001). This nomenclature of phenotypes is according to van der Lee et al. (2001). Strains NL80029 and T30-4 that are avirulent on plants carrying *R1* have the AVR1 phenotype whereas NL88133 is virulent and has the avr1 phenotype. The nomenclature of the AFLP markers is according to van der Lee et al. (1997).

AFLP DNA fingerprinting

DNA isolation and AFLP DNA fingerprinting was essentially performed as described by van der Lee et al. (1997) using the restriction enzyme combination *Pst*I/*Hha*I for the primary template preparations and primers with an extension of two bases for the selective amplification.

Bulked Segregant Analysis

To select AFLP markers linked to *Avr* genes bulked segregant analysis (BSA) was performed essentially as described by Michelmore et al. (1991). To find markers linked to *Avr1* we composed six pools of strains with different phenotypes. The pools consisting of F_1 progeny and parental strains of cross 71 are listed in Table 1. Poo1s 1, 3 and 5 represented the avirulent phenotype AVR1 and pools 2, 4 and 6 the virulent phenotype avr1. Primary templates of the 3 individuals that made up one pool were mixed in equal amounts based on the quantity and quality check, and served as template for the pre-amplification. In the selective amplification 240 *Pst*I+2/*Hha*I+2 primer combinations were used. Candidate markers were identified visually from the fingerprints obtained on the pooled DNA and were tested on the individual progeny of each pool. Markers showing good correlation were further tested on all available progeny of cross 71.

Table 1. Composition of DNA pools used for AFLP fingerprinting and RNA pools for hybridization of the *Phytophthora* GeneChip.

pool	1	2	3	4	5	6
DNA from strains	D12-9 T30-4 T15-7	E12-3 T20-2 T15-2	NL80029 D12-17 T15-9	NL88133 D12-21 T80-3	re11-16 T15-1 T30-2	D12-23 T35-3 T35-4 D12-2
phenotype	A*	V*	A	V	A	V
pool	1	2	3	4	5	6
RNA from strains	re11-16 T15-1 T30-2	D12-2 D12-23	D12-17 T15-9	T20-2 E12-3	T35-3	T30-2
phenotype	A	V	A	V	V	A

* A, Avirulent phenotype; V, Virulent phenotype.

BAC library screening

The *P. infestans* BAC library was screened with *Avr1*-linked AFLP markers obtained by van der Lee et al. (2001), and a three-dimensional pooling strategy previously described by Whisson et al.

(2001). Selected BACs were further analysed by restriction analyses and the BAC ends were sequenced according to standard procedures.

The *Phytophthora* GeneChip

The Syngenta custom designed *Phytophthora* GeneChip is an Affymetrix® array containing 19,324 unique sequences of which 18,256 represent unigenes. The sequences were generated from a large scale EST project and represent 75,757 ESTs obtained from libraries representing a wide range of growth conditions, stress responses, and developmental stages (Randall et al., 2005). Over 82% of the sequences on the GeneChip are from *P. infestans* (Judelson et al., 2008).

A BSA approach for selecting transcriptome markers

To select transcriptome markers associated with *Avr* genes the custom designed *Phytophthora* GeneChip was hybridized using a BSA approach. RNA of F_1 progeny with similar or overlapping AVR phenotypes was pooled. Six pools, composed as described in Jiang et al. (2006) and listed in Table 1, were used to probe the *Phytophthora* GeneChip. cDNA synthesis, array hybridization, and intensity normalization were performed similar to the methods described by Zhu et al. (2001) and Judelson et al. (2008). Array clones showing at least a two-fold induction of intensity with RNA pools derived from avirulent isolates as compared to RNA pools from virulent isolates were chosen as candidates.

Results and discussion

AFLP markers co-segregating with the *Avr1* locus

Previously van der Lee et al. (2004) identified two *Avr1*-linked AFLP markers in the cross 71 population at a distance of 8 and 9 cM, respectively, from the *Avr1* locus. To select additional *Avr1*-linked AFLP markers we adapted the AFLP procedure by changing the restriction enzymes to generate the primary templates for amplification. We choose a combination of *Pst*I and *Hha*I that reveals a higher level of polymorphisms in *P. infestans* coding regions than *Eco*RI and *Mse*I (Dong et al., 2004). AFLP fingerprints were generated from six pooled templates, three represented the avirulent phenotype, AVR1, and three the virulent phenotype, avr1. A total of 240 *Pst*I+2 / *Hha*I+2 primer combinations was used. Over 24,000 AFLP fragments were analysed and this resulted in five markers that showed association with *Avr1* in the pools (Figure 1). These five candidates, i.e. AP+GT/H+TAs161, AP+TA/H+GAs130, AP+GC/H+TGs340, AP+AC/H+CCs218 and AP+AC/H+CCs156, were then analysed on the individuals of the pools and on other F_1 progeny of cross 71. Two of the five showed absolute co-segregation with the AVR/avr phenotypes in the F_1 progeny. Markers AP+GT/H+TAs161 and AP+TA/H+GAs130 were present in the avirulent parent NL80029 and avirulent progeny but absent in the virulent parent NL8813 and the virulent progeny (Figure 1C). These two AFLP markers were cloned and sequenced. Linkage analysis between the two *Eco*RI/*Mse*I markers generated previously by van

der Lee et al. (2004) (HE+TG/M+TGf6 and AE+CG/M+TGs317) and the two markers selected here showed 100% co-segregation in 40 progeny.

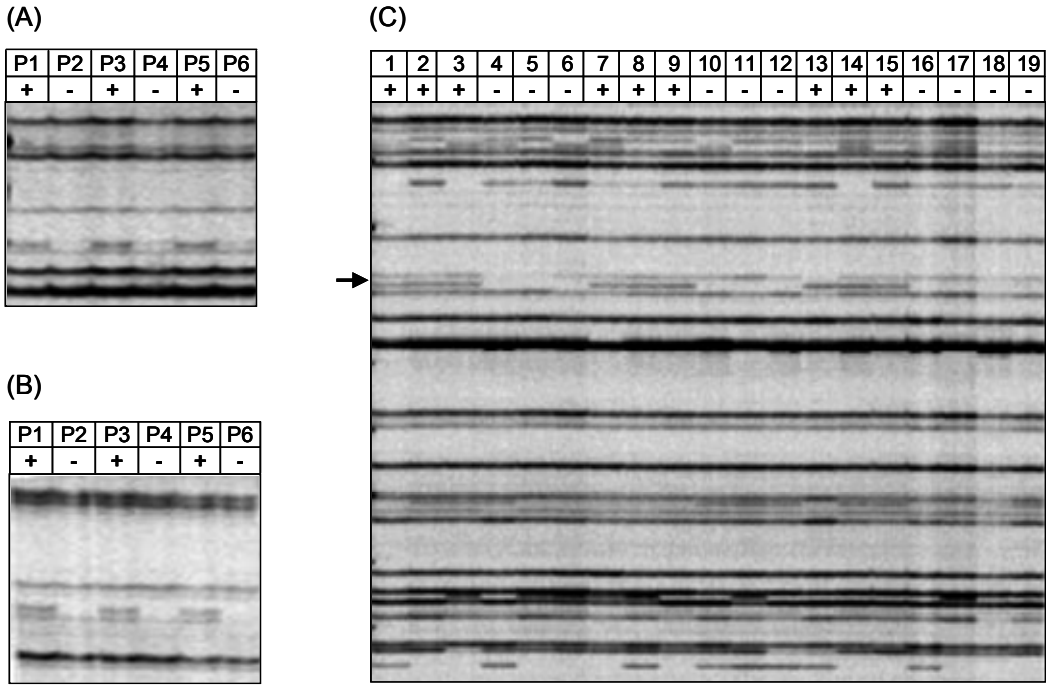

Figure 1. **Segregation of *Avr1*-linked markers in cross 71**. AFLP fingerprints of six BSA pools generated with primer combination P+GT and H+GA (A) and P+GT and H+TA (B). Markers AP+GT/H+TAs161 (A) and AP+TA/H+GAs130 (B) are present in the avirulent pools P1, P3 and P5 and absent in the virulent pools P2, P4 and P6. (C) shows AFLP fingerprints of individual strains generated with primer combination P+GT and H+TA. The templates are made from DNA isolated from the A1 parent NL80029 (in lane 7), the A2 parent NL88133 (in 10) and cross 71 F_1 progeny D12-9 (1), T30-4 (2), T15-7 (3), E12-3 (4), T20-2 (5), T15-2 (6), D12-17 (8), T15-9 (9), D12-21 (11), T80-3 (12), re11-16 (13), T15-1 (14), T30-2 (15), D12-23 (16), T35-3 (17), T35-4 (18) and D12-2 (19). Avirulent and virulent phenotypes are indicated by + and -, respectively. The arrow points to AFLP marker AP+GT/H+TAs161 that co-segregates with the AVR1 phenotype.

Avr1-linked AFLP markers land on one BAC contig

The two *Avr1*-linked AFLP markers that were previously generated by van der Lee et al. (2001; 2004) were used to screen a *P. infestans* BAC library of the AVR1 F_1 progeny T30-4 with a 10x genome coverage. Screening with AE+CG/M+TGs317 and HE+TG/M+TGf6 revealed three BACs carrying both markers and three BACs carrying one of the two. These six BACs (9I5, 11M15, 63M8, 64E1, 66P7 and 72B22) could be assembled into one contig of approximately 150 kb (data not shown). Of four of these BACs, the ends of the inserts were sequenced.

Transcriptome markers associated with *Avr1*

Previously, we used a cDNA-AFLP-based strategy to identify transcripts in germinating cysts that are associated with *Avr* genes in *P. infestans*. In a BSA approach pools of cDNA consisting

of F_1 progeny of a mapping population segregating for virulence and avirulence on *R1* plants, were used as template for cDNA-AFLP analysis. When the 28 Transcript Derived Fragments (TDFs) that were only present in the avirulent pools but not in the virulent pools were tested on the individuals in the pools and from the mapping population, none co-segregated with the AVR1 phenotype (Guo et al., 2006; **Chapter 3**). To find additional transcriptome markers for the *Avr1* locus we made use of the *Phytophthora* GeneChip (Judelson et al., 2008) and designed another BSA expression profiling experiment. Pooled RNA samples (Table 1), all derived from germinating cysts, were used as probes on the *Phytophthora* GeneChip. Array sequences with more than two fold higher expression in avirulent pools as compared to virulent pools were selected as *Avr1*-associated transcripts. Among 18,256 array sequences, three candidates Pi009420, Pi010367 and Pi015114 were found (Figure 2).

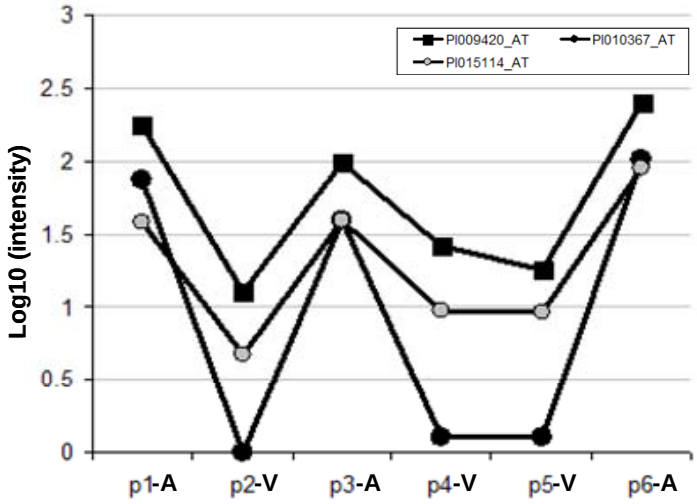

Figure 2. **Expression profiles of three array sequences with increased expression in AVR1 strains**. The *Phytophthora* GeneChip was hybridized with RNA from germinating cysts pooled (p1-p6) from virulent (V) and avirulent (A) strains. The strains represented in the six pools are listed in Table 1. The Y-axis shows the log10 of the hybridization intensity. The intensity fold change in p1, p3 and p6 (avirulent) as compared to p2, p4 and p5 (virulent) is 9.2 for PI00920, 7 for PI015114 and 63 for PI010367.

Multiple markers land on one supercontig

With the DNA sequences of the various *Avr1*-associated markers at hand we performed BLAST searches on the *P. infestans* genome sequence and found that the majority of the markers land on the same supercontig (Table 2). One AFLP marker, one BAC end sequence and two transcriptome markers all landed on supercontig 51. The genetic interval that spans the markers is around 800 kb (Figure 3) and it is very likely that the *Avr1* locus is located in this region.

Table 2. Sequences of multiple markers resulting from genetic mapping, transcriptional profiling and BAC marker landing anchor the *Avr1* locus to supercontig 51.

Markers	DNA sequence available	Size (bp)	Selection criteria	Targeted supercontig*
AFLP marker E+CG/M+TGs317	no		Linked to *Avr1* on linkage map	n.d.
AFLP marker E+TG/M+TGf6	no		Linked to *Avr1* on linkage map	n.d.
AFLP marker P+GT/H+TAs161	cloned AFLP fragment	161	co-segregates with AVR phenotype in BSA	51
AFLP marker P+TA/H+GAs130	cloned AFLP fragment	130	co-segregates with AVR Phenotype in BSA	32
BAC 72B22	BAC end sequence 72B22F	291	BAC selected with AFLP marker E+TG/M+TGf6	51
EST Pi009420	GeneChip array clone	150	differential expression in AVR pools	51
EST Pi010367	GeneChip array clone	157	differential expression in AVR pools	51
EST Pi015114	GeneChip array clone	134	differential expression in AVR pools	16

n.d. not determined

* see http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.html for the *P. infestans* genome sequence, assembly 1.

The 800 kb region carries seven RXLR effector genes

Supercontig 51 has a length of 1175kb. The 800 kb region that is delineated by the *Avr1*-associated markers on supercontig 51 carries 93 gene models of which 12 encode a secreted protein. Since all but one of the oomycete *Avr* loci that have been identified to date by positional cloning encode an RXLR effector we focused on finding all potential RXLR effector genes located within the 800 kb region. By using recursive BLAST searches and HMM screens we could identify seven RXLR-dEER effector genes (Table 3; Figure 3). Five are located within a 300 kb region and can be assigned to two subgroups based on high sequence homology (>70%). PiRXLRdEER_173, PiRXLRdEER_198, and PiRXLRdEER_179 form one subgroup and PiRXLRdEER_70 and PiRXLRdEER_300 another. PiRXLRdEER_386 is located at the very end of the 800 kb region and does not have sequence homology to any of the other RXLR effector genes in this region or on this supercontig. The latter is also true for PiRXLRdEER_301.

Table 3. The seven RXLR genes located within the targeted 800 kb region on supercontig 51.

Candidate *Avr1* gene	**Coordinates on supercontig 51**	**RXLR-dEER motif and surrounding amino acid residues**	**HMM score of RXLR deer motif** [1]	**Size of the protein (aa)**	**Number of W-Y-L motifs** [2]
PiRxLRdEER_386	374802-375425	RGGGARQLRTATMSDDEARVSKLP	9	208	W(2) Y(1)
PiRxLRdEER_173	708835-710868	ASSSTRLLRKNSTVDLVGEERAPSIV	16	678	W(7) Y(6) L(6)
PiRxLRdEER_198	757631-758125	QNSSTKLLRYTDAVDIVDEERAPILE	15	165	W(1) Y(1) L(1)
PiRxLRdEER_179	853408-854931	ASSSTRLLRKNSTVDLVGEERAPSVV	16	508	W(5) Y(5) L(3)
PiRxLRdEER_70	889786-890511	YATTERLLRAHSSDKEEQKEEEERAISIN	20	242	W(2) Y(1) L(1)
PiRxLRdEER_300	910183-910908	SVTTKRLLRAHISGKEEGTEQEEQRGISIN	12	242	Y(1) L(1)
PiRxLRdEER_301	943349-943864	AIGNTRYLRAQPSNQEDEDRSFAVL	12	172	none

[1] The HMM of the RXLRdEER motif is derived from RXLRdEER regions in 1307 RXLRdEER proteins in *P. sojae*, *P. ramorum* and *P. infestans*

[2] The number of each identified motif identified in the C terminal domain is listed in brackets.

The sizes of the proteins encoded by the seven RXLR genes vary from 165 to 678 amino acids. The size difference is mostly determined by the number W, Y and L motifs in the C terminal domain (Table 3). Most RXLR effectors have their own characteristic composition of W, Y and L motifs and these numbers can vary even between RXLR effectors that belong to the same subgroup (Jiang et al., 2008). PiRXLRdEER_173, for example, has as many as seven W domains whereas PiRXLRdEER_198 has only one.

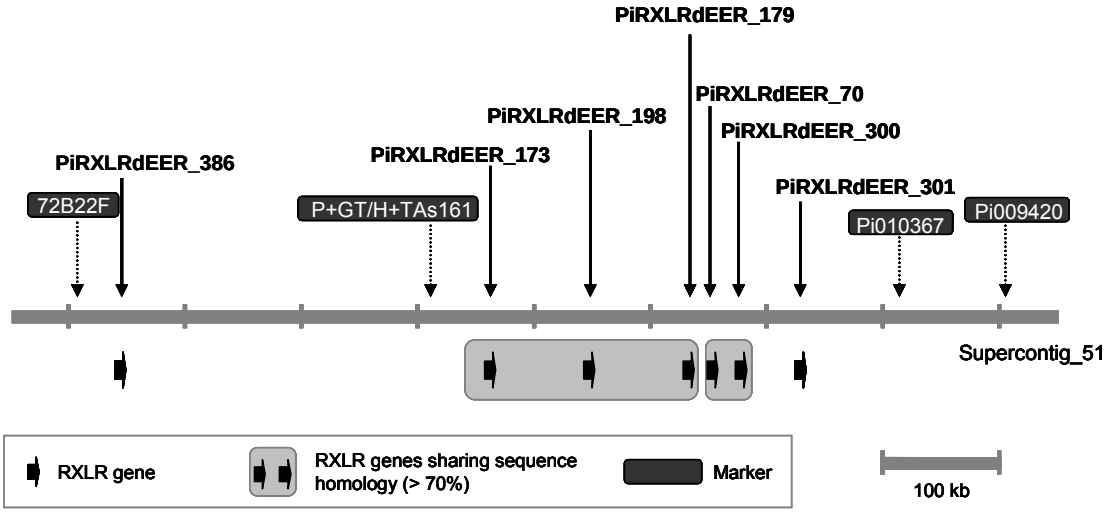

Figure 3. **Four *Avr1*-associated markers can be anchored to *P. infestans* supercontig 51 and delineate a region that contains seven RXLR genes**. The markers are a BAC end sequence (72B22F), an AFLP marker (P+GT/H+Tas161) and two array clones (Pi010367 and Pi009420). Two subgroups of RXLR genes (shaded) are assigned based on sequence similarity.

Conclusions

In this study, we successfully exploited the *P. infestans* genome sequence to assign candidate genes for the avirulence gene *Avr1*. Various types of *Avr1*-associated markers were targeted on the *P. infestans* genome and the genomic interval that covers the *Avr1* locus was narrowed down to 800 kb. By assuming that most, if not all, oomycete avirulence genes belong to the RXLR superfamily, we scanned the 800 kb region for RXLR genes. The seven candidates that were found, differ in length and in the number of W, Y and L motifs in their C terminal domains and also the HMM score of the RXLR-dEER motifs varies. T30-4, the strain that is sequenced, is avirulent on *R1* plants. Preliminary analyses of the seven candidate genes in the virulent and avirulent parent of the mapping population and in other strains with known AVR1 phenotype revealed that all seven are more or less polymorphic and none of the candidates stood out as the one being the most likely candidate for *Avr1*. One way to determine which of the seven is *Avr1* is a functional screening. To this end the various alleles of the seven candidate genes have to be cloned and sequenced, not only from T30-4 but also from the parental isolates and several field

isolates. Subsequently, the different alleles have to be expressed *in planta* using agroinfiltration (ATTA, *Agrobacterium tumefaciens* Transient Expression Assay) or agroinfection (with binary PVX constructs) and responses in the absence and presence of the *R1* gene have to be monitored. For the functional screening one can use agroinfiltration or agroinfection of potato differentials carrying *R1* or one can co-infiltrate *Nicotiana benthamiana* with two constructs, one carrying a cloned RXLR allele and the other carrying *R1*. These experiments are in progress.

Previous studies have demonstrated that combining genetic markers, transcriptome markers and BAC marker landing is a powerful approach to target *Avr* loci in *P. infestans* and to identify the genes (Jiang et al. 2006; van Poppel et al. 2008; **Chapter 4**). Here we focused on an *Avr* gene for which we could not easily find closely linked genetic markers (van der Lee et al., 2001; 2004) nor any transcriptome marker (Guo et al., 2006; **Chapter 3**). By taking advantage of a whole genome sequence, a GeneChip that allowed a more genome wide expression profiling, and a candidate gene approach focused on RXLR genes we have now narrowed down the number of candidate *Avr1* genes to seven and soon we will be able to report which of the seven is *Avr1*.

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Chapter 6

Phytophthora infestans isolates from Northern China show high virulence diversity but low genotypic diversity

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Phytophthora infestans isolates from Northern China show high virulence diversity but low genotypic diversity

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Abstract

Phenotypic and genotypic characteristics of 48 *Phytophthora infestans* isolates, collected in five provinces in Northern China during the period 1997 to 2003, were determined and compared with reference isolates. Characterization included mating type, virulence, mitochondrial DNA (mtDNA) haplotype and DNA fingerprinting patterns based on simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP). All isolates had the A1 mating type, mtDNA haplotype IIa and an identical SSR genotype (designated as SG-01-01) that differed from the SSR genotypes found in the reference isolates, including the ones representing the 'old' US-1 lineage that dominated the worldwide *P. infestans* population prior to 1980. In contrast, the virulence spectra were highly variable and virulence to all resistance genes present in the standard differential set (*R1* to *R11*) was found. AFLP analysis revealed some diversity; eight different AFLP genotypes were found that could be grouped into two major clusters. This study shows that there is very little genotypic diversity in the *P. infestans* population in Northern China. The occurrence of many different races within this rather uniform population is discussed in the framework of recently gained insights into the molecular determinants of avirulence in potato-*P. infestans* 'gene-for-gene' interactions.

Introduction

Late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is the most disastrous disease on potato worldwide and also the greatest threat to potato production in China (Govers and Latijnhouwers 2004; Zhang and Wang 2001). Before the 1980s, the global population of *P. infestans* was dominated by a single clonal lineage known as US-1 (Goodwin *et al.* 1994). Isolates with the US-1 genotype are of the A1 mating type, and have the mitochondrial DNA (mtDNA) haplotype Ib, allozymes *Gpi 86/100* and *Pep 92/100* and a characteristic RG57 pattern as deduced from RFLP analysis (Goodwin *et al.* 1994). The US-1 clonal population is commonly referred to as the 'old' population. In the early 1980s the first A2 mating type isolates were detected in Europe (Hohl and Iselin, 1984). This was due to migrations presumably initiated in 1976 by import into Europe of potatoes originating in Mexico, the center of diversity of *P. infestans* (Fry *et al.* 1993). In the eighties and nineties several studies revealed that the *P. infestans* population in Europe had become more diverse. Genotypic and phenotypic characterization of *P. infestans* isolates showed the coexistence of the two mating types, A1 and A2, within populations and the occurrence of many different genotypes (Drenth *et al.* 1994; Zwankhuizen *et al.* 2000; Cooke *et al.* 2003; Day *et al.* 2004). The 'new' population had rapidly replaced the 'old' US-1 population and the new and more diverse genotypes gradually migrated eastward to Eurasia and farther to the Far East and Japan (Fry *et al.* 1993; Koh *et al.* 1994; Elansky *et al.* 2001). Overall, the 'new' genotypes of *P. infestans* seem to be more aggressive than old US-1 isolates and, as a result, the epidemics have become more severe (Kadish *et al.* 1990; Day and Shattock 1997; Flier and Turkensteen 1999)

In recent years there were several studies focusing on characterizing the *P. infestans* population in Asian countries. Nishimura *et al.* (1999), for example, described the occurrence of A2 mating type isolates and Asian-specific allozyme genotypes in isolates collected in South-East Asia between 1992 and 1997. In Siberian populations sampled between 1997 and 1998 a dominant clonal lineage, SIB-1, was found (Elansky *et al.* 2001) and subsequently Akino *et al.* (2004) reported the occurrence of this genotype in China and Japan suggesting further spread of the SIB-1 lineage. Deahl *et al.* (2002) described the population displacement in Taiwan, and Ghimire *et al*. (2003) reported eleven diverse genotypes based on RG57 fingerprinting and mtDNA haplotype and a new dominant population with mtDNA Ia in Nepal. Gotoh *et al*. (2005) characterized isolates collected in eight South-East Asian countries between 1992 and 2000 and found twenty genotypes of which fourteen were new and not discovered elsewhere. Altogether these studies demonstrate that also in Asia a 'new' *P. infestans* population is widely distributed.

China has the biggest potato planting area in the world. In the year 2001, the total planting area had reached 4.72 million hectares, which is about 25% of the total planting area in the world, and about 60% of that in Asia (Qu *et al.* 2004). The province Inner Mongolia comprises one of the major seed potato production areas in China. Of the 22 Chinese provinces and autonomous regions that grow potatoes, Inner Mongolia has the largest area covered with potatoes (562.4

thousand hectares) and potato production has become a very important supporting industry for Inner Mongolia agriculture. Potato is also an important crop in a few provinces adjacent to Inner Mongolia such as Hebei, Heilongjiang, Shanxi and Jilin. Unfortunately, as in many potato-growing areas, the climate in Northern China is favorable for *P. infestans* and, as a result, potato late blight is the most serious threat for potato production in that area. In the past, some efforts were made to characterize the *P. infestans* population in Northern China. Mating type and virulence phenotypes were analyzed and also the sensitivity to metalaxyl was measured (Zhang *et al.* 1996; Wang *et al.* 2003). These studies, however, did not include any molecular characterization of the isolates.

The purpose of this study was to perform a more comprehensive survey of the *P. infestans* population in Northern China, in particular in Inner Mongolia, and to compare that population with reference isolates including a few isolates from Southern China. From isolates collected between 1997 and 2003 mating type and virulence phenotypes were determined and, in addition, the mtDNA haplotype, and AFLP and SSR genotypes were analyzed. The results show that all these isolates belong to a single SSR genotype that is not found in any of the tested reference isolates. In contrast, the virulence phenotypes are highly variable. The observation that a population with a low genotypic diversity has a high phenotypic diversity is discussed in the framework of the evolution of avirulence (*Avr)* genes in *P. infestans.*

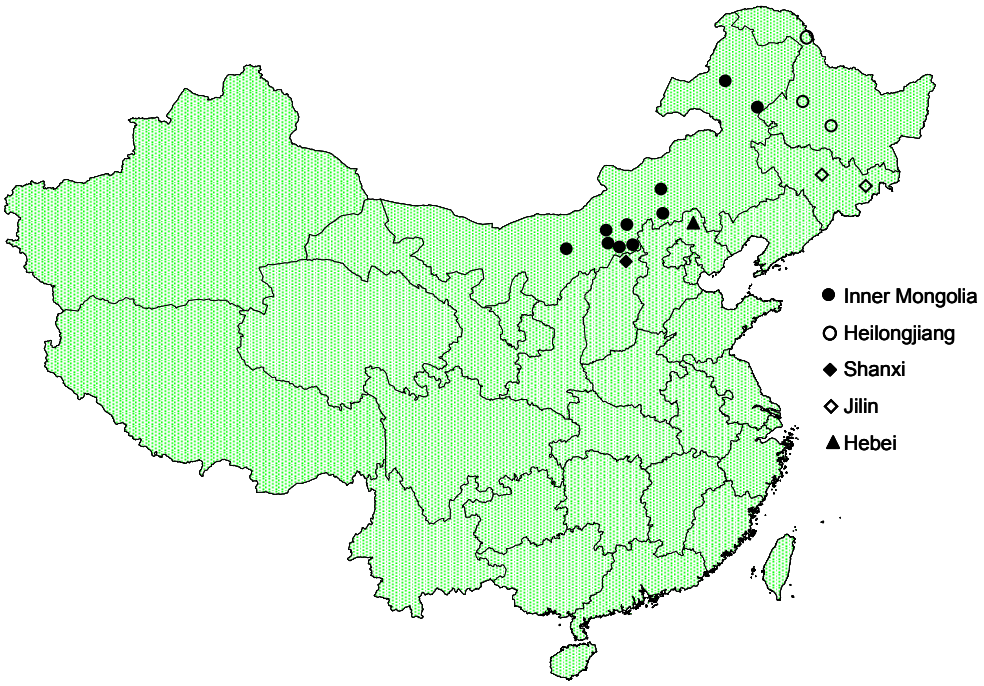


Fig. 1. Map of China showing the provinces and regions where the *P. infestans* isolates were sampled.

Table 1. Origin and characteristics of *P. infestans* isolates collected in Northern China.

Province	Region	Isolate	Year	Cultivar[a]	Mating type	mtDNA haplotype	SSR genotype
Inner	Huhhot	IM-Hh-201	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-202	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-204	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-207	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-208	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-209	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-210	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-211	1998	Braka	A1	IIa	SG-01-01
	Huhhot	IM-Hh-226	1998	Hu #1	A1	IIa	SG-01-01
	Huhhot	IM-Hh-231	1999	Favorita	A1	IIa	SG-01-01
	Huhhot	IM-Hh-233	1999	Zihuabai	A1	IIa	SG-01-01
	Huhhot	IM-Hh-234	1999	Neishu #3	A1	IIa	SG-01-01
	Huhhot	IM-Hh-236	1999	Zhengshu	A1	IIa	SG-01-01
	Huhhot	IM-Hh-237	1999	Desiree	A1	IIa	SG-01-01
	Huhhot	IM-Hh-238	1999	Zihuabai	A1	IIa	SG-01-01
	Huhhot	IM-Hh-267	2002	Shepody	A1	IIa	SG-01-01
	Huhhot	IM-Hh-268	2002	Zihuabai	A1	IIa	SG-01-01
	Huhhot	IM-Hh-269	2002	Favorita	A1	IIa	SG-01-01
	Huhhot	IM-Hh-272	2002	Atlantic	A1	IIa	SG-01-01
	Huhhot	IM-Hh-274	2003	Zihuabai	A1	IIa	SG-01-01
	Huhhot	IM-Hh-279	2003	Favorita	A1	IIa	SG-01-01
	Huhhot	IM-Hh-281	2003	97110	A1	IIa	SG-01-01
	Huhhot	IM-Hh-282	2003	53-109	A1	IIa	SG-01-01
	Huhhot	IM-Hh-283	2003	Kexin #1	A1	IIa	SG-01-01
	Huhhot	IM-Hh-285	2003	87110	A1	IIa	SG-01-01
	Huhhot	IM-Hh-287	2003	Zihuabai	A1	IIa	SG-01-01
	Zhalantun	IM-Z-214	1997	Zha #1	A1	IIa	SG-01-01
	Zhalantun	IM-Z-216	1999	J2	A1	IIa	SG-01-01
	Zhalantun	IM-Z-217	1999	J5	A1	IIa	SG-01-01
	Hailaer	IM-Hl-212	1998	Hongwenbai	A1	IIa	SG-01-01
	Hailaer	IM-Hl-213	1998	Favorita	A1	IIa	SG-01-01
	Taiqibaochang	IM-T-222	1998	Tai #1	A1	IIa	SG-01-01
	Baotou	IM-B-223	1998	Bao #1	A1	IIa	SG-01-01
	Baotou	IM-B-224	1998	Bao #3	A1	IIa	SG-01-01
	Yimengdaqi	IM-Y-225	1998	Da #4	A1	IIa	SG-01-01
	Wumeng	IM-W-240	1998	Zihuabai	A1	IIa	SG-01-01
	Fengzhen	IM-F-253	2002	Zihuabai	A1	IIa	SG-01-01
	Liangcheng	IM-L-262	2002	Shepody	A1	IIa	SG-01-01
	Liangcheng	IM-L-265	2002	Favorita	A1	IIa	SG-01-01
	Shangtuhai	IM-S-270	2002	Favorita	A1	IIa	SG-01-01
Shanxi	Youyu	SX-Y-256	2002	Zihuabai	A1	IIa	SG-01-01
Jilin	Changchun	JL-C-277	2003	Zihuabai	A1	IIa	SG-01-01
	Yanbian	JL-Y-278	2003	Zihuabai	A1	IIa	SG-01-01
Heilongjiang	Wangkui	HLJ-W-V1	2000	na	A1	IIa	SG-01-01
	Wangkui	HLJ-W-B3	2000	na	A1	IIa	SG-01-01
	Keshan	HLJ-K-B15	2000	na	A1	IIa	SG-01-01
	Huma	HLJ-H-220	2001	Kexin #1	A1	IIa	SG-01-01
Heibei	Weichang	HB-W-V2	2001	na	A1	IIa	SG-01-01

[a] The potato cultivars from which isolates were collected; na: not available.

Methods

Collection and maintenance of *P. infestans* isolates

Blighted potato material was collected in the major production areas of Northern China during the growing seasons of 1997-2003 (Table 1; Fig. 1). The geographical origin of the samples varied from year to year depending on the occurrence and development of late blight. Small pieces of leaflet excised from edges of single lesions were transferred to Rye A medium (Caten and Jinks 1968) amended with the antibiotics rifampicin (25 mg l^{-1}) and natamycin (25 mg l^{-1}). Following incubation at 15°C with 12 h light for several days, mycelium was transferred to fresh medium. All isolates were maintained on Rye A slants at 18°C in the dark. Three isolates from Yunnan province, located in Southwest China, and nine collected in other countries between 1958 and 2003 and with different RG57 genotypes (Drenth *et al.* 1994) were used as reference isolates (Table 2).

Table 2. Origin and characteristics of *P. infestans* reference isolates used in this study.

Country	Region	Isolate	Year	Mating type	Virulence[a]	mtDNA haplotype	SSR genotype
China	Yunnan	YN-B7[b]	1998	A1	1.3.4.5.7.9.10.11	Ia	SG-02-01
China	Yunnan	YN-B13[b]	1998	A1	1.3.4.5.7.9.10.11	IIb	SG-02-01
China	Yunnan	YN-V12[b]	1998	A2	1.3.4.5.6.7.9.10.11	Ia	SG-02-01
Ecuador	-	EC3417	2001	A1	3.4.5.7.9.10.11	IIa	SG-03-02
Mexico	Toluca	TV580	nd	A1	3.4.6.7.10	Ia	SG-04-03
Netherlands	-	VK1.4 (US-1)	1958	A1	1.4.7	Ib	SG-02-03
Netherlands	-	NL80029	1980	A1	2.3a.4.7	Ia	SG-03-01
Netherlands	-	NL88069	1988	A1	1.3a.3b.4.7	Ia	SG-02-02
Netherlands	-	NL88133	1988	A2	1.3a.3b.7.10.11	Ia	SG-04-01
Netherlands	-	NL90128	1990	A2	1.3a.3b.4.6.7.8.9.10.11	Ia	SG-02-04
na[c]	-	T30-4[c]	1992	A1	3a.7	Ia	SG-04-02
USA	-	IPO-0 (US-1)	before 1980	A1	3b	Ib	SG-02-05

[a] Not all isolates have been tested on differentials that carry either *R3a* or *R3b*; hence a scoring including '3' could be 3a or 3b or 3a.3b.

[b] YN-B7 and YN-V12 are from Kunming and YN-B1 from Qujing.

[c] na: not applicable; T30-4 is a F_1 progeny of cross 71 (NL80029 x NL88133) (van der Lee et al. 1997).

Mating type determination and virulence testing

Mating type was assessed by growing an isolate on clarified Rye A media in proximity to a strain with known mating type. The A1 tester strain was NL80029 and A2 NL88133 (van der Lee *et al.* 1997). After 7 to 10 days of incubation at 18 °C in dark, oospores were produced in the margins of opposite mating types. Positive and negative controls consisted of pairing tester isolates of opposite mating type and the same mating type, respectively.

The virulence phenotypes were determined by inoculation of detached leaflets of a differential set of potato lines carrying the major resistance (*R*) genes and cultivar Bintje, which has no known *R* genes. Each virulence assay was repeated at least twice. Differentials were

obtained from the International Potato Center in Lima, Peru: *R1* (CEBECO-43154-5; CIP 800986), *R2* (CEBECO-44158-4; CIP 800987), *R3* (CEBECO-4642-1; CIP 800988), *R4* (CEBECO-4431-5; CIP 800989), *R5* (Black 3053-18; CIP 800990), *R6* (Black XD2-21; CIP 800991), *R7* (Black 2182ef(7); CIP 800992), *R8* (Black 2424a(5); CIP 800993), *R9* (Black 2573; CIP 800994), *R10* (Black 3618ad(1); CIP 800995), and *R11* (Black 5008ab(6); CIP 800996). It should be noted that Huang *et al.* (2004) reported that the *R3* differential (CEBECO-4642-1; CIP 800988) carries more than one *R* gene at the *R3* locus. They distinguished *R3a* and *R3b* and found isolates with differential interaction with each of these two. Because we have not used differentials with only *R3a* or only *R3b* we can not distinguish whether an isolate is race 3a or race 3b or both.

Inoculum for virulence tests was obtained from sporulating potato tuber slices that were washed gently with water to liberate sporangia. The sporangial suspension was rinsed several times and placed in a refrigerator for 2 h to induce zoospore formation. Zoospores were separated from sporangia by filtration through a 12 µm mesh filter and diluted to a concentration of 10^5 ml^{-1}. Fully or near-fully expanded leaflets were picked from plants at early flowering stage that were grown in the greenhouse. Four detached leaflets of each differential were placed with abaxial side up on moist filter paper in Petri plates (150 mm diameter). Two 10 µl droplets of a zoospore suspension were placed on each leaflet. After seven days incubation at 18^{o}C under low light (16 h cold white fluorescent light and 8 h dark), the leaflets were examined with a stereo microscope. If sporulation was observed the interaction was rated compatible; if not, it was rated incompatible.

DNA isolation, mitochondrial DNA haplotyping, and SSR and AFLP analysis

Mycelium was cultured for 2 weeks in 9 cm Petri dishes containing 5 ml of liquid Rye A medium. Total DNA was extracted using the protocol described by van der Lee *et al.* (1997) and stored at -20^{o}C until use.

mtDNA haplotypes were determined by amplification of DNA using primer pairs P2 and P4 designed for specific regions of the mitochondrial genome of *P. infestans* (Griffith and Shaw 1998). Polymerase chain reaction (PCR) was performed in a thermocycler (PTC200; MJ Research, Waltham, MA, USA). Digestion of the amplified region with *Cfo*I, *Msp*I and *Eco*RI restriction enzymes yielded restriction patterns by which the isolates could be classified into four haplotypes: Ia, Ib, IIa and IIb.

SSR markers were developed based on *P. infestans* Expressed Sequence Tags (ESTs) and bacterial artificial chromosome (BAC) sequences (T. van der Lee *et al.* unpublished). For SSR analyses, the primer pairs PiAT0111 (forward: 5′-GTCCTCTTGCCGCAACTACG-3′; reverse: 5′-CGCAAGGCTTGTGGCATGG-3′) and PiGCA0012 (forward: 5′-CATGACGAGCGTGGCG AG-3′; reverse: 5′-AGGAATCATCGTGAAGGCAG-3′) were used to amplify polymorphic AT and GCA microsatellites in *P. infestans*, respectively. The PCR reaction started with 2 min at 94^{o}C, followed by 13 cycles at 94 ^{o}C for 30 s, 66^{o}C for 30 s and 72^{o}C for 30 s with a stepwise

lowering of the annealing temperature by 1°C in each cycle, and 28 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 30 s. At the two loci 11 alleles were identified (Table 3). For locus PiGCA-0012, three alleles were found of approximately 100, 103 and 106 base pairs and for locus PiAT-0111, eight alleles of approximately 278, 282, 284, 286, 288, 290, 292 and 296 base pairs. Genotypes are indicated by the prefix SG (SSR Genotype), followed by two numbers separated by a dash. The first number refers to the haplotypes detected with PiGCA-0012 (01-03) and the second number to the haplotypes detected with PiAT-0111 (01-05) (Table 3).

Table 3. SSR genotype (SG) classification based on SSR patterns and the frequency of each SG observed in 60 *Phytophthora infestans* isolates analysed in this study. For each of the two markers, PiGCA-0012 and PiAT-0111, multiple alleles of different sizes were detected varying from 100 to 106 bp and 278 to 296 bp, respectively.

	PiGCA-0012			PiAT-0111								
	100	103	106	278	282	284	286	288	290	292	296	frequency
SG-01-01	√		√	√	√							48
SG-02-01	√	√			√		√	√				3
SG-02-02	√	√		√		√		√				1
SG-02-03	√	√			√	√					√	1
SG-02-04	√	√					√	√				1
SG-02-05	√	√			√	√			√			1
SG-03-01		√		√				√				1
SG-03-02		√							√	√		1
SG-04-01		√	√		√					√		1
SG-04-02		√	√	√	√							1
SG-04-03		√	√		√		√					1

AFLP analysis was performed as described by van der Lee *et al.* (1997) with some modifications. The template was prepared using *Pst*I and *Hha*I. Sequences of primers and adapters were as follows: *Pst*I adapter top strand, 5′-CTCGTAGACTGCGTACATGCA-3′; *Pst*I adapter bottom strand, 5′-TGTACGCAG TCTAC-3′; *Hha*I adapter top strand, 5′-GACGATGAGTCCTGACG-3′; *Hha*I adapter bottom strand, 5′-TCAGGACTCATCG-3′; *Pst*I preamplification primer, 5′-GACTGC GTACATGCAG-3′; *Hha*I preamplification primer, 5′-CGATGAGTCCTGACGC-3′. The selective PCR was performed with primers containing two selective 3′-nucleotides, P+GC/H+TG and P+GT/H+TA. P+GC and P+GT primers were labeled with IRD700 for fluorescence detection using the LI-COR Global IR^2 system.

Data analysis

The calculations of genetic similarity and cluster analysis were performed with software NTSYSpc Version 2.1 (Rohlf 2000) using the SM coefficient and the unweighted pair group method algorithms (UPGMA).

Results

Origin of the isolates

In total, 48 isolates were collected from commercial potato fields in August and September during the years 1997-2003. Forty isolates came from Inner Mongolia, four from Heilongjiang Province, two from Jinlin Province and one from Hebei and Shanxi Provinces, respectively (Table 1; Fig. 1). In Inner Mongolia the samples were collected in 10 regions and from blighted leaves of 21 potato cultivars, mostly from Braka, Zihuabai and Favorita (Table 1). Isolates collected in the same year and the same region were obtained from different fields at distances that varied from 0.1 to 20 kilometers. The eight isolates originating from the other four provinces in Northern China were collected from seven regions. Only the isolates HLJ-W-V1 and HLJ-W-B3 are from the same region. For comparison we used isolates from Yunnan Province located in Southwest China and four other countries as reference isolates (Table 2).

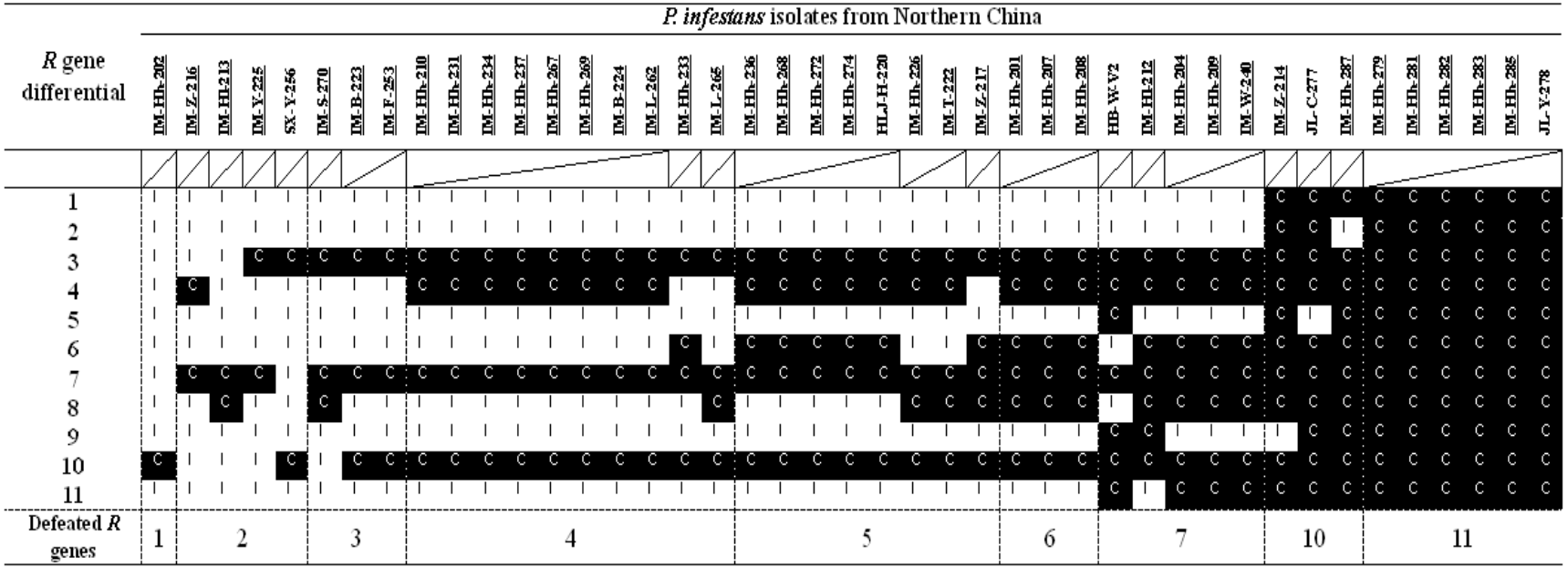
The A1 mating type is prevalent in Northern China

The 48 isolates from Northern China all had the A1 mating type and no self-fertile isolates were found (Table 1). Previously, the occurrence of A2 mating type isolates of *P. infestans* in China was reported by Zhu *et al.* (2000) for Hebei province and Sichuan province and by Zhao *et al.* (1999) for Yunnan Province and hence, the A2 mating type of *P. infestans* occurs in several of the major potato production areas in China. For Inner-Mongolia there are conflicting data. Wang *et al.* (2003) did not find any A2 mating type isolate in five provinces in Northern China including Inner Mongolia and this is in accordance with our own findings. They studied almost three times as many isolates as we did (143 versus 48) and sampled at different sites. In contrast, Zhang *et al.* (1996) reported that two out of the four isolates they analyzed from Inner Mongolia were A2. However, in that study the number of isolates was low and, more importantly, the identity of the A1 and A2 tester strains was not reported. Hence, it is not clear how reliable those data are. Taken together we conclude that the actual frequency of the A2 mating type in Inner Mongolia is very low.

The virulence phenotypes are highly diverse

The virulence spectrum in the isolates was highly variable. Of the 43 tested isolates six infected all eleven differentials. One infected only one of the eleven and there was no race 0 (Table 4). The majority was compatible with at least five differentials. A total of 21 different races was distinguished, of which race 3.4.7.10 (18.6%), race 1.2.3.4.5.6.7.8.9.10.11 (14.0%) and race 3.4.6.7.10 (11.6%) were the most frequent. In Inner Mongolia, eighteen of the 21 races were found (Table 4). Most isolates were virulent on the *R7* differential followed by the *R3*, *R10* and *R4* differential. The *R2* differential was capable of withstanding most isolates, 35 out of 43, followed by *R1* and *R5* (34 out of 43), and *R9* (33 out of 43). The two isolates from Jilin were among the most complex ones as was the one from Hebei. The most frequently occurring race,

Table 4. Virulence genotypes of 43 *Phytophtora infestans* isolates from Northern China; the majority of which is from Inner Mongolia (underlined). A black shading indicates that the isolate is compatible (C) with the potato *R* gene differential whereas 'I' indicates an incompatible interaction. The grouping into 21 races is represented by the diagonal lines that span one or more isolates.



i.e., race 3.4.7.10, was also found frequently in several European countries (Sujkowski *et al.* 1996; Lebreton *et al.* 1998; Hermansen *et al.* 2000; Knapova and Gisi 2002; Cooke *et al.* 2003).

The genotypes are not highly diverse

Genotyping of the mtDNA revealed that all isolates have the IIa haplotype (Table 1; Fig. 2). From the reference isolates only the Ecuadorian isolate EC3417 had the IIa haplotype (Table 2). SSR genotyping of the 48 isolates from Northern China and twelve reference isolates resulted in eleven genotypes at the two loci (Table 3). All isolates from Northern China had the same SSR genotype (Fig. 3). This genotype, designated SG-01-01, was not found for any of the reference isolates. More extensive SSR genotyping using an additional set of seven SSR markers did not reveal any polymorphism in the 48 isolates from Northern China (data not shown). Of the reference isolates the three from Yunnan Province shared a unique SSR genotype, SG-02-01; the other nine each had their own unique SSR genotype (Table 2).

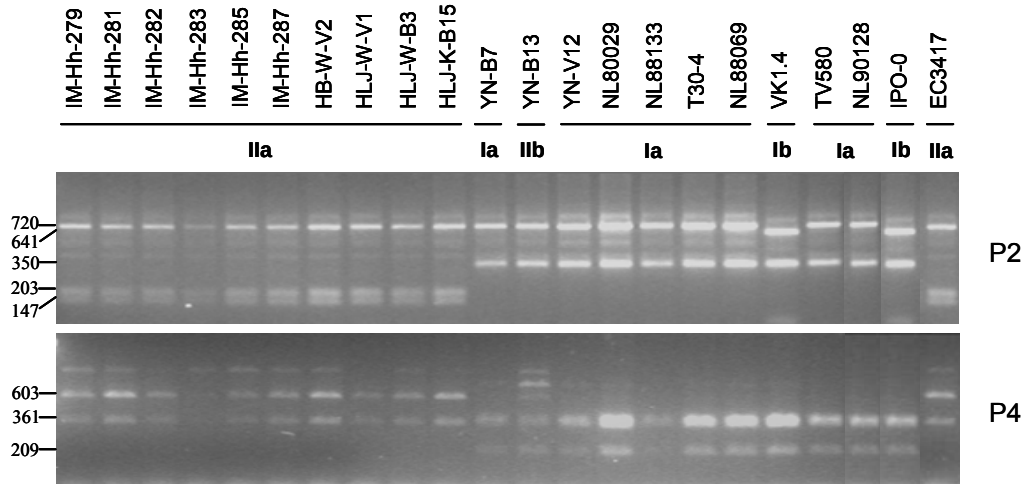


Fig. 2. The mtDNA haplotype of ten *P. infestans* isolates from Northern China and twelve *P. infestans* reference isolates. The mtDNA was amplified with P2 primers or P4 primers and digested with *Msp*I and *Eco*RI, respectively. The sizes of the restriction fragments (in bp) are indicated on the left.

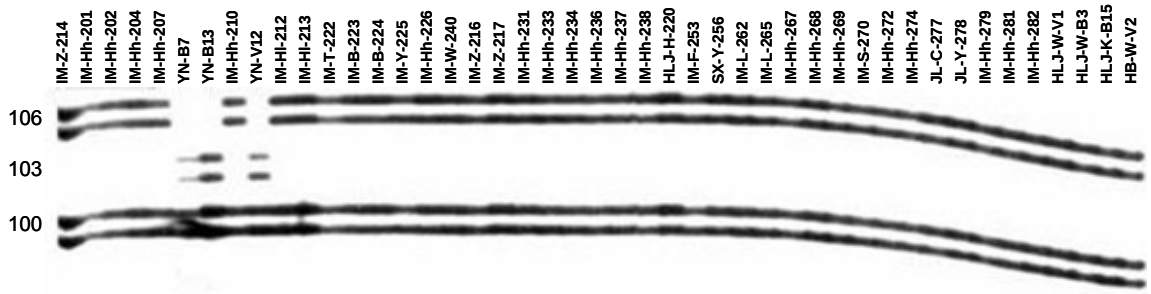


Fig. 3. SSR genotyping of *P. infestans* isolates from Northern China and three reference isolates from Yunnan Province (with prefix YN) with primer pair PiGCA-0012. The numbers on the left indicate the three alleles that can be distinguished with PiGCA-0012.

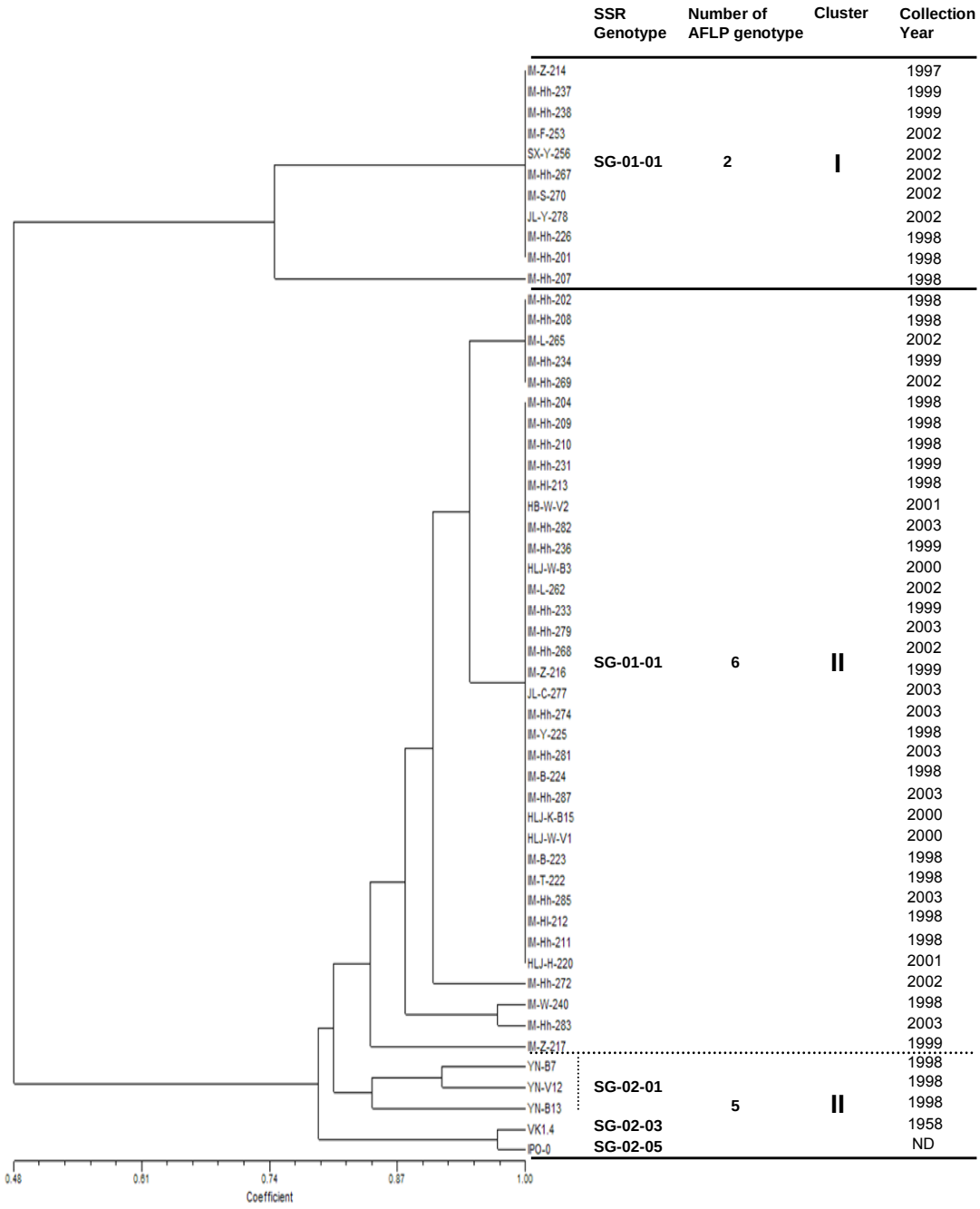


Fig. 4. Cluster analysis of 48 Chinese *P. infestans* isolates that have the same SSR genotype SG-01-01 and five reference isolates. The tree was inferred by bootstrap analysis of 35 AFLP markers. ND: no data.

To test for sub lineages within the SG-01-01 lineage we analyzed the isolates by AFLP fingerprinting. A typical AFLP fingerprint pattern of *P. infestans* generated with primers having two base extensions consisted of about 80 bands on average for each primer combination (van der Lee *et al.* 1997). With the two primer combinations used in this study we were able to score 35 bands that showed polymorphism in the Chinese isolates and reference isolates. Cluster analysis revealed two groups of which the smaller one, group I, comprised solely SG-01-01 genotypes (Fig. 4). The five tested reference isolates did not form a separate group but fell into

the larger group together with the majority of the Chinese isolates with the SG-01-01 phenotype. Within group II the three reference isolates with the same SG genotype (YN-B7, YN-B13 and YN-V12 from Yunnan) grouped together on one branch as did the two US-1 isolates IPO-0 and Vk1.4 (Fig. 4). Within the 48 isolates with the SG-01-01 genotype eight AFLP genotypes could be distinguished, 2 in group I and 6 in group II. More than half of the 48 SG-01-01 genotypes had an identical AFLP fingerprint. Clustering of the AFLP genotypes could not be associated with the collection year or the geographic origin of the isolates.

Discussion

The degree of diversity that can be detected within any population depends highly on the type of molecular marker that is used. An ideal marker system for population genetic analysis should be high throughput, robust, flexible, broadly applied, safe and suitable for rigorous genetic analysis (Cooke and Lees 2004). Of the three types of genotypic markers that were used in this study the mtDNA haplotyping is the least informative for the characterization of individuals within a population; in *P. infestans* it differentiates between only four genotypes. mtDNA is, however, suitable to track specific lineages within the population as has been nicely demonstrated by Ristaino *et al.* (2001). On the other hand, SSR and AFLP fingerprinting provide high levels of polymorphism and can detect gene or allele frequencies in populations rather easily. Because SSR markers are more robust than AFLP markers and more suitable for high throughput, they are ideal markers to study *P. infestans* populations. With the recent availability of large sequence data sets, microsatellites can be easily identified. As a result SSR genotyping has been adopted as the method of choice for analyzing *P. infestans* populations (Cooke and Lees 2004; Lees *et al.* 2006; Knapova and Gisi 2002). In this study, we found that just two SSR markers already distinguish nearly all reference isolates from each other. In contrast, all isolates from Northern China have the same, unique SSR genotype and even if we increase the number of SSR markers from two to nine, the SSR genotype in all 48 isolates is still identical. These data suggest that the *P. infestans* population in Northern China represents a clonal lineage, despite the fact that AFLP fingerprinting revealed some variation. Similar to our observations AFLP fingerprinting of *P. infestans* isolates from Scotland, England and Wales revealed many unique AFLP patterns (Cooke *et al.* 2003; Purvis *et al.* 2000) and not in all cases the AFLP genotypes were consistent with genotypes found with other fingerprint markers (Purvis *et al.* 2000).

The low genotypic diversity is in sharp contrast with the high level of virulence diversity. The 43 samples that were tested for virulence were derived from 21 potato cultivars, so on average there were two from each cultivar. Moreover, the six isolates that were virulent on all eleven *R* differentials were collected from six potato cultivars. In theory, it is possible that *R*-gene selection plays a role in generating race variation in *P. infestans* populations but it is more likely that random mutation plays a more important role (Goodwin 1997). In recent years insight into the molecular basis of compatibility and incompatibility and gene-for-gene

interactions has increased significantly and it is now generally accepted that new races appear because *Avr* genes encoding effector proteins undergo mutations so that the effectors are no longer recognized by the R protein. *Phytophthora* Avr proteins that have been indentified so far all share a conserved motif with the signature RXLR (Govers and Gijzen 2006) and belong to a highly dynamic superfamily consisting of around 560 members in *P. infestans* ((Jiang *et al.* 2008; R.H.Y. Jiang, personal communication). Two cloned *P. infestans Avr* genes interact with *R* genes that are represented in the differential set. In *Avr3a* point mutations leading to two amino acid changes result in a change in virulence phenotype (Armstrong *et al.* 2005). In *Avr4* a frame shift mutation results in absence of the effector and strains lacking Avr4 protein can infect *R4* plants (van Poppel *et al.* 2008; **Chapter 4**). Apparently there is no pressure for *P. infestans* to maintain an intact *Avr4* gene. Since the RXLR superfamily represents a rapidly evolving part of the genome (Jiang *et al.* 2008) it is likely that many of the effector genes are redundant. This rapid evolution may account for the highly diverse virulence phenotypes that we and others (Goodwin *et al.* 1995; Abu-El Samen *et al.* 2003) observed in *P. infestans* field populations that belong to one clonal lineage. SSR markers are very useful to trace the origin of isolates or the sources of infection but they are not suitable to reveal race structure. Now that the molecular basis underlying virulence in *P. infestans* is being unraveled it becomes feasible to develop marker systems that can diagnose the virulence phenotype based on Single Nucleotide Polymorphism (SNP) detection in RXLR effector genes. Compared to virulence testing in bioassays, virulence diagnostics based on SNPs has the advantage that it is less time consuming and has a high resolution. In theory, the allele distribution of every member of the RXLR superfamily can be diagnosed in one assay. Future efforts should be focused on more refined marker systems that can monitor the behavior of *P. infestans* populations with respect to characteristics that are relevant for proper disease management such as virulence and avirulence.

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Chapter 7

General discussion

General discussion

Cloning of avirulence genes in *Phytophthora infestans*

A major aim of the research described in this thesis was cloning and identification of race-specific avirulence (*Avr*) genes in the oomycete late blight pathogen *Phytophthora infestans*. In the past decade, cloning of resistance (*R*) genes and *Avr* genes in different plant species and microbial pathogens, respectively, has significantly improved our knowledge of the molecular and biochemical basis of gene-for-gene interactions (Bent and Mackey, 2007). To date, more than 55 *R* genes have been cloned from a number of model and crop plant species (van Ooijen et al., 2007). Although these genes encode proteins that can be separated into five main classes on the basis of the presence of a limited number of conserved domains, they were isolated from species representing a wide range of taxonomic origin (Martin et al., 2003). Their sequences suggest roles of the encoded proteins in both recognition of Avr proteins and signal transduction (Martin et al., 2003). Meanwhile, over 40 bacterial *Avr* genes have been cloned and the functional characterization of these genes has contributed to an increased knowledge of the interplay between host and pathogen during compatible and incompatible interactions (Van't Slot and Knogge, 2002; Mudgett, 2005). In addition, a few *Avr* genes were isolated from several fungal pathogens, among which the six ascomycete species *Cladosporium fulvum* (Rivas and Thomas, 2005; Thomma et al., 2005), *Magnaporthe grisea* (Orbach et al., 2000; Farman et al., 2002; Böhnert et al., 2004), *Rhynchosporium secalis* (Rohe et al., 1995), *Blumeria graminis* f. sp. *hordei* (Ridout et al. 2006), *Fusarium oxysporum* f.sp. *lycopersici* (Rep et al., 2004) and *Leptosphaeria maculans* (Gout et al., 2006), and the basidiomycete *Melampsora lini* (Dodds et al., 2004). Most bacterial *Avr* genes were isolated by shotgun cloning, a strategy that is feasible in bacteria because of their small genome sizes and high transformation efficiencies (Bonas and Van den Ackerveken, 1999). The isolation of *Avr* genes from fungi, however, has been less straightforward (Laugé and de Wit, 1998). In fungi, a reverse genetics strategy has been widely used to identify avirulence genes, such as *Avr9* and *Avr4* in *C. fulvum* (Van den Ackerveken *et al.*, 1992; Joosten et al., 1994). Additionally, positional cloning was used to isolate *Avr* genes in the rice blast fungus *M. grisea*, the stem canker pathogen *L. maculans* and the powdery mildew *B. graminis* f. sp. *horde* (Kang et al., 1994; Orbach et al., 2000; Gout et al., 2006; Ridout et al. 2006). An alternative method, i.e., a functional cloning strategy, has been successfully applied to clone *Avr2* from *C. fulvum* (Takken et al., 2000).

Functional cloning is hardly applicable in *P. infestans*. The efficiency of DNA transformation is too low and selection of complemented phenotypes (a change from virulent to avirulent) is technically impossible. Reverse genetics requires the availability of purified race-specific elicitors. Unfortunately, attempts to identify race-specific elicitors from *P. infestans* were unsuccessful (Alfonso & Govers, 1995). Therefore, positional cloning was considered to be

more promising and indeed it pushed the cloning of *Avr* genes in *P. infestans*. An AFLP linkage map of *P. infestans* was constructed (van der Lee et al., 1997) and on this map six dominant *Avr* genes were positioned (van der Lee et al., 2001). More markers were developed to enrich marker density and to identify new markers linked to *Avr* genes (van der Lee et al. 2004; **Chapter 2; Chapter 5**). Meanwhile, a *P. infestans* BAC library representing an estimated 10x *P. infestans* genome equivalents was constructed and this was an essential tool to accomplish positional cloning (Whisson et al., 2001). Despite all these efforts, positional cloning of *Avr* genes from *P. infestans* lagged behind. The first oomycete *Avr* genes were cloned from two other pathogens, *Phytophthora sojae* and *Hyaloperonospora parasitica*, as reported four years ago by Shan et al. (2004) and Allen et al. (2004), respectively. Two obstacles account for this. Firstly, unlike *P. sojae* and *H. parasitica*, *P. infestans* is heterothallic and it is not possible to generate sufficient inbred progeny to obtain recombinants in the *Avr* regions and to saturate the regions with markers. Secondly, the relatively large genome size of *P. infestans* (240 Mb) reduces the marker density and even with high-density linkage maps (van der Lee et al., 2004) it is difficult to generate enough markers for efficient landing.

To circumvent these obstacles in part, we adopted a combined cDNA-AFLP/BSA strategy with the aim to obtain transcriptome markers (**Chapter 3**). In this way, we obtained candidate transcripts associated with the *Avr4* locus and the *Avr3b-Avr10-Avr11* locus. With the help of the candidates, we succeeded in isolation of the avirulence gene *Avr4* (**Chapter 4**) and gene *Pi3.4* that is located at *Avr3b-Avr10-Avr11* locus (Jiang et al., 2006). To select *Avr*-associated transcript derived fragments (TDFs) we used four criteria. First of all, the TDFs had to be present in germinating cysts, a preinfection stage in which an *Avr* gene is most likely expressed. Secondly, the TDFs had to be present in pools consisting of strains having an AVR phenotype but not in pools consisting of virulent strains (avr phenotype). Thirdly, segregation of the TDFs in F_1 progeny had to be correlated entirely with segregation of the AVR/avr phenotypes and, fourthly, RT-PCR had to confirm the *Avr*-associated segregation in the F_1 progeny. In addition, a second transcriptional profiling strategy was exploited to generate more *Avr*-associated markers (Jiang et al., 2006; **Chapter 5**). For this strategy we used an Affymetrix® chip that was composed of a *Phytophthora* unigene set developed from ESTs (Randall et al., 2005; Judelson et al., 2008). The *Phytophthora* GeneChip was hybridized with RNA samples pooled from strains having the AVR phenotype and RNA samples pooled from strains having the avr phenotype. The transcriptome markers obtained by either cDNA-AFLP or GeneChip analyses proved to have added value for the positional cloning strategy.

Even in the genomics era, positional cloning of *Avr* genes from *P. infestans* is far from routine. Thus, a different strategy, i.e., association genetics, was exploited by Armstrong et al. (2005) to identify *P. infestans Avr* genes. Their approach was based on the assumption that AVR proteins should possess N-terminal type II signal peptides (SPs) for secretion and should exhibit significant sequence variation between pathogen races. This was indeed found for many fungal AVR proteins such as Avr4 and Avr2 from the tomato pathogen *Cladosporium fulvum* (Joosten

et al., 1997; Luderer et al., 2002) and NIP1 from the barley pathogen *Rhynchosporium secalis* (Rohe et al., 1995). A candidate gene approach starting off with screening *P.infestans* ESTs for genes encoding secreted proteins and followed by association genetic [linkage disequilibrium (LD)] studies, led to the successful identification of *P. infestans Avr3a* (Armstrong et al. 2005).

The discovery that all *Phytophthora* Avr proteins identified to date contain the conserved amino acid motif RXLR offers new perspectives for rapid cloning of additional *P. infestans Avr* genes. As described in **Chapter 5**, we used a whole range of *Avr1*-associated markers to narrow down a 800 kb genomic interval containing seven RXLR effector genes that are likely candidates for *Avr1*. Bioinformatic and functional analysis of these candidates, has meanwhile resulted in identification of the true *Avr1* gene (F. Govers et al., unpublished data). In the same way, *Avr2* was identified (P.R.J. Birch et al. Scottisch Crop Research Institute, personal communication). Another candidate approach was designed by Vleeshouwers et al. (2008) who used effector genomics to find novel *Avr-R* combinations. Screening for elicitor activity of *P. infestans* RXLR genes on *Solanum* accessions with resistance to late blight revealed that the cognate *R* genes of the in planta-induced gene *ipiO* (Pieterse et al., 1994; van West et al., 1998**)** are *Rpi-blb1* and *Rpi-sto1.* This effector genomics approach will not only allow the identification of *Avr* genes from candidate RXLR effector genes but also the corresponding *R* genes from *Solanum* species.

In the genomics era we have now reached the stage where the genome sequence of *P. infestans* is on line and that of potato in an advanced stage. By exploiting these resources more and more *Avr* genes from *P. infestans* and cognate *R* genes from potato will be isolated. This will provide us with more opportunities for detailed investigation of the earliest recognition events in potato-*P. infestans* R-AVR interactions and of subsequent signaling pathways leading to disease resistance.

RXLR effectors belong to a superfamily that is rapidly evolving

When it became clear that most oomycete avirulence loci that had been cloned by positional cloning or association genetics encode secreted proteins that share a conserved RXLR motif there was a rush to find more of such RXLR effector genes in oomycete pathogens (Birch et al., 2006; Kamoun, 2006; Tyler et al., 2006; Win et al., 2007). Genome mining revealed a total of 374, 396 and 563 RXLR effectors in *P. ramorum*, *P. sojae* and *P. infestans*, respectively (Jiang et al., 2008; Jiang R.H.Y., personal communication). The majority of these RXLR effectors belong to one large family. This family is a rapidly evolving superfamily that displays high sequence divergence in C-terminal domains, minimal paralog clustering, and frequent rearrangements (Jiang et al., 2008). Rapid gene sequence divergence between species can be caused by various mechanisms, such as relaxation of selection, frequent gene duplication, rapid gene loss, and/or positive selection. Rapid sequence divergence in newly formed RXLR genes would enable the number of effector genes in the pathogen to increase while minimizing the likelihood of host recognition. The genome distribution of RXLR effectors is mostly scattered and extensive

clustering of RXLR genes is rare. It means that newly formed genes are rapidly dispersed to other loci in the genome and this will result in expansion of the RXLR family. The frequent genomic rearrangements in the RXLR effector genes suggest that these genes are located within highly fluid regions of the genome. These features will facilitate the evolution of RXLR effector genes and, hence, of *Phytophthora* virulence.

Despite the high sequence divergence, motif searches and HMM screening revealed that more than half of the RXLR effectors do possess conserved motifs in the C-terminal domains. These were named W, Y and L motifs based on a conserved amino acid at a fixed position in the motif (Jiang et al., 2008). They occur in a repeated fashion, and in *P. infestans* RXLR effectors up to eleven repeats of W-Y-L can be found. The number of repeats correlates with the length of the RXLR effector. Avr4, for example, is 287 amino acids in length and has three W motifs followed by one Y motif. In constrast, *P. sojae* Avr1b and *P. infestans* IPI-O are smaller, 138 and 152 amino acids, respectively, and have only one W and one Y motif (Avr1b) or one W motif (IPI-O) (Bouwmeester et al., 2008). Also six of the seven candidates for *Avr1* have their own characteristic composition of of W, Y and L domains and the length correlates with the number of motifs (**Chapter 5**). These motifs presumably contribute to avirulence as well as virulence functions of RXLR effectors and likely result from some degree of purifying selection. A recent study by Dou et al. (2008) revealed that overexpression of *P. sojae Avr1b*-1 increased pathogen virulence on a compatible host, and that the W and Y motifs of *Avr1b* are required for suppression of programmed cell death triggered by the mouse BAX protein in yeast, soybean, and *N. benthamiana* and for interaction with the *Rps1b* resistance gene product. Functional analysis of truncated forms of Avr4 revealed that not all three W motifs in Avr4 are required for avirulence function (P.M.J.A van Poppel and F. Govers, unpublished data).

Prokaryotic and eukaryotic plant pathogens secrete effector proteins to different cellular compartments of their hosts to modulate plant defense circuitry and enable parasitic colonization (Birch et al., 2006; Chisholm et al., 2006; Kamoun, 2006; O'Connell et al., 2006). The current paradigm in the study of plant-microbe interactions is that unravelling the molecular function of effectors is central to a mechanistic understanding of virulence. Indeed, significant progress has been made in elucidating the virulence functions of bacterial effectors (Chisholm et al., 2006). Delivery is often carried out by specialized structures such as the type III secretion system (T3SS) of Gram-negative bacteria, which facilitates translocations of proteins across the host plasma membrane into the host cytoplasm. T3SS effectors have a range of biochemical activities that alter host cytoplasmic targets (Espinosa and Alfano, 2004). Other bacterial effectors that represent a diverse array of virulence determinants are also delivered to the plant apoplast through the pathogen type II secretion system (Jha et al., 2005). Fungi deliver effectors to both the inside and the outside of plant cells (Rooney et al., 2005; Jia et al., 2000; Dodds et al., 2004; Kemen et al., 2005). These studies indicated that fungi, similar to bacteria, translocate effectors to the inside of plant cells, although the molecular mechanism of such trafficking is unknown. Then how on earth are the RXLR effectors from oomycete pathogens delivered into the host?

Recent evidence revealed that the RXLR motif in *P. infestans* Avr3a is indeed required for targeting this effector into host plant cells, but not for effector activity (Whisson et al., 2007). A hypothetical model for RXLR effector delivery into the host, presented by Morgan and Kamoun (2007) (Figure 1), proposes that host translocation of RXLR effectors involves at least a RXLR leader binding protein, one or more additional chaperones, and a translocon, which could be of either pathogen or plant origin. Translocation into host cells initiates with the RXLR-binding protein recruiting mature effectors secreted via the general secretory pathway. In coordination with chaperones, the effector cargo is then transferred to a translocon embedded in the extrahaustorial membrane, and is then released across the membrane into the plant cytosol. Gouget et al. (2005) revealed that a lectin receptor kinase in Arabidopsis (LecRK) specifically binds to RGD-containing peptides including the oomycete RXLR effector IPI-O that has an RGD motif (Pieterse et al., 1994; Senchou et al., 2004). LecRK may be involved in protein-protein interactions with RGD-containing proteins as potential ligands and play a structural and signaling role at the plant cell surface. In the model (Figure 1) LecRK could be the RXLR binding protein or a component of the translocon that mediates uptake of IPI-O.

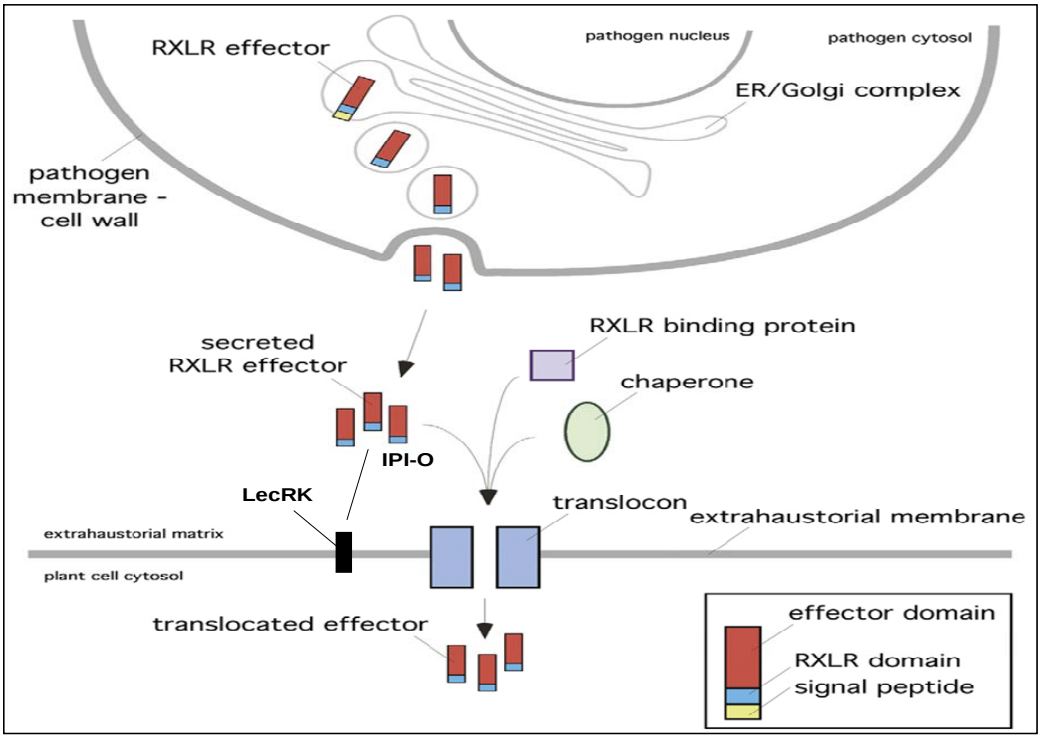

Figure 1. A hypothetical model for RXLR effector secretion and delivery into host cells. Adapted from Morgan and Kamoun (2007).

In our studies with *P. infestans Avr4* (**Chapter 4**), we observed a hypersensitive response (HR) when *Avr4* was transiently expressed in plants carrying *R4* and when PiAvr4 was targeted to the outside of the cell by fusion to a signal peptide (SP) sequence. Deletion of the

RXLR-dEER domain neither stimulated nor prevented elicitor activity of PiAvr4 but in the absence of the SP sequence *Avr4* expression mediated by a PVX vector did not elicit a HR. This suggested that Avr4 is not recognized inside the cell. In contrast, agroinfiltration of *R4* plants with *Avr4* constructs always resulted an a HR irrespective of the presence or absence of the SP. Based on these observations we can not exclude intracellular recognition of PiAvr4 but we can conclude that in all cases when there was a SP in the *in planta* expression construct there was also a specific response on *R4* plants, strongly suggesting extracellular recognition of PiAvr4. This is contradictory with the current thinking that all oomycete RXLR effectors are targeted into the host cell where they are recognized by intracellular NB-LRR proteins. As yet, the molecular structure of the R4 protein is not known and there is still a possibility that R4 is not a canonical intracellular NB-LRR protein, and that the recognition mechanism for Avr4 differs from that of other RXLR proteins. The emerging view that all oomycete RxLR-dEER effectors are massively targeted to the plant cell should be taken with caution. With more and more *R* genes and *Avr* genes cloned, the different interaction mechanisms between R proteins from potato and effectors from *P. infestans* will be uncovered and elucidated.

Virulence variability in *Phytophthora infestans*

Sexual reproduction of *P. infestans*, associated with genetic recombination during meiosis in the antheridium or the oogonium, is a major mechanism of genetic variation in this diploid organism. In addition, mutation, mitotic recombination, and parasexual recombination are the most common mechanisms of genetic variability in the absence of sexual reproduction (Goodwin, 1997). The most important aspect of genetic variability in plant pathogens is the variability in pathogenicity and virulence toward the host. Virulence variability in *P. infestans* populations is recognized as a major reason for failure of race-specific *R* genes in cultivated potato as a disease management strategy (Wastie, 1991).

Variability in virulence among single zoospore strains has been reported for some oomycetes including *P. sojae* (Rutherford et al., 1985) and *Aphanomyces euteiches* (Malvick and Percich, 1997). Abu-El Samen et al. (2003) reported different levels of virulence diversity among US-8 and US-1 asexual progenies of *P. infestans*. We also found high virulence diversity in *P. infestans* isolates of one clonal lineage collected in Northern China (**Chapter 6**). Three major mechanisms account for genetic variability during asexual reproduction in *Phytophthora* spp: mutation, mitotic crossing over, and extra-chromosomal elements (Shaw, 1983).

How are virulence phenotypes determined and what are the mechanisms underlying changes from avirulence to virulence in *P. infestans* populations? With a handful of cloned *Avr* genes it is now possible to trace the different mechanisms that cause the variation in virulence. The first *Phytophthora Avr* gene that was cloned, *Avr1b*-1, had numerous substitution mutations in some isolates of *P. sojae* virulent on cultivars containing the cognate *Rps1b* gene (Shan et al. 2004). In *P. infestans Avr3a*, point mutations leading to two amino acid changes result in a

change in virulence phenotype (Armstrong et al., 2005) and in *Avr4* a frameshift mutation results in absence of the Avr4 effector and thus, virulence on R4 plants (**Chapter 4**). Interestingly, the *Avr3b-Avr10-Avr11* locus shows copy number variation (CNV) with a high copy number in avirulent strains and a single copy in virulent strains (Jiang et al., 2006). Amplification generates modular diversity at the *Avr3b-Avr10-Avr11* locus and this modular diversity could be a novel mechanism for *P. infestans* to quickly adapt to changes in the enviroment. The gene *Pi3.4* that is amplified in avirulent strains, encodes a protein that has the characteristics of a transcription factor but how this putative transcription factor influences the avirulence phenotype remains to be determined. Jiang et al. (2006) postulated that it regulates the coordinated expression of at least three genes encoding secreted effectors, namely the cognate effectors of the resistance genes *R3b, R10* and *R11*, respectively. Also in *P. sojae* transcriptional regulation of an *Avr* gene has been found as a mechanism that causes variation in virulence. Virulent strains carrying a dominant avirulent allele of *Avr1b*-1, appeared to lack a functional copy of *Avr1b*-2, a gene required for the accumulation of *Avr1b*-1 mRNA (Shan et al., 2004).

Durable resistance to late blight: dream or reality?

Co-evolution is the process of reciprocal adaptive genetic change in two or more species (Woolhouse et al., 2002). The antagonism between host and pathogen leads to co-evolutionary arms races (Stahl and Bishop, 2000). The gene-for-gene model means co-evolution involving a single locus in the genomes of each of two interacting populations. Gene-for-gene resistance is often called major-gene resistance or pathotype-specific resistance because its effects are large and effective only against the portion of the pathogen population that produces the effector. When a plant cell receptor recognizes a pathogen effector, a defense response is activated that often leads to an HR and inhibition of the pathogen. Mutations from avirulence to virulence in the pathogen lead to a change in the effector, or failure to produce the effector at all, resulting in failure of recognition by the host receptor. In this gene-for-gene model, a breakdown in resistance is due to an increase in the frequency of pathogen strains that harbor a mutation from avirulence to virulence. It seems to be an inevitable event that *R* genes fail when pathogens evolve to escape detection. Nevertheless, many *R* genes have remained functionally effective despite decades of intensive use (Keen, 1982; McDonald and Linde, 2002). There are three factors that influence the durability of resistance, (i) the fitness penalty that is caused by loss of an effector, (ii) the ability of the effector to avoid recognition while maintaining its virulence function, and (iii) the broader evolutionary capacity of the pathogen. The latter is an important trait that strongly influences resistance durability. McDonald and Linde (2002) have proposed a flexible framework to predict the evolutionary potential of pathogen populations. According to this framework, pathogens that pose the greatest risk of breaking down *R* genes have a mixed reproduction system, a high potential for genotype flow, large effective population sizes, and high mutation rates. The low risk pathogens are those with strict asexual reproduction, low

potential for gene flow, small effective population sizes, and low mutation rates, such as the soilborne fungus *Fusarium oxysporum* f.sp. *lycopersici* causing vascular wilt in tomato. *P. infestans* has all the 'high risk' features and clearly belongs to the group of pathogens that can easily break down *R* genes. Therefore, of all pathogens *P. infestans* will require the greatest effort to achieve durable resistance because the mutations to virulence can be recombined into many genetic backgrounds until a pathogen clone with high fitness appears, and then this adapted genotype can be dispersed across long distances and into new populations.

Then how to achieve durable potato late blight resistance? As the 'highest risk' pathogen, McDonald and Linde (2002) suggested that breeding efforts should concentrate on quantitative resistance that needs to be renewed regularly to stay ahead of the pathogen. Huang et al. (2005), however, indicated that quantitative resistance is practically unsuccessful and unavailable and argued that major gene resistance should be managed aggressively, including development of cultivar mixtures and multilines that can be used in combination with regional and temporal deployment strategies. Indeed the major gene resistance from *Solamum demissum* has been exploited extensively. However, the *R* genes *R1-R11* introgressed from *S. demissum* were often already broken before they were introduced in commercial cultivars. Even for *Rpi-blb1*/*RB*, the potentially broad spectrum *R* gene from *S. bulbocastanum* (van der Vossen et al. 2003; Song et al., 2003), virlent *P. infestans* isolates have been found (K. Bouwmeester and F. Govers, personal communication). A few strategies have been tried to exploit the major resistance such as *R* gene pyramiding and *R* gene multiline cultivars. The process of pyramiding involves huge effort but only resulted in the release of a few cultivars such as the Dutch cv. Escort and the Scottish cv. Stirling with reasonably good resistance. Yet these cultivars are not grown in a considerably large acreage and in recent years the resistance in cv. Stirling has been overcome by a *P. infestans* strain that is quickly spreading over Europe and has the genotype notation #13 blue (D. Cooke, personal communication). Maybe mixing *R* gene multiline cultivars is a good approach to obtain durable resistance to *P. infestans,* but it will be a long way to clone a dozen *R* genes and to create a mixture of potato cultivars that only differ by the *R* genes.

According to records of the previous century it can be inferred that there were several periods during which potato could be grown without notable occurrence of late blight (Andrivon, 1996; Zwankhuizen and Zadoks, 2002) suggesting that the disease is, or at least was, manageable. At present however, *P. infestans* appears in a more aggressive form and it is more difficult than ever to control or eradicate this pathogen because of its worldwide occurrence. The challenge to control *P. infestans* more effectively is enormous, but it is difficult to predict which strategy will be successful at the end.

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Summary

Potato late blight, caused by the oomycete pathogen *Phytophthora infestans* (Mont.) de Bary, is the most disastrous disease on potato worldwide and also the greatest threat to potato production in China. Loss of yield and quality, and the costs of chemical control of potato late blight account for multi-billion US$ annually. Using host resistance is thought to be an economical and efficient approach to control potato late blight. To combat the disease breeders have introduced late blight resistance (*R*) genes from various *Solanum* species into the cultivated potato. The proteins encoded by these *R* genes can recognise specific races of the pathogen. This then triggers a cascade of defence responses ultimately leading to a localized programmed cell death called the hypersensitive response (HR) that arrests growth of the pathogen. However, once the resistant potato cultivars are released into the field, the resistance based on these genes is quickly overcome due to rapid evolution and adaption of *P. infestans*.

In the potato-*P. infestans* interaction race-specific recognition by *R* genes is based on the 'gene-for-gene' model which predicts that resistance is governed by the (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 for disease. Key to a better understanding of the molecular basis of resistance in the potato-*P. infestans* pathosystem is the unravelling of *R* protein-effector interactions and, hence, cloning of more *R* and *Avr* genes is a prerequisite to study these interactions. This thesis describes the mapping and cloning of *Avr* genes in *P. infestans*, and the phenotypic and genotypic diversity in *P. infestans* field isolates in Northern China.

To isolate *P. infestans Avr* genes a positional cloning strategy was adopted. **Chapter 2** presents a molecular-genetic linkage map of *P. infestans* that was constructed based on Single Nucleotide Polymorphism (SNP) markers and Amplified Fragment Length Polymorphism (AFLP) markers. The map was generated using a mapping population of 83 F_1 progeny derived from two Dutch field isolates, NL80029 and NL88133. Of 631 markers (398 SNP and 233 AFLP markers) that segregated in this population, 534 markers were positioned on 19 linkage groups spanning a total of 1144 cM and an average distance of 2.14 cM between adjacent markers. Fourteen of the linkage groups are major linkage groups that contain markers from both parents. The others are minor linkage groups with markers of only one of the two parents.

In parallel, a transcriptional profiling strategy was adopted to identify avirulence-associated transcripts (**Chapter 3**). cDNA-AFLP was used for comparing transcripts in *P. infestans* isolates with different virulence phenotypes. A large number of avirulence-associated TDFs (Transcript Derived Fragments) was cloned and sequenced, and EST and genome databases were mined to generate more sequence data. To identify promising candidates, bioinformatic predictions

such as the presence of signal peptides, number of cysteine residues and putative virulence functions were used as important selection criteria. Four TDFs associated with *Avr* loci were identified, two for *Avr4* and two for the *Avr3b-Avr10-Avr11* locus.

Chapter 4 describes how a combined approach of genetic mapping, transcriptional profiling and BAC marker landing resulted in isolation of the *P. infestans* avirulence gene *Avr4*. *PiAvr4* encodes a 287 amino acid protein that belongs to a superfamily of effectors sharing the putative host cell targeting motif RXLR-dEER. For the functional characterization *P. infestans* race 4 strains were transformed with *PiAvr4*. This resulted in transformants that were avirulent on *R4* potato plants, demonstrating that *PiAvr4* is responsible for eliciting *R4*-mediated resistance. Expression of *PiAvr4* in *R4* plants using PVX-agroinfection and agroinfiltration showed that PiAvr4 itself is the effector that elicits HR on *R4* plants. On potato plants lacking *R4,* like Bintje, there was no response. The presence of the RXLR-dEER motif suggested intracellular recognition of PiAvr4 but nevertheless a hypersensitive response was observed when PiAvr4 was targeted to the outside of the cell. Deletion of the RXLR-dEER domain neither stimulated nor prevented elicitor activity of PiAvr4. Race 4 strains have frame shift mutations in the *PiAvr4* gene that result in short truncated peptides, indicating that PiAvr4 is not crucial for virulence.

Chapter 5 describes *Avr1*-associated markers that resulted from genetic mapping, transcriptional profiling and BAC-end sequences. *In silico* landing of these markers on the *P. infestans* genome sequence narrows down a 800 kb genomic interval that carries seven genes that have the hallmarks of an oomycete *Avr* gene. They all encode a secreted protein with a conserved RXLR-dEER domain at the N-terminus and a divergent C-terminal region. Each of these seven could be a candidate for *Avr1*. The seven RXLR effector genes were further characterized by bioinformatic analyses such as HMM score of the RXLR motif, and prediction of the presence of W, Y, and L motifs in the C-terminal region. Cloning and functional analyses using transient expression assays in plants carrying the resistance gene *R1* should reveal whether any of the seven candidates is *Avr1.*

Chapter 6 describes the phenotypic and genotypic diversity of *P. infestans* isolates collected in Northern China between 1997 and 2003, especially in Inner Mongolia. Characterization included mating type, virulence, mitochondrial DNA (mtDNA) haplotype and DNA fingerprinting patterns based on simple sequence repeats (SSR) and amplified fragment length polymorphism (AFLP). All isolates had the A1 mating type, mtDNA haplotype IIa and an identical SSR genotype (designated as SG-01-01) that differed from the SSR genotypes found in the reference isolates, including the ones representing the 'old' US-1 lineage that dominated the worldwide *P. infestans* population prior to 1980. In contrast, the virulence spectra differed significantly and virulence to all *R* genes present in the standard differential set (*R1* to *R11*) was found. AFLP analysis revealed some diversity; eight different AFLP genotypes were found that could be grouped into two major clusters. This study shows that there is very little genotypic diversity in the *P. infestans*

population in Northern China. The occurrence of many different races within this uniform population is discussed in the framework of recently gained insights in the molecular determinants of avirulence in *P. infestans* and their role in the 'gene-for-gene' interaction with potato.

Finally, in **Chapter 7,** the implications of the findings described in this thesis are discussed with specific emphasis on *Avr* gene cloning, RXLR-dEER effectors, virulence diversity and durable late blight resistance. By combining various cloning strategies it becomes feasible to speed up the cloning of putative *P. infestans Avr* genes. Morover, the use of high throughput effector genomics screenings will allow the identification of the corresponding *R* genes. The high virulence diversity that is found in *P. infestans* field isolates, even within one clonal lineage, might be correlated to the observation that RXLR-dEER effector genes are the most rapidly evolving genes in the genome of *P. infestans.* Therefore, generating potato cultivars with durable resistance to late blight seems more challenging than anticipated.

Samenvatting

De aardappelziekte, veroorzaakt door de oomyceet *Phytophthora infestans* (Mont.) de Bary, is de meest verwoestende ziekte in aardappel wereldwijd en de grootste bedreiging voor de aardappelproductie in China. De kosten ten gevolge van verlies in opbrengst en kwaliteit, en voor chemische bestrijding lopen jaarlijks in de miljarden. Het gebruik van resistente aardappelplanten zou een efficiënte benadering kunnen zijn om de aardappelziekte onder controle te krijgen. Via veredeling zijn reeds verschillende resistentiegenen (*R*) uit wilde *Solanum* soorten in de gecultiveerde aardappel ingekruist. De eiwitten waarvoor deze *R* genen coderen herkennen specifieke fysio's van de ziekteverwekker. Deze herkenning leidt tot een cascade van afweerreacties en resulteert uiteindelijk in geprogrammeerde celdood en in een overgevoeligheidsreactie waardoor de ziekteverwekker stopt met groeien en zich niet verder in de plant kan verspreiden. Helaas is deze, op *R* gen gebaseerde resistentie vaak van korte duur. Binnen enkele jaren hebben de meeste nieuwe cultivars de resistentie verloren. *P. infestans* adapteert snel aan de nieuwe situatie en is dan in staat om de herkenning te vermijden.

In de aardappel-*P. infestans* interactie is de fysio-specifieke herkenning door *R* genen gebaseerd op het 'gen-om-gen' model. Volgens dit model wordt resistentie bepaald door de directe of indirecte interactie van een R eiwit met zijn corresponderende effector, het product van een avirulentiegen (*Avr*). Als het *R* gen of het *Avr* gen ontbreekt of niet functioneel is, is de interactie compatibel en de aardappelplant gevoelig voor de ziekte. Om een beter inzicht te verwerven in de moleculaire basis van resistentie tegen de aandappelziekte is het van cruciaal belang om de interacties tussen *R* eiwit en effector te ontrafelen. Een voorwaarde om deze interacties te kunnen bestuderen is de beschikbaarheid van meer gekloneerde *R* genen en *Avr* genen. Dit proefschrift beschrijft het karteren en kloneren van *Avr* genen in *P. infestans*, en de fenotypische en genotypische diversiteit in *P. infestans* veldisolaten in Noord China.

Voor het isoleren van *P. infestans Avr* genen werd gebruikt gemaakt van een positionele kloneringstrategie. **Hoofdstuk 2** beschrijft een moleculair-genetische koppelingskaart van *P. infestans* gebaseerd op 'Single Nucleotide Polymorphism' (SNP) merkers en 'Amplified Fragment Length Polymorphism' (AFLP) merkers. De kaart werd gegenereerd met een karteringspopulatie van 83 F_1 nakomelingen afkomstig van twee Nederlandse veldisolaten, NL80029 en NL88133. Van 631 merkers (398 SNP en 233 AFLP merkers) die uitsplitsten in deze populatie, werden 534 merkers gepositioneerd op 19 koppelingsgroepen met een totale lengte van 1144 centi-Morgan en een gemiddelde afstand van 2.14 centi-Morgan tussen twee flankerende merkers. Veertien van de koppelingsgroepen zijn samengestelde koppelingsgroepen die merkers van beide ouders bevatten. De andere zijn ouder-specifieke koppelingsgroepen met merkers afkomstig van slechts één van de twee ouders.

Gelijktijdig werd een zogenaamde ‘transcriptional profiling’ strategie toegepast om transcripten te identificeren die geassocieerd zijn met avirulentie (**Hoofdstuk 3**). cDNA-AFLP werd gebruikt om transcripten te vergelijken tussen *P. infestans* isolaten met verschillende virulentie fenotypes. Een groot aantal avirulentie-geassocieerde TDF’s (‘Transcript Derived Fragments’) werd gekloneerd en de DNA volgorde werd bepaald. Vervolgens werden EST- en genoom-databanken doorgespit om meer sequentiegegevens te verkrijgen. Om veelbelovende kandidaten te identificeren werden selectiecriteria gebruikt die met behulp van bioinformatica getoetst werden, zoals het vóórkomen van een signaalpeptide, het aantal cysteïne residuen en mogelijke virulentiefuncties. Er werden vier TDFs gevonden die geassocieerd zijn met *Avr* loci, twee voor *Avr4* en twee voor het *Avr3b-Avr10-Avr11* locus.

Hoofdstuk 4 beschrijft hoe merkers verkregen met genetische kartering en ‘transcriptional profiling’ gebruikt werden om BACs te selecteren en fysisch te karteren. Subklonering van de BACs resulteerde in de isolatie van het *P. infestans* avirulentiegen *Avr4*. *PiAvr4* codeert voor een eiwit van 287 aminozuren dat behoort tot de superfamilie van effectoren die allen een RXLR-dEER motief bevatten. Dit motief zorgt voor translocatie van de effector naar de gastheercel. Voor functionele karakterisering werden *P. infestans* fysio 4 isolaten getransformeerd met *PiAvr4*. Dit resulteerde in transformanten die avirulent zijn op aardappellijnen die het *R4* gen bevatten hetgeen aantoont dat *PiAvr4* verantwoordelijk is voor het opwekken van een resistentieresponse geïnitieerd door *R4.* Expressie van *PiAvr4* in *R4* planten met behulp van PVX-agroinfectie en agroinfiltratie toonde aan dat PiAvr4 inderdaad een effector is die een overgevoeligheidsreactie opwekt in *R4* planten. Dit gebeurde niet in aardappelplanten die geen *R4* gen bevatten zoals bijvoorbeeld Bintje. De aanwezigheid van een RXLR-dEER motief suggereert dat PiAvr4 in de cel herkend wordt; echter, ook als PiAvr4 naar de extracellulaire ruimte werd gedirigeerd trad overgevoeligheid op. Het verwijderen van het RXLR-dEER domein had geen effect op de activiteit van PiAvr4 als elicitor van de overgevoeligheidsreactie. Fysio 4 isolaten hebben mutaties in *PiAvr4* die een verstoring geven in het open leesraam hetgeen resulteert in een vroegtijdig afgekapt eiwit dat niet functioneel is als een avirulentiefactor. Dit suggereert dat PiAvr4 niet essentieel is voor virulentie.

Hoofdstuk 5 beschrijft *Avr1*-geassocieeerde merkers afkomstig van genetische kartering, ‘transcriptional profiling’ en DNA volgordes van BAC uiteindes. Deze merkers werden *in silico* gelanceerd op het genoom van *P. infestans*. Dit leidde tot de afbakening van een beperkt gebied van 800 kb waarop zeven genen liggen met eigenschappen die typisch zijn voor een oomyceet *Avr* gen. Deze zeven genen coderen voor een uitgescheiden eiwit met een geconserveerd RXLR-dEER motief in het amino-terminale deel en een variabel carboxy-terminaal deel. Elk van deze zeven RXLR-effectorgenen kan een kandidaat zijn voor *Avr1*. Ze werden nader gekarakteriseerd met bioinformatica analyses gericht op HMM scores van het RXLR motief, en voorspellingen over de aanwezigheid van W, Y, en L motieven in het carboxy -terminale deel.

Klonering en functionele analyses op basis van transiënte expressie-essays in planten die het resistentiegen *R1* hebben, kunnen aantonen of één van deze zeven kandidaten daadwerkelijk *Avr1* is.

Hoofdstuk 6 beschrijft de fenotypische en genotypische diversiteit van *P. infestans* isolaten die verzameld zijn in Noord China tussen 1997 en 2003, met name in Binnen-Mongolië. Alle isolaten hadden het A1 paringstype, het mitochondriale DNA haplotype IIa en eenzelfde DNA vingerafdruk op basis van 'Simple Sequence Repeat' (SSR) merkers. Het SSR genotype, SG-01-01, verschilde van dat van de referentie-isolaten. In DNA vingerafdrukken op basis van AFLP merkers werd wel wat variatie gevonden: acht AFLP genotypes verdeeld over twee clusters. In tegenstelling tot de redelijk uniforme DNA vingerafdrukken waren de virulentie-spectra zeer divers en er was virulentie voor alle *R* genen in de differentiële set (*R1 – R11)*. Deze studie toont aan dat de *P. infestans* populatie in Noord China waarschijnlijk tot een klonale lijn behoort die verschilt van de 'oude' US-1 lijn die tot 1980 wereldwijd dominant was. Het voorkomen van vele verschillende fysio's in een populatie die overwegend uniform is wat betreft DNA merkers wordt bediscussieerd in het kader van recent verworven inzichten in de moleculaire determinanten van avirulentie in *P. infestans* en hun rol in de 'gen-om-gen' interactie met aardappel.

In het laatste hoofdstuk, **Hoofdstuk 7,** worden de implicaties van de vindingen beschreven in dit proefschrift bediscussieerd met de nadruk op de klonering van *Avr* genen, op RXLR-dEER effectoren, op diversiteit van virulentie en op duurzame resistentie tegen de aardappelziekte. Door verschillende kloneringstrategiën te combineren is het mogelijk de isolatie van potentiële *P. infestans Avr* genen te versnellen. Bovendien zal het gebruik van 'high throughput' effector genomica screeningen de identificatie van de corresponderende *R* genen mogelijk maken. De hoge diversiteit in virulentie die gevonden wordt in *P. infestans* veldisolaten, zelfs binnen een klonale lijn, zou gecorreleerd kunnen zijn met het gegeven dat RXLR-effectorgenen de snelst evoluerende genen zijn in het genoom van *P. infestans.* Alles wijst erop dat het genereren van duurzame resistentie tegen de aardappelziekte een nog grotere uitdaging is dan eerder werd aangenomen.

中文摘要

马铃薯晚疫病菌无毒基因的定位、克隆和多样性分析

由卵菌 *Phytophthora infestans* 引起的马铃薯晚疫病，不仅是中国马铃薯产业最大的威胁，而且也是全世界马铃薯危害最严重的病害。全世界每年因马铃薯晚疫病造成的产量和质量的损失，以及用于防治马铃薯晚疫病的化学药剂费用多达数十亿美元。控制马铃薯晚疫病经济有效的办法是应用寄主抗性。为控制马铃薯晚疫病，育种家已经把来自 *Solanum* 属植物的抗病基因（*R* 基因）转化到栽培品种中加以利用。这些 *R* 基因编码的蛋白可以识别病原菌的不同生理小种，引发一系列的抗性反应，最终导致过敏性反应并阻止病原菌的生长。但是，一旦这些抗晚疫病马铃薯品种被栽种在大田里，以这些基因为基础的抗性，由于 *P. infestans* 的快速进化和适应而被迅速克服。

在马铃薯- *P. infestans* 互作中，寄主的抗病基因与病原菌的无毒基因 （*Avr* 基因）互作符合“基因对基因”假说，小种特异性抗性是由抗病基因编码的 R 蛋白与相应的无毒基因编码的效应子（effector）直接或间接相互作用的结果。假如 *R* 基因或是 *Avr* 基因一方缺失或不起作用，则表现亲和性互作，马铃薯被晚疫病菌侵染。为更好地了解马铃薯- *P. infestans* 互作中抗病的分子基础，需要揭示 R 蛋白与效应子的互作机制。因此，克隆更多的 *R* 和 *Avr* 基因是研究它们之间相互作用的先决条件。本论文阐述了 *P. infestans* 中无毒基因的定位和克隆，以及中国北方 *P. infestans* 菌株的表型和基因型的多样性。

首先，我们采用图位克隆法来分离*P. infestans* 的无毒基因。第二章介绍了利用单核苷酸多态性（SNP）和扩增片段长度多态性（AFLP）标记技术构建了*P. infestans*分子遗传连锁图谱。作图群体由来自荷兰菌株NL80029 和NL88133 的 83 个F_1子代组成。 利用Joinmap 3.0 对 631 个标记 （398 个SNP和 233 个AFLP标记）构建了连锁群，其中 534 个标记被定位在 19 个连锁群上，14 个是包含来自双亲标记的大连锁群，另 5 个为来自一个亲本标记的小连锁群。该遗传图谱连锁群总长 1144 cM，标记平均间距 2.14 cM。

同时，本论文利用转录表达谱策略获得了与 *P. infestans* 无毒基因相关的转录本（第3章）。利用具有6个无毒基因不同表现型，且处于萌发静孢子时期的马铃薯晚疫病菌构建4个混合池，通过cDNA-AFLP分析，获得了大量与无毒基因相关的差异表达片段，并进行了克隆测序。为了获得候选的无毒基因表达片断，我们利用了生物信息学预测如信号肽的有无、半胱氨酸残基的数量以及可能的毒性功能作为重要的筛选标准，最终获得了无毒基因位点*Avr3b-Avr10-Avr11*和*Avr4*的候选表达片段各2个。

第四章描述了如何结合遗传图谱、转录表达谱、细菌人工染色体标记着陆技术分离获得 *P. infestans* 无毒基因 *Avr4*。该基因编码 287 个氨基酸并且属于 RXLR-dEER 大家族。通过转化无毒基因 *Avr4* 到生理小种 4 的马铃薯菌株，获得的转化子在含 *R4* 的马铃薯植株表

现非亲和反应，表明无毒基因 *Avr4* 引发 *R4* 介导的抗性。借助马铃薯 X 病毒介导侵染和渗透的方法，让无毒基因 *Avr4* 在 *R4* 植株上表达，结果表明 PiAvr4 是引起 *R4* 植株过敏性反应的效应子；然而，侵染和渗透不含 *R4* 的马铃薯品种 Bintjie，没有观察到过敏反应的发生。RXLR-dEER 基序的存在表明 PiAvr4 是在细胞内被识别的，但是，当 PiAvr4 在胞外时，也能观察到过敏性反应的发生。生理小种 4 马铃薯晚疫病菌株含有的无毒基因 *Avr4* 具有移码突变的现象，导致编码产物缩短，表明 PiAvr4 对病菌毒性不是至关重要的。

第五章分析了来自遗传图谱、转录表达谱、细菌人工染色体末端序列的 *P. infestans* 无毒基因 *Avr1* 相关的标记。通过对 *P. infestans* 基因组进行电子着陆，在 800kb 的区域之间锚定了 7 个候选的卵菌无毒基因。这 7 个候选无毒基因编码的分泌蛋白 N-末端都具有保守的 RXLR-dEER 结构域，同时 C-末端区域变化多样。此外，利用生物信息学方法进一步预测了这 7 个候选无毒基因的特点，如 RXLR 结构域的 HMM 得分和 C-末端 W、Y、L 基序的存在情况。7 个候选无毒基因中任何一个可能为候选 *Avr1*。通过克隆并在含 *R1* 的马铃薯植株上进行瞬时表达分析将会从 7 个候选无毒基因中筛选获得 *Avr1*。

第六章研究了 1997 到 2003 年间采自中国北方地区，特别是内蒙古的马铃薯晚疫病菌株的表型和基因型多样性。多样性分析包括交配型、毒性、线粒体 DNA 单倍型、简单重复序列（SSR）、扩增片断长度多态性（AFLP）。分析表明：所有供试菌株具有 A1 交配型，线粒体 DNA 单倍型 IIA，相同的 SSR 基因型（SG-01-01）。SSR 基因型（SG-01-01）不同于参照菌株的 SSR 基因型，也不同于 1980 年前盛行的 US-1 无性系的 SSR 基因型。然而，病菌的毒性差异显著，并且发现了能克服所有 11 个抗病基因的菌株。AFLP 分析揭示了一定的多样性，共发现了 8 种不同的 AFLP 基因型。本研究表明中国北方的马铃薯晚疫病菌株表现较低的基因型多样性，同时通过近来的马铃薯晚疫病菌毒性的分子决定物以及它们在“基因对基因”互作中的作用的新观点，讨论了为何在同一无性系中存在大量的不同生理小种。

第七章在无毒基因的克隆、RXLR-dEER 效应子、毒性多样性和晚疫病持久抗性的基础上讨论了本论文的研究结果。通过结合不同的克隆策略加快 *P. infestans* 的无毒基因的克隆是可行的，而且，利用高通量的效应子基因组学筛选将会获得相应的抗病基因。即使在同一无性系，马铃薯晚疫病菌株也具有非常高的毒性多样性，这可能与 RXLR-dEER 基因是 *P. infestans* 基因组中进化最快的基因有关。因此，获得具有持久抗性马铃薯品种的困难远比我们想象的多。

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Jun Guo
Yangling, China
September 14, 2008

Curriculum Vitae

Jun Guo was born on the 10th of September 1974 in Zhucheng County, Shandong Province, China. He studied at Laiyang Agricultural College (Shandong Province, China) and obtained his B.Sc. degree with Vegetable Crops as major in 1998. In 2001, he received his M.Sc. degree at the College of Agriculture and Biotechnology of Zhejiang University (Hangzhou, Zhejiang Province, China) with Vegetable Crops as major and started his Ph.D. research at the Chinese Academy of Agricultural Sciences (CAAS) in Beijing (China). In 2002, he got the chance to enroll in the Joint Sandwich Ph.D. program from CAAS and Wageningen University (WU), funded by the WU-Interdisciplinary Research and Education Fund (INREF) and the Asian Facility Program. This thesis, that he will defend in Wageningen on the 20th of October 2008, describes the results obtained during his Ph.D. research at the Institute of Vegetables and Flowers (IVF) of CAAS, and the Laboratory of Phytopathology of WU, from 2001 to 2005. In July 2005 he joined the College of Plant Protection of Northwest A&F University (Yangling, Shaanxi Province, China) where he teaches courses in plant pathology and plant protection, and performs research on the interaction between the yellow rust fungus and wheat.

List of Publications

Guo J., van der Lee T., Qu D.Y., Yao Y.Q., Gong X.F., Liang D.L., Xie K.Y., Wang X.W. and Govers F. 2008. *Phytophthora infestans* isolates from Northern China show high virulence diversity but low genotypic diversity. Plant Biology, in press.

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Education Statement of the Graduate School
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1) Start-up phase	*date*
► **First presentation of your project**	
Identification of race-specific avirulence genes in *Phytophthora infestans*	Dec 20, 2002
► **Writing or rewriting a project proposal**	
Identification of race-specific avirulence genes in the potato late blight pathogen *Phytophthora infestans,* International Foundation for Science	Mar 15-25, 2003
► **Writing a review or book chapter**	
Advances in molecualr genetics of potato late blight pathogen *Phytophthora infestans*	Sep 2004
► **MSc courses**	
► **Laboratory use of isotopes**	
Safe handing with redioactive materials and sources	Nov 2002
Subtotal Start-up Phase	*9.0 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
► **EPS theme symposia**	
EPS theme 4 symposium 'Genome Plasticity', Leiden University (Netherlands)	Dec 07, 2007
► **NWO Lunteren days and other National Platforms**	
NWO Lunteren days	Oct 2002
Vegetable molecular breeding symposium at IVF, China	Jun 17-18, 2004
Annual meeting of Chinese Society for Plant Pathology In Yangling, China	Aug 06-07, 2007
► **Seminars (series), workshops and symposia**	
Progress in Agricultural Sciences, Graduate School of CAAS, China	Oct-Dec 2001
Seminar Sophien Kamoun	Jul 08, 2002
Seminar Andre Drenth	Oct 08, 2007
► **Seminar plus**	
► **International symposia and congresses**	
The 15th International Plant Protection Congress in Beijing	May 12-15, 2004
Plant Genomics in China VII in Harbin, China	Aug 08-10, 2006
► **Presentations**	
Poster presentation in EPS Autumn school ' Disease resistance in plants'	Oct 2002
Poster and oral presentation in CAAS-WUR autumn school; Satellite meeting *Phytophthora.* Beijing	Nov 2003
Oral presentation in Vegetable molecular breeding symposium at IVF, China	Jun 17-18, 2004
► **IAB interview**	
► **Excursions**	
Potato late blight investigation in Hebei Agricultural University and Inner Mongolia	Apr 2004
Subtotal Scientific Exposure	*8.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Cellular and Molecular biology (In gradulate school of CAAS)	Oct 06-15, 2001
EPS Autumn school ' Disease resistance in plants'	Oct 16-18, 2002
Autumn school CAAS-WUR 'Satellite meeting Phytophthora', Beijing, China	Nov 10-15, 2003
► **Journal club**	
Weekly literature discussion at IVF, CAAS and Wageningen university	2002-2007
► **Individual research training**	
Bioinformatics training in Zhejiang University, Hangzhou, China	Dec 02-06, 2002
Training intership of the NSF Phytophthora Molecular Genetics Network	Apr 20-24, 2003
Subtotal In-Depth Studies	*11.7 credits**

4) Personal development	*date*
► **Skill training courses**	
English (In gradulate school of CAAS)	Sep-Dec 2001
How to write scientific proposal (In graduate school of CAAS)	Oct 2001
Writing of scientific publication (In graduate school of CAAS)	Nov 2001
► **Organisation of PhD students day, course or conference**	
4th International ISHS Symposium on Edible Alliaceae in China	Apr 21-25, 2005
Annual meeting of Chinese Society for Plant Pathology In Yangling, China	Aug 06-07, 2007
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*7.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**36.8**

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

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